申报系列/类型 教师系列教学为主型

学科类型 自然科学类

□ 首聘

□ 博士后

√ 破格申请

□ 曾转过系列

□ 申请转系列

华南农业大学
职称评审表
（2024年）

申报者单位： 华南农业大学

申报者姓名： 张世海

现职称： 畜牧学 **专业** 副教授 **职称**

申报职称： 畜牧学 **专业** 教授 **职称**

华南农业大学人力资源处制

个人承诺

本人郑重承诺：本人对《华南农业大学职称评审表》所填写的内容及提交材料的真实性负责。如有虚假或不真实之处，按《华南农业大学职称评审办法》（华南农办［2022]9号)的相关规定处理。

填表人(签名)：张世海

年　　月　　日

个人情况

姓　名	张世海	工　号	30004371	性　别	男
出生年月	1989.01	政治面貌	群众	移动电话	13660346296
最高学历	博士研究生毕业	最高学位	农学博士学位	毕业时间	2016-06-01
所学专业	动物营养与饲料科学	现工作岗位	教学科研	参加工作时间	2016-10-08
是否曾转系列评审	否	转系列评审前职称		转系列评审前职称取得时间	
现职称名称	副教授	取得时间	2020-01-02	现职称取得方式	评审
聘任时间	2020-01-02	累计任职年限	5	获高校教师资格时间	2018-07-20
拟申报何职称	教授	所属专业	畜牧学		
是否首聘	否				
是否博士后	否				
是否破格申请	是				
本次是否转系列评审	否				

学习简历（从高中毕业以后填起）					
入学时间	毕业时间	毕业院校	所学专业	学历	学位
2007-09-01	2011-06-01	中国农业大学	动物科学	大学本科毕业	农学学士学位
2011-09-01	2016-06-01	中国农业大学	动物营养与饲料科学	博士研究生毕业	农学博士学位

工作简历			
开始日期	截至日期	任职单位名称	任职岗位（职务）
2016-10-01		华南农业大学	副教授

继续教育情况
2024年已经完成了专业技术人员继续学习的任务。

工 作 负 面 情 况 说 明					
本人负面情况申报	任职期间，是否出现下列情况：				
	负面情况	是否存在该情况	年份	处分时间	处分期限
	因师德问题受学校警告以上处分	否			
	因师德问题受学校记过以上处分	否			
	年度考核基本合格	否			
	年度考核不合格	否			
	受党纪、政纪处分	否			
	涉嫌违法违纪接受组织调查	否			
	受刑事处罚	否			
	发现并查证属实有伪造身份、学历、资历、业绩，剽窃他人成果等弄虚作假和违反学术道德行为，以及隐瞒事实真相未如实申报	否			
	指导研究生的学位论文，存在作假行为并造成严重不良影响，或在国家和省级学位论文抽检中定为“存在问题学位论文”	否			
	指导的学生参赛作品抄袭、伪造等情况	否			
	出现教学差错	否			
	出现教学事故	否			
	出现安全责任事故	否			
	其他	否			
本人对负面情况的陈述	本人签名：				
单位意见	（公章） 年 月 日				

注：1、申报人须如实填写上述各栏。若对现任职以来专业技术工作中既往过错隐瞒不报的，一经查实，按照《华南农业大学职称评审办法》（华南农办［2022]9号)的相关规定严肃处理。
2、“本人对负面情况的陈述”栏，如实填写出现负面情况的具体表述、出现原因、处理方式及本人的认识。
3、“单位意见”栏由单位针对申报人工作作风、态度、过失因果等，实事求是加具对其申报评审的意见；如有其他本人未申报的负面情况亦一并开列，并具公章。

思想政治素质和师德师风考核表

一、本人自述 **本人根据《新时代高校教师职业行为十项准则》《华南农业大学教师职业道德行为负面清单》，从政治表现、道德品质、师德师风、遵纪守法等方面进行陈述。（150个字符以内）**
坚持四项基本原则，政治立场坚定，积极学习习近平新时代中国特色社会主义思想；品行端正，忠诚敬业，为人师表；恪守师德师风，关爱学生，严谨治学；遵守法律法规，廉洁自律；严以律己、宽以待人，杜绝学术不端，维护良好校园风气。 **本人签名：** **年　月　日**
二、所在系（教研室、单位）的教工党支部意见 **所在系（教研室、单位）的教工党支部根据《新时代高校教师职业行为十项准则》《华南农业大学教师职业道德行为负面清单》，从政治表现、道德品质、师德师风、遵纪守法等方面考核并进行陈述。（100个字符以内）**
党支部书记签名： **年　月　日**
三、所在单位党组织综合意见 **所在单位党组织根据《新时代高校教师职业行为十项准则》《华南农业大学教师职业道德行为负面清单》，从政治表现、道德品质、师德师风、遵纪守法等方面进行考核，提出明确考核意见。（150个字符以内）**
考核结果：　合格 □　不合格 □ **二级党组织负责人（签名）：　　（盖章）：** **年　月　日**

相关经历与培训、实践情况

表1 学生工作等相关经历情况表

项目类型	起止时间		工作经历具体描述	考核结果	备注
	自	至			
班主任	2020-09	2023-07	担任动物科学学院2020级硕士动物营养与饲料科学专业4班班主任	合格	
班主任	2024-09	2028-06	担任动物科学学院2024级本科动物科学专业4班班主任		

表2 生产实践锻炼情况表

序号	起止时间		生产实践锻炼的项目内容	生产实践锻炼的单位或地点	生产实践锻炼单位的负责人	生产实践锻炼累计时间（单位/天）	备注
	自	至					
生产实践锻炼累计时间合计（单位/天）				0			

表3 担任科技推广专家情况（研究系列推广型申报人员必填）

序号	聘任时间	名称	具体业绩表述	级别	备注

表4 社会服务工作量情况（研究系列推广型申报人员必填）

序号	年度	服务概览	年度工作量	备注
社会服务工作量总计		0		

表5 思想政治理论课教师研修培训情况（思想政治理论课教师填报）

序号	起止时间		培训名称	具体业绩表述	备注
	自	至			

破格条件

教学成果奖或教学类比赛情况

获奖时间	项目类型	项目名称	奖励级别	成果授予部门	本人排名	证书号	备注
2024-07-01	教学比赛类	第四届全国高校教师教学创新大赛一等奖	国家级一等奖	中国高等教育学会	1	TIC2024NB1068	替代3个B类业绩（包括：1个B类教学改革论文，2个B类教学改革项目）

主持的科研项目情况

项目类型	项目名称	项目编号	项目来源	项目分类	实到经费(万)	经费卡号	立项时间	是否结题	结题时间	课题组总人数	项目等级	备注

发表本专业论文（著）情况

论文名称	刊物名称（刊号）	发表时间（年月）	作者类型	作者排名	文献类型	论文等级	备注

注：论文附件须包含期刊封面、目录（标注出所发论文）、论文全文、封底以及检索证明。

科研平台情况

立项时间	项目名称	项目来源	总经费额(万)	进展情况	本人排名	等级	备注

科技奖励情况

获奖时间	奖励名称+等级	成果名称	成果授予部门	本人排名	项目等级	备注

应用成果情况

获得时间	类型	名称	成果授予部门	本人排名	登记号/标准编号	项目等级	备注

科技成果转化项目情况

项目名称	实到经费（万元）	经费卡号	合同签订时间	本人排名	项目等级	备注

决策咨询报告采纳实施情况

采纳时间	采纳或实施部门	具体业绩表述	备注

教学任务

表6-A 讲授本科生课程情况-理论课程

学年学期	课程名称	授课对象	总学时	实际承担学时	是否合上课程	备注
2024-2025-1	动物营养学	22动物科学5	32	32	否	
2024-2025-1	动物营养学	22生物科学[1-2]班,22生科基地班[1-2]	32	32	否	
2024-2025-1	营养与美食探索之旅	通识选修（公选课）	32	32	否	
2024-2025-1	营养与美食探索之旅	通识选修（公选课）	32	32	否	
2024-2025-1	饲料学	22动物科学5	32	29	是	与邓跃林教师合上
2024-2025-1	智慧牧业导论	24智慧牧业1	16	2	是	与王燕,王丽娜,卫恒习,孙宝丽,闫希亮,张新珩,蔺文成教师合上
2023-2024-1	动物营养学	21动物科学6-7	32	32	否	
2023-2024-2	动物营养学	22动科温氏班1	40	40	否	
2023-2024-2	配合饲料生产学	21动物科学[5-7]班,21动科温氏班1	32	29	是	与邓跃林教师合上
2023-2024-2	智慧养殖概论	23智慧牧业1	16	2	是	与王燕,孙宝丽,闫希亮,张新珩,蔺文成,卫恒习,王丽娜教师合上
2022-2023-2	动物营养学	21动科温氏班1	40	40	否	

2022-2023-2	智慧养殖概论	22动科智慧牧业1	16	2	是	与王燕,孙宝丽,蔺文成,卫恒习,王丽娜,张新珩教师合上
2021-2022-2	动物营养学	20动物科学5-6	32	32	否	
2021-2022-1	动物营养学	19生科基地班[1-2]	32	32	否	
2020-2021-1	动物营养学	19动物科学5-8	32	26	是	与邓近平教师合上
2020-2021-2	动物营养学	19动物科学1班,19动物科学6	32	32	否	
2020-2021-1	动物营养学	18动物科学1-6	32	32	否	
2019-2020-2	动物营养学	18动物科学1-3	32	29	是	与管武太教师合上
总学时数	487	**年限** 5	**年均授课学时数**		97.4	

表6-B 讲授本科生课程情况-实验课程

学年学期	课程名称	授课对象	总学时	实际承担学时	是否合上课程	备注
2024-2025-1	精准动物营养综合实验	22动科智慧牧业1	32	12	是	与曹庆云,叶慧教师合上。
2024-2025-1	动物营养与饲料学综合实验	22动物科学1	48	8	是	与曹庆云,叶慧教师合上。
2024-2025-1	动物营养与饲料学综合实验	22动物科学2	48	8	是	与曹庆云,叶慧教师合上。
2024-2025-1	动物营养与饲料学综合实验	22动物科学3	48	8	是	与曹庆云,叶慧教师合上

			48	8		。
2024-2025-1	动物营养与饲料学综合实验	22动物科学4	48	8	是	与曹庆云,叶慧教师合上。
2024-2025-1	动物营养与饲料学综合实验	22动物科学5	48	8	是	与曹庆云,叶慧教师合上。
总学时数	52	**年限** 5	**年均授课学时数**		10.4	

表6-C 讲授本科生课程情况-教学实习、训练类课等

学年学期	课程名称	授课对象	天数	班级数	折算学时数	备注（是否与其他教师合上）
2023-2024-2	饲料学综合实习	21动物科学1	5	1	13	与朱勇文教师合上。
2023-2024-2	饲料学综合实习	21动物科学2	5	1	13	与朱勇文教师合上。
2023-2024-2	饲料学综合实习	21动物科学3	5	1	13	与朱勇文教师合上。
2023-2024-2	饲料学综合实习	21动物科学4	5	1	13	与朱勇文教师合上。
2023-2024-2	饲料学综合实习	21动物科学5	5	1	13	与朱勇文教师合上。
2023-2024-2	饲料学综合实习	21动物科学6	5	1	13	与朱勇文教师合上。
2023-2024-2	饲料学综合实习	21动物科学7	5	1	13	与朱勇文教师合上。
2022-2023-2	饲料学综合实习	20动物科学1	5	1	13	与朱勇文教师合上。
2022-2023-2	饲料学综合实习	20动物科学2	5	1	13	与朱勇文教师合上。
2022-2023-2	饲料学综合实习	20动物科学3	5	1	13	与朱勇文教师合上。
2022-2023-2	饲料学综合实习	20动物科学4	5	1	13	与朱勇文教师合上。
2022-2023-2	饲料学综合实习	20动物科学5	5	1	13	与朱勇文教师合上。
2022-2023-2	饲料学综合实习	20动物科学6	5	1	13	与朱勇文教师合上。
2022-2023-2	饲料学综合实习	20动物科学7	5	1	13	与朱勇文教师合上。
2022-2023-2	饲料学综合实习	20动物科学8	5	1	13	与朱勇文教师合上。
2021-2022-1	饲料学综合实习	19动物科学1	5	1	13	与朱勇文教师合上。
2021-2022-1	饲料学综合实习	19动物科学2	5	1	13	与朱勇文教师合上。

2021-2022-1	饲料学综合实习	19动物科学3	5	1	13	与朱勇文教师合上。
2021-2022-1	饲料学综合实习	19动物科学4	5	1	13	与朱勇文教师合上。
2021-2022-1	饲料学综合实习	19动物科学5	5	1	13	与朱勇文教师合上。
2021-2022-1	饲料学综合实习	19动物科学6	5	1	13	与朱勇文教师合上。
2021-2022-1	饲料学综合实习	19动物科学7	5	1	13	与朱勇文教师合上。
2021-2022-1	饲料学综合实习	19动物科学8	5	1	13	与朱勇文教师合上。
2020-2021-1	饲料学综合实习	18动物科学1	5	1	13	与朱勇文教师合上。
2020-2021-1	饲料学综合实习	18动物科学2	5	1	13	与朱勇文教师合上。
2020-2021-1	饲料学综合实习	18动物科学3	5	1	13	与朱勇文教师合上。
2020-2021-1	饲料学综合实习	18动物科学4	5	1	13	与朱勇文教师合上。
2020-2021-1	饲料学综合实习	18动物科学5	5	1	13	与朱勇文教师合上。
2020-2021-1	饲料学综合实习	18动物科学6	5	1	13	与朱勇文教师合上。
总学时数	377	**年限**	5	**年均授课学时数**		75.4

备注:
1.教学实习：含课程实习、生产实习、毕业实习等，每天按3学时计算；
2.参与农事训练类、通识管理训练类、工程基础训练类教学授课学时，按7学时/天/教学班计算；
3.参与军事技能训练、创新创业实践管理的教师，折算授课学时分别为32学时、7.5学时（不考虑班级数和天数因素）。

表6-D 讲授本科生课程情况-课程论文（设计）

学年学期	课程论文（设计）名称	授课对象		周数	折算学时数	是否合上课程	备注
总学时数		**年限**		**年均授课学时数**			

注：课程论文（设计）教学时数= 周数×5

表6-E 讲授本科生课程情况- 指导毕业论文（设计）

年度	指导毕业论文（设计）	指导人数	折算学时数	备注（是否与其他教师合上）
2021年	AMPK通过Prl1R-JAK2-STAT5通路调控乳蛋白合成的研究	1	5	
2021年	能量缺乏对乳腺上皮细胞氨基酸转运载体的调控作用	1	5	

2021年	mTORC1对乳脂和乳蛋白合成的影响	1	5		
2023年	妊娠后期添加乙酸钠对母猪繁殖性能和泌乳性能的影响	1	5		
2023年	妊娠后期添加纤维对母猪繁殖性能和泌乳性能的影响	1	5		
2023年	妊娠后期添加鱼油对母猪繁殖性能和泌乳性能的影响	1	5		
2023年	铁死亡对乳腺上皮细胞中乳脂和乳蛋白合成的影响	1	5		
2024年	母猪妊娠后期添加纤维对仔猪肠道健康的影响	1	5		
2024年	母猪妊娠后期添加鱼油对胎盘功能的影响	1	5		
2024年	母猪妊娠后期添加鱼油对仔猪肠道健康的影响	1	5		
2024年	白鸡青胫性状转录组和基因组学研究	1	5		
总学时数	55	**年限**	5	**年均授课学时数**	11

注：毕业论文（设计）教学时数= 指导学生数×5

表7-A 讲授研究生课程情况表

学期学年	课程名称	授课对象	课程总学时	本人承担学时	备注
2024-2025-1	饲料科学研究进展	饲料科学研究进展1班	36	3	
2023-2024-1	饲料科学研究进展	饲料科学研究进展1班	36	4	
2023-2024-2	猪营养研究专题	猪营养研究专题1班	40	6	
2023-2024-1	现代动物营养研究技术	现代动物营养研究技术1班	54	9	
2021-2022-2	猪营养研究专题	猪营养研究专题1班	40	6	
2020-2021-2	猪营养研究专题	猪营养研究专题1班	40	6	
2019-2020-2	猪营养研究专题	猪营养研究专题1班	40	6	
总学时数	40	**年限**	5	**年均授课学时数**	8

注：1.表7-A以研究生院下达教学任务的课程时数为准。
2.表7-A须提供证明材料，可导出打印本表，由本人签名确认、学院（单位）审核盖章后再上传附件

表7-B 指导毕业研究生折合教学时数

毕　业　年　度	2024年	2023年	2022年	2021年	

作为一导培养毕业全日制研究生人数	无二导	博士生					
		硕士生	1	2		1	
	有二导	博士生					
		硕士生	3		1		
作为二导培养毕业全日制研究生人数		博士生					
		硕士生					
折合学时数			136				
年均指导毕业研究生折合学时数			27.2				

备注：1.指导毕业研究生教学时数= 毕业全日制硕士人数×20+ 毕业全日制博士人数×35；若有二位指导教师，则第一导师占三分之二，第二导师占三分之一。
2.表7-B须提供证明材料，可导出打印本表，由本人签名确认、学院（单位）审核盖章后再上传附件。

表8 指导创新创业训练项目

学年学期	指导校级以上创新创业训练项目	项目数	折算学时数	备注（是否与其他教师合上，若合上备注合上教师姓名）

总学时数		年限		年均授课学时数	

注：创新创业训练项目教学时数= 指导项目数×5

表9-A 近五年本科生评教结果

学年学期	分数	参评人数	单位排名	排名占比	开课单位
2020-2021学年第一学期	97.3	95	67-15	22.39%	动物科学学院
2021-2022学年第二学期	95.71	59	69-8	11.59%	动物科学学院
2021-2022学年第一学期	96.67	32	73-1	1.37%	动物科学学院
2020-2021学年第二学期	95.48	23	64-4	6.25%	动物科学学院
2023-2024学年第一学期	97.81	29	75-18	24.00%	动物科学学院
2022-2023学年第二学期	99.57	30	70-1	1.43%	动物科学学院
2023-2024学年第二学期	99.67	31	71-1	1.41%	动物科学学院

2024-2025学年第一学期	98.97	119	74-7	9.46%	动物科学学院

表9-B 近五年研究生评教结果

学年学期	分数	参评人数	单位排名	排名占比	开课单位
2022-2023春季	95.24	21	49/56		动物科学学院
2023-2024秋季	97.04	27	38/116		动物科学学院
2023-2024秋季	97.06	9	37/116		动物科学学院
2019-2020春季	95.17	26	37/42		动物科学学院
2020-2021春季	97.28	30	12/46		动物科学学院
2020-2021秋季	96.12	33	36/92		动物科学学院
2021-2022春季	99.1	5	4/28		动物科学学院
2023-2024春季学期	94.74	29	45/57		动物科学学院
2024-2025秋季学期	96.69	13	92/135		动物科学学院

表9-C 评教结果排名情况

近五年，本科评教结果在本单位排名前10%的学期	2021-2022-1，2020-2021-2，2022-2023-2，2023-2024-2，2024-2025-1
近五年，本科评教结果在本单位排名前20%的学期	2021-2022-2
近五年，研究生评教结均在本单位排名前10%的学期	
近五年，研究生评教结均在本单位排名前20%的学期	2021-2022-2

表10 学工工作量情况统计表（仅限学生思想政治教育专业职称申报人员填报）

序号	年　度	项目清单	年度工作量	备注
年均学工工作量				

教研业绩

表11教学研究项目情况

序号	项目名称	项目编号	项目来源	实到经费（万元）	立项时间	是否结题	结题时间	主持人	本人排名	课题组总人数	项目级别	备注
1	《动物营养学》省级线下一流课程	202312626	广东省教育厅	3	2023-12-06	否		张世海	1	4	B	省级线下一流课程可认定一项B类
2	《动物营养学》省级一流本科课程（思政课程）	无	广东省教育厅	0	2024-11-12	否		张世海	1	4	B	省级一流思政课程可认定为B类教学研
3	《动物营养学》融入课程思政的教学改革探索	无	华南农业大学	1	2023-01-01	否		张世海	1	2	校	
4	“新农科”背景下《动物营养学》课程的改革与创新	无	华南农业大学	0.4	2023-01-01	否		张世海	1	2	校	

表12 以第一作者发表教改论文情况

序号	论文名称	刊物名称（刊号）	发表时间（年月）	作者排名	论文等级	备注

注：论文附件须包含期刊封面、目录（标注出所发论文）、论文全文、封底以及检索证明。

表13 教学成果情况

序号	获奖时间	项目类型	项目名称	奖励级别	成果授予部门	本人排名	证书号	备注

注：项目含教学成果奖、精品课程、一流课程、双语课程示范课、课程思政示范课程等。

表14 教学类比赛情况

序号	获奖时间	奖励名称	奖励级别	成果授予部门	证书号	备注
1	2024-07-01	第四届全国高校教师教学创新大赛（一等奖）	国家级	中国高等教育学会	TIC2024NB1068	国家级一等奖，替代三个B类业绩。选择替代2个B类教学研究改革项目，1个B类教学研究论文。
2	2023-04-01	第六届全国青年教师教学大赛（二等奖）	国家级	全国中华总工会/教育部	无	
3	2023-02-10	广东省第六届高校（本科）青年教师教学大赛（一等奖/第一名）	省级	广东省总工会/广东省教育厅	无	
4	2024-07-10	第四届广东省高校青年教师教学创新大赛（一等奖/特等奖）	省级	广东省教育厅/广东省高等教育学会	GDTIC2024041	
5	2022-07-02	本科课堂教学十佳	校级	华南农业大学	无	

注：项目含教学比赛、青年教师教学优秀奖、教学观摩奖、十佳教师等。

表15 编写教材情况

序号	教材名称	ISBN号	出版社	出版时间	教材性质	字数（万）	排名	备注

注：教材附件须包含封面、ISBN页、目录页。

科研项目

表16-A 科研项目情况-主持的项目

序号	类型	项目名称	项目编号	项目来源	实到经费（万元）	经费卡号	立项时间	是否结题	结题时间	课题组总人数	项目等级	备注
1	纵向项目	研发氧化应激/炎症反应防控技术与产品，缓解热应激母畜发情、	2024YFD1301004	农业部	62	C250013	2024-12-30	否		1	A	
2	纵向项目	母猪泌乳能力及初乳IgA含量提升的营养调控机制	2021YFD1300700	科技部	72	A220013	2021-12-01	否		1	A	
3	纵向项目	GPR43介导Gα信号通路调控母猪泌功能的机制研究	2021A1515010440	广东省基础与应用基础研究基金委员会	10	E21110	2021-03-15	否		5	B	
4	纵向项目	油酸通过GPR120调节母猪乳脂合成的机制研究	2023A1515012098	广东省基础与应用基础研究基金委员	10	E230137	2023-02-07	否		4	B	
5	纵向项目	FFAR2对母猪泌乳功能的调节作用及其机制研究	2021020200056	广州市科技局	5	F21309	2021-04-01	是		4	C	

表16-B 科研项目情况-主要参加的项目

序号	类型	项目名称	项目编号	项目来源	实到经费（万元）	经费卡号	立项时间	是否结题	结题时间	主持人	本人排名	课题组总人数	项目等级	备注

科研成果

表17-A 以第一作者发表本专业论文（著）情况

序号	论文名称	刊物名称（刊号）	发表时间（年月）	在第一作者中的排名	文献类型	论文等级	备注
1	Maternal supplementation with Artemisia annua L. ameliorates intestinal inflammation via inhibiting the TLR4/NF-kappa B and MAPK pathways and improves the oxidative stability of offspring	FOOD & FUNCTION	2022/09	1	Article	T2	
2	Combined yeast culture and organic selenium supplementation during late gestation and lactation improve preweaning piglet performance by enhancing the antioxidant capacity and milk content in nutrient-restricted sows	ANIMAL NUTRITION	2020/06	1	Article	T2	
3	Different Sources of High Fat Diet Induces	FRONTIERS		2			

3	Marked Changes in Gut Microbiota of Nursery Pigs	IN MICROBIOLOGY	2020/05	2	Article	A	
4	L-carnitine increases cell proliferation and amino acid transporter expression via the activation of insulin-like growth factor I signaling pathway in rat trophoblast cells	FOOD SCIENCE & NUTRITION	2020/07	1	Article	B	

注：论文附件须包含期刊封面、目录（标注出所发论文）、论文全文、封底以及检索证明。

表17-B 以通讯作者发表本专业论文（著）情况

序号	论文名称	刊物名称（刊号）	发表时间（年月）	在通讯作者中的排名	文献类型	论文等级	备注
1	Nutritional strategies to alleviate oxidative stress in sows	ANIMAL NUTRITION	2022/06	1	Review	T2	
2	Glyceryl butyrate attenuates enterotoxigenic Escherichia coli-induced intestinal inflammation in piglets by inhibiting the NF-kappa B/MAPK pathways and modulating the gut microbiota	FOOD & FUNCTION	2022/05	1	Article	T2	

3	Improved thermostability of lipase Lip2 from Yarrowia lipolytica through disulfide bond design for preparation of medium-long-medium structured lipids	LWT-FOOD SCIENCE AND TECHNOLOGY	2022/08	2	Article	T2	
4	Maternal sodium acetate supplementation promotes lactation performance of sows and their offspring growth performance	ANIMAL NUTRITION	2023/09	1	Article	T2	
5	Niacin/beta-hydroxybutyrate regulates milk fat and milk protein synthesis via the GPR109A/G(i)/mTORC1 pathway	FOOD & FUNCTION	2023/03	1	Article	T2	
6	Maternal microbe-specific modulation of the offspring microbiome and development during pregnancy and lactation	GUT MICROBES	2023/12	1	Review	T2	

7	Maternal supplementation with glycerol monolaurate improves the intestinal health of suckling piglets by inhibiting the NF-κB/MAPK pathways and improving oxidative stability	Food and Function	2023/04	2	Article	T2	
8	Amino acid transportation, sensing and signal transduction in the mammary gland: key molecular signalling pathways in the regulation of milk synthesis	NUTRITION RESEARCH REVIEWS	2020/12	1	Review	T2	
9	Dietary fiber and microbiota interaction regulates sow metabolism and reproductive performance	ANIMAL NUTRITION	2020/12	1	Review	T2	
10	A Combination of Formic Acid and Monolaurin Attenuates Enterotoxigenic Escherichia coli Induced Intestinal Inflammation	JOURNAL OF AGRICULTURAL AND	2020/04	2	Article	T2	

10	in Piglets by Inhibiting the NF-kappa B/MAPK Pathways with Modulation of Gut Microbiota	FOOD CHEMISTRY		2			
11	Maternal Nutrition During Late Gestation and Lactation: Association With Immunity and the Inflammatory Response in the Offspring	FRONTIERS IN IMMUNOLOGY	2022/01	1	Review	A	
12	Dietary Supplementation of Enzymatically Treated Artemisia annua L. Improves Lactation Performance, Alleviates Inflammatory Response of Sows Reared Under Heat Stress, and Promotes Gut Development in Preweaning Offspring	FRONTIERS IN VETERINARY SCIENCE	2022/03	1	Article	A	
13	A critical role of AMP-activated protein kinase in regulating intestinal nutrient absorption, barrier function, and intestinal diseases	JOURNAL OF CELLULAR PHYSIOLOGY	2022/07	1	Review	A	

14	Effects of Dietary Monoglyceride and Diglyceride Supplementation on the Performance, Milk Composition, and Immune Status of Sows During Late Gestation and Lactation	FRONTIERS IN VETERINARY SCIENCE	2021/08	1	Article	A	
15	Exploring the Benefits of Probiotics in Gut Inflammation and Diarrhea-From an Antioxidant Perspective	ANTIOXIDANTS	2023/07	2	Review	A	
16	Energy deprivation-induced AMPK activation inhibits milk synthesis by targeting PrIR and PGC-1 alpha	CELL COMMUNICATION AND SIGNALING	2022/03	1	Article	A	
17	Novel advances in understanding fatty acid-binding G protein-coupled receptors and their roles in controlling energy balance	NUTRITION REVIEWS	2022/02	1	Review	A	
18	Sodium acetate regulates milk fat synthesis through the activation	FRONTIERS IN NUTRITION	2023/02	1	Article	A	

18	of GPR41/ GPR43 signaling pathway			1			
19	Dietary Supplementation with Lysozyme-Cinnamaldehyde Conjugates Enhances Feed Conversion Efficiency by Improving Intestinal Health and Modulating the Gut Microbiota in Weaned Piglets Infected with Enterotoxigenic ＜i＞ Escherichia coli＜/i＞	ANIMALS	2023/11	2	Article	A	
20	Leucine and arginine enhance milk fat and milk protein synthesis via the CaSR /Gi/mTORC1 and CaSR/Gq/ mTORC1 pathways	EUROPEAN JOURNAL OF NUTRITION	2023/10	1	Article	A	
21	Maternal Selenium-Enriched Yeast Supplementation in Sows Enhances Offspring Growth and Antioxidant Status through the Nrf2/Keap1 Pathway	ANTIOXIDANTS	2023/12	2	Article	A	
22	Regulation of the JAK2-STAT5 Pathway by	FRONTIERS IN CELL AND	2020/11	1	Review	A	

22	Signaling Molecules in the Mammary Gland	DEVELOPMENTAL BIOLOGY		1			
23	Fat Encapsulation Reduces Diarrhea in Piglets Partially by Repairing the Intestinal Barrier and Improving Fatty Acid Transport	ANIMALS	2021/01	2	Article	A	
24	Anethole Attenuates Enterotoxigenic Escherichia coli-Induced Intestinal Barrier Disruption and Intestinal Inflammation via Modification of TLR Signaling and Intestinal Microbiota	FRONTIERS IN MICROBIOLOGY	2021/03	2	Article	A	
25	Artemisinin Protects Porcine Mammary Epithelial Cells against Lipopolysaccharide-Induced Inflammatory Injury by Regulating the NF-kappa B and MAPK Signaling Pathways	ANIMALS	2021/06	1	Article	A	

26	Dietary enzymatically-treated Artemisia annua L. supplementation could alleviate oxidative injury and improve reproductive performance of sows reared under high ambient temperature	JOURNAL OF THERMAL BIOLOGY	2020/12	2	Article	A	
27	Ferroptosis Regulation by Nutrient Signalling	NUTRITION RESEARCH REVIEWS	2022/12	1	Review	A	
28	Current Evidences and Future Perspectives for AMPK in the Regulation of Milk Production and Mammary Gland Biology	FRONTIERS IN CELL AND DEVELOPMENTAL BIOLOGY	2020/06	1	Review	A	
29	氧化应激对母畜乳腺功能的影响及其营养调控策略	动物营养学报	2020/08	1	Review	B	
30	Excessive BCAA regulates fat metabolism partially through the modification of m(6)A RNA methylation in weanling piglets	NUTRITION & METABOLISM	2020/01	1	Article	B	

注：1.论文附件须包含期刊封面、目录（标注出所发论文）、论文全文、封底以及检索证明。 2.“在通讯作者中的排名”，排名最后的通讯作者在此栏填1，排名倒数第2的通讯作者在此栏填2，以此类推。

表18 以第一作者发表理论文章情况

序号	文章名称	发表载体	发表版面/栏目	发表时间（年月）	发表卷期	字数（千）	备注

备注：含在《求是》《人民日报》《光明日报》《经济日报》上发表的理论文章，或在省级党报理论版上发表的理论文章，或在人民网、新华网、求是网、光明网发表的理论文章。

表19 学术专著、工具书等情况

序号	著作名称	出版社	出版时间	著作性质	字数（万）	作者排名	备注

注：附件须包含封面、目录页。

表20-A 科技奖励

序号	获奖时间	奖励名称+等级	成果名称	奖励授予部门	本人排名	项目等级	备注

备注：项目含《华南农业大学学术业绩评价体系》中的科技奖励和科研成果获奖。

表20-B 获得知识产权情况

序号	获得时间	知识产权类型	知识产权名称	成果授予部门	本人排名	登记号/专利号	项目等级	备注

知识产权类型选项：1.发明专利、实用新型专利、外观设计专利；2.软件著作权；3.植物新品种权；4.审定植物新品种；5.新兽药（一类、二类、三类、四类、五类）；6.其他（在备注中说明）

表20-C 标准情况

序号	获得时间	标准类型	标准名称	发布部门	本人排名	标准号	项目等级	备注

表20-D 科技成果转化项目情况

序号	项目名称	实到经费（万元）	经费卡号	合同签订时间	本人排名	项目等级	备注

表20-E 决策咨询报告采纳实施

序号	采纳时间	项目类型	采纳或实施部门	具体业绩表述	备注

表20-F 科研平台

序号	立项时间	项目名称	项目来源	总经费额（万元）	进展情况	本人排名	项目等级	备注

其他

表21 指导学生参加学科竞赛

序号	获奖时间	奖励名称+等级	成果授权部门	本人在指导老师中的排名	项目等级	备注

表22 艺术类成果

序号	获得时间	项目类型	具体业绩表述	主办单位	本人排名	项目等级	备注

表23 体育类指导学生比赛获奖情况

序号	获奖时间	项目类型	获奖情况	主办单位	是否为主教练	备注

表24 个人荣誉

序号	获奖时间	项目类型	奖励名称	奖励级别	授予部门	备注
1	2023-12-10	综合类个人荣誉	广东省五一劳动奖章	省部级	广东省总工会	
2	2024-06-10	教育教学个人荣誉	教学名师	校级	华南农业大学	
3	2024-07-10	教育教学个人荣誉	教书育人—先进个人	校级	华南农业大学	

备注：项目含教育教学个人荣誉、综合类个人荣誉称号、学生思政类个人荣誉等。

表25 其他业绩

序号	时间	项目名称	具体业绩表述	备注

单位推荐意见及结果

所在学院（系、部、所）的评价意见

（对申报人的政治思想、职业道德、专业技术工作、业绩负责核实，并对其水平、能力、业绩作出客观、公正的评价。）

单位（公章）：

年　月　日

学院（教学部）推荐委员会推荐结果：

推荐委员人数	到会人数	推荐结果				备注
		同意人数		不同意人数		

评委会评前公示情况	年　月　日

<table>
<tr><td rowspan="6">职称评审委员会意见</td><td>评议组
专家数</td><td>到会人数</td><td colspan="4">表决结果</td><td>备注</td></tr>
<tr><td></td><td></td><td>同意人数</td><td></td><td>不同意人数</td><td></td><td></td></tr>
<tr><td colspan="7">学科组评审委员会结果：</td></tr>
<tr><td>高评委会
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<tr><td></td><td></td><td>同意人数</td><td></td><td>不同意人数</td><td></td><td></td></tr>
<tr><td colspan="7">高评委会评审意见及结果：

评委会公章
主任委员签章：
年　月　日</td></tr>
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评审结果公示情况：

职称审核确认意见：

华南农业大学（公章）

年　月　日

代表作鉴定意见

代表作的鉴定意见装订或在此页

（由单位负责办理，注意保密，不得将鉴定意见外泄给其本人或其他人员）

申报	系列：教师系列教学为主型
	专业：畜牧学
	职称：教授

业绩成果材料

（申报人的业绩成果材料包括论文、科研项目、获奖以及其他成果等）

单　位（二级单位）动物科学学院

姓　名　张世海

材料核对人:　　单位盖章:

核对时间:

华南农业大学制

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广东省一流本科课程

证书

★ ★ ★ ★ ★

课程名称：动物营养学

课程类别：线下一流课程

课程负责人：张世海

课程团队其他主要成员：管武太、陈芳、谭成全

主要建设单位：华南农业大学

广东省教育厅

2023年12月

证书编号：202312626

广 东 省 教 育 厅

粤教高函〔2024〕27 号

广东省教育厅关于公布 2024 年度本科高校课程思政改革示范项目认定名单的通知

各本科高校:

根据《广东省教育厅关于开展 2024 年课程思政改革示范项目遴选认定工作的通知》，经学校推荐、专家评审及公示，拟认定课程思政改革示范项目共计 379 项，包括课程思政改革示范高校 4 所、课程思政教学研究示范中心 5 个、课程思政示范团队 76 个、课程思政示范课程 120 门、课程思政示范课堂 174 个（名单详见附件），现予以公布。

本次发文认定的课程思政改革示范项目有效期至 2027 年 12 月 31 日，有效期内，示范项目原则上不允许更换负责人或大幅度变更项目团队成员，否则予以撤项处理。获评为课程思政示范课程的，同时认定为省级一流本科课程（课程思政）。上述示范项目将优先选送参评教育部课程思政示范项目。

各高校要充分认识课程思政改革的重要意义，统筹本校资金、创造必要条件，支持各示范项目后续建设、应用与更新，

充分发挥其辐射引领作用，不断加强对学校教师的相关培训、指导和支持，引导教师积极自觉投身课程思政改革，持续深入抓典型、树标杆、推经验，全面提升本科人才培养质量。

附件：广东省本科高校2024年课程思政改革示范项目认定名单

广东省教育厅

2024年11月27日

附件

广东省本科高校2024年课程思政改革示范项目认定名单

一、示范高校

序号	示范高校名称
1	暨南大学
2	南方医科大学
3	广州大学
4	广州中医药大学

二、教学研究示范中心

序号	建设单位	教学研究示范中心名称	负责人
1	广东外语外贸大学	广东外语外贸大学本科课程思政教学研究示范中心	陈金诗
2	韩山师范学院	韩山师范学院课程思政教学研究中心	刘海春
3	仲恺农业工程学院	仲恺农业工程学院课程思政教学研究示范中心	朱立学
4	广州医科大学	广州医科大学课程思政教学研究中心	唐小平
5	东莞理工学院	东莞理工学院课程思政教学研究中心	王红成

三、示范团队

序号	建设单位	示范团队名称	负责人
1	中山大学	大学体育——奥运冠军课程思政示范团队	张新萍
2	中山大学	公共卫生与预防医学专业课程思政示范团队	夏敏
3	中山大学	经济学专业课程思政示范团队	黄新飞
4	中山大学	数值分析课程思政示范团队	任传贤
5	华南理工大学	媒介文化与素养课程群课程思政示范团队	陈刚
6	华南理工大学	工程训练公共教学课程思政示范团队	郑志军
7	华南理工大学	化工原理系列课程思政示范团队	郑大锋
8	华南理工大学	基础力学公共教学课程思政示范团队	张晓晴
9	华南理工大学	产品设计专业系列核心课程思政示范团队	张瑞秋

10	华南理工大学	城乡规划原理课程群课程思政示范团队	王世福
11	暨南大学	全媒体传播课程群课程思政示范团队	罗昕
12	暨南大学	中医外科学课程思政示范团队	马民
13	暨南大学	理解当代中国多语种课程思政示范团队	朱红强
14	暨南大学	医学影像课程思政示范团队	张水兴
15	华南农业大学	行政管理专业课程思政示范团队	唐斌
16	华南农业大学	无机及分析化学课程思政示范团队	刘英菊
17	华南农业大学	数字电路与逻辑设计课程思政示范团队	黄琼
18	南方医科大学	医学特色的英语专业课程思政示范团队	邓文华
19	广州中医药大学	医学信息工程专业课程思政示范团队	陈平平
20	广州中医药大学	中药学课程思政示范团队	李海燕
21	广州中医药大学	内科临床技能课程思政示范团队	杨晓军
22	华南师范大学	传播学专业前沿课程思政示范团队	刘兢
23	华南师范大学	教育心理学课程思政示范团队	何先友
24	华南师范大学	中国文化概论课程思政示范团队	孙雪霞
25	广东工业大学	电子信息类课程思政示范团队	蔡述庭
26	广东外语外贸大学	寰球人文中国课程思政示范团队	杨静
27	广东外语外贸大学	刑事诉讼法学课程思政示范团队	杨帆
28	广东外语外贸大学	财务管理专业课程思政示范团队	龙文滨
29	汕头大学	组织学与胚胎学课程思政示范团队	苏中静
30	广东财经大学	会计学专业课程思政示范团队	雷宇
31	广东财经大学	产教创融合的影视类专业课程思政示范团队	贾毅
32	广东医科大学	“德行兼备：中华体育精神文化”课程思政示范团队	张枝尚
33	广东海洋大学	水产食品加工学课程思政示范团队	刘书成
34	广东海洋大学	船舶电气自动化类课程思政示范团队	边莉
35	仲恺农业工程学院	能源与动力工程专业核心课程思政示范团队	沈向阳
36	广东药科大学	内科学课程思政示范团队	谢文瑞
37	广州美术学院	公共艺术课程思政示范团队	张健
38	广州体育学院	运动训练专业课程思政示范团队	孙健
39	广东技术师范大学	网络工程专业课程思政示范团队	单纯
40	广东技术师范大学	学前教育专业课程思政示范团队	熊华军
41	岭南师范学院	制药工程工艺设计课程群课程思政示范团队	周中流
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54	嘉应学院	中国古代文学课程思政示范团队	汤克勤
55	惠州学院	学前教育专业课程思政示范团队	谢桂新
56	东莞理工学院	基础力学课程思政示范团队	张伟伟
57	五邑大学	数学与应用数学专业课程思政示范团队	黄金锐
58	佛山大学	“服务地方、融合产业”药学专业课程思政示范团队	郭嘉亮
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60	肇庆学院	AI赋能+党建引领的无机化学课程思政示范团队	吴燕妮
61	北京理工大学（珠海）	数字化制造课程思政示范团队	刘检华
62	广东科技学院	国际经济与贸易核心课程思政示范团队	姚文戈
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64	广州理工学院	人工智能课程群课程思政示范团队	王煜林
65	珠海科技学院	国际贸易课程思政示范团队	杨帅
66	广州应用科技学院	刑法学课程思政示范团队	刘湘廉
67	东莞城市学院	汉语言文学专业课程思政示范团队	张红运
68	广州工商学院	物流管理专业课程思政示范团队	乔鹏亮
69	广东理工学院	中级财务会计课程思政示范团队	赵燕
70	广州城市理工学院	税收学专业课程思政示范团队	王仁曾
71	广州软件学院	物联网工程专业课程思政示范团队	袁志
72	广州软件学院	数智物流课程群课程思政示范团队	王春生
73	广东白云学院	产品设计专业课程思政示范团队	方建松
74	广东外语外贸大学南国商学院	基础朝鲜语课程思政示范团队	池圣女
75	广州新华学院	人文地理与城乡规划专业课程思政示范团队	唐波
76	广州南方学院	大学英语课程思政示范团队	管乐

四、示范课程

序号	建设单位	示范课程名称	负责人
1	中山大学	眼科临床见习	李轶擎
2	中山大学	儿科学	梁立阳
3	中山大学	海洋油气地质学	万志峰
4	中山大学	重症医学初级探索	管向东
5	华南理工大学	人工智能：思维方式、理论基础及实践应用	张俊勃
6	华南理工大学	实变函数	李兵
7	华南理工大学	基因工程	王斌
8	华南理工大学	物理化学I	刘琳琳
9	暨南大学	医学职业素养与能力	尹海燕
10	暨南大学	人机交互	龙锦益
11	暨南大学	微观经济学	郑贤
12	暨南大学	媒介文化	张潇潇
13	华南农业大学	动物营养学	张世海
14	华南农业大学	植物检疫学	何晓芳
15	华南农业大学	电机学	孙振刚
16	华南农业大学	土壤学	吴道铭
17	南方医科大学	人力资源管理学	李贝
18	南方医科大学	法医物证学	朱波峰
19	南方医科大学	心肺物理治疗	曾庆
20	南方医科大学	流行病学	毛琛
21	广州中医药大学	中医骨伤科学	张顺聪
22	广州中医药大学	中医护理学基础	施珍妮
23	广州中医药大学	临床微生物学检验技术	蓝锴
24	广州中医药大学	发展心理学	任滨海
25	华南师范大学	环境工程学与实验	王熙
26	华南师范大学	物理光学	邓冬梅
27	华南师范大学	即兴弹唱	陈雪慧
28	华南师范大学	跨文化交际	许丽玲
29	广东工业大学	经济学原理	刘艳
30	广东工业大学	运筹学	贺勇
31	广东工业大学	工程化学	阳香华
32	广东工业大学	结构力学	袁兵
33	广东外语外贸大学	中国古代文学（三）	张向荣
34	广东外语外贸大学	跨文化传播学	王雪晔
35	广东外语外贸大学	英语文学导论	付晶晶
36	汕头大学	艺术美学导论	黄继刚

37	汕头大学	妇产科学	刘伟
38	汕头大学	线性代数	单丽
39	广东财经大学	经济学原理	柴盈
40	广东财经大学	财产保险	李亚青
41	广东财经大学	中级财务会计	孔令辉
42	广东医科大学	病理生理学	李蓉
43	广东医科大学	护理伦理学	王丽萍
44	广东海洋大学	波谱分析	胡章
45	广东海洋大学	数字电子技术基础	任肖丽
46	仲恺农业工程学院	经济法	张眉
47	仲恺农业工程学院	液压与气压传动	张日红
48	广东药科大学	临床血液学检验技术	杨兆收
49	广东药科大学	卫生微生物学	曾转萍
50	星海音乐学院	中国古典舞身韵	肖蕊恋
51	星海音乐学院	岭南风格艺术歌曲钢琴伴奏	郭洁
52	广州美术学院	设计与自然	刘再行
53	广州美术学院	陶瓷文化记录与研究	谭红宇
54	广州体育学院	体育经纪导论	周良君
55	广东技术师范大学	经济法	林蔚
56	广东技术师范大学	自动控制原理	徐辰华
57	岭南师范学院	武术基础Ⅱ	刘舟
58	岭南师范学院	通信原理	费娟
59	韩山师范学院	面向对象分析与 UML	李旅军
60	韩山师范学院	“过程→生成”教学论	张磊
61	广东石油化工学院	混凝土结构设计原理	金焕
62	广东石油化工学院	审计学	张芹秀
63	广东金融学院	金融学	李日新
64	广东金融学院	金融数据挖掘	骆世广
65	广东警官学院	反黑概论	周心捷
66	广东警官学院	大数据技术与应用	文硕
67	广东第二师范学院	景观设计	钟香炜
68	广州航海学院	装卸工艺	易燕
69	广州大学	英语课程标准与教材分析	陈丽虹
70	广州大学	材料力学	燕乐纬
71	广州大学	环境学概论	吴卓
72	广州大学	城市管理学	刘晓洋
73	广州医科大学	生理学	董颀
74	广州医科大学	临床技能学	张作鹏
75	广州医科大学	卫生统计学	姜劲

76	深圳大学	混凝土与砌体结构设计	熊琛
77	深圳大学	软件工程	杜文峰
78	深圳大学	普通心理学	刘金婷
79	南方科技大学	普通生物学实验	生悦
80	韶关学院	商务统计与软件应用	胡春春
81	韶关学院	茶文化与茶艺基础	隋春花
82	韶关学院	体育心理学	范锦勤
83	嘉应学院	财务管理学	郭伊楠
84	嘉应学院	天然药物化学	廖梅
85	惠州学院	概率统计	周大镯
86	惠州学院	工程项目审计	熊明良
87	东莞理工学院	建设法规概论	吴晓伟
88	东莞理工学院	社会研究方法	黄俊辉
89	五邑大学	电商项目创新创业综合实践	王天擎
90	五邑大学	高等数学 A	王鹏
91	佛山大学	计算机网络原理	杨文茵
92	佛山大学	无机化学	樊婷
93	佛山大学	中级财务会计	古颖妮
94	肇庆学院	食品分析	张素斌
95	肇庆学院	软件工程	熊芳敏
96	北京理工大学（珠海）	运筹学	郝佳
97	哈尔滨工业大学(深圳)	电路	王灿
98	北京师范大学珠海校区	中国文学海外传播研究	姚建彬
99	广东科技学院	国际市场营销学	刘琦
100	广州商学院	资产评估	宋敏
101	广州理工学院	培训与开发	梁金如
102	广州理工学院	工程力学	高波
103	珠海科技学院	大学生职业生涯规划	刘冰
104	珠海科技学院	冲压工艺及模具设计	李晓达
105	广州应用科技学院	微观经济学	褚霞
106	东莞城市学院	插画设计	何帅森
107	广东东软学院	软件工程	李晶
108	广东东软学院	电子商务数据分析	何明慧
109	广东培正学院	景观与园林设计	冷凝
110	广州工商学院	通信原理	唐江波
111	广东理工学院	软件工程及应用	林显宁

112	广州城市理工学院	建筑设计原理	刘佳琼
113	湛江科技学院	宏观经济学	杨艳
114	广东开放大学	创业案例分析	赵海霞
115	广州软件学院	计算机图形学	罗林
116	广东白云学院	家居产品设计	潘文芳
117	电子科技大学中山学院	公共政策学	程宇
118	广东外语外贸大学南国商学院	综合英语I	方慧
119	广州新华学院	天然药物化学	王有娣
120	广州南方学院	网络营销	戴国良

五、示范课堂

序号	建设单位	示范课堂名称	负责人
1	中山大学	皮肤性病学（第十四章第二节 特应性皮炎）	谢阳
2	中山大学	生理学（第五章第四节 氧气在血液中的运输）	崔宇
3	中山大学	牙体牙髓病学（第三章 龋齿的临床表现与诊断）	黄绮婷
4	中山大学	专业小组课（琵琶）（第一章节 中国琵琶艺术之美）	胡妍璐
5	中山大学	知识产权（第五章 著作权内容与限制）	韦景竹
6	中山大学	肿瘤学理论（第十四章 胃癌）	梁垚
7	华南理工大学	材料与社会	薛锋
8	华南理工大学	运动控制系统	许玉格
9	华南理工大学	德语初级	黄婷
10	暨南大学	《 麻醉与健康 —— 一场梦与醒的旅行》（心肺脑复苏）	聂偲
11	暨南大学	出镜记者实务（出镜报道的细节表达）	王媛
12	暨南大学	数据科学导论（关联分析商业实践案例）	刘潇
13	华南农业大学	《世界古代史I》第六章第一节（从希腊到中国——丝绸之路上的文化交流）	邵兆颖
14	华南农业大学	《森林培育学》第九章第2节（修枝抚育）	邱权

15	华南农业大学	《海洋生物学基础》第四章第5节（海洋节肢动物）	严慕婷
16	华南农业大学	《土壤生物学》第一章绪论第2节（土壤生物：土壤健康的基石）	张池
17	华南农业大学	《发展经济学》第十一章第1节	张沁岚
18	华南农业大学	《概率论》第一章第4节（条件概率与乘法公式）	周燕
19	南方医科大学	《内科学》- 急性早幼粒细胞白血病	张延清
20	南方医科大学	《医古文》—大医精诚（第二段）	安海燕
21	南方医科大学	《内科学》-糖尿病	邹梦晨
22	南方医科大学	《Clinical Procedures》-基础生命支持	李薇
23	南方医科大学	《呼吸系统》-肺结核	刘来昱
24	南方医科大学	《临床营养学》-绪论	杨力
25	广州中医药大学	中医临床护理学 第四章 第七节 胎漏、胎动不安	何婉婉
26	广州中医药大学	伤寒论 阳明病湿热发黄证	徐笋晶
27	广州中医药大学	中医伤科学 第四章第二节 颈椎病	杨伟铭
28	华南师范大学	中世纪欧洲美术	王金玲
29	华南师范大学	高温超导特性的测量	符斯列
30	华南师范大学	《视听新闻图像》	彭柳
31	华南师范大学	《中国古代文学（I-3）》	黎思文
32	华南师范大学	电解质溶液	左晓希
33	华南师范大学	改革开放以来中国的工业化进程	王颖
34	广东工业大学	基于专思创三融合的课程教学改革研究与实践——以经济社会调查课程为例	黄丹宇
35	广东工业大学	环境生态工程微生物学——第四章 第一节 病毒的繁殖与灭活	陈姗姗
36	广东工业大学	《国际经济法》课程思政示范课堂	甘玉环
37	广东外语外贸大学	“理解当代中国”葡萄牙语演讲(1)——第四单元绿色中国	杨菁
38	广东外语外贸大学	金融风险管理——第十五章金融衍生品市场风险管理	邓超
39	广东外语外贸大学	综合英语（7）——Unit 1 Text A	范栩颖
40	广东外语外贸大学	跨境电子商务——第四章第一节跨境电商通关政策及监管模式	冯然
41	汕头大学	概率论——连续型随机变量及其正态分布	陈晓鹏
42	汕头大学	急危重症医学-心肺脑复苏	朱文捷

43	广东财经大学	景观专题设计第二章第四节形式美感策略	王仲伟
44	广东财经大学	证据法学第十六章第二节司法认知	丁朋超
45	广东财经大学	数学建模第五章第十节传染病模型	陈冰川
46	广东医科大学	《卫生统计学》（非参数检验）课程思政示范课	于海兵
47	广东医科大学	《医学传播学》（医学传播的艺术）课程思政示范课堂	陈欣欣
48	广东医科大学	《平面设计》(海报设计)课程思政示范课堂	郑金秋
49	广东医科大学	《临床药物治疗学》（糖尿病的药物治疗）课程思政示范课堂	付晖
50	广东医科大学	《外科学总论》（创伤）课程思政示范课堂	刘宏伟
51	广东海洋大学	《C语言程序设计》一维数组排序	石丽梅
52	广东海洋大学	《综合英语II》- Unit 3 春之祭	许静
53	广东海洋大学	《现代物流设施与规划》—选址问题建模	徐晓鸣
54	广东海洋大学	课程思政示范课堂《房屋建筑学》---楼梯构造与设计	李雅婷
55	仲恺农业工程学院	普通生态学-厄尔尼诺	李江涛
56	仲恺农业工程学院	《会展策划与管理》第一章第一节《什么是会展》	王婧
57	仲恺农业工程学院	翻译理论与实践（Ⅰ） 第二章第二节日汉同形汉字词的翻译	陈姗姗
58	仲恺农业工程学院	景观规划与设计（第六章 植物种植设计）	曾皓玲
59	广东药科大学	《数据库原理》第一章 绪论	彭柳芬
60	广东药科大学	《物流学》第五章第二节 物流信息技术（无线射频识别）	沈凯
61	广东药科大学	《组织学与胚胎学》第二十章 女性生殖系统	王欣
62	广东药科大学	《临床中药学》第二十四章第三节 补血药	林健
63	广东药科大学	《基因组导向下健康管理》第七章 基因与心理健康	王珊珊
64	星海音乐学院	弦意岭南	龙壬哲
65	星海音乐学院	古舞今韵	赵文婷
66	广州美术学院	交互界面与系统设计第二章第一节（交互界面设计理论与方法）	郜洵

67	广州美术学院	系统素描第二章第一节（国家形象系统与人因设计）	林畅
68	广州美术学院	场地设计第二章第二节(“在场”的解构与身体力行)	张莎玮
69	广州体育学院	《户外运动概述》第一章	崔洪成
70	广州体育学院	《运动生物力学》 第五章第二节	吕长生
71	广州体育学院	《休闲体育概论》第一章第一节	栗燕梅
72	广东技术师范大学	数字艺术与平面设计第二章第一节（点亮你的设计思维）	王竹君
73	广东技术师范大学	创新与创业基础第一章第一节(国际创新创业的认知)	陈晓桁
74	岭南师范学院	1.2节逻辑函数图形化简法	刘瑶
75	岭南师范学院	中外设计史（教学专题十五：黑暗中的新曙光——破解达芬奇的设计密码）	魏晓
76	岭南师范学院	地图学第二章第2节	罗松英
77	岭南师范学院	《智力障碍教育概论》第一章第二节智力障碍儿童	卢祖琴
78	韩山师范学院	设计素描——主题绘画的创新与艺术实践	冯菡子
79	韩山师范学院	广东省本科高校课程思政示范课堂--数字电子技术	谢森林
80	韩山师范学院	大学英语A2第五单元飞行先驱	彭汪鑫
81	韩山师范学院	蛋白质和氨基酸分析	王军
82	广东石油化工学院	《土木工程施工（一）》5.3 预应力的形成——取上得中的超张拉策略	杨云英
83	广东石油化工学院	人因工程学——人体测量	赵晶英
84	广东石油化工学院	《线性代数》第二章第二节：矩阵的运算	梁燕
85	广东金融学院	国际贸易地理	李健欣
86	广东警官学院	高级程序设计语言第四章选择结构程序设计第八节选择结构程序综合举例	毛莉
87	广东警官学院	《警察文化学》 第九章警察电影 第三节主流警匪片	韩帮文
88	广东第二师范学院	无机化学(第五章第二节影响化学反应速率的因素)	王延惠
89	广东第二师范学院	钢琴第八章第一节《保卫黄河》	魏鹂娆
90	广州航海学院	船舶原理(上) 第五章第一节 船舶抗沉性原理	唐伟炜
91	广州航海学院	建筑结构试验与检测技术 第二章第三	范杰

		节 液压加载法	
92	广州航海学院	集装箱运输与多式联运C 第五章 集装箱船舶及班轮航线	孙战秀
93	广州大学	国际法学第六章第二节（国家管辖权）	隽薪
94	广州大学	冷藏运输技术与管理第四章第四节（影响易腐货物物流品质的因素）	刘广海
95	广州大学	电力系统继电保护原理第三章第四节（距离保护的整定计算与对距离保护的评价）	王晓刚
96	广州大学	人文地理学第四章第三节（转变中的中国人口）	袁振杰
97	广州大学	化学反应工程第三章第三节（非理性流动模型）	邹汉波
98	广州医科大学	《信号与系统》第三章第2.3节-系统的线性与非线性	习佳宁
99	广州医科大学	《中医学》下篇第一章第二节——清热药及清热方剂模块	陈文君
100	广州医科大学	《医学统计学》第十五章第一节——实验设计的基本原则	赵倩
101	广州医科大学	《医学导论》——病人告知实践	陈嘉慧
102	深圳大学	播音语言表达（第三章第二节 播音的内在语）	陈昕瑜
103	深圳大学	水污染控制工程	毛艳萍
104	深圳大学	感染免疫	陈思
105	深圳大学	人格心理学第二编第三章	蒋多
106	南方科技大学	教育社会学导论	刘绪
107	南方科技大学	形势与政策——深圳改革开放专题	马俊军
108	南方科技大学	人体解剖学（关节学）	任铭新
109	深圳技术大学	广东省本科高校课程思政示范课堂-形态基础1	王文静
110	韶关学院	《PLC系统综合设计与实训》第一章认识物料分拣自动控制系统	刘文秀
111	韶关学院	《无机化学》第五章第四节缓冲溶液	余静
112	韶关学院	《数学建模》第六章第三节——市场经济中的物价波动	罗亮
113	嘉应学院	社会学概论——第七章　第三节 社会互动的主要理论视角	杨文苑
114	嘉应学院	文学概论第一章第一节文学是社会意识形态	石朝辉

115	惠州学院	儿童亲社会行为	金艳
116	惠州学院	《生态学》第七章第二节：生态位和捕食	解欣斐
117	惠州学院	基于“全球定位系统”内容授课的课程思政示范课堂	王少君
118	惠州学院	测量学-水准测量	王盾
119	东莞理工学院	固体废物处理与处置	花开慧
120	东莞理工学院	《市场营销》课程思政建设改革示范课堂	方向
121	东莞理工学院	基础设计	罗瑜斌
122	东莞理工学院	概率论与数理统计	王立本
123	五邑大学	中国建筑史--第三章住宅与聚落	周宏
124	五邑大学	结构力学——第二章第五节	王小蔚
125	佛山大学	《兽医免疫学》模块二专题三II型超敏反应-课程思政示范课堂	梅堃
126	肇庆学院	《园林规划设计》植物景观设计与生态文明建设	谢秀丽
127	肇庆学院	《单片机原理与应用——汽车仪表盘显示控制》思政示范课堂	王丹
128	北京理工大学（珠海）	就业创业1：大学生职业生涯规划与发展	刘丽
129	北京理工大学（珠海）	对外汉语教学导论	孙燕琳
130	北京理工大学（珠海）	纳米材料基本效应	张加涛
131	哈尔滨工业大学(深圳)	《现代汉语》第一章第一节	张莹
132	北京师范大学珠海校区	中国概况课之当代中国专题课程思政示范课堂	吴成年
133	北京师范大学珠海校区	世界之窗-生态之美之湿地与人类福祉课程思政示范课堂	唐海萍
134	广东科技学院	统计学（数据的误差）	陈晶晶
135	广东科技学院	无线传感器网络第1章（无线传感器网络概述）	张笑晨
136	广州商学院	经济法	陈思思
137	广州商学院	《兼并与收购》（反收购措施）示范课堂	张若凡
138	广州理工学院	英汉笔译（翻译的定义与本质）	周杨
139	珠海科技学院	《工业机器人离线编程与仿真》课程思政示范课堂	张恩光

140	珠海科技学院	基础英语II Unit 8 The Kindness of Strangers（陌生人的友善）	鲍海春
141	广州应用科技学院	岭南美术	王佳丽
142	东莞城市学院	《仓储管理》课程思政示范课堂	赵影
143	广东东软学院	高等数学I（理工类）：导数概念	曾荣
144	广东东软学院	会计学基础：财产清查——财产清查的基本方法	张雯琰
145	广东培正学院	管理信息系统（第三章第二节信息理论）	曾兰
146	广东培正学院	大学物理（第九章第五节 驻波）	马昕瑶
147	广州工商学院	网络营销：直播营销的具体实施	赖洁瑜
148	广州工商学院	税法：个人所得税法律制度	聂秀萍
149	广东理工学院	机械制图与CAD（二）第八章第二、三节	冯丹艳
150	广州城市理工学院	材料力学	肖新瑜
151	广州城市理工学院	PLC控制技术	朱万浩
152	广州城市理工学院	财务大数据分析技术	王一明
153	华南农业大学珠江学院	《纪录片创作》第三章第一节（选题调研—解读中国故事）	王欣颖
154	华南农业大学珠江学院	《景观设计》第二章第二节(主景营造手法--家国情怀，传承传统文化）	龙珍文
155	广州华商学院	《资本市场运作》第二章第二节股票的上市和退市	姚永红
156	广州华商学院	《财务共享服务》第五篇第十章 出纳与总账共享实践	陈平
157	广州华商学院	《统计学》第一章第一节:统计学的含义和作用	金莹
158	湛江科技学院	营销策划管理与实践第十章第一节	肖荆
159	湛江科技学院	军事理论第四章第四节信息化战争的发展历程	雷振文
160	广东白云学院	税法第一章第一节税收的内涵与分类	贾文博
161	广东白云学院	土木工程材料-水硬性胶凝材料-硅酸盐水泥	张凤
162	广东白云学院	学前教育专业与职业发展导论-学前教育本科专业的优势与不足	李亚玲
163	电子科技大学中山学院	电机学（三相感应电机的结构、工作原理及运行状态）	程羽佳
164	电子科技大学中山学院	高频电子线路（通信系统的基本组成、无线信道及其传播方式）	陈又鲜
165	电子科技大学中山	公司治理(党组织嵌入的中国公司治理	郭彬彬

	学院	模式)	
166	广州华立学院	《畲族纹样的构成设计》	吴念姿
167	广州华立学院	《第二外语（日语）》	潘永萍
168	广州华立学院	非关税措施的新发展	黄小燕
169	广东外语外贸大学南国商学院	美国文学（1.3.4 托马斯·潘恩）	宋文玲
170	广州新华学院	《中国古典园林史》第七章第二节从“颐和园”到“岭南园林”	张琳
171	广州新华学院	《经济法》第一章第二节经济法律关系	邵咪咪
172	广州南方学院	会计学原理及其实验	胡成艳
173	广州南方学院	大学英语（三）	李琳
174	广州南方学院	税务筹划	陈苏阳

公开方式：依申请公开

校对人：李成军

华南农业大学
课程思政示范课程申报书

课程名称：《动物营养学》

课程负责人：张世海

课程类别：□通识课　□ 公共基础课

√专业课　□ 实验课　□ 实践课

课程形式：□ 在线开放课程　√线上线下混合课程

□ 线下课程　□ 社会实践课程

所在学院：动物科学学院

申报日期：2022.9.5

本科生院制

二〇二二年

填 写 要 求

1.以 word 文档格式如实填写各项。

2.表格文本中外文名词第一次出现时，要写清全称和缩写，再次出现时可以使用缩写。

3.本表栏目未涵盖的内容，需要说明的，请在说明栏中注明。

4.有可能涉密和不宜大范围公开的内容不可作为申报内容填写。

5.如表格篇幅不够，可另按所填表格格式附纸。

一、课程基本信息

1.课程负责人信息	姓名	张世海	性别	男	年龄	33
	专业技术职称	副教授	行政职务	无	移动电话	13660346296
	本科教龄	6		院系	动物科学学院	

2.课程基本信息	课程名称	动物营养学
	面向专业	动物科学学院（必修）；生物学院（选修）
	课程学时	32 学时（提供教务系统截图） 动物营养学 2022202... 张世海 可选 否 选修 19生物科学1-2 1 2 32 1-11 3 33(1-11)
	课程学分	2 学分
	主要教材	动物营养学、9787109265165、陈代文、中国农业出版社、2020-10-1
	已开课周期数	12 期
	最近两期开课时间	2021 年 9 月—2021 年 12 月 2022 年 1 月—2022 年 1 月 12 日（提供教务系统截图） 2021-2022-1 611621 动物营养学 2021202... 张世海 可选 否 选修 19生科基地班[1-2] 34 2021-2022-2 611621 动物营养学 2021202... 张世海 可选 否 必修 20动物科学5-6 61 2
	最近两期学生总人数	95
	是否为省级以上课程	□是（包括精品课程、精品资源共享课、精品视频公开课、在线开放课程等。） √ 否

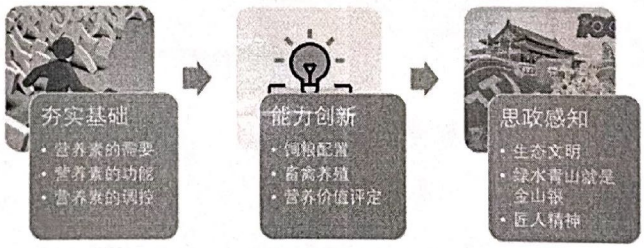
3.课程团队主要成员	序号	成员姓名	所在单位	学科背景	职称	承担课程任务
	1	管武太	华南农业大学	动物科学	教授	教学思政指导

二、课程设计

（举证说明本课程教学设计及实施过程中采用的教育思想或理念、课程思政的主要设计思路和实施方式，以及如何与专业教育融合，说明本课程的目标、适用对象、选取内容、学习资源建设与使用的原则等，800 字以内，可以插图。）

《动物营养学》是作为畜牧兽医专业的专业基础课，是学生从事本专业工作必须掌握的课程。本课程争对“两性一度”标准，在高阶性、创新性和挑战度等方面进行设计，通过在课堂中，引入学科前沿知识和行业发展需求。

落实立足畜牧、聚焦生态、志在强国的育人方案。2016 年，习近平总书记指出，要坚持把立德树人作为中心环节，把思想政治工作贯穿教育教学全过程。高校思想政治工作，要因事而化、因时而进、因势而新。2020 年，教育部发布《高等学校课程思政建设指导纲要》指出，农学类专业课程的重要思政元素为生态文明教育、绿水青山就是金山银山、“大国三农”情怀、服务农业农村现现代化、服务乡村全面振兴的使命感和责任感等。对照上述要求，在教学过程中，全程围绕全方位育人为核心。培养学生以“推动畜牧业绿色发展，解决养殖场发展难题”为目标的学习态度，为美丽乡村建设贡献自己的力量。



1. 课前思政元素凝练——深度挖掘

挖掘动物营养从业者的匠人精神，培养同学们作为畜牧人的职业担当；挖掘与动物营养相关的绿色畜牧理念，培养学生的生态文明意识，让学生深入理解“绿水青山就是金山银山”；挖掘动物营养领域中研究者的创新突破精神，培养学生独立思考与逻辑思辨的能力。

2. 课中思政案例结合——隐形融入

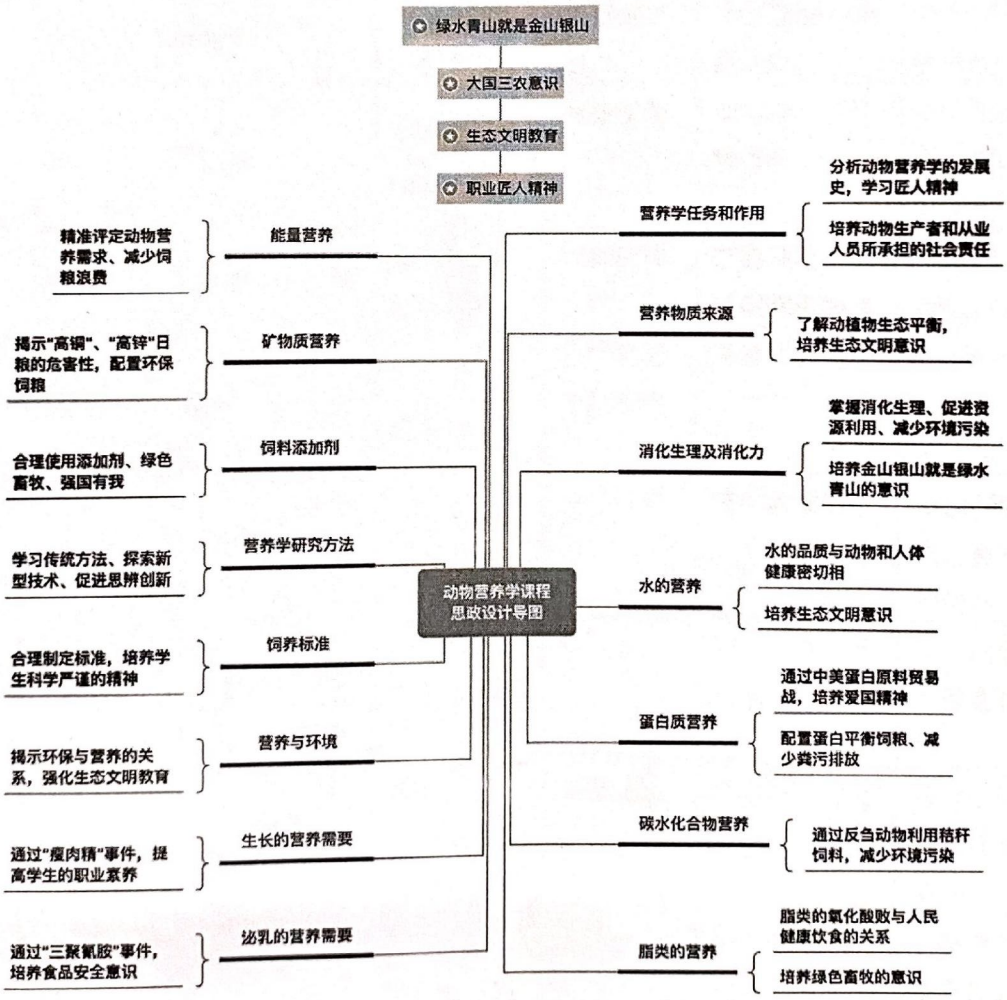
课程中通过事实案例、名人故事、伟人格言等手段，把该章节最核心思政要素融入课堂。让学生深刻理解“营养与生态文明教育”、“畜牧大国三农”、“绿水青山就是金山银山”、“匠人精神”、“创新性意识”等思政元素。具体章节案例，见下图。

3. 课后思政作业强化——提升效果

通过让学生在课后配制环保饲粮，并在课堂案例分享的形式，隐形强化“绿色畜牧的理念”；通过学生独立设计消化代谢笼，培养学生的创新突破精神；通过完成院士的成长故事读后感，树立学生的职业情怀。

4. 思政教学基地建设

建设《动物营养学》绿色环保样板实践猪场和饲料厂。通过亲身体验，让学生了解如何进行绿色畜牧，培养学生生态文明的意识。并把相关的绿色畜牧相关的理念带入到未来的工作企业和单位中。



三、教学内容（须列出每周详细的教学活动计划：以周为单位安排教学活动，每周有若干次课堂教学，每次课堂教学中课程思政点的融入方式和教学方法，以及预期达成效果等。）

课程学分：32 学分		共 11 个教学周	每周 1 次课		每次课 3 学时	
教学周次	课时安排	主讲教师	教学进度（章节讲/知识单元）	课程思政点	融入方式与教学方法	思政育人预期成效
1	1	管武太	第一章 营养物质及其来源	关注民生食品安全	三聚氰胺事件举例	让学生遵守职业道德，保证食品安全
1	1	管武太	第二章 动物的消化生理	个人成长	因材施教案例	根据个人特点，制定学习规划
1	1	张世海	第三章 水的营养	环境保护	水污染事件	激发同学水源保护精神
2	3	张世海	第四章 蛋白质营养	强国意识	中美贸易战	提升民族意识
3	3	张世海	第五章 碳水化合物	环境保护意识	秸秆焚烧，温室效应	激发同学的环保意识
4	3	张世海	第六章 脂类营养	食品安全	地沟油 垃圾猪事件	让学生遵守职业道德，保证食品安全
5	2	张世海	第七章 能量的营养	创新精神	李德发院士突破创新的精神	培养学生突破创新的精神
6	3	张世海	第八章 矿物质元素	强化思想意识形态	思想“补钙”	激发同学们参加青年大学习的热情
7	3	张世海	第九章 维生素的营养	突破精神	维生素发展史	激发不断求真、勇于创新的精神
8	2	张世海	第十章 动物营养学研究方法	绿色畜牧	消化不良对环境造成污染	激发同学们的环保意识
8	1	张世海	第十一章 动物营养与饲养标准	遵守法规	无规律不方圆	让学生明白规章制度的重要性
9	1	张世海	第十二章 动物的采食	绿水青山就是金山	过度采食 消化不良	激发学生的环境保护意识

			量	银山	污染环境	
9	1	张世海	第十三章 营养与环境	绿水青山 就是金山 银山	畜牧与环保的 关系	激发学生的环 境保护意识
10	2	管武太	第十四章 维持的营养 需要	维持需要 的变化	变则通 通则久	让学生学会变 通
10	2	管武太	第十五章 生长育肥的 营养需要	食品安全	瘦肉精事件	激发民生食品 安全意识
11	2	管武太	第十六章 繁殖的营养 需要	母爱传承	妊娠不易 母爱伟大	让学生体会母 爱
11	2	管武太	第十七章 泌乳的营养 需要	食品安全	黄曲霉事件	激发民生食品 安全意识

*教学活动计划不适用于本表格式表述的内容部分，可另加附页加以描述。

四、课程应用及评价情况

4.1 课程的主要特色及亮点自评（500 字以内）

《动物营养学》是动物科学专业的核心基础课程。其在不同历史时期，承担了前后相继的历史任务，其研究重点从早期的“满足动物生产、节约饲料成本”，演变到中期的“提升畜禽产能、优化畜禽产品品质”，再变革到当代的“替抗减抗、低氮排放、绿色畜牧”。为了适应我国新时代“三农大国”的建设和国家“十四五”发展计划，本团队计划对《动物营养学》课程的教学目标、教学内容、教学方法和教学评价进行创新重组，做到以下“四个突破”。 **1. 教学目标突破**——放眼世界，展望未来——从“传统养殖人”转变为“现代畜牧者”；**2 课程内容突破**——聚焦前沿，突破创新——从“畜禽营养素功能”到“前沿畜禽营养素功能与绿色畜牧”；**3. 教学手段突破**——打破传统，优化平台——从“《动物营养学》PPT 讲解”到“线上线下混合+翻转课堂+案例讨论+特邀专家+跨学科交流的多元教学”**4. 教学评价突破**——多层检测，多维评价——从“《动物营养学》知识机械记忆考核”到“《动物营养学》课前预习情况、课中学习态度和课后知识应用的综合评价”。通过以上四个突破，在课前、课中和课后融入思政元素。打造《动物营养学》思政金课。

4.2 课程同行专家或社会评价情况（请据实列明同行专家的基本信息、评价时间、评价内容或媒体报道版面等。500 字以内）

媒体报道

2022 年 广东省第六届高校（本科）青年教师教学大赛 工科组一等奖（冠军）

新闻：https://m.thepaper.cn/baijiahao_19689488

冠军席位2/5，全省第一！华农"青椒"，真棒！

澎湃 政务：华南农业大学 2022-08-30 21:49

原创 第一的 华南农业大学 收录于合集#奋进华农 150 个 #学高为师，身正为范 49 个

2022 华南农业大学十佳教师

新闻：https://view.inews.qq.com/a/20220708A0B9IQ00

这些华农老师，课讲得真好！

华南农业大学
2022年7月8日20:30 广东 华南农业大学官方账号

卷帙浩繁的世界

教学同行专家评价：

适当增加板书，富有感染力，上课效果好！

在授课过程注意价值观的引导，育人效果较好；备课认真，讲授状态好，精神饱满；师生互动多，有效调动学生主动思考。

优点：
1、声音洪亮，吐字清晰，PPT图文并茂；
2、采用课堂软件随机点名回答问题，效果较好；
3、能较好地结合学科前言和产业实际。
建议：适当板书。降低语速。

4.3 课程校内评价情况（包括学校、院系和学生整体评价情况。500 字以内）

教学评价：
教学排名在本教学单位常年处于前 10%。

2021 年 上半年 学院教评排名第 4
2021 年 下半年 学院教评排名第 1

2022 年 华南农业大学 教学十佳
2018 年 华南农业大学 青教赛一等奖

2022 年广东省第六届高校（本科）青年教师教学大赛 工科组一等奖（第一）

教学效果：
1、教学效率高。
2、学生受益面大，让不同程度的学生在原有基础上都有进步。知识的增长、情感的陶冶、学习方法的获得、能力素质的发展、良好习惯的养成、学习品德的升华、学习兴趣和信心的培养、思想情操目标的达成等，这是课堂教学的实质所在。
3、有效利用 45 分钟，学生学得轻松愉快，积极性高，当堂问题当堂解决。

9. 您对课程教学的建议是 (样本数=12)

序号	回答
1	继续保持
2	老师很帅也很负责，这学期收获很大，谢谢老师
3	老师太好了呜呜呜呜吹爆，还想上老师的课，老师你太帅了吧，不知道怎么夸了
4	张帅nice
5	无
6	暂无
7	谢谢老师，新年快乐

*有关课程评价的佐证材料可据实另附。

五、附件材料清单

1.教学设计样例说明（必须提供）

（提供一节代表性课程的完整教学设计和教学实施流程说明，尽可能细致地反映出教师的思考和教学设计，在文档中应提供不少于5张教学活动的图片。要求教学设计样例应具有较强的可读性，表述清晰流畅，课程负责人签字。）

2.最近一学期的课程教案（必须提供）

（课程负责人签字。）

3.最近一次学校对课堂教学评价（必须提供）

（申报学院审核盖章。）

4.最近一次学生评教情况（必须提供）

（申报学院审核盖章。）

5.教学（课堂或实践）实录视频（选择性提供）

（完整的一节课堂实录，至少40分钟，技术要求：分辨率720P及以上，MP4格式，图像清晰稳定，声音清楚。教师必须出镜，视频中需标注教师姓名、单位；要有学生的镜头，并须告知学生可能出现在视频中，此视频会公开。）

6.其他材料，不超过2份（选择性提供）

以上材料均可能在网上公开，请严格审查，确保不违反有关法律及保密规定。

六、课程承诺与保证

1.课程团队保证本团队所有成员不存在师德师风问题；

2.课程团队保证课程资源内容不存在政治性、思想性、科学性和规范性问题；

3.保证申报所使用的课程资源知识产权清晰，无侵权使用的情况；

4.保证课程资源及申报材料不涉及国家安全和保密的相关规定，可以在网络上公开传播与使用；

5.课程如若获评为校级级课程思政示范课程，将继续提供课程教学服务不少于5年。

课程团队全体成员签字：张世海 [illegible]

2022年9月16日

七、所在学院审查意见与承诺

我院已按有关规定对申请课程进行了资格审查，对申报书及所使用的课程内容进行了审核，并保证：

1.课程团队所有成员不存在师德师风问题；

2.课程资源内容不存在政治性、思想性、科学性和规范性问题；

3.学院将给予课程必要的经费支持并按期排课，为课程开展教学活动提供便利；

4.严格遵守省教育厅课程建设项目管理等有关规定，积极开展校际的课程共享、互选和学分互认等工作；

5.督促项目团队按照要求提供教学服务。

主管院领导签字：

（学院公章）

2022年 9月16日

项目编号： JG22 _ _ _

华南农业大学教育教学研究和改革项目

申 报 书

项目类别 一般项目

项目名称 “新农科”背景下《动物营养学》课程的改革与创新

项目负责人 张世海

职 称 副教授

手机号码 13660346296

所在单位 （公章）

申报日期 2022.10.8

华南农业大学 本科生院 制

2022 年 9 月

申请者的承诺与成果使用授权

本人自愿申报华南农业大学教育教学改革项目，承诺对所填写的《申报书》所涉及各项内容的真实性负责，保证没有知识产权争议。课题申请如获准立项，在研究工作中，接受华南农业大学教务处及本人所在单位的管理，并对以下约定信守承诺：

1. 遵守相关法律法规。遵守我国著作权法和专利法等相关法律法规；遵守我国政府签署加入的相关国际知识产权规定。

2. 遵循学术研究的基本规范，恪守学术道德，维护学术尊严。研究过程真实，不以任何方式抄袭、剽窃或侵吞他人学术成果，杜绝伪注、伪造、篡改文献和数据等学术不端行为；成果真实，不重复发表研究成果；维护社会公共利益，不以项目名义牟取不当利益。

3. 遵守华南农业大学教育教学改革项目有关管理规定以及华南农业大学财务规章制度。

4. 凡因项目内容、成果或研究过程引起的法律、学术、产权或经费使用问题引起的纠纷，责任由相应的项目研究人员承担。

5. 项目获批后务必按项目计划要求及时开展研究工作，确保研究工作如期完成。

6. 同意华南农业大学或其授权（委托）单位有权基于公益需要公布、使用、宣传《项目申请·评审书》内容及相关成果。

项目负责人（签章）：张世海[1]

2022 年 10 月 8 日

[1]此页须手写签字后扫描成 PDF 电子版。

一、项目及项目负责人、项目组简况

项目简况	项目名称	"新农科"背景下《动物营养学》课程的改革与创新				
	项目类别	□1.招标项目 □2.重点项目 √3.一般项目 □4.青年项目 □5.自筹项目				
	起止年月	2022.12 月-2024.12 月				
项目申请人	姓名	张世海	性别	男	出生年月	1989．01.02
	专业技术职务/行政职务	副教授/无		最终学位/授予国家		博士/中国
	所在单位及联系方式	单位名称	动物科学学院		手机号码	13660346296
		电子邮箱	zhangshihai@scau.edu.cn			
	主要教学工作简历	时间	课程名称	授课对象	学时	所在单位
		2022 上	动物营养学	大三	32	动科学院
		2021 下	动物营养学	大三	32	生命学院
		2021 上	动物营养学	大三	32	动科学院
		2020 上	动物营养学	大三	32	动科学院
	主要教学改革和科学研究工作简历	时间	项目名称			获奖情况
		2018	华南农业大学青教赛			一等奖
		2022	华南农业大学教学十佳			十佳
		2022	广东省第六届高校（本科）青年教师教学大赛			工科组 一等奖（第一）
项目组	总人数	职称			学位			
		高级	中级	初级	博士后	博士	硕士	参加单位数
	2	2				2		1
	主要成员（不含申请者）	姓名	性别	出生年月	职称	工作单位	分工	签名[2]
		管武太	男	1963.6	教授	动科	课程指导	管武太

[2]此页须成员手写签字后扫描成 PDF 电子版。

二、立项依据（项目研究的意义、现状分析）[3]

1.课程概况

1.1 课程简介

本校动物科学专业是**国家级一流专业**,而《动物营养学》是动物科学专业的核心基础课之一。主要通过介绍动物所需各种营养物质的营养作用与营养缺乏症、饲料营养价值与饲料加工利用、畜禽饲养标准及日粮/配合饲料产品设计等内容，培养畜牧、饲料业所需的专业复核型人才。学生在学习本课程过程中需掌握《动物生理学》和《动物生物化学》专业基础课程的相关知识。随着时代发展和历史变迁,《动物营养学》的任务从**早期的“满足动物生产、节约饲料成本；演变到中期的“提升畜禽产能、优化畜禽产品品质”；再变革到当代的“替抗减抗、低氮排放、绿色畜牧”**，是一门关系我国畜牧生产的重要课程。

1.2 学情分析、课情分析和教情分析

表 1 学情分析

项目	学情情况	改善途径
基础知识	基础课程《动物生物化学》和《动物生理学》容易遗忘	课前对学生知识体系进行课前调研、课中进行补充
心理特点	对《动物营养学》中文字描述类和记忆类知识类抵触	增加图文比例和案例分析
实践应用	缺乏把《动物营养学》应用于实践生产的能力	在课程教学中，以“生产问题”为导向背景
创新突破	拘泥于《动物营养学》课本，对前沿知识无从下手	在课程中导入前沿知识，课后提供辅助材料
专业成长	对畜牧业比较抵触，怕苦、怕累，不愿从事本专业	增加课程思政、大国三农情怀

[3] 表格不够，可自行拓展加页；但不得附其他无关材料。下同。

表 2 课情分析

项目	课情情况	改善途径
课程地位	《动物营养学》是动物科学专业的核心基础课之一	需传递专业知识也传递思政元素
课程作用	培养畜牧、饲料业所需的专业复核型人才	教学过程中增加案例分析与实践教学
课程内容	《动物营养学》知识枯燥乏味、记忆难度大、容易混淆 重理论、轻实践 科技前沿与行业动态融入较少 课程思政薄弱	重组教学内容，调整教学结构、拓展教学前沿、引入课程思政
依托平台	广东省动物营养调控重点实验室、农业部华南动物营养与饲料科学观测实验站、国家生猪种业工程技术研究中心、畜禽育种国家地方联合工程研究中心、农业部鸡遗传育种与繁殖重点实验室、广东省农业动物基因组学与分子育种重点实验室	充分利用平台资源，拓展教学应用

表 3 教情分析

项目	教情情况	改善途径
教学目标	专注《动物营养学》基础知识教学，缺乏职业素养培养和创新意识塑造	增加案例教学、实践环节、和思政环节
手段与方法	传统教学模式，主要局限于《动物营养学》课本知识的讲述。缺乏现代教学手段，以及以学生为中心的思想。	应用手机电脑等现代教学工具、增加课前、课中和课后师生互动
教学资源	广东省动物营养调控重点实验室、农业部华南动物营养与饲料科学观测实验站、国家生猪种业工程技术研究中心、畜禽育种国家地方联合工程研究中心、农业部鸡遗传育种与繁殖重点实验室、广东省农业动物基因组学与分子育种重点实验室	充分利用平台资源，拓展教学应用
教学能力	对新的教学方法、理念和思想接受较快。但缺乏畜牧学生产一线知识，以及对复杂前新沿知识的凝练描述能力。	深入生产一线、教学科研两手抓

1.3. 课程核心问题

通过本课程学情、课情和教情研究，发现《动物营养学》教学中存在五大短板——“五重五轻”，具体问题如下表：

表 4 课程核心问题

课程问题	“五重五轻”	问题解析
传统教学思政	重：畜牧生产成果和企业效益” 轻：畜禽粪污处理与环保减排”	《动物营养学》作为畜牧科学的重点专业核心课，核心思想是给畜禽营养均衡饲粮、提高企业更高利润。但是，传统教学内容忽视部分营养元素过量问题（如：氮元素、磷元素）。虽可以提高畜禽生产性能，但其添加剂量过高会对环境污染产生一定影响。
传统教学内容	重：《动物营养学》基础知识与概念 轻：《动物营养学》应用能力与科技创新	《动物营养学》中需记忆的知识点多，概念描述复杂。但往往忽略了如何指导学生把营养学知识应用到畜牧学中。同时，课本中没有没有营养学最新的前沿知识，学生也缺乏对营养学知识探索和创新的能力。
传统教学方式	重《动物营养学》术语和概念的抽象描述 轻知识的感官体验、案例分析和课堂互动	《动物营养学》中有许多抽象的概念描述和定义（如畜禽生理构造、营养物质消化和吸收过程），以及枯燥的动物营养学研究的方法和手段（消化试验、代谢试验等）。教师主要采用讲述法，而学生多数采用机械记忆，缺乏对知识掌握与了解。
传统教学环境	重：《动物营养学》PPT讲解和板书 轻：现代技术平台教学工具与模式的应用	在传统《动物营养学》课室教学环境中，教师依赖的主要教学工具为PPT。在教学过程中，不会应用以手机、ipad为载体的现代教学工具。同时，也忽视了在线课程、在线教学平台对学生预习和课后知识的巩固的作用。
传统教学评价	重：《动物营养学》课堂出勤与期末成绩 轻：课中态度评价和课后应用评价	传统《动物营养学》教学评价方式，主要以平时作业和闭卷考试对学生知识记忆和掌握情况进行单一评价。学生学习以考前突击为主、课堂学习积极性不高，对知识以强行记忆为主，缺乏相关理解。课堂学习挑战度较弱，学生课堂投入不足。

三、项目实施方案及实施计划

1.具体改革内容、改革目标和拟解决的关键问题

《动物营养学》是动物科学专业的核心基础课程。其在不同历史时期，承担了前后相继的历史任务，其研究重点从早期的“满足动物生产、节约饲料成本”，演变到中期的“提升畜禽产能、优化畜禽产品品质”，再变革到当代的“替抗减抗、低氮排放、绿色畜牧”。为了适应我国新时代“三农大国”的建设和国家“十四五”发展计划，本团队计划对《动物营养学》课程的教学目标、教学内容、教学方法和教学评价进行创新重组，做到以下“四个突破”。

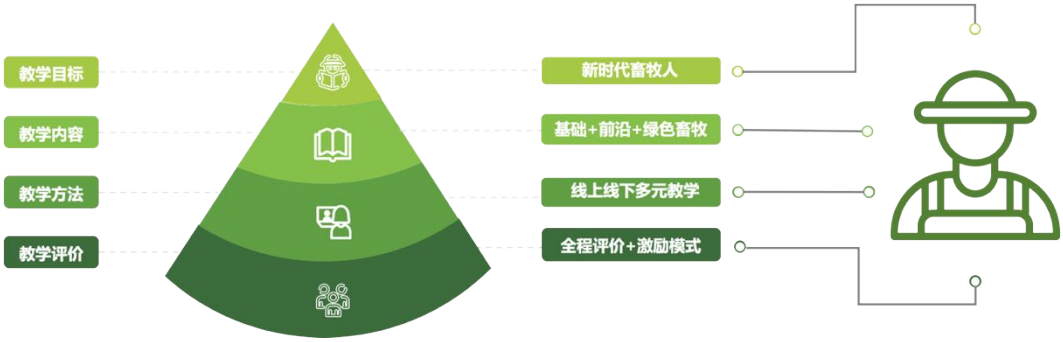


图 4 课程改革理念和思路

2.实施方案、实施方法、具体实施计划（含年度进展情况）及可行性分析

2.1 教学目标突破——放眼世界，展望未来

——从“传统养殖人”转变为“现代畜牧者”

根据新的时代对畜牧人的要求，课程培养目标进行修正。(1)在知识目标方面，掌握常量营养元素和微量营养元素的功能和缺乏症；动物不同生理阶段对于营养素的需求；以及饲料营养价值，理解《动物营养学》学科前沿动态。(2)在技能目标方面，锻炼学生对常规畜禽饲粮以及节能减排饲粮的配置。能设计科学合理的营养素和添加剂的动物试验评定方案。(3)在情感目标方面，培养“绿色环保畜牧人”，能够适应和引领未来畜牧业发展需要的新型人才。

	传统课堂	创新课堂
培养目标	知识目标： 1. 掌握常量营养元素和微量营养元素的功能和缺乏症 2. 动物不同生理阶段对于营养素的需求 3. 饲料营养价值评定方法 技能目标： 1. 锻炼学生对常规畜禽饲粮配置 2. 能设计科学合理的营养素和添加剂的动物试验评定方案 情感目标： 培养高效、敬业的畜牧工作者	知识目标： 1. 掌握常量营养元素和微量营养元素的功能和缺乏症 2. 动物不同生理阶段对于营养素的需求 3. 饲料营养价值方法 4. 《动物营养学》前沿动态 5. 营养素过度排放与环境污染 技能目标： 1. 锻炼学生对常规畜禽饲粮及节能减排饲粮的配置 2. 在考虑环保情况下，能设计科学合理的营养素和添加剂的动物试验评定方案，并能对传统试验技术提出改良策略。 情感目标： 培养“绿色环保畜牧人”，能够适应和引领未来畜牧业发展需要的新型人才

图 5 课程培养目标突破前后对比图

2.2 课程内容突破——聚焦前沿，突破创新

——从“畜禽营养素功能”到“前沿畜禽营养素功能与绿色畜牧”

课程内容改革方向，围绕培养“营养专业知识强、营养创新能力强、环保思维执行强、三农服务意识强”的“四强”现代畜牧工作者，重组教学模块。首先、帮助学生了解现代营养学和畜牧学知识体系；第二、让学生构建系统营养学思维，能运用营养学知识，能判断常见的动物营养缺乏症，并进行饲料营养价值评定；第三、让学生树立“金山银山就是绿水青山”的环保意识，在追求企业效益的同时，也注重畜禽环保减排；第四、培养学生的继续学习和创新意识，能自己通过查阅文献，了解动物营养学和饲料学的行业最新动态。在课堂上，让同学们分享学习的新技术和新观点。

图 6 课程教学内容重组四大模块

图 7 课程教学内容突破前后对比图

2.3 教学手段突破——打破传统，优化平台

——从“《动物营养学》PPT 讲解”到“线上线下混合+翻转课堂+案例讨论+特邀专家+跨学科交流的多元教学”

利用现代教学工具，打造信息化课堂。学生通过手机或平板电脑与教师进行互动、交流和反馈。完成课前预习过程、课中问题讨论与课后作业布置等过程。借助现代教学平台，让教学过程智能化，师生沟通便捷化，学习体验先进化。连线企业和专家进行现场教学。全面打造多元教学资源和手段。

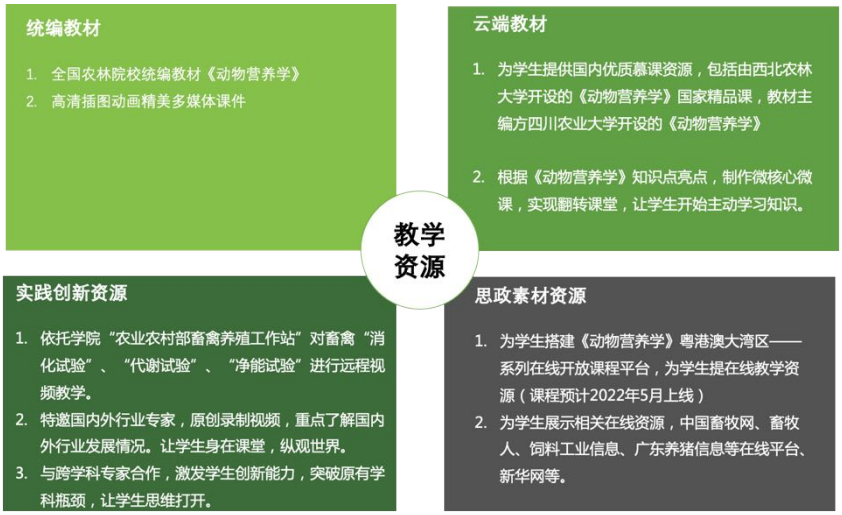


图 8 多元教学模式图

	传统课堂	创新课堂
教学手段	1. PPT讲解+板书	1. PPT讲解+板书 2. MOOC 3. 手机+ipad教学（学习通、雨课堂） 4. 线上线下混合 5. 翻转课堂 6. 案例讨论 7. 特邀企业专家互动 8. 问题导向教学 9. 跨学科专家交流

图 9 课程教学手段突破前后对比图

2.4 教学评价突破——多层检测，多维评价

——从“《动物营养学》知识机械记忆考核”到“《动物营养学》课前预习情况、课中学习态度和课后知识应用的综合评价”

打破传统评价模式，课程评价由课前准备性、课中过程性和课后总结性评价构成。课前准备性评价根据学生的参与课程预习的情况来评价;课中过程性评价根据学生的随堂表现和课后线上学习来评价;课后总结性评价根据期末考试成绩和知识应用来评价整体教学效果。课程实行“多维度考核”。**创新提出“三轮”评价模式，参与得分制和积分制，**掌握学生真实情况，促进学生学习。

图 10 “三轮”评价体系

	传统课堂	创新课堂
教学评价	1. 平时作业 2. 期末考试	1. 课前预习评价（考察参与度） 2. 课中学习评价（注重考勤+互动+答题） 3. 课后效果评价（聚焦考试+应用）

图 11 课程教学评价突破前后对比图

3.项目预期成果及其实践运用预期（包括成果形式，预期推广、应用范围、受益面等，须详述在我校应用情况）

使得学生从机械记忆动物营养学概念到灵活应用动物营养学知识；从简单追求畜禽养殖的饲粮基本配置，到关注节能减排的环保饲粮；为国家培养创新拔尖畜牧人才奠定基础。课程创新成果为课将程和学科建设等提供了重要支撑，在农林院校中起到良好示范作用。

4.本项目的特色与创新点

《动物营养学》课程教学改革旨在解决传统教学中存在的“五轻五重”现象。针对畜牧学在“十四五”建设新时期承担的历史任务，创新性的从培养目标——“培育现代畜牧者”、教学内容——“聚焦前沿畜禽营养素功能与绿色畜牧”、教学手段——“线上线下混合+翻转课堂+案例讨论+特邀专家+跨学科交流的多元教学”、评价方法——“从课程前、中、后多维度对《动物营养学》知识掌握评价”四个维度提出了“四突破”的创新思路，并将“绿色畜牧”思政元素系统融入《动物营养学》课程教学。此外，依托现代信息技术和教育平台，创新性在课堂中引入“院士专家连线”和“交叉学科互动”的课堂活动以及“课前参与得分”和“课中互动积分”评分制度的“双革新”手段。

思政融合创新

课前思政元素凝练——深度挖掘，挖掘动物营养从业者的匠人精神，培养同学们作为畜牧人的职业担当；挖掘与动物营养相关的绿色畜牧理念，培养学生的生态文明意识，让学生深入理解“绿水青山就是金山银山”；挖掘动物营养领域中研究者的创新突破精神，培养学生独立思考与逻辑思辨的能力。

课中思政案例结合——隐形融入，课程中通过事实案例、名人故事、伟人格言等手段，把该章节最核心思政要素融入课堂。让学生深刻理解“营养与生态文明教育”、“畜牧大国三农”、“绿水青山就是金山银山”、“匠人精神”、“创新性意识”等思政元素。具体

章节案例，见下图。

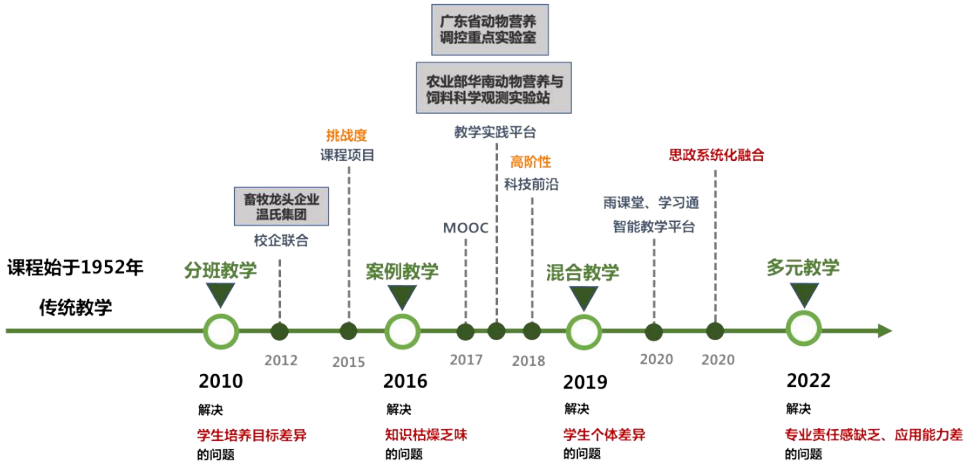
课后思政作业强化——提升效果，通过让学生在课后配制环保饲粮，并在课堂案例分享的形式，隐形强化“绿色畜牧的理念”；通过学生独立设计消化代谢笼，培养学生的创新突破精神；通过完成院士的成长故事读后感，树立学生的职业情怀。

4．思政教学基地建设

建设《动物营养学》绿色环保样板实践猪场和饲料厂。通过亲身体验，让学生了解如何进行绿色畜牧，培养学生生态文明的意识。并把相关的绿色畜牧相关的理念带入到未来的工作企业和单位中。

四、项目建设基础

1.与本项目有关的工作积累和已取得的工作成绩



课程建设与改革简史

2.单位对项目的支持情况（含有关政策、经费及其使用管理机制、保障条件等，可附有关文件），尚缺少的条件和拟解决的途径 2015 年，畜牧学一级学科被列入我校高水平大学重点建设学科群；2017 年，在教育部第四轮学科评估中我院畜牧学进入全国八强；2019 年，畜牧学被列入我校重点建设的八大学科、广东省“冲一流”重点建设学科。具备了良好的教学改革环境。
3.项目负责人和项目组成员所承担的教学改革和科研项目情况 **教改论文** 《动物营养学》教学案例初探，课程教育研究，(2095-3089)，2019 年，21 期 翻转课堂在《动物营养学》课程中的应用策略，教育教学论坛，(1674-9324)，25 期 **校级教改项目** 动物营养学科方向课程组的改革与建设—基于《动物营养学》、《饲料学》和《饲料添加剂学》的探索研究

五、经费预算

预算经费总额		10000（元）	
序号	支出科目	预算	支出用途
1	出版/文献/信息传播/知识产权事务费		
2	数据采集费		
3	设备费及材料费	2000 元	打印材料
4	差旅费		
5	咨询费	2000 元	专家咨询
6	其他支出		

六、单位意见[4]

所在单位意见：

同意推荐。

（公章）

单位负责人签字：

2022 年 10 月 8 日

[4]此页须签字盖章后扫描成 PDF 电子版。

七、学校主管部门意见

学校主管部门意见：

签章：

年　　月　　日

NATIONAL TEACHING INNOVATION CONTEST FOR COLLEGE TEACHERS
全国高校教师教学创新大赛

第四届全国高校教师教学创新大赛

获奖证书

张世海 老师：

荣获第四届全国高校教师教学创新大赛新农科副高组“一等奖”，特颁发此证。

工作单位：华南农业大学

课程名称：动物营养学

团队成员：张永亮、张常明、曹庆云

证书编号：
TIC2024NB1068

中国高等教育学会

2024年7月

第六届全国高校青年教师教学竞赛 2022

荣誉证书

张世海 同志：

在中华全国总工会、教育部联合主办的第六届全国高校青年教师教学竞赛中荣获工科组二等奖，特发此证。

中华全国总工会办公厅　教育部办公厅

二〇二三年四月

荣誉证书

张世海 同志：

在广东省第六届高校（本科）青年教师教学大赛总决赛（工科组）中表现优异，荣获总决赛第一名。

特发此证，以资鼓励。

广东省总工会　广东省教育厅

二〇二三年二月

主讲教师：张世海

团队成员：张永亮、张常明、曹庆云

主讲课程：动物营养学

获奖等级：新农科 一等奖

证书编号：GDTIC2024041

NATIONAL TEACHING INNOVATION CONTEST FOR COLLEGE TEACHERS
全国高校教师教学创新大赛

第四届全国高校教师教学创新大赛广东分赛
暨广东省高校教师教学创新大赛

获奖证书

广东省教育厅　　广东省高等教育学会

二〇二四年七月

荣誉证书

张世海 老师：

荣获华南农业大学2021年度“本科课堂教学十佳教师”。

特发此证，以资鼓励！

华南农业大学

2022年7月2日

国家重点研发计划项目
子课题任务书

子课题名称/编号：	研发氧化应激/炎症反应防控技术与产品，缓解热应激母畜发情、妊娠障碍
子课题承担单位：	华南农业大学
子课题负责人：	张世海
课题名称/编号：	热应激母畜发情、妊娠障碍防控技术和产品研发/2024YFD1301004
课题承担单位：	山西农业大学
所属项目：	家畜繁殖障碍调控技术研究与示范应用
所属专项：	畜禽新品种培育与现代牧场科技创新
执行期限：	2024 年 12 月至 2027 年 11 月

填 写 说 明

一、任务书甲方即课题牵头承担单位，乙方即子课题承担单位。

二、任务书中的单位名称，请按规范全称填写，并与单位公章一致。

三、任务书中文字须用宋体小四号字填写。

四、凡不填写内容的栏目，请用“无”表示。

五、任务书用 A4 纸打印、装订、签章。一式四份报课题承担单位，其中课题承担单位两份，子课题承担单位两份。

六、《课题任务书》是本任务书填报的重要依据，任务书填报不得降低考核指标，不得自行对主要研究内容作大的调整。《课题任务书》和本任务书将共同作为子课题过程管理、验收和监督评估的重要依据。

子课题基本信息表

<table>
<tr><td colspan="2">子课题名称</td><td colspan="5">研发氧化应激/炎症反应防控技术与产品，缓解热应激母畜发情、妊娠障碍</td></tr>
<tr><td colspan="2">所属课题</td><td colspan="5">热应激母畜发情、妊娠障碍防控技术和产品研发</td></tr>
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<tr><td colspan="2">所属专项</td><td colspan="5">畜禽新品种培育与现代牧场科技创新</td></tr>
<tr><td colspan="2">密级</td><td colspan="5">■公开　□秘密　□机密</td></tr>
<tr><td colspan="2">经费预算</td><td colspan="5">总预算 100 万元，其中中央财政专项经费 100 万元</td></tr>
<tr><td rowspan="5">子课题承担单位</td><td>单位名称</td><td colspan="2">华南农业大学</td><td>单位性质</td><td>大专院校</td></tr>
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<tr><td>单位开户名称</td><td colspan="4">华南农业大学</td></tr>
<tr><td>开户银行（全称）</td><td colspan="4">中国工商银行股份有限公司 广州五山支行</td></tr>
<tr><td rowspan="6">子课题负责人</td><td>姓 名</td><td>张世海</td><td>性 别</td><td>■男□女</td><td>出生日期 1989-01-02</td></tr>
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<tr><td>职 称</td><td colspan="2">□正高级 ■副高级 □中级 □初级 □其他</td><td colspan="2">职务</td></tr>
<tr><td>电子邮箱</td><td colspan="2">zhangshihai@scau.edu.cn</td><td>移动电话</td><td>13660346296</td></tr>
<tr><td rowspan="3">子课题联系人</td><td>姓 名</td><td>张世海</td><td>电子邮箱</td><td colspan="2">zhangshihai@scau.edu.cn</td></tr>
<tr><td>固定电话</td><td>13660346296</td><td>移动电话</td><td colspan="2">13660346296</td></tr>
<tr><td>证件类型</td><td>身份证</td><td>证件号码</td><td colspan="2">350783198901020259</td></tr>
<tr><td rowspan="3">子课题财务负责人</td><td>姓 名</td><td>袁思雨</td><td>电子邮箱</td><td colspan="2">2815129864@qq.com</td></tr>
<tr><td>固定电话</td><td>15183017906</td><td>移动电话</td><td colspan="2">15183017906</td></tr>
<tr><td>证件类型</td><td>身份证</td><td>证件号码</td><td colspan="2">510503200112300933</td></tr>
<tr><td rowspan="2">子课题参加人数</td><td colspan="2" rowspan="2">9 人。其中：</td><td colspan="3">高级职称 1 人，中级职称 ___ 人，初级职称 ___ 人，其他 8 人；</td></tr>
<tr><td colspan="3">博士学位 1 人，硕士学位 2 人，学士学位 6 人，其他 ___ 人。</td></tr>
</table>

一、子课题研究目标、内容及考核指标

（一）主要研究内容及目标

(拟解决的关键科学问题、关键技术问题，针对这些问题拟开展的主要研究内容。研发主要针对什么问题和需求；将要解决哪些科学问题、突破哪些核心/共性/关键技术；预期成果及应用等。)

1. **拟解决的关键科学问题、关键技术问题**

针对我国缺乏缓解热应激的特效药物，亟待研发绿色新型抗炎药物等产业问题，开发新型青蒿酶解产物等热应激缓解关键技术。

2. **主要研究内容**

1）基于青蒿富含多酚类化合物具有抗炎和抗氧化功能，开发新型青蒿酶解产物

针对南方湿热气候，建立母猪热应激模型，分析氧化应激和炎症反应与发情、妊娠障碍的关联，并揭示肠道微生物在其中的调控作用；结合生物工程和发酵工程，优化青蒿酶解工艺，开发新型青蒿酶解产物，评估其对母畜发情和妊娠的影响，确定最适添加剂量和时间。

2）**基于优化母畜肠道屏障功能的发情、妊娠调控产品开发**

探究热应激下肠道屏障破坏如何诱发机体系统性炎症及其对母畜发情的影响。研究功能性低聚糖（甘露寡糖）对母畜系统性炎症、肠道屏障、肠道微生物群及代谢物的调控作用，开发改善肠道菌群及其代谢物，增强肠道屏障的功能性低聚糖（甘露寡糖）调控产品，降低热应激对母畜发情、妊娠的影响，评估其在发情和妊娠中的应用效果，并确定其在饲粮中的适宜添加时间和剂量。

3. **主要研究目标**

提升热应激母畜繁殖性能，解决规模养殖场热应激母畜繁殖效率低的问题。

（二）考核指标

（考核指标：指相应成果的数量指标、技术指标、质量指标、应用指标和产业化指标等，其中，数量指标可以为论文、专利、产品等的数量；技术指标可以为关键技术、产品的性能参数等；质量指标可以为产品的耐震动、高低温、无故障运行时间等；应

用指标可以为成果应用的对象、范围和效果等；产业化指标可以为成果产业化的数量、经济效益等。）

① 研发降低母畜热应激的防控技术与产品 1-2 项；

② 提高热应激下母畜夏季发情率 5%-10%；

③ 建立热应激调控技术及产品应用示范场 1 个；

④ 发表相关科研论文 2-3 篇；

⑤ 培养研究生 2-3 名。

二、子课题年度计划

按每6个月制定形成课题的计划进度，应将课题的考核指标分解落实到年度计划中。

1、2024-2025 年度：2024 年 12 月—2025 年 05 月

任务：揭示热应激母畜代谢异常通过氧化应激/炎症反应致使发情障碍机制，系统研究其在热应激母畜发情/妊娠障碍中的代谢规律。

考核指标：检测血液的激素、炎症因子及抗氧化因子的变化。

成果形式：形成课题方案。

2、2025 年度：2025 年 06 月—2025 年 11 月

任务：揭示热应激母畜代谢异常通过氧化应激/炎症反应致使妊娠障碍机制，系统研究其在热应激母畜发情/妊娠障碍中的代谢规律。

考核指标：检测血液的激素、炎症因子及抗氧化因子的变化；挖掘相关差异代谢物，挖掘生长因子等功能蛋白 1-2 个，培养研究生 1 名。

成果形式：年度报告 1 份。

3、2025-2026 年度：2025 年 12 月—2026 年 05 月

任务：研发氧化应激/炎症反应防控技术与产品。开发新型青蒿酶解产物等，评估其对母畜发情和妊娠的影响，确定最适添加剂量和时间。

考核指标：研发降低母畜热应激的防控技术 1 项，评测方法以发表研究论文或试验报告为准。投稿相关科研论文 1 篇。

成果形式：论文投稿证明。

4、2026 年度：2026 年 06 月—2026 年 11 月

任务：开发改善肠道菌群及其代谢物，增强肠道屏障的功能性低聚糖（甘露寡糖）调控产品，并研究适宜添加剂量和时间。

考核指标：投稿相关科研论文 1 篇，培养研究生 1 名，年度报告 1 份。

成果形式：论文投稿或录用证明，年度报告。

5、2026-2027 年度：2026 年 12 月—2027 年 05 月

任务：研究热应激母畜繁殖性能调控技术，开发新型青蒿酶解产物热应激缓解关键技术。

考核指标：建立青蒿酶解产物缓解热应激高效调控技术，在示范场中进行效果评价，使母畜夏季发情率、受胎率提高 5%-10%，；获得缓解热应激繁殖障碍产品 1 个，培养研究生 1 名。

成果形式：应用证明。

6、2027 年度：2027 年 06 月—2027 年 11 月

任务：总结子课题科研成果，准备子课题综合绩效评价的相关材料。优化发情障碍调控方案并形成相应技术和产品（青蒿酶解产物等），在示范场进行应用效果示范。

考核指标：投稿相关科研论文 1 篇，结题报告 1 份，科技报告 1 份。

成果形式：论文，科技报告，结题报告。

三、知识产权对策、成果管理及合作权益分配

限 500 字以内。

双方在研发过程中应加强研究成果管理，严格执行《中华人民共和国科学技术进步法》、《关于加强国家科技计划知识产权管理工作的规定》、《中华人民共和国专利法》和《中华人民共和国著作权法》等有关规定。研究成果在申报国家及有关部门、地方的科技成果奖励时，需由甲乙双方共同协商成果署名等相关事宜。甲乙双方应根据有关法律法规，及甲方与项目组织单位签订的任务书规定，协商确定课题研究成果的收益分配等事宜并签订知识产权合作协议。

四、需要约定的其他内容

限 500 字以内。

1.项目未完成任务目标，任务验收结论为结题或不通过的，项目下所有课题结余资金由项目牵头单位统一组织上交专业机构。

2.在子课题实施中，课题法人单位负责建立本单位生物安全管理体系，落实生物安全管理责任部门或责任人，定期召开生物安全管理会议，对实验室生物安全相关的重大事项做出决定，批准和发放实验室生物安全手册、生物危害评估等重要文件，为本单位废弃物处理提供处置条件，定期生物防护、实验室安全、废弃物处置等规章制度实施进行监督、检查，负责制定和实施实验室应急处置预案和安全事故现场处置和调查。课题负责人全面负责课题实施过程中的生物安全工作，决定并授权并培训实验室工作人员，监督有关法规和标准操作规程的执行，协助安全事故的调查。

五、子课题参加人员基本情况表

填表说明： 1.职称分类：A、正高级 B、副高级 C、中级 D、初级 E、其他；
2.投入本子课题的全时工作时间（人月）是指在子课题实施期间该人总共为子课题工作的满月度工作量；累计是指子课题组所有人员投入人月之和；
3.子课题固定研究人员需填写人员明细；
4.是否有工资性收入：Y、是 N、否；
5.人员分类代码：A、子课题负责人 B、子课题骨干 C、其他研究人员；
6.工作单位：填写单位全称，其中高校要具体填写到所在院系。

序号	姓名	性别	出生日期	身份证号码（军官证、护照）	技术职称	职务	学位	专业	投入本子课题的全时工作时间（人月）	人员分类	是否有工资性收入	工作单位
1	张世海	男	1989-01-02	350783198901020259	副高级	无	博士	动物营养与饲料科学	15	项目骨干	是	华南农业大学动物科学学院
2	陈佳明	男	1995-08-18	352201199508183611	其他	无	硕士	动物营养与饲料科学	24	其他研究人员	否	华南农业大学动物科学学院
3	李其晖	男	1998-12-03	141182199812030014	其他	无	硕士	动物营养与饲料科学	24	其他研究人员	否	华南农业大学动物科学学院
4	董世奇	男	2000-01-02	412821200001023635	其他	无	学士	动物营养与饲料科学	24	其他研究人员	否	华南农业大学动物科学学院
5	单嘉学	男	2000-06-15	440183200006151710	其他	无	学士	动物营养与饲料科学	24	其他研究人员	否	华南农业大学动物科学学院
6	何振霆	男	2000-10-21	441622200010213713	其他	无	学士	动物营养与饲料科学	24	其他研究人员	否	华南农业大学动物科学学院
7	苏森林	男	2001-05-02	420881200105023337	其他	无	学士	动物营养与饲料科学	24	其他研究人员	否	华南农业大学动物科学学院

8	李保锋	男	2000-02-19	360421200002194411	其他	无	学士	动物营养与饲料科学	24	其他研究人员	否	华南农业大学动物科学学院
9	张倩姿	女	2000-02-20	370682200002208428	其他	无	学士	动物营养与饲料科学	24	其他研究人员	否	华南农业大学动物科学学院
		固定研究人员合计							207	/	/	/
		流动人员或临时聘用人员合计							0	/	/	/
		累计							207	/	/	/

六、经费预算

子课题预算表

子课题名称：研发氧化应激/炎症反应防控技术与产品，缓解热应激母畜发情、妊娠障碍

金额单位：　万元

序号	预算科目名称	金额
	（1）	（2）
1	**一、中央财政专项资金**	**100**
2	（一）直接费用	**77**
3	1.设备费	
4	其中：购置设备费	
5	2.业务费	**59**
6	3.劳务费	**18**
7	（二）间接费用（自动计算）	**23**
8	**二、其他来源资金**	
9	**三、合计**	**100**

注：1.间接费用无需编制预算说明；2.绩效支出在间接费用中无比例限制。绩效支出安排与科研人员在课题工作中的实际贡献挂钩。

设备费——购置/试制设备预算明细表

子课题名称：研发氧化应激/炎症反应防控技术与产品，缓解热应激母畜发情、妊娠障碍　　金额单位：　　万元

填表说明：1.设备分类：购置、试制；
2.购置设备类型：通用、专用；
3.试制设备不需填列本表（9）列、（10）列、（11）列、（12）列；
4.设备单价的单位为万元/台套，设备数量的单位为台套；
5.单价 50 万元以下的设备不用填写；
6.本表只填写中央财政资金购置（试制）的设备。

序号	设备名称	设备分类	功能和技术指标	单价	数量	金额	购置或试制单位	安置单位	购置设备类型	主要生产厂家及国别	规格型号	拟开放共享范围
	（1）	（2）	（3）	（4）	（5）	（6）	（7）	（8）	（9）	（10）	（11）	（12）
单价 50 万元以上购置设备合计							/	/	/	/	/	/
单价 50 万元以上试制设备合计							/	/	/	/	/	/
累计							/	/	/	/	/	/

预算说明

一、中央财政资金

预算的编制要坚持任务相关性、政策相符性和经济合理性，实事求是编制提出课题预算。填报时，直接费用应按设备费、业务费 、劳务费三个类别填报，每个类别结合科研任务按支出用途进行说明。除 50 万元以上的设备外，其他费用只提供基本测算说明，不需要提供明细。

本子课题直接费用预算 77.00 万元，其中设备费 0 万元，业务费 59 万元，劳务费 18 万元。

1. **设备费**（是指项目实施过程中购置或试制专用仪器设备，对现有仪器设备进行升级改造，以及租赁外单位仪器设备而发生的费用等。计算类仪器设备和软件工具可在设备费科目编列。填报时，50 万元以上的设备详细说明，50 万元以下的设备费用分类说明）

设备费 0.00 元。

2. **业务费**（是指在项目实施过程中消耗的各种材料、低值易耗品等、发生的测试化验加工、燃料动力、出版文献、信息传播、知识产权事务、会议、差旅、国际合作与交流以及其他与项目实施直接相关的各项费用。编报时，对单笔大额支出、对外委托支出重点说明）

预算业务费 59 万元，其中开展研究消耗的各种材料、低值易耗品等 40.53 万元，测试化验加工支出 11 万元，出版/ 文献/信息传播/知识产权事务支出 2.50 万元，会议/差旅/国际合作交流支出 4.475 万元，其他支出 0.495 万元。

2.1．材料费 40.53 万元

（1）试验动物补偿费用：7.75 万元。

用于研发不同产品和繁殖技术应对热应激导致的猪繁殖障碍所需试验动物补偿或屠宰等费用。本项目拟初建猪热应激模型，评估青蒿提取物对热应激的防控效果，并进行体外验证，需要 3.875 万元；

本项目拟初建猪热应激模型，评估青蒿提取物对热应激的防控效果，并进行体外验证，需要 3.875 万元。

（2）热应激防控药物研发费用：8.00 万元。

药学研究，优化高效绿色中草药（青蒿、甘露寡糖）的提取或合成，需要 2.00 万元；

药物结构分析，需要 1.00 万元；

功能检测等研究，需要 5.00 万元；

（3）细胞培养所用试剂及耗材：2.00 万元。

开发新型抗炎药物对热应激母畜发情/妊娠障碍的防控药用效果和构建家畜热应激模型所需试剂及耗材：主要包括 DMEM 培养基、MEMα 培养基、DMSO、台盼蓝、双抗、进口胎牛血清、培养皿等，需要 2.00 万元。

（4）分子类试剂耗材：20.59 万元。

家畜热应激造模过后，用于检测生殖激素、抗氧化因子、炎症因子等生理生化指标，挖掘差异生物标志物，研究应对热应激的抗炎药物。生殖激素检测试剂盒：促卵泡激素(FSH)、雌激素(E2)、孕酮、睾酮、血浆 HSPs、皮质醇、红细胞钾离子浓度酶联免疫吸附测定试剂盒，用于检测母猪血液及细胞等样品中的 FSH、E2、孕酮、睾酮含量，HSPs、皮质醇、红细胞钾离子浓度的变化， 需要 3.69 万元;

细胞分子水平检测试剂盒：活性氧(ROS)、白介素(IL)酶联免疫吸附测定试剂盒、细胞凋亡检测试剂盒、线粒体染色试剂盒检测不同试验处理后卵子或胚胎的代谢水平、氧化还原平衡状态、凋亡水平、线粒体含量等，需要 3.15 万元;

免疫荧光染色及蛋白质表达水平检测试剂：用于课题开展猪卵子成熟和胚胎发育过程中关键调控基因、蛋白等表达检测，包括 BCA 蛋白浓度测定试剂盒、Western blot 相关试剂、4%多聚甲醛、组织包埋用相关试剂、一抗、荧光二抗等，需要 8.75 万元;

mRNA 表达水平检测所需的试剂及耗材，用于检测卵子、颗粒细胞、各个阶段早期胚胎基因表达定量检测，包括 RNA 提取试剂盒、RNA 反转录试剂盒、定量试剂 Sofast EvaGreen Supermix 等，需要 5.00 万元。

（5）其他常规试剂耗材等：2.19 万元。

课题开展研究过程中试验操作、样本处理及保存等相关的常规试剂耗材。液氮：用于试验样品的冷冻保存，需要 0.5 万元；

样品保存及处理所需的试剂耗材：用于卵巢、细胞等样品的保存及处理，包括移液管、细胞冻存管、细胞冻存液、国产及进口无 RNA 酶吸管，各类注射器、各型号移液枪头，需要 0.85 万元；

常用试剂：课题实施中所需生理盐水、无水乙醇、盐酸、DPBS、PBS、玻璃瓶等，需要 0.34 万元；

体外培养所需各类气体：用于卵母细胞、颗粒细胞及胚胎培养过程中所需的高纯度 CO_2 气体等，需要 0.8 万元。

2.2 测试化验加工费 11 万元

（1）样品测试测序费：总计 7.00 万元。

检测影响卵巢、子宫功能，分析母畜卵巢、子宫的热应激差异标志物，确定关键标志物组成及阈值，揭示热应激母畜代谢异常通过氧化应激/炎症反应致使发情、妊娠障碍机制。检测正常组、热应激组和热应急条件下药物处理后颗粒细胞中关键差异代谢物，旨在从代谢物层面揭示热应激对母畜卵巢的影响机制。

非靶向代谢物测试，每例 0.05 万元，需要 1.25 万元；

靶向代谢物测试，每例 0.05 万元，需要 1.25 万元。

基于前期非靶向代谢物筛选出来的药物靶点，针对这一特定的代谢物及代谢途径进行热应激的干预治疗，更有针对性的阐述热应激对母畜卵巢的影响机制,以及使用不同的药物治疗效果。

mRNA、非编码 RNA 测试，每例 0.40 万元，需要 3.00 万元；

蛋白水平测试，每例 0.10 万元，需要 1.25 万元；

引物加工测试，每例 50 元，需要 0.25 万元；

（2）大型仪器使用费：4.00 万元。

激光扫描共聚焦显微镜用于检测细胞和组织中氧化应激指标 ROS 的产生以及关键蛋白的定位等仪器使用费，需要 1.00 万元；

扫描电镜和投射电镜用于检测热应激对卵巢组织和卵泡颗粒细胞中线粒体、内质网以及自噬小体的损伤，同时用于观察使用不同的药物和繁殖技术对热应激损伤的缓解效果等的仪器使用费, 需要 2.00 万元；

流式细胞仪用于检测正常组、热应激组和热应急条件下药物处理后颗粒细胞细胞周期、凋亡等指标的仪器使用费，需要 1.00 万元。

2.3 出版/文献/信息传播/知识产权事务费 2.50 万元

（1）出版 SCI 论文 2 篇，单价 2.00 万元，小计 2.00 万元；

（2）出版中文核心论文 1 篇，单价 0.50 万元，小计 0.50 万元。

2.4 会议/差旅/国际合作交流费 4.475 万元

（1）差旅费：4.475 万元。

课题成员参加畜牧兽医学会、繁殖学分会学术交流会及理事会等国内会议，共 5 人次，差旅小计 1.275 万元；

课题人员拟每年 1 人次参加国际动物繁殖学术会议等，人均费用 10000 元/次，1 年合计 1.00 万元；

课题研究人员赴广东等地的猪场开展热应激条件下氧化应激、炎症反应和肠道屏障损伤对母畜发情影响的机制，并开发新型饲用抗炎、抗氧化剂（青蒿酶解产物）和功能性纤维（甘露寡糖），以缓解热应激对母畜发情和妊娠的负面影响。收集数据母猪肠道微生物群、生理生化以及血液参数等数据，预计 10 人/次，需要 2.20 万元。

2.5 其他支出 0.495 万元

3.劳务费（是指在项目实施过程中支付给参与项目的研究生、博士后、访问学者以及项目聘用的研究人员、科研辅助人员、科研（财务）助理等的劳务性费用；支付给临时聘请的咨询专家的费用等。项目聘用人员由单位缴纳的社会保险补助、住房公积金等可纳入劳务费列支。）

3.1 研究生劳务费 18 万元

课题需要 3 名博士参与，揭示热应激对猪发情妊娠障碍的影响机制，通过多组学联合分析揭示找到药物治疗靶点及作用机制，并优化繁殖生物技术缓解热应激对母畜发情障碍的影响；开发缓解热应激对猪发情妊娠障碍的药物以及临床效果验证；完成数据分析及论文发表等，按照博士 2500 元/月，工作 8 月/年，3 年需要 18.00 万元。

二、其他来源资金

对其他来源资金主要用途、支出预算做简要说明。

无。

任务书签署

甲乙双方根据《国务院印发关于深化中央财政科技计划（专项、基金）管理改革方案的通知》（国发〔2014〕64 号）、《国务院关于优化科研管理提升科研绩效若干措施的通知》（国发〔2018〕25 号）、《国务院办公厅关于改革完善中央财政科研经费管理的若干意见》（国办发〔2021〕32 号）、《科技部 财政部关于印发<国家重点研发计划管理暂行办法>的通知》（国科发资〔2017〕152 号）、《财政部 科技部关于印发<国家重点研发计划资金管理办法>的通知》（财科教〔2016〕113 号）、《科技部财政部关于印发<中央财政科技计划（专项、基金等）监督工作暂行规定>的通知》（国科发政〔2015〕471 号）等有关文件规定，以及有关法律、政策和管理要求，依据项目立项通知，签署本任务书。

同时，子课题负责人**郑重承诺**：对本子课题所有成果产出（包括但不限于新产品、新技术、标准、论文、专利等）的真实性、与项目（课题）的关联性等负责，将按要求落实科研作风学风和科研诚信主体责任；项目经费全部用于与本项目研究工作相关的支出，不截留、挪用、侵占，不用于与科学研究无关的支出；接受并积极配合相关部门的监督检查。如有违反，子课题负责人以及相关成果产出者愿接受项目管理专业机构和相关部门做出的各项处理决定，包括但不限于终止课题执行、追回课题经费，取消一定期限国家科技计划项目（课题）申报资格，记入科研诚信严重失信行为数据库以及主要负责人接受相应党纪政纪处理等。

课题承担单位（甲方）：山西农业大学

单位负责人签字（签章）：孔胜印照

（公章）

2025 年 1 月 23 日

课题负责人签字（签章）：吕丽华

2025 年 1 月 20 日

子课题承担单位（乙方）：华南农业大学

单位负责人签字（签章）薛红已

（公章）

2024年12月30日

子课题负责人签字（签章）：

2024年12月30日

项目编号：2021YFD1300700

国家重点研发计划青年科学家项目
任务合同书

项目名称：母猪泌乳能力及初乳 IgA 含量提升的营养调控机制

项目起止时间：2021 年 12 月至 2026 年 11 月

项目负责人：任文凯　　联系电话：18613955926

项目承担单位：华南农业大学

任务名称：日粮营养结构调控初产母猪初乳 IgA 水平的机制研究

任务负责人：张世海　　联系电话：13660346296

任务承担单位：华南农业大学

华南农业大学 2021 年制

一、项目年度研究内容与任务

年度	主要工作内容	任务及年度考核指标
第一年	解析营养结构调控初乳 IgA 水平的影响；	1. 总结年度报告 1 份。
第二年	解析营养结构对初乳 IgA 调控的机制；	1. 发表 SCI 论文 1 篇； 2. 总结年度报告 1 份。
第三年	研究候选代谢产物对乳腺上皮细胞 pIgR 表达的影响	1. 总结年度报告 1 份。
第四年	研究候选代谢物对乳腺上皮细胞 pIgR 表达的调控机制	1. 总结年度报告 1 份。
第五年	验证营养结构对 IgA 的调控机制	1. 发表 SCI 论文 1 篇； 2. 总结年度报告 1 份。

二、项目验收考核指标

1. 解析营养结构调控初乳 IgA 水平的影响；
2. 揭示候选代谢物对乳腺上皮细胞 pIgR 表达的调控机制；
3. 发表 SCI 论文 2-3 篇；
4. 申请或授权专利 1-2 件。

三、参与人员信息

序号	姓名	性别	年龄	职务	职称	学位	本课题承担任务	所在单位
1	张世海	男	32	无	副教授	博士	课题主持	华南农业大学
2								
3								
4								

四、项目经费

序号	预算科目名称	金额
	（1）	（2）
1	一、中央财政专项资金	72.00
2	（一）直接费用	60.00
3	1.设备费	0.00
4	其中：购置设备费	0.00
5	2.业务费	52.00
6	3.劳务费	8.00
7	（二）间接费用（自动计算）	12.00
8	二、其他来源资金	0.00
9	三、合计	72.00

五、知识产权对策、成果管理及合作权益分配

1. 项目实施过程中所产生的知识产权，优先执行任务下达单位的知识产权管理政策，在此前提下，作如下规定： ①本项目各方独立完成的所有权归各自所有；双方共同完成的由双方共享，具体按照双方的贡献大小进行分配或双方另行商定。 ②双方共有的知识产权（含成果、专利等）的转让，须在双方同意的前提下进行，任何一方不得私自转让或许可实施。 2. 项目成果申报各级奖项，双方单位排名根据具体情况另行商定，人员排名原则上按贡献大小先后排名。 3. 与本项目相关的论文、论著等成果公开发表时，应将本项目名称或项目编号进行标注。

六、任务书条款

第一条	甲乙双方根据《国务院印发关于深化中央财政科技计划（专项、基金）管理改革方案的通知》（国发〔2014〕64 号）、《国务院关于优化科研管理提升科研绩效若干措施的通知》（国发〔2018〕25 号）、《国务院办公厅关于改革完善中央财政科研经费管理的若干意见》（国办发〔2021〕32 号）、《科技部 财政部关于印发<国家重点研发计划管理暂行办法>的通知》（国科发资〔2017〕152 号）、《财政部 科技部关于印发<国家重点研发计划资金管理办法>的通知》（财教〔2021〕178 号）、《科学技术活动违规行为处理暂行规定》（科学技术部令第 19 号）、《科技部财政部关于印发<中央财政科技计划（专项、基金等）监督工作暂行规定>的通知》（国科发政〔2015〕471 号）、《科技部 自然科学基金委关于进一步压实国家科技计划（专项、基金等）任务承担单位科研作风学风和科研诚信主体责任的通知》（国科发监〔2020〕203 号）等有关文件规定，以及有关法律、政策和管理要求，依据项目立项通知，签署本任务书。
第二条	甲方的权利和义务： 1. 按任务书规定进行经费核拨的有关工作协调。 2. 根据甲方需要，在不影响乙方工作的前提下，定期或不定期对乙方项目的实施情况和经费使用情况进行检查或抽查。 3. 根据《广东省科技计划项目信用管理办法（试行）》对乙方进行科技计划信用管理。
第三条	乙方的权利义务： 1. 确保落实自筹经费及有关保障条件。 2. 按任务书规定，对甲方核拨的经费实行专款专用，单独列账，并随时配合甲方进行监督检查。 3. 使用财政资金采购设备、原材料等，按照《广东省实<中华人民共和国招标投标法>办法》有关规定，符合招标条件的须进行招标。 4. 项目实施完成或实施到一定程度，须按照《广东省省级科技计划项目结题管理的实施细则（试行）》提出验收或终止结题的申请，并按

	甲方要求做好项目结题工作。 5. 在每年的12月向甲方如实提交上年度工作情况报告，报告内容包含上年度项目进展情况、经费决算和取得的成效等。 6. 按照国家和省有关规定，每年须提交年度科技报告；项目验收时，须提交验收科技报告。
第四条	在履行本任务书的过程中，如出现广东省相关政策法规重大改变等不可抗力情况，甲方有权对所核拨经费的数量和时间进行相应调整。
第五条	在履行本任务书过程中，需要对项目起止时间、项目经费使用（包括自筹经费、经费分配及经费支出预算等）、项目内容（包括研发内容、技术指标、经济指标及成果指标等）、项目名称、项目承担单位（包括承担单位更名、承担单位替换）、参与单位、项目负责人和成员等进行变更的，甲乙双方须按照《广东省省级科技计划项目合同书管理的实施细则（试行）》有关规定执行。
第六条	在履行本任务书的过程中，当事人一方发现可能导致项目整体或部分失败的情形时，应及时通知另一方，并采取适当措施减少损失，没有及时通知并采取适当措施，致使损失扩大的，应当就扩大的损失承担责任。
第七条	本项目技术成果的归属、转让和实施技术成果所产生的经济利益的分享，除双方另有约定外，按国家和广东省有关法规执行。
第八条	属技术保密的项目，甲乙双方应另行订立技术保密条款，作为本任务书正式内容的一部分，与本任务书具有同等效力。
第九条	根据项目具体情况，经双方另行协商订立的附加条款，作为本任务书正式内容的一部分，与本任务书具有同等效力。
第十条	本任务书的争议应由双方本着协商一致的原则解决，如双方协商不成的，则应向甲方所在地法院提起诉讼。
第十一条	保密条款： 1. 本任务书保密内容范围为： 无。 2. 本任务书保密期限为： 无。 3. 乙方应与可能知悉保密内容的人员签订技术秘密保护协议。 4. 各方应建立技术保密保护制度。 5. 属技术保密的项目必须经省负责技术保密部门的审查后，确定可否发表或用于国际合作和交流。
第十二条	甲方可根据具体情况决定乙方是否需要单位担保，若需要保证单位，应订立担保条款，作为本任务书正式内容一部分。当乙方不履行或不完全履行本任务书，以及没有或没有完全承担违约责任时，乙方的保证单位承担连带保证责任。
第十三条	本任务书一式两份，各份具有同等效力。甲、乙方各留存一份，本任务书自签订之日起生效，有效期至项目结题后一年内。各方均应负任务书的法律责任，不应受机构、人事变动的影响。

七、签约各方

项目承担单位（甲方）：华南农业大学（盖章） 项目负责人： 年　月　日
任务承担单位（乙方）：华南农业大学（盖章） 任务负责人： 年　月　日

受理编号：c21140500001015

项目编号：2021A1515010440

文件编号：粤基金字〔2021〕4号

广东省基础与应用基础研究基金项目

合同书

项目名称：GPR43介导Gα信号通路调控母猪泌功能的机制研究

项目类别：广东省自然科学基金-面上项目

项目起止时间：2021-01-01　至　2023-12-31

管理单位（甲方）：广东省基础与应用基础研究基金委员会

依托单位（乙方）：华南农业大学

通讯地址：广东省广州市天河区五山路483号

邮政编码：510642　　单位电话：020-85283435

项目负责人：张世海　　联系电话：13660346296

2021A1515010440

（广东科技微信公众号）　（查看合同书信息）　（受理纸质材料二维码）

广东省基础与应用基础研究
基金委员会
二〇二〇年制

填写说明

一、项目合同书内容原则上要求与申报书相关内容保持一致，不得无故修改。

二、项目承担单位通过广东省科技业务管理阳光政务平台下载项目合同书，按要求完成签名盖章后提交至省科技厅受理窗口。

三、签名盖章说明。请分别在单位工作分工及经费分配情况页、人员信息页、签约各方页等地方按要求签字或盖章，签章不合规或错漏将不予受理。其中，人员信息页要求所有参与人员本人亲笔签名，代签或印章无效，漏签将不予受理。

四、本合同书自签字并加盖公章之日起生效，各方均应负本合同书的法律责任，不应受机构、人事变动影响。

2021A1515010440

一、主要研究内容和要达到的目标

研究内容

（1）泌乳期和非泌乳期GPR43在母猪乳腺组织的表达和分布

采用qPCR和Western Blot等手段，研究母猪泌乳期（泌乳启动期和泌乳高峰期）和非泌乳期（空怀期和妊娠期）等不同生理阶段，乳腺中GPR43的mRNA和蛋白表达水平；采用活体采样法，制备母猪乳腺切片，采用免疫组化法研究GPR43在母猪乳腺不同细胞（乳腺上皮细胞，脂肪细胞，肌细胞）中的定位和分布。

（2）GPR43调控母猪乳腺上皮细胞泌乳的信号通路

以母猪乳腺上皮细胞为研究模型，采用过表达（质粒转染）和干扰（siRNA）技术，研究GPR43表达调控对母猪乳腺上皮细胞泌乳（乳脂和乳蛋白合成）的影响；检测Gαi （AC/cAMP/PKA）、Gαq（PLCβ/IP3/Ca2+）、JAK2/STAT5、和mTORC1信号通路的变化；同时，研究GPR43对Raptor（Ser791）、HSL（Ser563）、perilipin（Ser517）等参与泌乳和增殖调控重要信号通路和酶的影响，并通过免疫共沉淀（IP）技术挖掘新的调控蛋白。

（3）短链脂肪酸对母猪乳腺上皮细胞泌乳功能的影响

以母猪乳腺上皮细胞为研究模型，研究不同剂量GPR43营养素配体（乙酸、丙酸和丁酸）对猪乳腺上皮细胞Gαi（AC/cAMP/PKA）、Gαq（PLCβ/IP3/Ca2+）、JAK2/STAT5、和mTORC1信号通路以及母猪乳腺上皮细胞泌乳的影响；探索不同FFAR2配体配伍对母猪乳腺上皮细胞功能的调控；通过shRNA转染建立GPR43母猪乳腺上皮细胞稳定敲除细胞系，检测GPR43配体，在母猪乳腺上皮细胞GPR43敲除细胞系中的作用。

（4）日粮中添加短链脂肪酸盐对母猪泌乳性能的影响

在母猪日粮（妊娠后期和泌乳期）中添加不同剂量的短链脂肪酸，研究其对母猪乳腺发育、泌乳功能（泌乳量和乳成分）、仔猪断奶前体重以及血液的催乳素、雌激素、孕激素水平的变化；采用qPCR、Western Blot和免疫组化法检测乳腺GPR43表达，以及AC/cAMP/PKA、PLCβ/IP3/Ca2+（或PLCβ/DAG/PKC）、JAK2/STAT5、和mTORC1信号通路的影响。

研究目标

（1）揭示GPR43是否是调节乳腺增殖和泌乳功能的关键GPCR受体

（2）短链脂肪酸盐调控母猪乳腺功能的最佳剂量及其生理调控机制

（3）在国内外高水平期刊发表论文2篇

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二、项目预期获得的研究成果及形式

<table>
<tr><td rowspan="3">论文及专著情况</td><td colspan="2">国家统计源刊物以上刊物发表论文（篇）</td><td colspan="2">2</td><td colspan="2">科技报告（篇）</td><td colspan="2">1</td></tr>
<tr><td colspan="2">其中被SCI/EI/ISTP收录论文数（篇）</td><td colspan="2">2</td><td colspan="2">培养人才（人）</td><td colspan="2">2</td></tr>
<tr><td colspan="2">专著（册）</td><td colspan="2"></td><td colspan="2">引进人才（人）</td><td colspan="2"></td></tr>
<tr><td rowspan="3">专利情况(项)</td><td colspan="2">发明专利</td><td colspan="2">实用新型专利</td><td colspan="2">外观设计专利</td><td colspan="2">国外专利</td></tr>
<tr><td>申请</td><td>授权</td><td>申请</td><td>授权</td><td>申请</td><td>授权</td><td>申请</td><td>授权</td></tr>
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三、项目进度和阶段目标

（一）项目起止时间： 2021-01-01 至 2023-12-31

（二）项目实施进度及阶段主要目标:

开始日期	结束日期	主要工作内容
2021-01-01	2021-12-31	（1）GPR43在母猪乳腺不同生理阶段的表达与分布； （2）研究GPR43过表达和干扰对母猪乳腺上皮细胞泌乳功能的影响； （3）参加学术会议一次。
2022-01-01	2022-12-31	（1）GPR43配体（短链脂肪酸）对母猪乳腺上皮细胞泌乳功能的影响； （2）参加学术会议一次； （3）撰写并发表论文一篇。
2023-01-01	2023-12-31	（1）研究日粮中添加短链脂肪酸盐对母猪泌乳和繁殖性能的影响； （2）参加学术会议一次； （3）总结材料，撰写并发表论文一篇。

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四、项目总经费及省基金委经费预算

1.省基金委经费下达总额：　（大写）壹拾万圆整；（小写　）10万元；

2.省基金委经费年度下达计划：

年度	2021 年	年	年	年	年
经费(万元)	10.00				

3.总经费及省基金委经费开支预算计划：

经费筹集情况：　（单位：万元）

省基金委经费	自筹资金				合计
	自有资金	贷款	地方政府投入	其它	
10.00				0	10.00
政府部门、境外资金及其他资金投入情况说明：					

与本项目相关的其他经费来源	（单位：万元）
其他计划资助经费：	
单位配套经费：	
其他经费资助：	
其他经费来源合计：	

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五、人员信息

项目负责人								
姓名	证件号码	年龄	性别	职称	学历	在项目中承担的任务	所在单位	签名
张世海	350783198901020259	32	男	副教授	博士研究生	项目负责人	华南农业大学	

项目组主要成员								
姓名	证件号码	年龄	性别	职称	学历	在项目中承担的任务	所在单位	签名
管武太	14242919631105081X	58	男	教授	博士研究生	试验设计	华南农业大学	
田敏	431121199302030010	28	男	未取得	博士研究生	乳腺细胞分子试验	华南农业大学	
恒景会	411422199401214842	27	女	未取得	硕士研究生	乳腺细胞分子试验	华南农业大学	
吴志辉	440902199607100871	25	男	未取得	硕士研究生	母猪饲养试验	华南农业大学	

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六、工作分工及经费分配

承担/参与单位名称（盖章）	工作分工	总经费分摊（万元）	省基金委经费分配（万元）
华南农业大学	项目执行	10.00	10.00
	合计	10.00	10.00

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七、合同条款

第一条 甲方与乙方根据《中华人民共和国合同法》及国家有关法规和规定，为顺利完成（2021）年GPR43介导Gα信号通路调控母猪泌功能的机制研究 专项项目（文件编号： 粤基金字〔2021〕4号）经协商一致，特订立本合同，作为甲乙双方在项目实施管理过程中共同遵守的依据。

第二条 甲方的权利义务：

1.按合同书规定进行经费核拨的有关工作协调。

2.根据甲方需要，在不影响乙方工作的前提下，定期或不定期对乙方项目的实施情况和经费使用情况进行检查或抽查。

3.根据《广东省科技计划项目信用管理办法(试行)》对乙方进行科技计划信用管理。

第三条 乙方的权利义务：

1.确保落实自筹经费及有关保障条件。

2.乙方是项目资金管理的责任主体，应当建立健全科研项目资金管理制度，严格按照省科技经费使用范围和有关规定管好用好财政资金；应当按合同书规定，对甲方核拨的经费实行专款专用，单独列账，并随时配合甲方进行监督检查。

3.实施“包干制”的面上项目及青年基金项目，依托单位应参照国家杰出青年科学基金试点项目经费使用“包干制”要求，制定经费使用“包干制”内部管理规定。项目经费支出应实际用于研发活动相关支出，使用范围限于设备费、材料费、测试化验加工费、燃料动力费、差旅/会议/国际合作与交流费、出版/文献/信息传播/知识产权事务费、劳务费、专家咨询费、依托单位管理费用、绩效支出以及其他合理支出。依托单位管理费用由依托单位根据实际管理支出情况与项目负责人协商确定。绩效支出由项目负责人根据实际科研需要和相关薪酬标准自主确定，依托单位按照现行工资制度进行管理。其余用途经费无额度限制，由项目负责人根据实际需要自主决定使用。项目验收时应提交经费决算表。

4.项目负责人是项目资金使用的直接责任人，对资金使用的合规性、合理性、真实性和相关性承担法律责任。

5.使用财政资金采购设备、原材料等，按照《广东省实施〈中华人民共和国招标投标法〉办法》有关规定，符合招标条件的须进行招标。

6.项目合同任务完成后，或合同书规定的任务、指标及经费投入等提前完成的，乙方可按照《广东省省级科技计划项目结题管理实施细则（试行）》提出验收结题申请，并按甲方要求做好项目验收结题工作。

7.若项目发生需要终止结题的情况，乙方须按照《广东省省级科技计划项目结题管理的实施细则（试行）》提出终止结题申请，并按甲方要求做好项目终止结题工作。

8.在每年规定时间内向甲方如实提交上年度工作情况报告，报告内容包含上年度项目进展情况、经费决算和取得的成果等。

9.按照国家和省有关规定，提交科技报告及其他材料。

10.利用甲方的经费获得的研究成果，项目负责人和参与者应当注明获得“广东省基础与应用基础研究基金（英文：Guangdong Basic and Applied?Basic Research Foundation）（项目编号）”资助或作有关说明。

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11.乙方要恪守科学道德准则，遵守科研活动规范，践行科研诚信要求，不得抄袭、剽窃他人科研成果或者伪造、篡改研究数据、研究结论；不得购买、代写、代投论文，虚构同行评议专家及评议意见；不得违反论文署名规范，擅自标注或虚假标注获得科技计划（专项、基金等）等资助；不得弄虚作假，骗取科技计划（专项、基金等）项目、科研经费以及奖励、荣誉等；不得有其他违背科研诚信要求的行为。

12.确保本项目开展的研究工作符合我国科研伦理管理相关规定。

第四条 在履行本合同的过程中，如出现广东省相关政策法规重大改变等不可抗力情况，甲方有权对所核拨经费的数量和时间进行相应调整。

第五条 在履行本合同的过程中，当事人一方发现可能导致项目整体或部分失败的情形时，应及时通知另一方，并采取适当措施减少损失，没有及时通知并采取适当措施，致使损失扩大的，应当就扩大的损失承担责任。

第六条 本项目技术成果的归属、转让和实施技术成果所产生的经济利益的分享，除双方另有约定外，按国家和广东省有关法规执行。

第七条 根据项目具体情况，经双方另行协商订立的附加条款，作为本合同正式内容的一部分，与本合同具有同等效力。

第八条 本合同一式三份，各份具有同等效力。甲、乙方及项目负责人各执一份，三方签字、盖章后即生效，有效期至项目结题后一年内。各方均应负合同的法律责任，不应受机构、人事变动的影响。

第九条 乙方必须接受甲方聘请的本项目合同监理单位的监督和管理。监理单位按照甲方赋予的权利对本项目合同的履行进行审核、进度调查，对项目合同变更、经费使用情况进行监督管理及组织项目验收。

说明：1.本合同书中，凡是当事人约定无需填写的内容，应在空白处划（/）。

2.委托代理人签订本合同书的，应出具合法、有效的委托书。

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八、本合同签约各方

管理单位（甲方）：　　广东省基础与应用基础研究基金委员会　（盖章）

法定代表人（或法人代理）：　曾路　（签章）

2021 年　03 月　24 日

依托单位（乙方）：　华南农业大学　（盖章）

法定代表人（或法人代理）：　刘雅红　（签章）

联系人（项目主管）姓名：　安沛（基金类基础科、成果登记成果科、实验室平台办、海外名师人事处）　（签章）

Email：　414484016@qq.com

电话：　020-85283435　/　13535577893

开户单位名称：　华南农业大学

开户银行名称：　广东广州工行五山支行

开户银行帐号：　3602002609000310520

年　月　日

联系人（项目负责人）姓名：　张世海　（签名）

Email：　zhangshihai@scau.edu.cn

电话：　13660346296

年　月　日

2021A1515010440

受理编号：c23140500000638

项目编号：2023A1515012098

文件编号：粤基金字〔2023〕2号

广东省基础与应用基础研究基金项目

任务书

项目名称：油酸通过GPR120调节母猪乳脂合成的机制研究

项目类别：广东省自然科学基金-面上项目

项目起止时间：2023-01-01　至　2025-12-31

管理单位（甲方）：广东省基础与应用基础研究基金委员会

依托单位（乙方）：华南农业大学

通讯地址：广东省广州市天河区五山路483号

邮政编码：510642　　单位电话：020-85283435

项目负责人：张世海　　联系电话：13660346296

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（广东科技微信公众号）　（查看任务书信息）　（受理纸质材料二维码）

广东省基础与应用基础研究
基金委员会
二〇二〇年制

填写说明

一、项目任务书内容原则上要求与申报书相关内容保持一致，不得无故修改。

二、项目承担单位通过广东省科技业务管理阳光政务平台下载项目任务书，按要求完成签名盖章后扫描上传到广东省科技业务管理阳光政务平台。

三、签名盖章说明。请分别在单位工作分工及经费分配情况页、人员信息页、签约各方页等地方按要求签字或盖章，签章不合规或错漏将不予受理。其中，人员信息页要求所有参与人员本人亲笔签名，代签或印章无效，漏签将不予受理。

四、本任务书自签字并加盖公章之日起生效，各方均应负本任务书的法律责任，不应受机构、人事变动影响。

五、根据《广东省科学技术厅广东省财政厅关于深入推进省基础与应用基础研究基金项目经费使用“负面清单+包干制”改革试点工作的通知》(粤科规范字〔2022〕2号)，2022年度及以后立项资助的全部省基金项目(包括省自然科学基金、省市联合基金、省企联合基金项目等)均适用“负面清单+包干制”，项目提交申请书和任务书时无需编制费用明细科目预算。

2023A1515012068

一、主要研究内容和要达到的目标

1. 研究内容

（1）泌乳期不同阶段GPR120在母猪乳腺组织中的表达

研究母猪泌乳期不同生理阶段，乳腺组织中GPR120的mRNA和蛋白表达水平；采用活体采样法，制备母猪乳腺切片，采用免疫组化法研究GPR120在母猪乳腺不同细胞（乳腺上皮细胞、脂肪细胞和肌细胞）中的定位和分布。

（2）GPR120调控母猪乳腺上皮细胞乳合成的作用及其信号通路

分离原代母猪乳腺上皮细胞，采用过表达和干扰技术，研究GPR120表达调控对母猪乳腺上皮细胞乳脂合成的影响；检测Gαi、Gαq、JAK2/STAT5和mTORC1信号通路的变化；同时，研究GPR120对乳脂合成过程中参与泌乳调控限速酶的影响，并以免疫共沉淀为手段挖掘新调控分子。

（3）油酸对母猪乳腺上皮细胞和母猪乳腺切片乳合成的作用

以母猪乳腺上皮细胞和母猪乳腺切片为研究模型，研究不同剂量油酸对母猪乳腺上皮细胞和乳腺切片中Gαi、Gαq、JAK2/STAT5和mTORC1信号通路的影响；探索油酸对母猪乳腺上皮细胞泌乳功能的调控机制；建立GPR120母猪乳腺上皮细胞稳定敲减细胞系，检测油酸在母猪乳腺上皮细胞GPR120敲减细胞系中的作用。在母猪乳腺切片中，研究对油酸对其相关信号通路的作用，并通过C13标记油酸，检测油酸在乳腺切片中的代谢情况，探索油酸是否作为底物参与乳成分的合成。

（4）长链脂肪酸组合对母猪泌乳性能以及哺乳仔猪生长发育调控的验证

在泌乳期母猪饲粮中添加不同剂量的油酸，研究其对母猪乳腺发育、泌乳功能、仔猪断奶前体重以及血液中催乳素、雌激素、孕激素水平的变化；采用不同生物学手段，检测乳腺GPR120的表达以及泌乳相关信号通路的影响。验证油酸对母猪泌乳性能以及哺乳仔猪生长性能的作用。

2. 研究目标

（1）以母猪乳腺上皮细胞、乳腺切片和活体母猪为模型，探索GPR120及其相关GPCR信号通路对乳脂合成的调控机制。

（2）阐明母猪泌乳期日粮中添加油酸对乳脂合成的生物学作用，并揭示其生理调控机制。

（3）发表高水平论文1-2篇

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二、项目预期获得的研究成果及形式

<table>
<tr><td rowspan="3">论文及专著情况</td><td colspan="2">国家统计源刊物以上刊物发表论文（篇）</td><td colspan="2">2</td><td colspan="2">科技报告（篇）</td><td colspan="2">1</td></tr>
<tr><td colspan="2">其中被SCI/EI/ISTP收录论文数（篇）</td><td colspan="2">2</td><td colspan="2">培养人才（人）</td><td colspan="2">2</td></tr>
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<tr><td rowspan="3">专利情况(项)</td><td colspan="2">发明专利</td><td colspan="2">实用新型专利</td><td colspan="2">外观设计专利</td><td colspan="2">国外专利</td></tr>
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</table>

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三、项目进度和阶段目标

（一）项目起止时间： 2023-01-01 至 2025-12-31		
（二）项目实施进度及阶段主要目标:		
开始日期	结束日期	主要工作内容
2023-01-01	2023-12-31	（1）解析GPR120在母猪乳腺不同生理阶段的表达； （2）构建GPR120过表达和干扰载体（siRNA和shRNA）； （3）参加学术会议一次。
2024-01-01	2024-12-31	（1）研究GPR120过表达和干扰对母猪乳腺上皮细胞乳脂合成的影响； （2）揭示油酸对母猪乳腺上皮细胞乳和乳腺切片中乳脂合成的影响和信号通路的调控作用； （3）参加学术会议一次。
2025-01-01	2025-12-31	（1）油酸对母猪泌乳性能以及哺乳仔猪生长发育调控的验证； （2）发表SCI论文； （2）总结材料。

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四、项目总经费及省基金委经费预算

1.省基金委经费下达总额：　（大写）壹拾万圆整；（小写 ）10万元；					
2.省基金委经费年度下达计划：					
年度	2023 年	年	年	年	年
经费(万元)	10.00				

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五、人员信息

项目负责人								
姓名	证件号码	年龄	性别	职称	学历	在项目中承担的任务	所在单位	签名
张世海	350783198901020259	34	男	副教授	博士研究生	项目负责人	华南农业大学	

项目组主要成员								
姓名	证件号码	年龄	性别	职称	学历	在项目中承担的任务	所在单位	签名
管武太	14242919631105081X	60	男	教授	博士研究生	试验设计	华南农业大学	
田敏	431121199302030010	30	男	未取得	博士研究生	乳腺细胞分子试验	华南农业大学	
亓迎澳	410329199808201013	25	男	未取得	本科	母猪饲养试验	华南农业大学	

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六、工作分工及财政经费分配

承担/参与单位名称（盖章）	工作分工	省级财政科技资金分配（万元）
华南农业大学	项目执行	10.00
	合计	10.00

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七、任务书条款

第一条 甲方与乙方根据《中华人民共和国民法典》及国家有关法规和规定，按照《广东省科学技术厅关于广东省基础与应用基础研究基金（省自然科学基金、联合基金等）项目管理的实施细则（试行）》《广东省省级科技计划项目验收结题工作规程（试行）》等规定，为顺利完成（2023）年油酸通过GPR120调节母猪乳脂合成的机制研究专项项目（文件编号：粤基金字〔2023〕2号 ）经协商一致，特订立本任务书，作为甲乙双方在项目实施管理过程中共同遵守的依据。

第二条 甲方的权利义务：

1.按任务书规定进行经费核拨的有关工作协调。

2.根据甲方需要，在不影响乙方工作的前提下，定期或不定期对乙方项目的实施情况和经费使用情况进行检查或抽查。

3.根据《广东省科研诚信管理办法(试行)》等规定对乙方进行科技计划信用管理。

第三条 乙方的权利义务：

1.确保落实自筹经费及有关保障条件。

2.按任务书规定，对甲方核拨的经费实行专款专用，单独列账，并随时配合甲方进行监督检查。

3.经费使用按照广东省级财政科研项目经费使用等有关规定进行管理。

4.项目依托单位应制定经费使用“负面清单+包干制”内部管理制度并报甲方备案。

5.使用财政资金采购设备、原材料等，按照《广东省实施〈中华人民共和国招标投标法〉办法》有关规定，符合招标条件的须进行招标。

6.项目任务书任务完成后，或任务书规定的任务、指标及经费投入等提前完成的，乙方可提出验收结题申请，并按甲方要求做好项目验收结题工作。

7.若项目发生需要终止结题的情况，乙方须提出终止结题申请，并按甲方要求做好项目终止结题工作。

8.在每年规定时间内向甲方如实提交上年度工作情况报告，报告内容包含上年度项目进展情况、经费决算和取得的成果等。

9.按照国家和省有关规定，提交科技报告及其他材料。

10.利用甲方的经费获得的研究成果，项目负责人和参与者应当注明获得“广东省基础与应用基础研究基金（英文：Guangdong Basic and Applied Basic Research Foundation）（项目编号）”资助或作有关说明。

11.乙方要恪守科学道德准则，遵守科研活动规范，践行科研诚信要求，不得抄袭、剽窃他人科研成果或者伪造、篡改研究数据、研究结论；不得购买、代写、代投论文，虚构同行评议专家及评议意见；不得违反论文署名规范，擅自标注或虚假标注获得科技计划（专项、基金等）等资助；不得弄虚作假，骗取科技计划（专项、基金等）项目、科研经费以及奖励、荣誉等；不得有其他违背科研诚信要求的行为。

12.确保本项目开展的研究工作符合我国科技伦理管理相关规定。

第四条 在履行本任务书的过程中，如出现广东省相关政策法规重大改变等不可抗力情况，甲方有权对所核拨经费的数量和时间进行相应调整。

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第五条 在履行本任务书的过程中，当事人一方发现可能导致项目整体或部分失败的情形时，应及时通知另一方，并采取适当措施减少损失，没有及时通知并采取适当措施，致使损失扩大的，应当就扩大的损失承担责任。

第六条 本项目技术成果的归属、转让和实施技术成果所产生的经济利益的分享，除双方另有约定外，按国家和广东省有关法规执行。

第七条 根据项目具体情况，经双方另行协商订立的附加条款，作为本任务书正式内容的一部分，与本任务书具有同等效力。

第八条 本任务书一式三份，各份具有同等效力。甲、乙方及项目负责人各执一份，三方签字、盖章后即生效，有效期至项目结题后一年内。各方均应负任务书的法律责任，不应受机构、人事变动的影响。

第九条 乙方必须接受甲方聘请的本项目任务书监理单位的监督和管理。监理单位按照甲方赋予的权利对本项目任务书的履行进行审核、进度调查，对项目任务书变更、经费使用情况进行监督管理及组织项目验收。

说明：1.本任务书中，凡是当事人约定无需填写的内容，应在空白处划（/）。
2.委托代理人签订本任务书的，应出具合法、有效的委托书。

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八、本任务书签约各方

管理单位（甲方）：　　广东省基础与应用基础研究基金委员会　（盖章）

法定代表人（或法人代理）：　曾路　（签章）

2023 年　05 月　25 日

依托单位（乙方）：　华南农业大学　（盖章）

法定代表人（或法人代理）：　刘雅红　（签章）

联系人（项目主管）姓名：　倪慧群　（签章）

Email：　kjcgxk@scau.edu.cn

电话：　020-85283435　/　15920301530

开户单位名称：　华南农业大学

开户银行名称：　广东广州工行五山支行

开户银行账号：　3602002609000310520

年　月　日

联系人（项目负责人）姓名：　张世海　（签名）

Email：　zhangshihai@scau.edu.cn

电话：　13660346296

年　月　日

2023A1515012098

项目编号： 202102020056

广州市科技计划项目

合同书

项目名称： FFAR2对母猪泌乳功能的调节作用及其机制研究

计划类别： 基础研究计划

专题名称： 基础与应用基础研究项目（博士青年科技人员类）

起止时间： 2021年04月01日 至 2023年03月31日

承担单位： 华南农业大学

组织单位： 华南农业大学

责任处室： 基础研究处

填表日期： 2021年04月09日

202102020056

广州市科学技术局制

（2021年版）

填 写 说 明

一、本合同书的项目编号由市科学技术局（以下简称市科技局）统一确定。

二、本合同书由申报书在后台自动转换生成，如有错漏之处需修正，请联系市科技局项目责任处室退回承担单位修正。

三、本项目如涉及多家（包含两家）单位参加，乙方应在签订本合同书前与合作单位就任务分工、经费和知识产权分配等问题签订有关合同或协议，作为本合同书的附件。

四、本合同书适用于广州市事前资助类科技计划项目，有特殊要求另行制定的除外。

202102020056

一、项目基本信息

<table>
<tr><td>项目名称</td><td colspan="4">FFAR2对母猪泌乳功能的调节作用及其机制研究</td></tr>
<tr><td>技术领域</td><td colspan="4">农业与食品-畜牧业-营养与控制</td></tr>
<tr><td rowspan="8">承担单位</td><td>名　　称</td><td colspan="3">华南农业大学</td></tr>
<tr><td>通信地址</td><td colspan="3">广东省广州市天河区五山路483号</td></tr>
<tr><td>邮政编码</td><td>510642</td><td>传　　真</td><td>85281885</td></tr>
<tr><td>单位特性</td><td></td><td>单位类型</td><td>高等院校</td></tr>
<tr><td>统一社会信用代码
或组织机构代码</td><td colspan="3">12440000455416563 4</td></tr>
<tr><td>法定代表人</td><td>刘雅红</td><td>电子邮箱</td><td>gale@scau.edu.cn</td></tr>
<tr><td>联系人</td><td>侯建国</td><td>联系电话</td><td>02038632413</td></tr>
</table>

202102020056

二、项目组人员信息

项目负责人	姓名	张世海	证件类型	身份证	证件号码	350783198901020259	性别	男
	出生年月	1989年01月02日	民族	汉族	国籍	中国	学历	博士研究生
	学位	博士	学位授予国家（或地区）	中国	职务	无	职称	副教授
	所学专业	动物营养与饲料科学	手机号码	13660346296	办公电话	13660346296	电子邮箱	zhangshihai@scau.edu.cn

项目组成员（含项目负责人）

序号	姓名	证件类型	证件号码	年龄	性别	职务	职称	学历	现从事专业	分工	所在单位
1	张世海	身份证	350783198901020259	31	男	无	副教授	博士研究生	动物营养与饲料科学	项目负责人	华南农业大学
2	田敏	身份证	431121199302030010	27	男	无	未取得	博士研究生	动物营养与饲料科学	乳腺细胞分子试验	华南农业大学
3	恒景会	身份证	411422199401214842	26	女	无	未取得	硕士研究生	动物营养与饲料科学	乳腺细胞分子试验	华南农业大学
4	吴志辉	身份证	440902199607100871	23	男	无	未取得	硕士研究生	动物营养与饲料科学	母猪饲养实验	华南农业大学

2021020200

三、项目实施内容

（一）项目概述

母乳是仔猪早期生长发育的决定性因素。本实验室发现GPCR信号通路在乳腺中具有重要生理功能。本项目以母猪乳腺上皮细胞为模型，研究FFAR2过表达和干扰以及短链脂肪酸处理对乳腺泌乳功能的影响。最后，通过母猪试验验证相关结果。该研究可阐明短链脂肪酸通过FFAR2对乳腺功能的调控机制。拟发表SCI 2篇。

（二）项目研究内容

4.1 研究目标和内容

4.1.1 研究目标：

（1）以母猪乳腺上皮细胞和活体母猪为模型探索FFAR2对母猪乳腺增殖和泌乳的调控机制。

（2）阐明母猪妊娠后期和泌乳期日粮中添加短链脂肪酸盐对母猪乳腺增殖和泌乳调控的作用，并揭示其生理调控机制。

4.1.2 研究内容：

（1）泌乳期和非泌乳期FFAR2在母猪乳腺组织的表达和分布

采用qPCR和Western Blot等手段，研究母猪泌乳期（泌乳启动期和泌乳高峰期）和非泌乳期（空怀期和妊娠期）等不同生理阶段，乳腺中FFAR2的mRNA和蛋白表达水平；采用活体采样法，制备母猪乳腺切片，采用免疫组化法研究FFAR2在母猪乳腺不同细胞（乳腺上皮细胞，脂肪细胞，肌细胞）中的定位和分布。

（2）FFAR2调控母猪乳腺上皮细胞泌乳的信号通路

以母猪乳腺上皮细胞为研究模型，采用过表达（质粒转染）和干扰（siRNA）技术，研究FFAR2表达调控对母猪乳腺上皮细胞泌乳（乳脂和乳蛋白合成）的影响；检测Gαi （AC/cAMP/PKA）、Gαq（PLCβ/IP3/Ca2+）、JAK2/STAT5、和mTORC1信号通路的变化；同时，研究FFAR2对Raptor（Ser791）、HSL（Ser563）、perilipin（Ser517）等参与泌乳和增殖调控重要信号通路和酶的影响，并通过免疫共沉淀（IP）技术挖掘新的调控蛋白。

（3）短链脂肪酸对母猪乳腺上皮细胞泌乳功能的影响

以母猪乳腺上皮细胞为研究模型，研究不同剂量FFAR2营养素配体（乙酸、丙酸和丁酸）对猪乳腺上皮细胞Gαi（AC/cAMP/PKA）、Gαq（PLCβ/IP3/Ca2+）、JAK2/STAT5、和mTORC1信号通路以及母猪乳腺上皮细胞泌乳的影响；探索不同FFAR2配体配伍对母猪乳腺上皮细胞功能的调控；通过shRNA转染建立FFAR2母猪乳腺上皮细胞稳定敲除细胞系，检测FFAR2配体，在母猪乳腺上皮细胞FFAR2敲除细胞系中的作用。

（4）日粮中添加短链脂肪酸盐对母猪泌乳性能的影响

在母猪日粮（妊娠后期和泌乳期）中添加不同剂量的短链脂肪酸，研究其对母猪乳腺发育、泌乳功能（泌乳量和乳成分）、仔猪断奶前体重以及血液的催乳素、雌激素、孕激素水平的变化；采用qPCR、Western Blot和免疫组化法检测乳腺FFAR2表达，以及AC/cAMP/PKA、PLCβ/IP3/Ca2+（或PLCβ/DAG/PKC）、JAK2/STAT5、和mTORC1信号通路的影响。

4.2 拟解决的关键技术问题

（1）FFAR2是否是调节乳腺增殖和泌乳功能的关键GPCR受体？

已经有大量研究表明，短链脂肪酸可通过FFAR2调节许多重要的生理功能。但是，目前FFAR2在乳腺中的生物学功能尚不明确。本项目若能阐明FFAR2在母猪乳腺中的生理功能，不但具有重要的生理学价值，同时也能为以FFAR2为营养素调控靶点，调控母猪的泌乳功能，打下理论基础。

（2）短链脂肪酸盐调控母猪乳腺功能的最佳剂量及其生理调控机制？

短链脂肪酸包括乙酸、丙酸和丁酸，理论上它们都可以激活FFAR2。然而，近年来研究发现，不同的配体可激活同一个GPCR受体不同的下游信号通路（如Gαi偏好或Gαq偏好）。目前，已有研究报道FFAR2可同时激活Gαi和Gαq信号通路。而不同短链脂肪酸对母猪乳腺Gαi或Gαq的偏好性激活，尚不明确。探索调节乳腺功能的最佳短链脂肪酸具有重要意义。同时，探索短链脂肪酸是否通过卵巢等生殖器官间接调控乳腺功能也具有重要的学术价值。

4.3 主要创新点

（1）项目选题新颖，内容独特。GPCR信号通路在机体中具有重要的生理功能。目前，约有三分之一FAD认证药物的调控靶点都集中在GPCR受体上。近年来，研究发现部分营养素也可激活GPCR受体并调控下游信号通路。本实验室前期发现GPCR信号通路在母猪乳腺具有重要调控功能（增殖和泌乳）。但是，目前尚不清楚乳腺中核心的GPCR受体。已有报道发现，母猪血液短链脂肪酸水平和母猪泌乳功能密切相关。因此，FFAR2可能是介导乳腺功能的重要受体分子。本项目的研究结果不仅可以揭示FFAR2在乳腺中的调控机制；也

可以为生产实践中，母猪饲粮中添加短链脂肪酸调控母猪泌乳功能，提供一定理论依据。
（2）研究策略有新意。母猪的泌乳性能是影响仔猪早期生长发育的决定性因素。为了更系统研究母猪乳腺功能，本课题从母猪乳腺上皮细胞、母猪乳腺切片和母猪活体试验三个维度共同探讨FFAR2在乳腺中的作用。使用siRNA、shRNA、质粒过表达等分子生物学手段精准调控FFAR2在母猪乳腺上皮细胞中的表达。同时，使用免疫共沉淀（IP）-Mass Spec技术，探索短链脂肪酸激活乳腺GPCR受体的重要下游信号分子，揭示GPCR参与调控的信号通路在乳腺中的作用。

（三）项目预期风险及规避措施

（1）预期风险
中国是养殖业大国。正常情况下，我国生猪出栏约7亿头，其中母猪5000万头。根据农业部统计数据，2018年猪饲料产量为9720万吨。2019-2020年，受非洲猪瘟和COVID-2019“双非”影响。我国养猪业和猪肉进口业收到重创。目前，国家开始鼓励企业大力建猪场。国内即将迎来养猪的新一轮高峰。新型母猪饲料的研发，在国内市场具有巨大潜力。预计可达到200多万吨。因此，拟报项目产品市场广阔，市场风险小。
（2）规避措施
本项目拥有良好试验平台，试验条件完善，研究方法可行。本课题所在的实验室，与广州温氏集团有紧密合作，具有良好的动物试验条件基础，属于“国家生猪种业工程技术研究中心”、农业部“华南动物营养与饲料科学观测实验站”、及广东省教育厅“现代生物技术重点开放实验室”。项目组成员主要由长期在科研一线工作的高学历青年教师和研究生组成，长期从事与本项目相关的课题。因此，在团队全体成员合作努力下，本项目可以顺利完成。

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四、项目主要验收指标

（一）主要技术指标

通过营养素调控提高母猪的泌乳力。

（二）主要技术成果

序号	成果形式		成果数量
1	新产品（或新材料、新装备、新品种/系）	无	0
2	新工艺（或新方法、新模式、新技术、新服务）	无	0
3	发明专利（件）	申请	0
		授权	0
4	实用新型专利（件）	申请	0
		授权	0
5	外观设计专利（件）	申请	0
		授权	0
6	国外专利（件）	PCT受理	0
		授权	0
7	技术标准制定（个）	牵头	0
		参与	0
8	软件著作权（项）		0
9	论文论著（篇）	SCI/EI/ISTP	2
		其他	0
10	创新平台（载体）项目	技术服务数量（项）	0
		服务企业数量（家）	0
11	获得国家级奖项（项）		0
12	获得省级奖项（项）		0
13	科技人才奖励（人）		0
14	引进人才（人）		0
15	培养人才（人）	博士	0
		硕士	2

其他成果及形式说明（新药证书、动植物新品种、创新特色、成果宣传推介措施等）

提供一个改善母猪泌乳性能的营养素调控方案

（三）主要经济指标及社会效益

序号	指标名称	指标值
1	实施期内项目销售收入（万元）	/
2	实施期内项目出口创汇（万美元）	/
3	实施期内项目新增就业人数（人）	/

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其他经济指标及社会效益说明
无

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五、项目经费预算

（单位：万元）

项目经费： 15.00				
资金来源	小计	市科技局经费	其他财政经费	自筹资金
2021年	5.00	5.00	0.00	0.00
2022年	5.00	0.00	0.00	5.00
2023年	5.00	0.00	0.00	5.00
合计	15.00	5.00	0.00	10.00

注：项目经费实行“包干制”，经费使用按项目承担单位或组织单位相关科研项目经费管理办法执行。

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六、共同条款

第一条　甲、乙、丙三方根据《中华人民共和国科学技术进步法》《广东省自主创新促进条例》《广州市科技创新促进条例》及《中华人民共和国民法典》等国家有关法规和规定，经协商一致，特订立本合同，作为甲、乙、丙三方在合同执行中共同遵守的依据。

第二条　甲、乙、丙三方应当严格履行《广州市科技计划项目管理办法》《广州市级财政科研项目资金绩效提升和管理监督办法》《广州市科技创新发展专项资金管理办法》《广州市科技计划项目经费“包干制”改革试点工作方案》《广州市科技创新发展专项项目全过程管理简政放权改革试点工作方案》的规定要求。

第三条　甲方应：

1.根据财政经费预算安排，及时拨付项目经费。

2.赋予乙方和丙方广州市科技业务管理阳光政务平台（以下简称阳光政务平台）的使用权限，保障丙方进行项目全过程管理的使用需求。

3.按时拨付项目经费。

4.审核丙方提交的年度工作报告，制定下一年度的资金切块方案。

5.对丙方进行周期绩效考核和检查评估，重新评估丙方资格。

第四条　乙方应：

1.作为项目具体组织实施的责任主体，为本单位提供的与本项目有关的全部材料真实、合法、有效性负责；

2.按照《合同书》规定的内容组织实施项目，接受并配合甲方、丙方以及各级财政、审计部门，或上述部门委托的机构进行评估、稽查、审计、检查和绩效评价，并按要求提供项目任务与预算执行情况和有关财务资料；

3.按照市财政科技经费管理“包干制”相关要求对项目经费单独设账，专款专用；

4.保证自筹资金按时到位和其它配套条件的落实；

5.在项目研究开发过程中优先考虑使用“广东省科技资源共享服务平台”的仪器设备，项目购置的设备仪器若符合入网条件应及时办理入网手续对社会共用共享，提高设备仪器的使用率。按照《中华人民共和国采购法》要求，对符合政府采购范围的设备仪器，执行政府采购；

6.项目合同执行期内需进行变更的，按照《广州市科技计划项目管理办法》《广州市级财政科研项目资金绩效提升和管理监督办法》《广州市科技创新发展专项资金管理办法》《广州市科技创新发展专项项目全过程管理简政放权改革试点工作方案》相关程序办理；

7.项目合同执行期满后3个月内向丙方提出验收申请，提前完成合同规定任务的可提前申请验收；

8.按照相关规定，在项目验收时提交科技报告，办理《验收证书》和科技成果登记手续；

9.配合甲方开展对财政资金绩效的跟踪，在项目实施期内及实施期结束后按照甲方要求提供相关信息和数据；

10.对合作单位承担监管责任，与合作单位签署合作协议，作为本合同的附件，因合作单位违反合作协议或其他导致本合同书项目建设未能按约定完成的行为，应向甲方承担违约责任。

第五条　丙方应：

1.明确项目管理依据的管理办法或管理规程，承担项目全过程管理职责；

2.自主安排立项评审和结题验收工作，充分利用阳光政务平台，推进项目全过程管理的网络化电子化，主动配合推行合同电子签章；

3.严格落实信息公开制度，公示遴选和结题验收结果，并及时处理异议；

4.及时报送相关材料，按广州市科学技术局要求，每年按时提交拟立项项目清单，报送年度工作总结；

5.按广州市科学技术局要求配合开展绩效评价和监督检查工作；

6.主动追回终止项目未使用和不合规支出的市财政科技经费；

7.按照本单位相关项目管理办法组织项目验收工作，并按相关规定做好存档工作；

8.协助甲方对项目的实施过程进行跟踪、检查和提供相关信息，并对所提供信息的客观真实性负责；

9.负责监管乙方严格遵守《合同书》规定的任务；

10.督促乙方按时到位自筹资金并保证和落实其他配套条件。

第六条　甲方同意给予乙方人民币（¥5.00万）的资助，立项后一次性拨付。

第七条　合同终止：

1.项目因故无法继续进行的，按照相关规定实施合同终止。

2.发现存在以下情况之一的，立即启动终止程序：

①因不可抗拒因素导致项目无法继续进行、没有必要继续进行或无法完成合同预期目标任务的；

②不接受项目监督检查、检查不合格限期整改后仍未通过的或拒不配合项目验收工作的；

③无正当理由项目合同执行期满后3个月以后仍未提交验收申请的；

④项目承担单位已迁出本市，或已停止经营活动，或已注销的；

⑤发现在项目申报、实施过程中有违法、欺骗等事实的；

⑥存在其他导致项目不能正常实施的原因。

3.合同终止由乙方提出申请，丙方审定。也可由丙方强制实施。

4.合同终止后，乙方应停止使用该项目财政经费；上缴尚未使用和使用不符合规定的财政经费。

第八条 对合同正常执行期及项目整改期之外的经费开支，不属于财政项目经费列支范围。

第九条　在履行本合同的过程中，乙方发现可能导致项目失败或部分失败的情形时，应及时通知甲方和丙方，并采取适当措施减少损失，没有及时通知并采取适当措施，致使损失扩大的，应当就扩大的损失承担责任。

第十条　在履行本合同的过程中，如遇到市财政计划改变等不可抗力情况，甲方对所核拔经费的数量和时间可进行相应变更。

第十一条　成果转化：本项目技术成果及知识产权的归属、转让和实施技术成果所产生的经济利益的分享，除另有约定外，按国家和省、市有关规定执行；正式发表的论文、论著应标注“广州市科技计划项目资助”字样及项目编号；项目所取得的技术成果和知识产权应优先广州产业化或推广转让。

第十二条　属技术保密的项目，经协商订立如下技术保密条款：

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1.本合同书保密内容范围为：本合同及其补充协议和附件、乙方因履行本合同所接触或知晓的甲方工作秘密（包括但不限于甲方的任何技术性资料、以及甲方为完成本合同提供的任何其他信息资料并且在提供时未说明是公开信息的）；

2.本合同书保密期限为 ：\；

3.乙方（包括但不限于乙方雇员、代理人、顾问等人员，下同）采取有效的保密措施以避免泄露给任何第三方；在本合同有效存续期间及合同终止后，未经甲方事先的书面同意，不得以任何方式公布、发表、公开、披露、散播、复制此种保密信息的任何部分，或对其加以任何形式的利用或使用；如甲方要求，乙方必须签署甲方提供的保密协议。乙方应与可能知悉保密内容的人员签订技术保密保护协议，保密义务不得低于本合同书的约定；

4.双方应建立技术保密制度；

5.属技术保密的项目必须经市负责技术保密部门审查后,方可确定可否发表或用于国际合作与交流。

第十三条　廉洁责任

甲方、丙方、评审机构及其工作人员不得索取、收受利益相关方财物或其他不正当利益，严格遵守中央八项规定精神及其实施细则。

乙方应严格遵守国家、省、市关于科技专项经费使用的有关法律、法规，相关政策以及廉洁建设的各项规定，积极开展人员廉洁从业教育，防范科技项目组成员在科研活动中出现出现“法律、行政法规、部门规章或规范性文件规定的其他相关违规行为。

第十四条 科研诚信和科技伦理要求

乙方应建立健全促进科研诚信和科技伦理的规章制度，落实以下职责：

1.建立健全本单位学术论文发表诚信承诺制度、科研过程可追溯制度、科研成果检查和报告制度等成果管理制度。对本项目形成的科研成果的署名、研究数据真实性、实验可重复性等进行诚信审核和学术把关。防范科技项目组成员在项目申报、研发过程中提供虚假信息或材料，抄袭、剽窃他人科研成果，捏造、变造或篡改科研数据；

2.加强对科技项目参加人员的科研诚信和科技伦理教育,督促科技项目组成员恪守科学道德准则,遵守科研活动规范。对在科研诚信和科技伦理方面存在问题情节较严重的,应及时调整出项目团队并及时以书面形式报告甲方；

3.加强对项目合作单位的科研诚信管理,正确履行管理、指导、监督职责,全面落实科研诚信和科技伦理要求；

4.乙方或项目合作单位及其相关人员被纳入科研严重失信行为记录或相关社会领域信用“黑名单”，　乙方应及时以书面形式报告甲方；

在项目实施过程中,对乙方或项目合作单位及其相关人员有严重违背科研诚信和科技伦理要求的行为,甲方和相关部门可对乙方采取约谈主要负责人、停拨或核减经费、记入科研诚信严重失信行为数据库、将不良行为向社会公开、移送至有管理权限的纪检监察部门等处理处罚措施。

第十五条　争议解决

因本合同书所产生的争议，各方应友好协商解决；协商不成的，各方同意由本合同签订地人民法院管辖。

第十六条　书面通知与送达

甲方在本合同履行过程中向乙方或丙方发出或者提供的所有书面通知、文件、文书、资料等，均以本合同所列明的乙方或丙方地址送达。乙方或丙方如果迁址，应当书面通知甲方；未履行书面通知义务的，甲方按原地址邮寄相关材料即视为已履行送达义务。

本合同一式四份，各份具有同等效力。甲方和丙方各存一份，乙方存二份。本合同签订各方均负有相应的法律责任，不受机构、人事变动而影响。

说明：本《合同书》中，凡是三方约定无需填写的条款，在该条款的空白处划（\）。

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附件：承诺书

承诺书

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2021年04月20日

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二级部门：华南农业大学动物科学学院

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1	Maternal supplementation with Artemisia annua L. ameliorates intestinal inflammation via inhibiting the TLR4/NF-κB and MAPK pathways and improves the oxidative stability of offspring	FOOD & FUNCTION 出版年：2022 出版日期：SEP 22 卷期：13 18 页码：9311-9323 文献类型：Article	第一作者	T2 类	华南农业大学动物科学学院	SCI	IF2-year=6.1 IF5-year=6.5 （2022）	农林科学 1区 Top 期刊：是 （2022）
2	Combined yeast culture and organic selenium supplementation during late gestation and lactation improve preweaning piglet performance by enhancing the antioxidant capacity and milk content in nutrient-restricted sows	ANIMAL NUTRITION 出版年：2020 出版日期：JUN 卷期：6 2 页码：160-167 文献类型：Article	第一作者	T2 类	华南农业大学动物科学学院	SCI	IF2-year=6.383 IF5-year=5.497 （2020）	农林科学 1区 Top 期刊：是 （2020）
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Cite this: DOI: 10.1039/d2fo00675h

Maternal supplementation with *Artemisia annua* L. ameliorates intestinal inflammation *via* inhibiting the TLR4/NF-κB and MAPK pathways and improves the oxidative stability of offspring

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Artemisia annua L. (*A. annua*) contains artemisinin, which attracts attention on account of its anti-inflammatory and anti-oxidant effects. Increased intestinal inflammation, oxidative stress, and hypoimmunity commonly occur in neonatal and early-weaning piglets. Abundant evidence suggests that maternal nutritional interventions during pregnancy modify the offspring's long-term gut development. The present study was conducted to investigate the effects of maternal *A. annua* extract (AAE) supplementation on the offspring's intestinal inflammation and redox status. A total of 90 pregnant sows were assigned randomly and equally into the control (CON) group (fed with a basal diet) and the 0.1% (AAE) group (basal diet with 1.0 g kg^{-1} AAE) during late gestation and lactation. The results showed that 0.1% AAE supplementation significantly decreased the contents and relative mRNA expressions of interleukin (IL)-1β, IL-6, and IL-12, and tumor necrosis factor-α in the small intestine of the newborn and weaned piglets (offspring) ($P < 0.05$). There were higher activities of total antioxidant capacity and total superoxide dismutase, whereas a lower concentration of malondialdehyde in the small instestine of offspring in the 0.1% AAE group than that in the CON group ($P < 0.05$). Furthermore, the 0.1% AAE group decreased the mRNA and protein expressions of Toll-like receptor 4 (TLR4) and inhibited the activation of TLR4-mediated nuclear factor kappa B (NF-κB) and mitogen-activated protein kinase (MAPK) signaling pathways ($P < 0.05$). The mRNA expression of peroxisome proliferator activated receptor γ (PPARγ), porcine beta-defensin (PBD)-1, PBD-2, PBD-3, mucin (MUC)-1, MUC-2 and MUC-4 was significantly enhanced in the small intestine of both neonatal and weanling piglets ($P < 0.05$). Together, these results showed that maternal 0.1% AAE supplementation improved the redox status and attenuated the neonatal and early-weaning associated inflammatory response in the offspring's small intestine, possibly by suppressing the activation of the TLR4/NF-κB and MAPK inflammatory pathways, and stimulated expressions of beta-defensins, mucins, and PPARγ to promote inflammation resolution and innate immunity response.

Received 9th March 2022,
Accepted 6th June 2022

DOI: 10.1039/d2fo00675h

rsc.li/food-function

Published on 21 June 2022. Downloaded on 9/9/2022 8:23:56 AM.

Introduction

Neonatal piglets suffer from serious oxidative stress in the gut due to the sudden uprush in oxygen partial pressure and consumption caused by the fetus–neonate transition.[1] Early-weaning piglets sustain continuous external stressors, including changing dietary habits, gut microbiota, and environmental factors, which lead to gastrointestinal inflammation, oxidative injury, and diarrhea.[2] Thereafter, intestinal inflammation and oxidative injury commonly result in low feed efficiency and growth performance in piglets. Therefore, improving the redox homeostasis of piglets at critical periods is of great significance for their gut health and growth performance.[3] Previous studies indicated that antioxidant protection and immune agents could be transferred from mother to infants during pregnancy or *via* maternal colostrum and milk supply.[4,5] In addition, supplementation of antioxidant nutrients could improve the redox status in colostrum and milk, further alleviating the oxidative damage

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and promoting the antioxidant capacity in newborn and 21 day-old weaned piglets.[3,6]

Artemisia annua L. (*A. annua*) is an annual, overgrown, and natural herb belonging to the Asteraceae family, which is used in folk Chinese medicine because of the artemisinin composition and other crucial bioactive components, mainly including vitamins, minerals, sesquiterpenes, flavonoids, and phenolic compounds.[7] Artemisinin characterizes the biological functions of *A. annua*, which has anti-inflammatory, anti-viral, and anti-oxidant activities.[8,9] Besides, phenolics and flavonoids, collectively known as polyphenols, can modulate the inflammatory pathways such as nuclear factor kappa B (NF-κB) and mitogen-activated protein kinase (MAPK) to restrain the generation of inflammatory cytokines. Furthermore, polyphenols promote the inactivation of free radicals through the synthesis of antioxidants, including total superoxide dismutase (T-SOD) and glutathione peroxidase (GSH-Px).[10] Previous studies have reported that the *A. annua* extract (AAE) could increase the redox status and innate immunity in mice[11] and alleviate the intestinal inflammatory response[12] and liver oxidative injury in broilers.[13] Moreover, earlier work on weaned piglets showed that *A. annua*, as a feed supplement, could decrease the concentrations of interleukin (IL)-1β, IL-6, and tumor necrosis factor (TNF)-α in the jejunum.[14]

Recently, compelling evidence indicated that maternal *A. annua* intake is beneficial for their offspring. For instance, dietary supplementation of the enzymatically treated AAE at 1.0 g kg^{-1} greatly alleviated the oxidative stress in sows, and improved the activity of T-SOD and total antioxidant capacity (T-AOC) in piglet serum.[15] However, whether AAE supplementation in the maternal diet could affect the intestinal health of offspring is still unknown. The primary aim of the current study is to investigate the effect of maternal AAE supplementation during late pregnancy and lactation on the small intestinal inflammatory and redox status as well as expression in Toll-like receptor 4 (TLR4) mediated NF-κB and MAPK signalling pathways in neonatal and early-weaning piglets.

Materials and methods

Preparation of *Artemisia annua* L. extract (AAE) by enzymatical treatment

A. annua was produced in China, and was harvested in the flourishing stage. The AAE was provided by Guangzhou Cohoo Biotechnology Research and Development Center (Guangzhou, P. R. China). Studies reported that enzymatic hydrolysis technology has some positive effects, including (i) hydrolyzing plant cell wall components, which leads to higher extraction efficiency of bioactives; (ii) enhanced biosecurity and absorption efficiency in animals compared with physical and chemical extraction; and (iii) higher yield and quality of the product extract and without the requirement for the removal of waste which is hazardous to animals.[16] Cellulase and pectinase were used as the main hydrolytic enzymes to treat the entire *A. annua* herb, then filtered and dried. The AAE contained 45.02, 972.96, and 335.17 g per kg CP (AOAC, 2005, method 991.20), DM (AOAC, 2005, method 926.12), and ether extract (AOAC, 2005, method 920.39), respectively. The total phenolic content and total flavonoid content were measured according to the method described in our previous study.[15] The bioactive ingredients of the AAE mainly consist of 4.77 ± 0.22 mg of gallic acid equivalents per g phenols, 1.68 ± 0.13 mg rutin equivalents per g flavonoids, 156.2 ± 0.20 mg per kg arteannuinic acid, 62.0 ± 0.08 mg per kg deoxyartemisinin, and 26.0 ± 0.06 mg per kg artemisinin.

Experimental design and animals

All animal procedures performed for animal care and use during this study were approved by the South China Agricultural University Animal Care and Animal Use Committee (Guangzhou, China) and the application number of ethics review is 2021g017. This trial was carried out in a commercial pig breeding farm in Jiangmen, Guangdong, China, from July to October 2018. A total of 90 multiparous sows (Landrace × Large white) were randomly allocated by parity, body condition, and historical reproductive performance to 2 groups (45 sows per treatment) as follows: the CON group (basal diet) and the 0.1% AAE group (basal diet + 0.1% AAE). The dietary nutrient levels were referenced to the NRC (2012) recommendation. No antibiotics were used during the entire trial. The feed composition and nutrient levels of the basal diet are shown in Table 1.

The experiment lasted from d 85 of gestation until d 21 of lactation according to the daily management and routine immunization procedure of the pig farm. In the late gestation

Table 1 Ingredient composition and nutritional levels of the basic diet (g kg^{-1}, as-fed basis; %)

Ingredient	Content	Nutritional level[b]	Content
Maize	52.20	Dietary energy, MJ kg^{-1}	14.31
Soyabean meal	24.00	Dry matter %[c]	89.96
Wheat meal	6.00	Crude protein %[c]	18.13
Fish meal	2.50	Crude ash %[c]	5.76
Wheat flour	7.50	Crude fiber %[c]	3.31
Soybean oil	4.00	Crude fat %[c]	6.50
Dicalcium phosphate	1.20	Calcium %[c]	0.77
Stone powder	0.90	Total phosphorus %[c]	0.66
Salt	0.30	Digestible phosphorus %[c]	0.41
Premix[a]	0.30	Digestible lysine %[c]	0.86
Choline chloride (50%)	0.20	Digestible methionine + cystine %[c]	0.51
Sodium bicarbonate	0.20	Digestible threonine %[c]	0.60
Mildew remover	0.05	Digestible tryptophan %[c]	0.18
Mildew preventive	0.20		
Carrier	0.45		
Total	100.00		

[a] Premix provided the following per kg of diet: vitamin A 13 000 IU, vitamin D 4000 IU, vitamin E 30 IU, vitamin K_3 4 mg, vitamin B_1 4 mg, vitamin B_2 10 mg, vitamin B_6 4.8 mg, vitamin B_{12} 34 μg, nicotinic acid 40 mg, pantothenic acid 20 mg, folic acid 2.0 mg, biotin 0.16 mg; Fe 80 mg, Cu 5.0 mg, Zn 50 mg, Mn 20.0 mg, iodine 0.14 mg, and Se 0.3 mg. [b] Nutrient levels were the calculated values. [c] Analyzed values.

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period (from d 85 to d 107 of gestation), every sow was housed in an individual crate (2.10 × 0.6 m^2) in an environmentally controlled facility, had *ad libitum* access to clean water and was fed twice daily (6:30 and 14:00). Sows were feed restricted, and the total daily rations per sow were 3.0–3.5 kg d^{-1}. On d 108 of gestation, all sows were transferred into the respective farrowing houses, and had limited access to food for 5 days after the delivery. Within 48 h postpartum, the piglets were cross-fostered only within the same dietary treatment, and the litter sizes were standardized to 10–12. There was no milk replacer or creep feed provided to the piglets in the present study.

Sample collection

A total of 12 newborn piglets (1 piglet per litter, n = 6 for each treatment, 3 males and 3 females) were immediately slaughtered to collect the intestinal sample without suckling colostrum after delivery on the first postnatal day (<12 h). Similarly, at the weaning age (d 21 of lactation), 12 weaning piglets (n = 6 per group) with an average body weight were randomly selected for sample collection. The neonatal piglets and weaning piglets were slaughtered by an injection of pentobarbital (200 mg per kg body weight) after general anesthesia. Then the abdominal cavity was immediately opened and the entire small intestine from the pylorus to the ileocecal valve was removed and divided into three segments. Thereafter, segments of approximately 3 cm in length of the middle of the duodenum, jejunum, and ileum were immediately isolated and flushed with ice-cold normal saline to remove luminal contents. The intestinal tissues were snap-frozen in liquid nitrogen and stored at −80 °C until further analysis.

Laboratory analysis

Inflammatory factors. Approximately 1.0 g (accurate to 0.001 g) of tissue samples were homogenized with 1 ml of ice-cold PBS and then the homogenate was centrifuged at 5000*g* for 5 min at 4 °C to form a supernatant. The supernatant was collected to assay the concentrations of IL-1β, IL-6, IL-12, and TNF-α in the small intestinal tissues using commercial ELISA kits (Huamei Biotechnology, Wuhan, China) according to the manufacturer's protocols.

Redox status. Approximately 1.0 g (accurate to 0.001 g) of intestinal tissue samples were homogenized (1 : 9, wt/vol) with ice-cold normal saline solution to obtain tissue homogenates (10%) and then centrifuged at 2500*g* for 10 min. The supernatant was taken to determine the concentrations of glutathione (GSH) and malonaldehyde (MDA), as well as the enzyme activities of GSH-Px, T-SOD, and T-AOC. The protein concentration of the homogenate was quantified by the BCA method using commercial kits (Nanjing JianCheng Bioengineering Institute, Nanjing, China) following the manufacturer's protocol. All results were normalized against total protein concentration in each sample for inter-sample comparison.

Reverse transcriptase-quantitative PCR (RT-qPCR). Total RNA from duodenum, jejunum, and ileum liquid nitrogen-pulverized tissues was extracted with an EZ-press RNA purification kit (#B0004DP, EZBioscience) as recommended by the manufacturer. The cDNA was obtained from the RNA reverse transcription reaction using a Color Reverse Transcription Kit (with a gDNA Eraser) (EZBioscience) according to the manufacturer's instructions. RT-qPCR was performed on an ABI Prism 7500 sequence detection system (Applied Biosystems, Carlsbad, CA, USA). The oligonucleotide primers were designed and synthesized by Shanghai Sangon Biotech Co., Ltd (Shanghai, China) and are shown in Table 2. The target genes analyzed included IL-1β, IL-6, and IL-12, TNF-α, TLR4, myeloid differentiation factor 88 (MyD88), inhibitor of NF-κB kinase α (IKKα), inhibitor of NF-κB kinase β (IKKβ), (TGF-β)-activated kinase 1 (TAK1), extracellular signal-regulated kinase (ERK), c-Jun N-terminal kinase (JNK), p38, peroxisome proliferator activated receptor γ (PPARγ), transforming growth factor-β (TGF-β), three porcine beta-defensins (PBD-1, PBD-2, and PBD-3), and three mucins (MUC1, MUC2, and MUC4). Target genes of each sample were analyzed in duplicate. The endogenous control, β-actin, was confirmed to be a suitable housekeeping gene, as it was not affected by the diets. The thermal cycling conditions used were as per the protocol: denaturation at 95 °C for 30 s, and amplification and quantification were executed for 40 cycles (95 °C for 5 s and 60 °C for 30 s for annealing in each cycle), and then melting curve analysis was conducted from 60 °C to 95 °C. All samples were tested in triplicate. The relative mRNA expression levels of the selected genes normalized against the reference gene (β-actin) were calculated using the $2^{-\Delta\Delta CT}$ method.

Total protein extraction and western blotting. Total proteins from duodenum, jejunum, and ileum tissues were extracted using RIPA lysis buffer containing 1% PMSF (100 mM) and 1% phosphatase inhibitor (100×). After incubating on ice for 15 min, the samples were centrifuged at 13 000 rpm for 15 min at 4 °C. The concentration of the acquired protein was detected using a BCA protein assay kit (P0012B, Beyotime Institute of Biotechnology, Nantong, Jiangsu, China). Equal amounts of protein were separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) according to the size of the target protein and then were blotted onto polyvinylidene difluoride (PVDF) membranes. The membranes were blocked using 6% skim milk for 2 h to avoid the nonspecific binding, and then washed with 1× TBST. Subsequently, the membranes were incubated with primary antibodies against target proteins at 4 °C overnight, and then incubated with the corresponding secondary antibodies for 1.5 h at room temperature followed by washing with 1× TBST three times. Finally, the obtained target protein bands were detected using a chemiluminescent ECL western blot detection system (ProteinSimple, Santa Clara, CA, USA). The grayscale of the target protein bands was analyzed using ImageJ software.

Statistical analysis

The data obtained from each experiment were preliminarily sorted using Excel 2010, and SPSS 22.0 software (SPSS Inc., Chicago, USA) was used for the independent samples' T-test. $P < 0.05$ was considered to confirm statistical significance. $P < 0.01$ indicates that the difference is extremely significant. Trends were identified when $0.05 < P < 0.10$. All data were expressed as the mean ± standard error of the mean (SEM).

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Table 2 Sequences of primers used in real-time PCR

Gene	Accession number	Sequence (5′-3′)	
IL-1β	NM_214055.1	F: CCAAAGAGGGACATGGAGAA	R: TTATATCTTGGCGGCCTTTG
IL-6	AF518322.1	F: TGGCTACTGCCTTCCCTACC	R: AGAGCCTGCATCAGCTCAGT
IL-12	NC_010458.4	F: CAACCCTGTGCCTTAGCAGT	R: AGAGCCTGCATCAGCTCAGT
TNF-α	NM_214022.1	F: CCACCAACGTTTTCCTCACT	R: TAGTCGGGCAGGTTGATCTC
TLR4	NM_001113039.1	F: CAACCCTGTGCCTTAGCAGT	R: AGAGCCTGCATCAGCTCAGT
MyD88	NM_001099923	F: CGCATGGTGGTGGTTGTT	R: GCCTTCTTCATCGCCTTGTATTT
IKKα	NM_001114279.1	F: TCTTGATCCTCGGAAACCAG	R: TGCTTCGGCCCATACTTTAC
IKKβ	NM_001099935.1	F: CCTCACCTTGCTGAGTGACA	R: TCCCCACAAAGGAGGTACAG
TAK1	NM_001114280.1	F: CATGTGGGCTGTTCATAACG	R: GAGTTGCTCTGGCCTTCATC
ERK	XM_021088019.1	F: CAGTCTCTGCCCTCCAAGAC	R: GGGTAGATCATCCAGCTCCA
JNK	XM_001929166.6	F: TGGATGAAAGGGAACACACA	R: ATGATGACGATGGATGCTGA
p38	XM_021091323.1	F: CCCTGAGGTTCTAGCGAAGA	R: TCTCATCGTAGGGCTCTGCT
PPAR-γ	NM_214379.1	F: AAGACGGGGTCCTCATCTCC	R: CGCCAGGTCGCTGTCATCT
TGF-β	NM_214015.1	F: GAAGCGCATCGAGGCCATTC	R: GGCTCCGGTTCGACACTTTC
PBD-1	NM_213838.1	F: ACCGCCTCCTCCTTGTATTC	R: CACAGGTGCCGATCTGTTTC
MUC1	XM_021089730.1	F: TTCTTCGGGCTGTTGCTACT	R: ACTGTCTTGGAAGGCCAGAA
MUC2	NM_010843.1	F: CTCCATCCGCTTCAGAAAAG	R: ACTACCTGGGGCCTCTGAAT
MUC4	XM_021068274.1	F: CCTCCCAAGCAGATGTCAAT	R: CTGGGATAAGAATGCCTCCA
β-Actin	397563	F: TGCGGGACATCAAGGAGAAG	R: AGTTGAAGGTGGTCTCGTGG

IL-1β, interleukin 1β; TNF-α, tumor necrosis factor-α; TLR4, Toll-like receptor 4; NF-κB, nuclear factor kappa B; MyD88, myeloid differentiation factor 88; TAK1, transforming growth factor β (TGF-β)-activated kinase 1; ERK1, extracellular signal-regulated kinase 1; JNK, c-Jun N-terminal kinase; IKKα, inhibitor of NF-κB kinase α; IKKβ, inhibitor of NF-κB kinase β; NF-κB, nuclear factor kappa B; ERK, extracellular signal-regulated kinase; JNK, c-Jun N-terminal kinase; PPARγ, peroxisome proliferator activated receptor γ; TGF-β, transforming growth factor-β; PBD, porcine beta-defensin; and MUC, mucin.

Results

Redox status in the small intestine

As listed in Table 3, the 0.1% AAE group had higher T-AOC and T-SOD activities in the duodenum, T-AOC activity in the jejunum, and T-SOD activity in the ileum ($P < 0.05$), while a lower concentration of MDA in the jejunum and ileum of newborn piglets than those in the CON group ($P < 0.05$); besides the GSH-Px activity ($P = 0.08$) and GSH content ($P = 0.05$) in the duodenum, and T-SOD activity ($P = 0.07$) in the jejunum had an uptrend, nevertheless, the MDA content ($P = 0.05$) had a downtrend in the duodenum in the 0.1% AAE group of newborn piglets. Table 4 shows that the 0.1% AAE group had higher T-AOC and T-SOD activities in the duodenum, jejunum, and ileum ($P < 0.05$), but a lower MDA content in the duodenum, jejunum, and ileum of weaned piglets ($P < 0.05$).

Concentrations of inflammatory cytokines in the small intestine

Compared with the CON group, the contents of IL-1β, IL-6, IL-12, and TNF-α in the duodenum, IL-1β and TNF-α in the jejunum, and IL-6 and TNF-α in the ileum of newborn piglets were significantly decreased ($P < 0.05$), as well as the IL-6 concentration in the jejunum tended to decrease in the 0.1% AAE group ($P = 0.06$; Table 5); additionally, the contents of IL-1β, IL-12, and TNF-α in the duodenum, IL-1β and IL-6 in the jejunum and IL-6 and IL-12 in the ileum were also significantly decreased in weaned piglets ($P < 0.05$), and the content of IL-6 in the duodenum tended to decrease ($P = 0.07$; Table 6).

Table 3 Effects of maternal *Artemisia annua* L. extract (AAE) supplementation on the small intestinal redox status of newborn piglets

	Newborn piglets		
Items	CON	0.1% AAE	*P*-Value
Duodenum			
T-AOC (U mg^{-1} protein)	11.05 ± 0.29[b]	12.83 ± 0.36[a]	<0.01
T-SOD (U mg^{-1} protein)	68.15 ± 3.09[b]	79.76 ± 2.19[a]	0.012
GSH-Px (U mg^{-1} protein)	616.19 ± 8.80	639.92 ± 8.20	0.077
GSH (mg g^{-1} protein)	12.57 ± 0.66	15.00 ± 0.88	0.052
MDA (nmol mg^{-1} protein)	3.66 ± 0.15	3.28 ± 0.07	0.050
Jejunum			
T-AOC (U mg^{-1} protein)	12.10 ± 0.30[b]	13.62 ± 0.52[a]	0.030
T-SOD (U mg^{-1} protein)	66.92 ± 2.12	72.16 ± 1.47	0.070
GSH-Px (U mg^{-1} protein)	579.60 ± 11.35	590.28 ± 13.98	0.567
GSH (mg g^{-1} protein)	17.51 ± 1.32	18.14 ± 1.76	0.782
MDA (nmol mg^{-1} protein)	4.97 ± 0.41[b]	3.62 ± 0.18[a]	0.014
Ileum			
T-AOC (U mg^{-1} protein)	13.13 ± 0.44	13.79 ± 0.57	0.383
T-SOD (U mg^{-1} protein)	78.41 ± 1.00[b]	84.72 ± 1.32[a]	<0.01
GSH-Px (U mg^{-1} protein)	629.84 ± 8.16	639.41 ± 13.00	0.547
GSH (mg g^{-1} protein)	18.60 ± 0.49	19.26 ± 0.58	0.492
MDA (nmol mg^{-1} protein)	5.17 ± 0.45[b]	3.45 ± 0.29[a]	<0.01

All values are expressed as means ± SEM. The data presented are obtained from 6 animals in each group ($n = 6$). Differences were statistically significant when $P < 0.05$. Values with different superscripts (a and b) were statistically significant. CON, control group; 0.1% AAE, 1.0 g kg^{-1} AAE group; T-AOC, total antioxidant capacity; T-SOD, total superoxide dismutase; GSH-Px, glutathione peroxidase; GSH, glutathione; and MDA, malondialdehyde.

Relative gene expression levels of inflammatory cytokines in the small intestine

The effect of 0.1% AAE on the relative gene expression levels of inflammatory factors in the small intestine of piglets is

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Table 4 Effects of maternal *Artemisia annua* L. extract (AAE) supplementation on the small intestinal redox status of weaned piglets

Items	Weaned piglets		*P*-Value
	CON	0.1% AAE	
Duodenum			
T-AOC (U mg^{-1} protein)	13.82 ± 0.40[b]	15.21 ± 0.35[a]	0.025
T-SOD (U mg^{-1} protein)	66.72 ± 2.00[b]	75.47 ± 2.05[a]	0.012
GSH-Px (U mg^{-1} protein)	550.75 ± 11.58	561.24 ± 13.63	0.570
GSH (mg g^{-1} protein)	31.67 ± 1.82	33.18 ± 1.20	0.502
MDA (nmol mg^{-1} protein)	5.15 ± 0.20[b]	4.59 ± 0.12[a]	0.040
Jejunum			
T-AOC (U mg^{-1} protein)	12.48 ± 0.33[b]	13.77 ± 0.39[a]	0.030
T-SOD (U mg^{-1} protein)	72.33 ± 1.66[b]	78.48 ± 2.14[a]	0.046
GSH-Px (U mg^{-1} protein)	579.46 ± 12.22	580.39 ± 9.83	0.954
GSH (mg g^{-1} protein)	20.05 ± 1.12	22.55 ± 1.25	0.161
MDA (nmol mg^{-1} protein)	2.54 ± 0.08[b]	2.22 ± 0.09[a]	0.026
Ileum			
T-AOC (U mg^{-1} protein)	12.33 ± 0.37[b]	14.72 ± 0.83[a]	0.026
T-SOD (U mg^{-1} protein)	70.33 ± 2.48[b]	75.41 ± 1.47[a]	0.015
GSH-Px (U mg^{-1} protein)	536.66 ± 8.38	557.45 ± 10.86	0.161
GSH (mg g^{-1} protein)	18.12 ± 0.97	19.00 ± 0.95	0.536
MDA (nmol mg^{-1} protein)	2.75 ± 0.06[b]	2.61 ± 0.01[a]	0.041

All values are expressed as means ± SEM. The data presented are obtained from 6 animals in each group (n = 6). Differences were statistically significant when P < 0.05. Values with different superscripts (a and b) were statistically significant. CON, control group; 0.1% AAE, 1.0 g kg^{-1} AAE group; T-AOC, total antioxidant capacity; T-SOD, total superoxide dismutase; GSH-Px, glutathione peroxidase; GSH, glutathione; and MDA, malondialdehyde.

Table 5 Effects of maternal *Artemisia annua* L. extract (AAE) supplementation on the small intestinal inflammatory cytokines of newborn piglets

Items	Newborn piglets		*P*-Value
	CON	0.1% AAE	
Duodenum			
IL-1β (pg g^{-1} protein)	700.43 ± 33.55[b]	268.87 ± 6.13[a]	<0.01
IL-6 (pg g^{-1} protein)	172.12 ± 10.42[b]	79.64 ± 4.92[a]	<0.01
IL-12 (pg mg^{-1} protein)	243.33 ± 11.36[b]	151.74 ± 13.89[a]	0.003
TNF-α (pg mg^{-1} protein)	16.72 ± 1.23[b]	12.26 ± 0.80[a]	0.012
Jejunum			
IL-1β (pg g^{-1} protein)	567.72 ± 25.63[b]	247.41 ± 9.02[a]	<0.01
IL-6 (pg g^{-1} protein)	117.52 ± 7.51	82.37 ± 6.97	0.06
IL-12 (pg mg^{-1} protein)	90.69 ± 4.43	78.11 ± 5.69	0.112
TNF-α (pg mg^{-1} protein)	27.48 ± 1.27[b]	16.48 ± 1.15[a]	<0.01
Ileum			
IL-1β (pg g^{-1} protein)	385.61 ± 19.34	363.49 ± 15.99	0.399
IL-6 (pg g^{-1} protein)	255.32 ± 12.32[b]	81.88 ± 4.73[a]	<0.01
IL-12 (pg mg^{-1} protein)	124.80 ± 12.09	105.67 ± 3.32	0.165
TNF-α (pg mg^{-1} protein)	23.58 ± 1.53[b]	7.44 ± 0.69[a]	<0.01

All values are expressed as means ± SEM. The data presented are obtained from 6 animals in each group (n = 6). Differences were statistically significant when P < 0.05. Values with different superscripts (a and b) were statistically significant. CON, control group; 0.1% AAE, 1.0 g kg^{-1} AAE group; IL-1β, interleukin 1β; and TNF-α, tumor necrosis factor-α.

observed in Fig. 1. Compared with the CON group, the 0.1% AAE group markedly decreased the relative mRNA expression levels of IL-6, IL-12, and TNF-α in the duodenum, IL-1β, IL-6, and TNF-α in the jejunum and IL-6 and TNF-α in the ileum of newborn piglets (P < 0.05). Moreover, the 0.1% AAE group also decreased the relative mRNA expression levels of IL-1β, IL-6, IL-12, and TNF-α in the duodenum, IL-1β and IL-6 in the jejunum, and IL-6 and IL-12 in the ileum of weaned piglets (P < 0.05).

Table 6 Effects of maternal *Artemisia annua* L. extract (AAE) supplementation on the small intestinal inflammatory cytokines of weaned piglets

Items	Weaned piglets		*P*-Value
	CON	0.1% AAE	
Duodenum			
IL-1β (pg g^{-1} protein)	350.43 ± 13.92[b]	310.21 ± 10.96[a]	0.024
IL-6 (pg g^{-1} protein)	94.23 ± 5.11	80.63 ± 4.23	0.068
IL-12 (pg mg^{-1} protein)	156.86 ± 6.82[b]	112.75 ± 7.01[a]	0.001
TNF-α (pg mg^{-1} protein)	17.93 ± 1.22[b]	11.95 ± 0.75[a]	<0.01
Jejunum			
IL-1β (pg g^{-1} protein)	244.86 ± 5.81[b]	221.07 ± 7.03[a]	0.031
IL-6 (pg g^{-1} protein)	92.69 ± 7.55[b]	65.71 ± 4.80[a]	0.015
IL-12 (pg mg^{-1} protein)	469.99 ± 22.72	403.53 ± 35.57	0.164
TNF-α (pg mg^{-1} protein)	8.73 ± 0.34	8.62 ± 0.64	0.886
Ileum			
IL-1β (pg g^{-1} protein)	305.54 ± 15.11	276.08 ± 14.52	0.190
IL-6 (pg g^{-1} protein)	88.62 ± 2.22[b]	76.42 ± 1.91[a]	0.002
IL-12 (pg mg^{-1} protein)	96.03 ± 6.95[b]	76.33 ± 1.25[a]	0.036
TNF-α (pg mg^{-1} protein)	10.59 ± 0.39	10.16 ± 0.59	0.561

All values are expressed as means ± SEM. The data presented are obtained from 6 animals in each group (n = 6). Differences were statistically significant when P < 0.05. Values with different superscripts (a and b) were statistically significant. CON, control group; 0.1% AAE, 1.0 g kg^{-1} AAE group; IL-1β, interleukin 1β; and TNF-α, tumor necrosis factor-α.

Gene expressions in the TLR4/NF-κB and MAPK signaling pathways of the small intestine

To further investigate the mechanism of maternal 0.1% AAE supplementation to protect piglets from inflammation, we performed qRT-PCR experiments (Fig. 2). Compared with those in the CON group, the 0.1% AAE group downregulated the relative mRNA expressions of duodenal TLR4, NF-κB, TAK1, ERK, and JNK (P < 0.05); jejunal TLR4, MyD88, NF-κB, ERK, and TAK1 (P < 0.10); and ileal TLR4, NF-κB, TAK1, ERK, JNK, and p38 (P < 0.05) in newborn piglets. As expected, the 0.1% AAE group also downregulated the relative mRNA expressions of duodenal MyD88, IKKβ, IKKα, TAK1, ERK, TLR4, NF-κB, and JNK (P < 0.10); jejunal IKKα, NF-κB, TAK1, and p38 (P < 0.05); and ileal TLR4, MyD88, NF-κB, TAK1, ERK, JNK, and p38 (P < 0.05) in weaned piglets.

Protein expressions in the TLR4/NF-κB and MAPK signaling pathways of the small intestine

In the present study, western blot results showed that maternal 0.1% AAE supplementation downregulated the expression of proteins related to TLR4/NF-κB and MAPK pathways in the small intestine of piglets (Fig. 3). Compared with the CON group, the 0.1% AAE group reduced the relative protein expressions of duodenal phosphorylated NF-κB (p-NF-κB),

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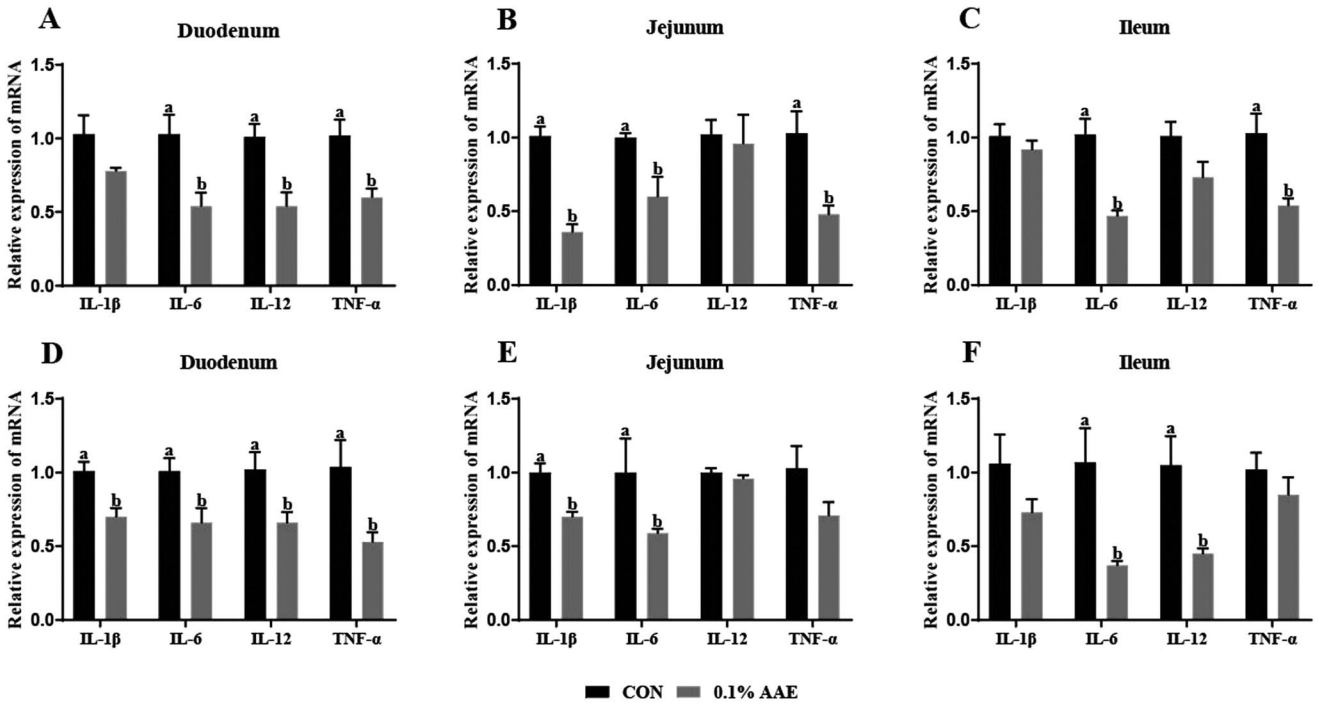

Fig. 1 Effects of maternal *Artemisia annua* L. extract (AAE) supplementation on the relative mRNA expression levels of the inflammatory factor in the duodenum (A), jejunum (B), and ileum (C) of newborn piglets and the duodenum (D), jejunum (E), and ileum (F) of weaned piglets. CON, control group; 0.1% AAE, 1.0 g kg^{-1} AAE group; IL-1β, interleukin 1β; and TNF-α, tumor necrosis factor-α.

p-ERK, and p-JNK; jejunal p-NF-κB and p-ERK; and ileal p-NF-κB, p-ERK, p-JNK, and p-p38 in newborn piglets ($P < 0.05$). Furthermore, the 0.1% AAE group also reduced the relative protein expressions of duodenal p-NF-κB, p-ERK, and p-JNK, jejunal p-NF-κB and p-p38, and ileal p-NF-κB, p-ERK, p-JNK, and p-p38 in weaned piglets ($P < 0.05$).

Gene expressions in the endogenous synthesis of PBDs, PPARγ, and TGF-β in the small intestine

Fig. 4 presents the changes in the gene expressions of small intestinal PBD, PPARγ, and TGF-β between both the CON group and the 0.1% AAE group in piglets. Specifically, the 0.1% AAE group upregulated the mRNA expressions of duodenal PBD-1 and PBD-2; jejunal PPARγ, TGF-β, PBD-2, and PBD-3; and ileal PPARγ, TGF-β, PBD-2, and PBD-3 in newborn piglets ($P < 0.05$). Besides, the 0.1% AAE group also upregulated the mRNA expressions of duodenal PPARγ, PBD-1, and PBD-2; jejunal PPARγ, PBD-2, and PBD-3; and ileal PPARγ and PBD-2 of weaned piglets ($P < 0.05$), and duodenal PBD-3 and jejunal TGF-β tended to increase ($P < 0.10$).

Gene expressions of MUCs in the small intestine

The small intestinal gene expressions of MUCs in piglets are described in Fig. 5. Maternal 0.1% AAE supplementation had higher mRNA expressions of duodenal MUC-1 and MUC-4 and jejunal and ileal MUC-1, MUC-2, and MUC-4 in newborn piglets than those fed with a maternal CON diet ($P < 0.05$). As envisaged, the 0.1% AAE group also upregulated the relative mRNA expressions of duodenal MUC-1 and MUC-4 and jejunal and ileal MUC-1 and MUC-2 in weaned piglets ($P < 0.05$).

Discussion

The plant *A. annua* draws wide attention because of its antimalarial compound artemisinin. However, plant cytoderm restricts the release of phytochemicals inside the cytoplasm, which causes inefficient nutritional ingredient utilization in natural herbs.[16] *A. annua* was treated with enzymatic hydrolysis technology which led to enzymolysis in the cytoderm, and thus the extraction and purification of bioactive compounds would be easier than before.[17] Previously, maternal supplementation of 0.1% AAE has been demonstrated to significantly increase the average daily feed intake in sows and the average daily gain in body weight in piglets.[15,18] However, the effects of maternal supplementation of 0.1% AAE on the health of the offspring's intestines are still largely unknown. The interactions between maternal nutrition and gut development start when fetuses begin to swallow amniotic fluid and extend past weaning.[6] Intestinal inflammation, oxidative stress, and infection could easily destroy small intestinal epithelial cells during early life.[19,20] Thus, the primary objective of this study is to uncover the potential role of AAE in the inflammation and redox biology in the offspring's intestine.[18]

The antioxidant defense systems, including SOD, GSH-Px, CAT, and GSH, efficiently transform ROS into H_2O and O_2 to protect the organism against ROS-induced oxidative injury.[21]

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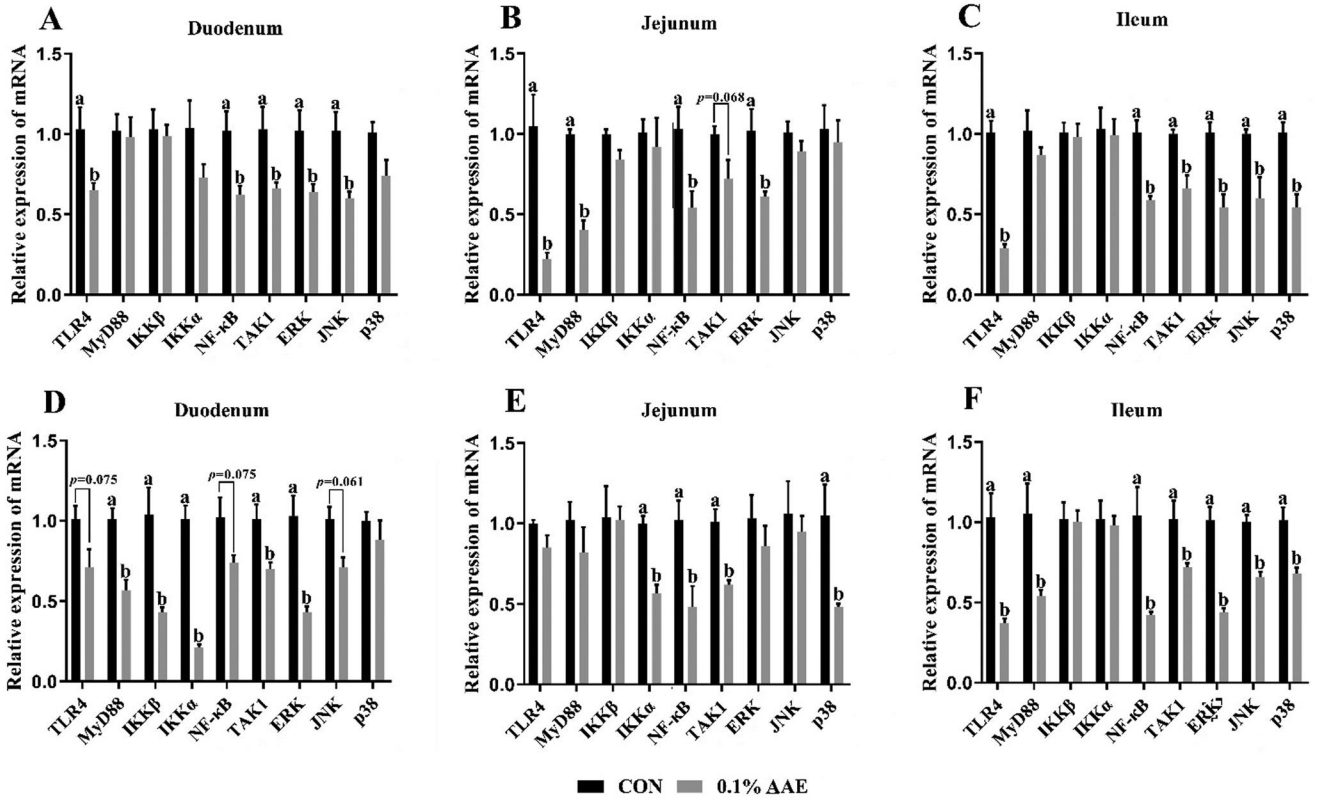

Fig. 2 Effects of maternal *Artemisia annua* L. extract (AAE) supplementation on the relative mRNA expression levels of TLR4/NF-κB and MAPK signalling pathways in the duodenum (A), jejunum (B), and ileum (C) of newborn piglets and the duodenum (D), jejunum (E), and ileum (F) of weaned piglets. CON, control group; 0.1% AAE, 1.0 g kg^{-1} AAE group; TLR4, Toll-like receptor 4; NF-κB, nuclear factor kappa B; MyD88, myeloid differentiation factor 88; TAK1, transforming growth factor β (TGF-β)-activated kinase 1; ERK, extracellular signal-regulated kinase; JNK, c-Jun N-terminal kinase; IKKα, inhibitor of NF-κB kinase α; and IKKβ, inhibitor of NF-κB kinase β.

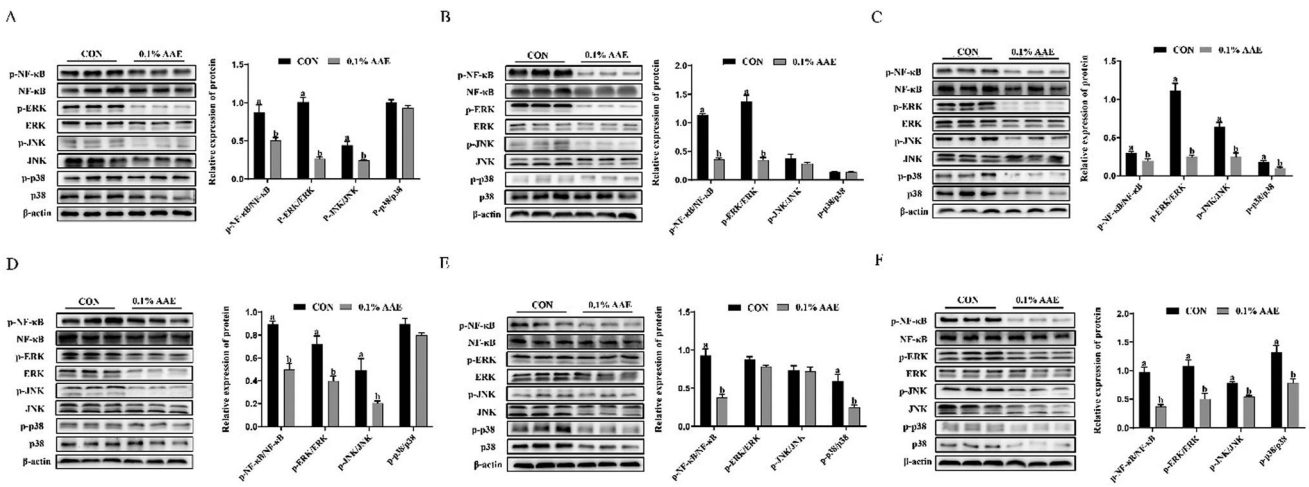

Fig. 3 Effects of maternal *Artemisia annua* L. extract (AAE) supplementation on the protein expression levels of TLR4/NF-κB and MAPK signalling pathways in the duodenum (A), jejunum (B), and ileum (C) of newborn piglets and the duodenum (D), jejunum (E), and ileum (F) of weaned piglets. CON, control group; 0.1% AAE, 1.0 g kg^{-1} AAE group; NF-κB, nuclear factor kappa B; ERK, extracellular signal-regulated kinase; and JNK, c-Jun N-terminal kinase.

The SOD catalyzes the dismutation of O_2^- into H_2O_2, which is obliterated by GSH-Px and impedes free radical production *in vivo*.[22] The GSH acts as a free radical scavenger, and GSH-Px further catalyzes GSH into GSSG in the cell.[23] MDA, a decomposition product of lipoperoxidation, can inhibit the activities of antioxidant enzymes.[24] Similar to previous studies,

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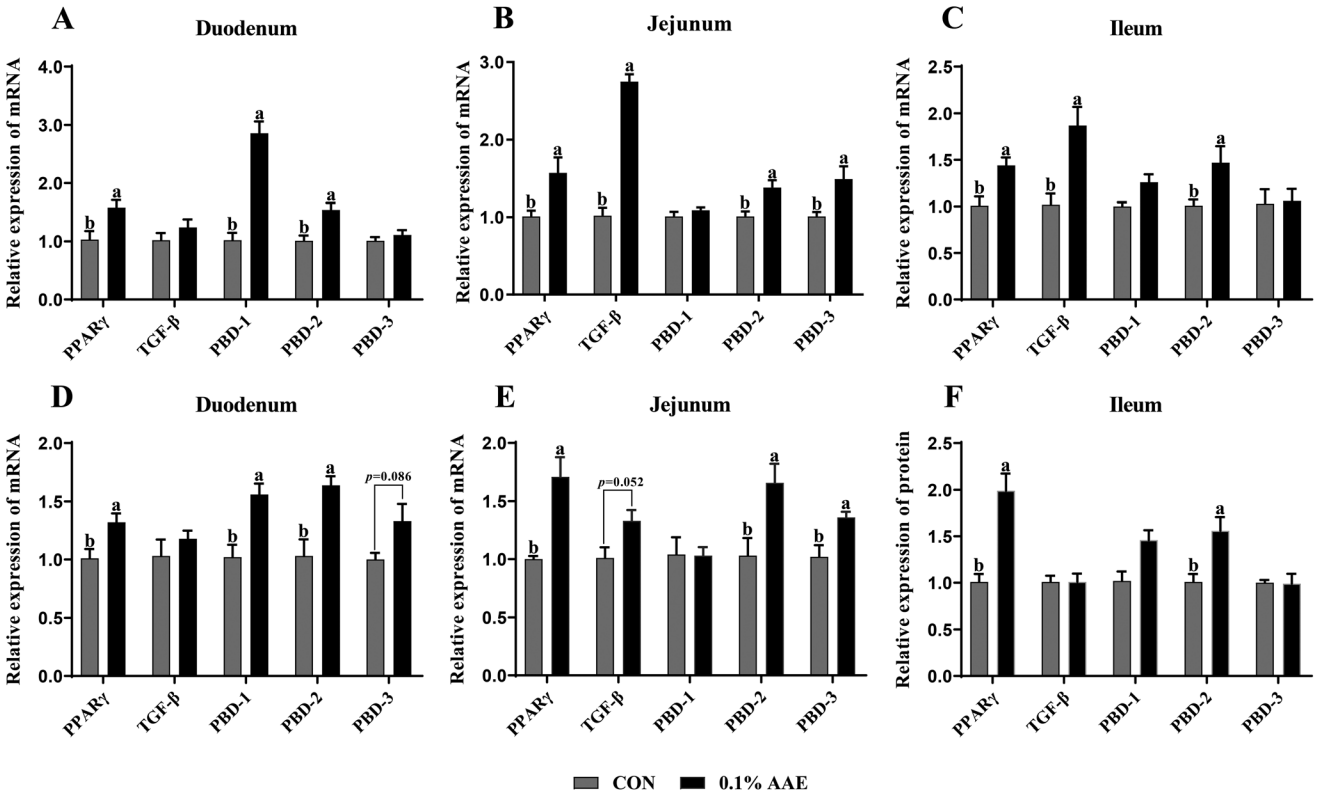

Fig. 4 Effects of maternal *Artemisia annua* L. extract (AAE) supplementation on the relative mRNA expression levels of PPARγ, TGF-β, and PBDs in the duodenum (A), jejunum (B), and ileum (C) of newborn piglets and the duodenum (D), jejunum (E), and ileum (F) of weaned piglets. CON, control group; 0.1% AAE, 1.0 g kg^{-1} AAE group; PPARγ, peroxisome proliferator activated receptor γ; TGF-β, transforming growth factor-β; and PBD, porcine beta-defensin.

dietary AAE supplementation reduced MDA concentrations in muscle[25] and serum,[26] and improved oxidative stability including the T-SOD and GSH-Px activities as well as the GSH levels in the liver[9] and jejunal mucosa[27] of broilers. Niu *et al.*[28] also reported that AAE supplementation increased the activities of GSH-Px and T-SOD as well as T-AOC levels, whereas it decreased the concentrations of MDA in the serum and both the jejunum and ileum of weaned piglets. Our lab recently found that 0.1% AAE supplementation reduced the MDA content, and increased the activities of T-SOD and T-AOC in the serum, colostrum, and milk of heat-stressed sows and in piglet serum.[15] Similar to our previous study, this study indicated that maternal 0.1% AAE increased the activities of T-AOC and T-SOD, whereas it decreased the MDA concentration in the small intestine of both newborn and weaning piglets. Combining the results of our two experiments, the improved intestinal antioxidant defense system in the offspring caused by maternal 0.1% AAE might be relevant to fetus–neonate transition and milk intake effects. The elevated antioxidant status might be one reason why AAE supplementation benefits the growth performance of piglets. Artemisinin, arteannuinic acid, and polyphenols in the added AAE offer antioxidant protection in sows and offspring,[9] which could be transferred from the mother to neonates during gestation or *via* maternal colostrum supply to improve the intestinal antioxidant status in the piglets.

Oxidative stress causes injury in the mucosal layer in the gastrointestinal (GI) tract and results in pathogenic invasion which furthermore decomposes and releases lipopolysaccharide (LPS), and stimulates the inflammatory and immune responses.[29] Hence, we also determined key gene expression related to proinflammation considering that oxidative stress has been reported to be a crucial regulatory factor for the intestinal proinflammatory reaction. The essential proinflammatory cytokines, including TNF-α, IL-1β, IL-6, and IL-12, mediate the inflammatory and immune responses.[30] Previous research reported that AAE treatment significantly decreased the IL-1β concentration and mRNA abundances of TLR4, IL-6, and IL-1β in the jejunum and ileum of broilers.[12] Dietary AAE addition also decreased the levels of IL-6, IL-1β, and TNF-α in the jejunum of weaned piglets.[31] These results were based on the direct AAE addition; thus, we conducted a maternal AAE supplementation to investigate the vertically transmitted effect of the maternal effect on offspring's small intestinal health. Moreover, our recent research also showed that 0.1% AAE addition in sow diets effectively reduced the inflammatory cytokine levels in the serum, colostrum, and milk of sows and further relieved the inflammation.[18] The results of the present study demonstrated that maternal 0.1% AAE obviously suppressed the concentrations and mRNA expressions of inflammatory cytokines (TNF-α, IL-1β, IL-6, and IL-12) in the small

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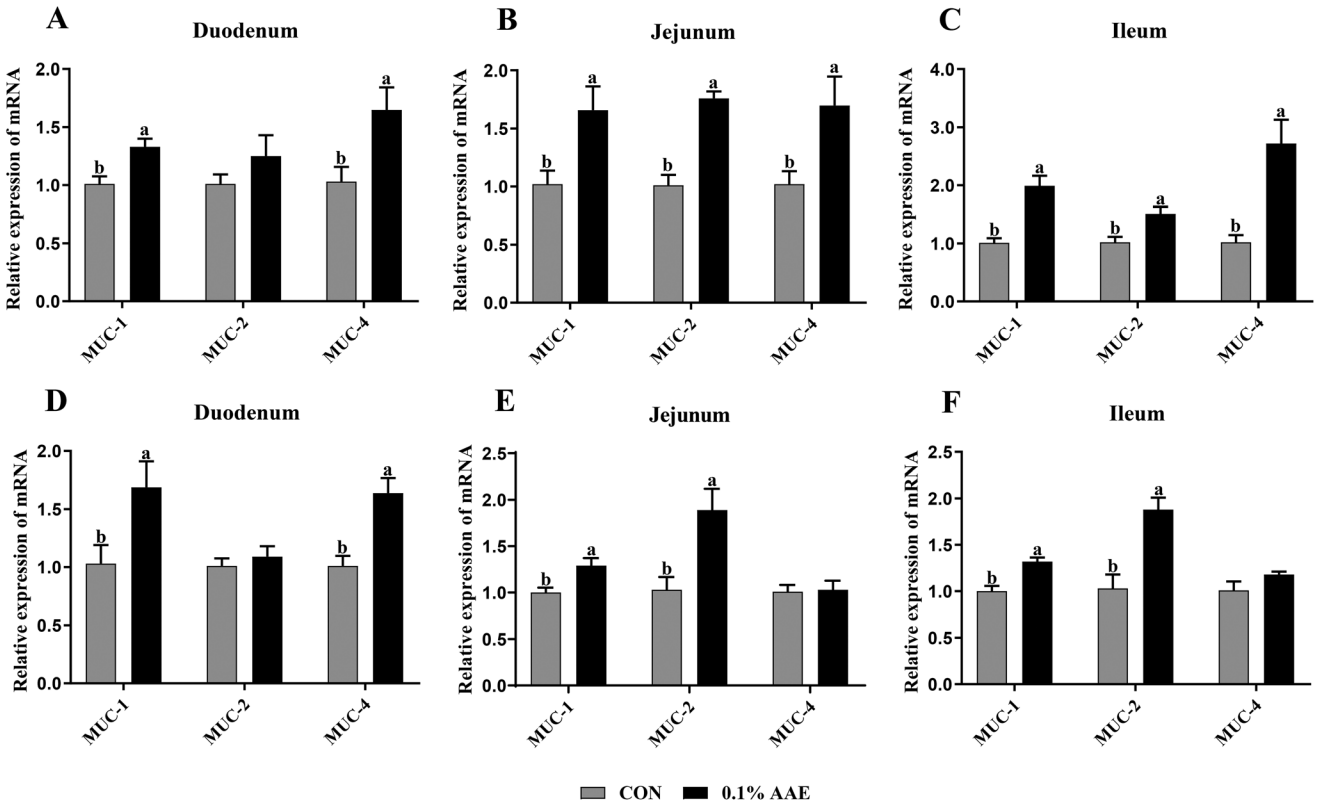

Fig. 5 Effects of maternal *Artemisia annua* L. extract (AAE) supplementation on the relative mRNA expression levels of MUCs in the duodenum (A), jejunum (B), and ileum (C) of newborn piglets and the duodenum (D), jejunum (E), and ileum (F) of weaned piglets. CON, control group; 0.1% AAE, 1.0 g kg^{-1} AAE group; and MUC, mucin.

intestine of both neonatal and weaned piglets. It is noteworthy that neonates in our study were immediately slaughtered without suckling colostrum after delivery, indicating that the effect of maternal AAE supplementation on the intestinal cytokine levels of fetal pigs has already occurred *via* the placenta and maternal–fetal interface. Research studies have demonstrated that artemisinin and polyphenols (including curcumin, resveratrol, and chlorogenic acids) in AAE prevent the inflammatory response prompted by LPS which is a cytoderm component of Gram-negative bacteria such as *E. coli.*[8] In addition, it is worth noting that much higher concentrations of IL-1β were observed in the proximal and middle parts of the small intestine in CON neonatal piglets. This might partially be caused by a higher concentration of environment-derived LPS in the front part of the gut. Thus, maternal AAE addition mainly attenuated the LPS-induced inflammatory pathways in the proximal and middle intestine (but not the distal part) to alleviate inflammation in neonatal and weaning piglets through the placenta and milk communication.

To further investigate the molecular mechanism of maternal AAE addition in mitigating the intestinal inflammatory response, we detected key mRNA and protein expressions related to TLR4/NF-κB and MAPK signaling pathways. TLR4 acts as a member of the TLR family and exists in almost all kinds of intestinal epithelial cells.[32] LPS could stimulate TLR4 and subsequently activate the NF-κB and MAPK signaling pathways.[33] Our results showed that the 0.1% AAE group had decreased mRNA levels of TLR4, indicating that AAE is capable of inhibiting the expression of TLR4 in neonatal and weaned pigs. Of note, we also found that the level of MyD88, an important adaptor protein in the TLR4 signal transduction process, was also decreased.[34] MyD88 activates the transforming TAK1, which leads to the excitation of the inhibitor of the NF-κB (IκB) kinase complex existing as IKKα and IKKβ.[35] The cytoplasmic binding of the inhibitor IκB with NF-κB protein results in the inhibition of NF-κB protein activity.[36] Then, IκB is phosphorylated and ubiquitylated by IκB kinase, which prompts NF-κB to separate from inhibitors IκB and translocates into the nucleus, where it induces the proinflammatory responses.[37] And the translocation increases the expression of various inflammatory cytokine genes, including IL-1β, IL-6, and TNF-α in the porcine intestine.[38] Assays for p-NF-κB can measure NF-κB activation.[39] In the current study, maternal 0.1% AAE significantly decreased the gene expression of NF-κB as well as the protein level of p-NF-κB in the small intestinal mucosa of offspring. Substantial research studies verified that the active ingredients in maternal AAE such as sesquiterpenes (artemisinin and arteannuinic acid) as well as flavonoids (casticin and chrysosplenol D) could inhibit TLR4/NF-κB and MAPK signaling pathways.[40–42] Therefore, maternal 0.1% AAE indeed attenu-

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ated the inflammatory response mediated by the TLR4/NF-κB signaling pathway, which was similar to our study in porcine mammary epithelial cells.[42]

TLR4 also triggers the downstream MAPK signaling pathway, and TAK1 also plays a key role in the intermediation of MAPK cascades. TAK1 recruits MAPK kinases, which phosphorylates and activates the MAPKs further.[43] There are three kinds of MAPKs, including the ERKs, JNK, and p38, which in turn phosphorylates AP-1 transcription factors.[44] Intranuclear AP-1 is composed of heterodimers of Fos and Jun subunits. Activation of AP-1 also facilitates the biosynthesis of inflammatory cytokines, including IL-1β, TNF-α, and IL-6.[45] The present study found that the mRNA expression levels of TLR4 and its downstream signals, including MyD88, TAK1, and MAPK, significantly decreased in the small intestine of offspring in the 0.1% AAE group. Apart from these, western blot results showed that 0.1% AAE obviously downregulated the protein levels of p-p38, p-JNK, and p-ERK in the MAPK signaling pathway in the small intestine of offspring. Based on these results, we speculated that in neonatal and weaned piglets, maternal 0.1% AAE could alleviate the overstimulation of intestinal inflammatory responses, partly *via* inhibiting the activation of TLR4/NF-κB and MAPK inflammatory pathways, which at least partly accounts for the inhibitory effect of AAE on the excessive release of inflammatory cytokines (TNF-α, IL-1β, IL-6, and IL-12) as mentioned above. In addition, PPARγ is a nuclear receptor secreted by intestinal epithelial cells and exhibits anti-inflammatory and antioxidant effects.[46] Numerous reports have documented that activated PPARγ can restrain major inflammation signaling pathways including the TLR4/NF-κB signaling pathway.[47] In our research, the 0.1% AAE group had a higher level of PPARγ in the small intestine of neonatal and weaned piglets. Thus, the upregulation in PPARγ transcripts indicated that maternal AAE could also mediate anti-inflammatory effects through activation of PPARγ. Moreover, previous studies showed that the ERKs/MAPK cascade signaling pathway suppressed activation in PPARγ either by inhibitory phosphorylation or by modulating nucleo-cytoplasmic compartmentalization,[48,49] suggesting that the effects of maternal AAE treatment on both stimulatory PPARγ expression and prohibitive ERK activation could be linked.[50]

Nutritional intervention in endogenous antimicrobial peptide (AMP) secretion is considered an efficient nonantibiotic approach to restrain pathogenic intestinal infection.[51] *Ex vivo* studies have shown that defensins act as AMPs and have a broad-spectrum bacteriostasis and anti-inflammatory effect.[52] To date, only β-defensins have been found to exist in swine.[53] As described previously, defensins can neutralize endotoxin and therefore restrain the secretions of proinflammatory cytokines such as TNF-α, IL-1β, and IL-6.[54] Previous research reported that porcine beta-defensin 2 (PBD-2) can promote the growth rate, alleviate diarrhea in piglets[55] and decrease the expression of inflammatory cytokines in weaned pigs.[56] PBD-2 also supplies protection against pathogenic infection and alleviates inflammation *via* interference with the TLR4/NF-κB signalling pathway.[57] Human β-defensin3 (hBD3) also suppresses TNF-α and IL-6 biosynthesis to promote an anti-inflammatory reaction.[58] The current results revealed that compared to the CON group, the 0.1% AAE group had higher concentrations of targeted PBD-2 in the small intestine, PBD-1 in the duodenum, and PBD-3 in the jejunum of neonatal and weaned piglets. The increase caused by 0.1% AAE treatment might be related to the inhibition of the TLR4/NF-κB pathway and cytokine

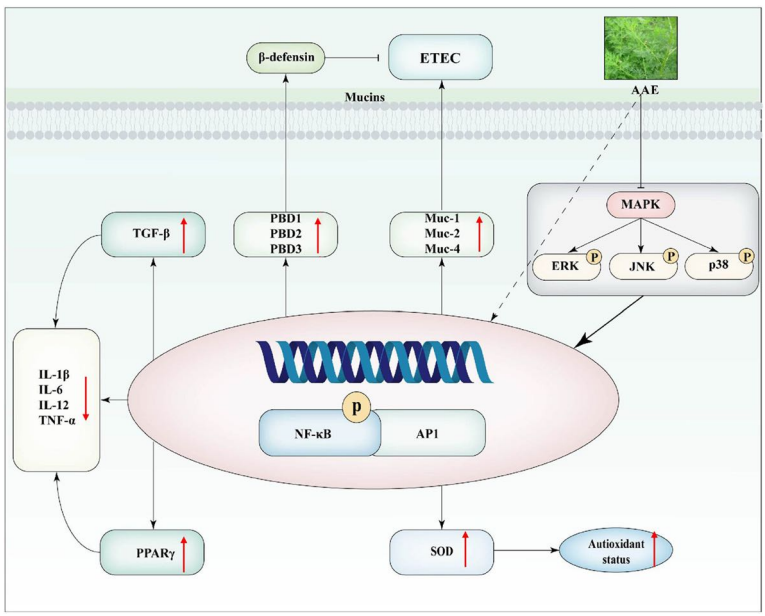

Fig. 6 Study design and schematic diagram of the possible mechanisms of maternal *Artemisia annua* L. extract (AAE) supplementation against inflammation and oxidative stress of the offspring.

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secretion and provided a higher defense and immunoregulation for the invasion of pathogens in the gut.

Mucins (MUCs), the secretory products of intestinal goblet cells, are the front line of innate host defense lining the GI tract.[59] MUC2 is the primary gel-forming component of intestinal mucus, which was secreted by goblet cells. Ueno *et al.*[60] reported that MUC1 restrained the activation of the TLR4/NF-κB signaling pathway, suggesting its anti-inflammatory impact on the gut. In our research, the levels of MUC1 in the small intestine, MUC2 in the jejunum, and MUC4 in the duodenum were all obviously enhanced in the offspring in the 0.1% AAE group. The results indicated that maternal 0.1% AAE supplementation might suppress the NF-κB/TLR4 activation through induction of the endogenous synthesis of MUCs.

Conclusions

To summarize, as shown in Fig. 6, the current study indicated that maternal 0.1% AAE supplementation during late gestation and lactation attenuated the offspring's small intestinal inflammatory response, which is possibly mediated by suppressing the activation of the TLR4/NF-κB and MAPK inflammatory pathways. Besides, 0.1% AAE supplementation significantly stimulated the expression of β-defensins, mucins, and PPARγ to promote the inflammation resolution and innate immunity response. Moreover, maternal AAE supplementation significantly improved the offspring's small intestinal redox status. Collectively, these findings have direct implications for refining dietary recommendations in pregnancy and lactation and provide insight into mother–infant nutrition in humans.

Ethics review

All animal procedures performed for animal care and use during this study were approved by the South China Agricultural University Animal Care and Animal Use Committee (Guangzhou, China) and complied with relevant laws or guidelines. The application number of ethics review on the institutional committee is 2021g017 and the ethics review had been offered.

Conflicts of interest

The authors declared no conflict of interest.

Author contributions

F. Chen, S. H. Zhang, and W. T. Guan designed the experiments. L. Xiong drafted the manuscript. Y. F. Zhang and L. Xiong carried out the experiments. Y. F. Zhang and L. Xiong performed the analysis of all data. F. Cheng, S. H. Zhang, M. Ren, and W. T. Guan. conducted the final proofreading. All authors have read and agreed to the published version of the manuscript.

Acknowledgements

The authors thank the Guangzhou Cohoo Biotechnology Research and Development Center (Guangzhou, P. R. China) for the preparation of the *Artemisia annua* L. extract (AAE). The present study was jointly supported by the National Natural Science Foundation of China (31872364) and the Guangdong Natural Science Foundation (2021A1515010944).

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Animal Nutrition 6 (2020) 160–167

Contents lists available at ScienceDirect

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Original Research Article

Combined yeast culture and organic selenium supplementation during late gestation and lactation improve preweaning piglet performance by enhancing the antioxidant capacity and milk content in nutrient-restricted sows

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ARTICLE INFO

Article history:
Received 6 November 2019
Received in revised form
14 December 2019
Accepted 14 January 2020
Available online 23 January 2020

Keywords:
Yeast culture
Organic selenium
Reproductive performance
Antioxidant status
Sow

ABSTRACT

This study was conducted to investigate the effects of dietary supplementation with yeast culture (YC) and organic selenium (Se) during late gestation and lactation on reproductive performance, milk quality, piglet preweaning performance, antioxidant capacity, and secretion of immunoglobulin in multiparous sows. A total of 160 healthy cross-bred sows (Landrace × Yorkshire, mean parity 4.1 ± 0.3) were randomly assigned to 4 groups as follows: 1) high nutrient (HN), 3,420 kcal/kg digestible energy (DE) and 18.0% crude protein (CP); 2) low nutrient (LN), 3,240 kcal/kg DE and 16.0% CP; 3) LN + YC, LN diet + 10 g/kg YC; 4) LN + YC + Se, LN diet + 10 g/kg YC + organic Se (1 mg/kg Se). Feeding trials of sows started from d 85 of pregnancy to d 35 of lactation. Compared with sows in the LN group, sows fed the LN + YC + Se diet had greater litter weaning weight, average litter gain, and milk fat content (14-d and 25-d milk) ($P < 0.05$). The content of malonaldehyde (MDA) (colostrum and 14-d milk) was lesser, and the activity of glutathione peroxidase (GSH-Px) (colostrum and 25-d milk) was greater when sows were fed the LN + YC + Se diet, compared with sows fed the LN diet ($P < 0.05$). Supplementation of YC and organic Se in the nutrient-restricted diet improved sows' reproductive performance and pig weaning body weight by enhancing the antioxidant capacity and fat content in milk.

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1. Introduction

Yeast culture (YC) is a dried fermented product containing a small amount of active yeast cells and various metabolites. Current studies suggest that YC is a promising alternative to antimicrobial growth promoter (AGP), because of β-glucan and mannan oligosaccharides existing in its cell wall (Ganner and Schatzmayr, 2012). Supplementation of YC has been shown to improve growth performance, increase milk production, and stimulate immune systems in pigs (Van Heugten, Funderburke & Dorton, 2003) and ruminants (Nocek et al., 2011). Furthermore, dietary supplementation of YC has also been shown to improve the digestibility of dry matter, gross energy, and crude protein (CP) in pigs, indicating YC supplementation could improve the dietary feed conversion ratio (Shen et al., 2009). Recently, more attention has been focused on the function of YC in diets of sows. Some studies have reported that yeast products supplementation in gestation and lactation diets of sows increased the litter weight and litter weight gain of weaned

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Peer review under responsibility of Chinese Association of Animal Science and Veterinary Medicine.

Production and Hosting by Elsevier on behalf of KeAi

https://doi.org/10.1016/j.aninu.2020.01.004

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piglets (Shen et al., 2011), which might be the result of higher amounts of total solids, CP, and γ-globulin in milk of sows supplemented with yeast products (Jurgens et al., 1997).

Selenium (Se) is an important trace nutrient for many different animal species, as it is an essential component of selenoproteins, such as the antioxidative enzyme glutathione peroxidase (GSH-Px) (Brown and Arthur, 2001). Besides playing a vital role in cell antioxidant defense, Se also participates in the reduction of inflammation and immune regulation. In the past few years, several reports highlighted the function of high dose Se supplementation in animals (higher than the up-limit level of selenium [0.5 mg/kg] in Food and Drug Administration [FDA] in USA and standards in China). This level is greater than that required for optimal expression of selenoproteins, but less than maximum tolerable level. In ruminants, feeding Se at supranutritional levels to pregnant or lactating females alleviated heat-induced oxidative stress (Chauhan et al., 2014c), down-regulated inflammatory genes (Chauhan et al., 2014b), and altered colostrum volume and composition including immunoglobulin G (IgG) (Stewart et al., 2013b). Providing Se to the females during gestation and lactation is an effective way to transfer Se to the infants and newborns, because Se can cross the placental barrier and is excreted in colostrum and milk (Stewart et al., 2013b).

Generally, feed cost accounts for approximately 70% of the total cost of animal husbandry. Reasonable reduction of dietary nutrient levels with an increase in nutrient digestibility is a strategy to reduce expenditure and decrease environmental pollution. As mentioned above, YC is a functional feed additive with the ability to increase the digestibility of nutrients in pigs. However, it is unknown if the effects of feeding sows nutrient-limited diets could be ameliorated by supplementation with YC. Furthermore, oxidative stress during gestation and lactation have a strong negative influence on reproductive performance, which might require a stronger antioxidant defense system (like GSH-Px) to protect females from oxidative stress. Therefore, the objective of this study was to determine whether supplementation of dietary YC and Se in nutrient-limited diets during late gestation and lactation could regulate piglet performance, reproductive performance, milk quality, antioxidant capacity, and secretion of immunoglobulin in multiparous sows.

2. Materials and methods

The animal use and care protocol was approved by the South China Agricultural University Animal Care and Use Committee.

2.1. Experimental design

This experiment was conducted as a completely randomized design. A total of 160 multiparous sows (Landrace × Yorkshire, mean parity 4.1 ± 0.3) were allocated equally by parity and body condition to 1 of 4 groups (40 replicates per group and one sow per replicate) as follows: 1) high nutrient (HN) group, a corn-soybean meal basal diet containing 3,420 kcal/kg digestible energy (DE) and 18.0% CP; 2) low nutrient (LN) group, a corn-soybean meal basal diet containing 3,240 kcal/kg DE and 16.0% CP; 3) LN + YC group, LN diet supplemented with 10 g/kg YC (*saccharomyces cerevisiae*); 4) LN + YC + Se group, LN diet supplemented with 10 g/kg YC (*saccharomyces cerevisiae*) and 1 mg/kg Se (SelenoSource AFTM2000, 2,000 mg/kg, 98% of total Se are organic Se). Yeast culture and Se were supplemented on top of basal formula. Sows were fed the experimental diets from d 85 post-coitus until d 35 postpartum (piglets were weaned at 28 d of age).

2.2. Diets and management

In the HN group, a corn-soybean meal based diet was used and formulated to meet or exceed the nutrient requirements of gestating and lactating sows (NRC, 2012). Compared with the HN group, DE and CP in LN groups were decreased by 180 kcal/kg and 2%, respectively. The composition and nutrient content of basal diets are presented in Table 1. After breeding, sows were housed individually in gestation crates and allowed *ad libitum* access to water. The total daily rations per sow were 3.5 kg from d 85 post-coitus until 112 d post-coitus. At 112 d post-coitus, sows were moved into an environmentally controlled farrowing house, with feed restricted (3.5 kg) until 5 d postpartum. From 5 d postpartum to the end of this experiment (35 d postpartum), sows had *ad libitum* access to experimental diets and water. Creep feed was provided to the piglets from 7 d postpartum. Sows experiencing abnormal health conditions (abortion, severe lameness, metritis, respiratory disease, death, etc.) during the experiment were culled with numbers listed in Table 2. The average temperatures in the farrowing house and the gestation room were 30.8 and 27.2 °C, and the humidity averages were 82.78% and 77.89%, respectively.

2.3. Data and sample collection

Six sows per treatment were randomly selected and 10 mL blood was sampled via ear venipuncture at 85 d post-coitus, and 1, 14 and 25 d postpartum. The same subset of sows was bled at each

Table 1
Ingredients and nutrient content of basal diets (as-fed basis, %).

Item	High nutrient diet	Low nutrient diet
Ingredients		
Maize	58.70	64.42
Soybean meal (42% crude protein)	23.70	19.58
Wheat bran	8.00	8.00
Fish meal (65% crude protein)	2.00	2.00
Palm oil	4.00	2.00
Limestone	1.35	1.35
Dicalcium phosphate	0.75	0.83
Vitamin premix[1]	0.10	0.10
Mineral premix[2]	0.04	0.04
Salt	0.33	0.33
L-Lys·HCl	0.10	0.21
Vitamin E (50%)	0.01	0.01
Biotin (2%)	0.005	0.005
Folic acid (98%)	0.005	0.005
Choline chloride (50%)	0.30	0.30
Phytase	0.02	0.02
Fungicide	0.05	0.05
Antioxidant (ethoxyquin >6.5%, butylated hydroxytoluene > 2.5%)	0.04	0.04
Carrier	0.50	0.71
Total	100.00	100.00
Nutrient content (calculated)		
Digestible energy, kcal/kg	3,420	3,240
Crude protein	18.00	16.00
Calcium	1.02	1.02
Total phosphorus	0.79	0.79
Available phosphorus	0.48	0.49
NaCl	0.41	0.41
Lys	0.96	0.96
Met + Cys	0.58	0.59
Thr	0.62	0.63
Trp	0.21	0.21

[1] Provided the following per kilogram of diet: Fe, 80 mg; Cu, 5 mg; Zn, 51 mg; Mn, 20.5 mg; I, 0.14 mg; Se, 0.15 mg.
[2] Provided the following per kilogram of diet: vitamin A, 13,000 IU; vitamin D_3, 4,000 IU; vitamin E, 60 mg; vitamin K_3, 4 mg; vitamin B_1, 4 mg; vitamin B_2, 10 mg; vitamin B_6, 4.8 mg; vitamin B_{12}, 0.034 mg; niacin, 40 mg; pantothenic acid, 20 mg; folacin, 2 mg; biotin, 0.16 mg.

Table 2
Sample size before and after exclusions from trial.

Item	Groups[1]			
	HN	LN	LN + YC	LN + YC + Se
No. of initial sows	40	40	40	40
Diseases[2]	2	6	6	1
Sows for analysis	38	34	34	39

[1] High nutrient (HN) group: 3,420 kcal/kg digestible energy (DE) and 18.0% crude protein (CP); low nutrient (LN) group: 3,240 kcal/kg DE and 16.0% CP; LN + yeast culture (YC) group: LN diet + 10 g/kg YC; LN + YC + selenium (Se) group: LN diet + 10 g/kg YC + 1 mg/kg Se.
[2] Diseases refer to lameness, metritis, poor health, etc.

time period. The blood was collected in sterile vacutainer tubes and remained at room temperature for 1 h to allow clotting. Samples were centrifuged in a clinical centrifuge at 3,000×g for 10 min, and the serum was pipetted into micro-tubes. All the serum samples were immediately frozen at −20 °C until subsequent chemical analysis.

After intramuscular injection of 20 IU of oxytocin, samples of colostrum, 14-d milk, and 25-d milk were collected from anterior, middle and posterior teats of the same subset of sows for blood sample collection. Colostrum was sampled within 12 h postpartum. Milk samples were collected into 30 mL tubes and stored at −20 °C until analysis.

The number of piglets born (total, stillborns, live, weak [body weight < 0.8 kg], qualified [number of live piglets minus number of weak piglets]), litter weight at birth, and individual weight at birth were recorded at farrowing. The number of pigs weaned, litter weight and individual pig weight were all recorded at weaning.

2.4. Milk composition

Colostrum and milk samples were analyzed for solids-not-fat, fat, protein, and lactose using a fully automated milk composition analyzer (MilkoScan® FT + Analyzer, Foss, Hillerød, Denmark).

2.5. Immunoglobulin

The concentrations of IgG, immunoglobulin A (IgA) and immunoglobulin M (IgM) IgM were analyzed by turbidimetric inhibition immunoassay in the laboratory of Anda Clinical Inspection Center, Sun Yat-Sen University, using a Hitachi 7600 automatic biochemical analyzer (Hitachi Co., Tokyo, Japan).

2.6. Determination of redox status of serum and milk

Total antioxidant capability (T-AOC), and the activities of superoxide dismutase (SOD), GSH-Px, glutathione (GSH) and malonaldehyde (MDA) in serum and milk were estimated using commercial kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, China), according to the manufacturer's protocols with a V1600 Split Beam Visible Spectrophotometer (Meipuda Co., Shanghai, China). The results were expressed as units per milliliter serum or milk.

2.7. Statistical analysis

Statistical analyses were performed using the General Linear Model procedure of SPSS 17.0 software (SPSS, INC., Chicago, IL, USA) in a completely randomize design. The individual sow and its litter were used as an experimental unit for the analysis of sow and litter performance. Milk composition and redox status in serum and milk were analyzed as repeated measures. Pair-wise tests of hypotheses were done using LSD multiple range analysis. Probabilities less than 0.05 were used for determination of significant differences and probabilities less than 0.10 were considered to represent tendencies among treatments.

3. Results

3.1. Sow reproductive performance

Sow reproductive performance is presented in Table 3. There was little evidence of differences among the 4 groups in the number of pigs born in total, live pigs born, stillbirths, and qualified pigs ($P > 0.10$). Similarly, treatment differences in pig weight at birth, litter weight at birth, and number of pigs weaned were small and not important ($P > 0.10$). Compared with pigs from sows in the LN group, there was a trend for individual weaning weights of pigs from sows fed LN + YC diet to be greater ($P < 0.10$); and litters from sows fed LN + YC + Se diet were greater in litter weaning weight ($P < 0.01$), pig weaning weight ($P < 0.01$), average litter gain ($P < 0.05$) and average daily gain per piglet ($P < 0.05$) compared to pigs from sows fed the LN diet. Compared with pigs from sows fed HN diet, pigs from sows fed LN + YC + Se diet had greater pig weaning body weight ($P < 0.01$) and tended to have greater average litter gain ($P < 0.10$) and average daily pig weight gain ($P < 0.10$).

3.2. Milk components

The effect of dietary supplementation of YC and organic Se on the composition of colostrum, 14-d milk, and 25-d milk is shown in Fig. 1 Compared with sows in the HN group, sows fed LN and LN + YC diets tended to have less concentrations of protein (9.19% vs. 8.24% and 8.29%, $P < 0.10$), lactose (12.90% vs. 11.80% and 11.81%, $P < 0.10$) and solids-not-fat (24.07% vs. 21.80% and 21.87%, $P < 0.10$) in colostrum. Sows fed LN + YC + Se diet had greater concentrations of milk fat in 14-d (6.88% vs. 7.71%, $P < 0.05$) and 25-d (5.54% vs. 6.80%, $P < 0.05$) milk, compared with sows fed LN diet. The concentration of solids-non-fat from 25-d milk in the HN group were greater than that in the LN group (6.18% vs. 5.54%, $P < 0.05$) and the LN + YC group (6.18% vs. 5.69%, $P < 0.10$).

3.3. Antioxidant status in colostrum and milk

Fig. 2 shows the effect of yeast culture and organic Se on antioxidant status in colostrum and milk. Compared with sows fed the HN diet, sows fed the LN diet had a trend for decreased activity of SOD in colostrum (356 vs. 326 U/mL, $P < 0.10$). Compared to sows in the LN group, sows in the LN + YC group had comparatively lower MDA content in colostrum (13.59 vs. 10.98 nmol/mL, $P < 0.05$) and greater activity of GSH-Px in 25-d milk (79.47 vs. 64.34 U/mL, $P < 0.05$). Additionally, sows in the LN + YC group tended to have increased activity of SOD in colostrum (326.27 vs. 350.28 U/mL, $P < 0.10$), compared with the sows in the LN group. Compared with sows in the LN group, the MDA content was less in colostrum (13.59 vs. 9.63 nmol/mL, $P < 0.05$) and 14-d milk (8.41 vs. 7.01 nmol/mL, $P < 0.05$) in LN + YC + Se group. When sows were fed LN + YC + Se diet, the activity of GSH-Px was greater in colostrum (146.59 vs. 181.23 U/mL, $P < 0.05$) and 25-d milk (64.43 vs. 79.47 U/mL, $P < 0.05$) compared to sows fed the LN diet. Additionally, sows fed the LN + YC + Se diet tended to have decreased MDA content in colostrum (12.30 vs. 9.63 nmol/mL, $P < 0.10$) compared with sows fed the HN diet.

Table 3
Effects of yeast culture (YC) and supranutritional organic selenium (Se) supplementation during late gestation and lactation on the reproductive performance of sows.

Item	Groups[1]				Pooled SEM	*P* value
	HN	LN	LN + YC	LN + YC + Se		
Parturition						
Total No. of pigs born per litter	11.13	11.21	11.06	10.90	0.21	0.92
No. of live pigs born per litter	10.42	10.47	10.18	10.08	0.19	0.82
No. of stillbirths per litter	0.71	0.74	0.88	0.82	0.08	0.69
No. of qualified piglets	10.30	10.00	9.82	9.64	0.19	0.84
No. of weak pigs per litter	0.26	0.29	0.21	0.31	0.02	0.80
Pig weight at birth, kg	1.57	1.50	1.47	1.55	0.02	0.30
Litter weight at birth, kg	16.29	15.36	14.91	15.24	0.27	0.58
Weaning (21 d of age)						
No. of pigs weaned per litter	8.63	8.38	8.47	8.53	0.16	0.73
Litter weight, kg	50.84[ab]	47.16[b]	52.11[ab]	54.53[a]	1.22	<0.01
Pig weight, kg	5.86[b]	5.65[b]	6.14[ab]	6.47[a]	0.09	<0.01
Average litter gain, kg	29.36[ab]	27.21[b]	29.72[ab]	34.17[a]	1.40	0.03
Average daily gain per piglet, g	150[ab]	145[b]	155[ab]	167[a]	3.04	0.02

[a, b] Means in the same row with different superscripts differ ($P < 0.05$).
[1] High nutrient (HN) group: 3,420 kcal/kg digestible energy (DE) and 18.0% crude protein (CP); low nutrient (LN) group: 3,240 kcal/kg DE and 16.0% CP; LN + yeast culture (YC) group: LN diet + 10 g/kg YC; LN + YC + selenium (Se) group: LN diet + 10 g/kg YC + 1 mg/kg Se.

3.4. Antioxidant status in serum of sows

The effect of YC and organic Se on antioxidant status in serum of sows is presented in Fig. 3. Sows fed the LN diet had a trend for lower activity of serum GSH-Px at 25 d postpartum (948 vs. 885 U/mL, $P < 0.10$) than sows fed the HN diet. Compared to the sows in the LN group, sows fed the LN + YC diet showed a trend for greater activity of serum SOD at 1 d postpartum (174 vs. 195 U/mL, $P < 0.10$), but there was little evidence of differences in other serum indexes. When compared with the sows in the LN group, sows in the LN + YC + Se group tended to exhibit greater activity of T-AOC at 1 d postpartum (1.57 vs. 2.18 U/mL, $P < 0.10$) and 25 d postpartum (0.99 vs. 1.35 U/mL, $P < 0.10$). Sows in the LN + YC + Se group had trends for comparatively greater GSH concentration at 1 d postpartum (5.80 vs. 6.65 μmol/L, $P < 0.10$) and greater activity of GSH-Px at 25 d postpartum (885.35 vs. 911.61 U/mL, $P < 0.10$) than sows in the LN group. Additionally, compared to sows in the LN group, sows in the LN + YC + Se group had greater SOD activity at 1 d postpartum (174 vs. 198 U/mL, $P < 0.05$) and a trend at 14 d postpartum (141 vs. 154 U/mL, $P < 0.10$).

3.5. Concentrations of immunoglobulin in serum and colostrum

The concentrations of immunoglobulins in serum and colostrum are shown in Fig. 4. There was little evidence of differences in serum IgG concentration among the 4 groups ($P > 0.10$). On d 85 of gestation and d 1, 14, and 25 of lactation, there was little evidence of differences in concentrations of IgG, IgA and IgM in sow colostrum among the groups ($P > 0.10$).

4. Discussion

The performance of breeding sows affects the entire productivity of pig operations. Deficiency in either protein or energy during gestation can have negative effects on sow reproductive performance. It has been demonstrated that sows fed an adequate-energy diet during gestation gained more weight from the start of the experiment to weaning and farrowed heavier pigs than those fed energy-restricted diet (Clawson et al., 1963; Frobish et al., 1966). Furthermore, milk yield in sows is depressed by a deficiency of protein or energy during gestation and lactation and this would be reflected in lower litter weights (Pond, 1973). Similarly, in this study, sows fed the nutrient-limited diet had lower litter weight, which might be caused by the decrease in fat, protein, lactose, and non-fat-milk content in milk. Although we did not measure milk yield, it is reasonable to assume that lower milk yield in the nutrient-limited sows was also a contributing factor. Our research also demonstrated that feeding sows with a nutrient-limited diet had adverse effects on the protein, lactose and solids-not-fat concentrations in colostrum but not in 14-d milk and 25-d milk (except

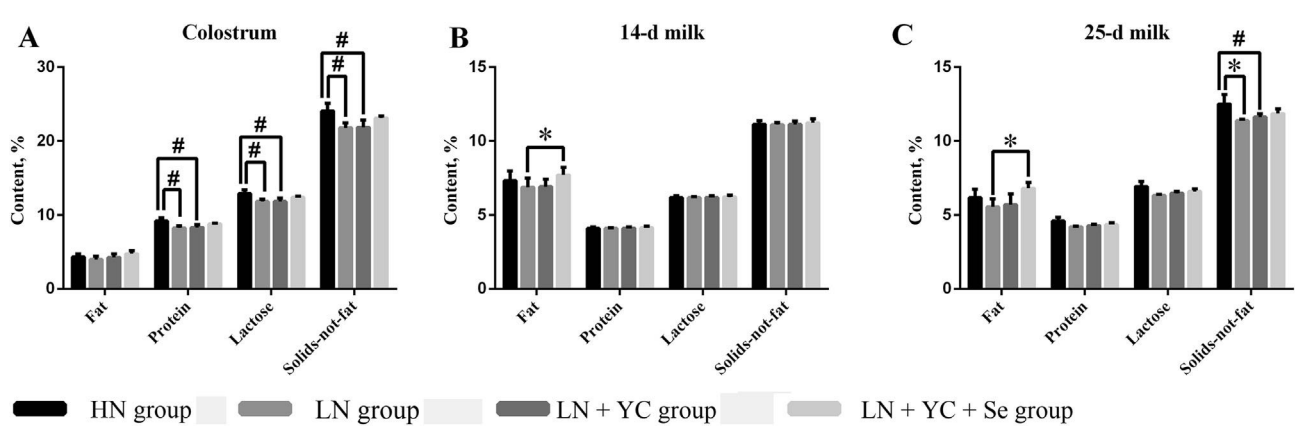

Fig. 1. Effects of dietary supplementation of yeast culture (YC) and supranutritional organic selenium (Se) on the composition of colostrum (A), 14-d milk (B), and 25-d milk (C). High nutrient (HN) group: 3,420 kcal/kg digestible energy (DE) and 18.0% crude protein (CP); low nutrient (LN) group: 3,240 kcal/kg DE and 16.0% CP; LN + YC group: LN diet + 10 g/kg YC; LN + YC + Se group: LN diet + 10 g/kg YC + 1 mg/kg Se. The asterisk (*) indicates that there are significant differences ($P < 0.05$) between 2 groups. Pound sign (#) represents tendencies between 2 groups ($P < 0.10$).

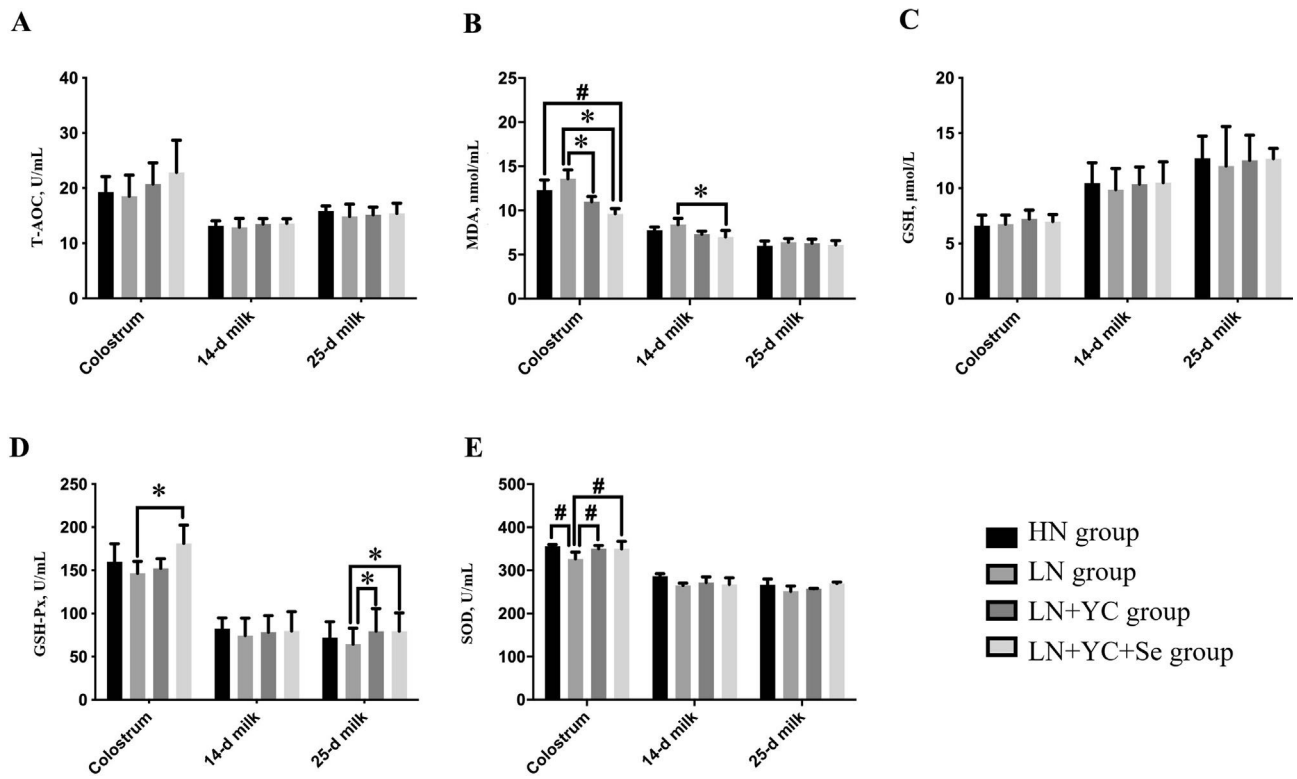

Fig. 2. Effects of dietary supplementation of yeast culture (YC) and supranutritional organic selenium (Se) on T-AOC (A), MDA (B), GSH (C), GSH-Px (D), and SOD (E) in colostrum and milk. T-AOC = total antioxidant capability; MDA = malonaldehyde; GSH = glutathione; GSH-Px = glutathione peroxidase; SOD = superoxide dismutase. High nutrient (HN) group: 3,420 kcal/kg digestible energy (DE) and 18.0% crude protein (CP); low nutrient (LN) group: 3,240 kcal/kg DE and 16.0% CP; LN + YC group: LN diet + 10 g/kg YC; LN + YC + Se group: LN diet + 10 g/kg YC + 1 mg/kg Se. The asterisk (*) indicates that there are significant differences between 2 groups ($P < 0.05$). Pound sign (#) represents tendencies between 2 groups ($P < 0.10$).

the solids-not-fat content in 25-d milk). This might be related to the early limited feed intake of sows (3.5 kg from d 85 post-coitus to 5 d postpartum, ad libitum thereafter). Colostrum was sampled under limited intake, and 14-d milk and 25-d milk were collected during *ad libitum* feeding. Sows appear to maintain the output of energy and protein in milk and protect the offspring against nutritional protein and energy deficiency by mobilizing their own reserves to allow pig survival, unless undergoing severe deficiencies in either class of nutrients (Pond, 1973; Noblet and Etienne, 1986). Thus, the sufficient feed intake after 5 d postpartum might allow sows to mobilize more energy and maintain the nutrient content in 14-d milk and 25-d milk.

Reports in the literature of the effect of YC supplementation on growth performance are inconsistent. Some studies reported supplementation of live yeast or YC improved post-weaning performance (Van Heugten, Funderburke & Dorton, 2003; Shen et al., 2009), whereas one report observed no benefits of YC supplementation (Kornegay et al., 1995). Others reported that yeast product supplementation in gestation and lactation diets of sows increased the litter weight and litter weight gain of weaned piglets (Sungwoo et al., 2008). In this study, YC supplementation in nutrient-limited diet numerically increased the litter weight, pig weight, average litter gain and average daily gain for the piglet. Yeast culture supplementation has resulted in higher contents of total solids, CP, and γ-globulin in milk (Jurgens et al., 1997), which might be partly caused by the increase of nutrient digestibility in the sow. The results from our study agreed with the hypothesis that supplementation of YC in gestation and lactation diets of sows significantly increased the digestibility of ether extract, dry matter, organic matter, and gross energy (unpublished data).

Sufficient Se must be provided to prevent Se-related diseases in animals. High dose organic Se was selected to supplement the sow diet during late gestation and lactation in this trial. Commonly, organic forms of Se have higher bioavailability and antioxidant properties and are more friendly to the environment than inorganic forms of it (Kim and Mahan, 2001). Supplementation of Se at high levels has resulted in an improvement in animal performance and immune function in ruminants (Rooke et al., 2004), which might have been caused by increases of ruminal fermentation and nutrient digestibility (Xun et al., 2012). Maternal nutrition during gestation and lactation plays an important role in neonatal health as it regulates both colostrum volume and composition, which is required for offspring in an early stage (Lilja et al., 1991). High dose of Se has been reported to result in a greater milk yield and higher butterfat, solids-not-fat, lactose and protein content in colostrum (Meyer et al., 2011), which is consist with our results that supplementation of a high dose Se numerically increased solids-not-fat, lactose and protein content in colostrum, and fat content in 14-d milk and 25-d milk.

Supranutritional supplementation has been shown to enhance humoral immune function and increase IgG absorption (Kamada et al., 2007). Stewart et al. (2013a) found supranutritional Se supplementation in diets of ewes and cows during pregnancy increased IgG concentrations in colostrum and promoted IgG transfer from mother to offspring. Additionally, supranutritional Se status has been shown to influence the vascularity of the mammary gland and enhance nutrient content level in milk (Vonnahme et al., 2011). However, in this study, no difference was observed in concentrations of IgG, IgA and IgM in colostrum and sow serum when supranutritional Se was supplemented in the diet. Species

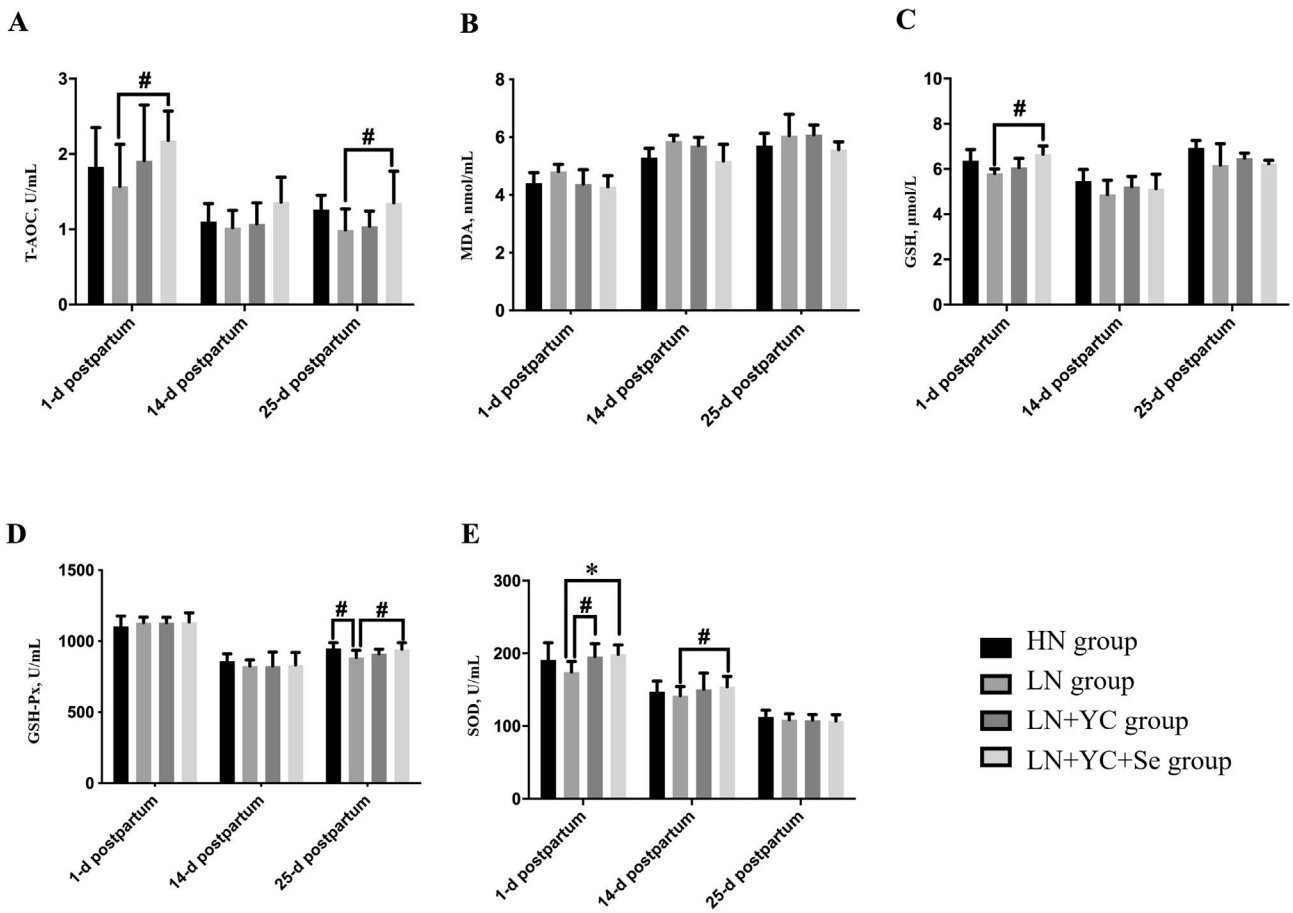

Fig. 3. Effects of dietary supplementation of yeast culture (YC) and supranutritional organic selenium (Se) on T-AOC (A), MDA (B), GSH (C), GSH-Px (D), and SOD (E) in serum of sows. T-AOC = total antioxidant capability; MDA = malonaldehyde; GSH = glutathione; GSH-Px = glutathione peroxidase; SOD = superoxide dismutase. High nutrient (HN) group: 3,420 kcal/kg digestible energy (DE) and 18.0% crude protein (CP); low nutrient (LN) group: 3,240 kcal/kg DE and 16.0% CP; LN + YC group: LN diet + 10 g/kg YC; LN + YC + Se group: LN diet + 10 g/kg YC + 1 mg/kg Se. The asterisk (*) indicates that there are significant differences between 2 groups ($P < 0.05$). Pound sign (#) represents tendencies between 2 groups ($P < 0.10$).

differences in effects of supranutritional Se on immunoglobulin secretion and transportation might lead to these differences between ruminants and non-ruminants. More studies are needed to fully elucidate species differences in response to supranutritional Se supplementation.

The current nutrient requirements of swine (NRC, 2012) recommend 0.15 mg/kg Se for sows during gestation and lactation. Some scientists hypothesized that pigs are more sensitive to insulin than ruminants, and supranutritional Se supplementation might interfere with insulin homeostasis with the result of negative effects on pig performance (Pinto et al., 2012). However, in our experiment, when the dose of Se was increased to 1 mg/kg, supranutritional Se supplementation significantly enhanced piglet performance (litter weight, pig weight, average litter gain and average daily gain) and had no apparent adverse effects.

Pregnancy is considered as a state of oxidative stress. Metabolic burdens on sows can increase lipid and protein oxidation and

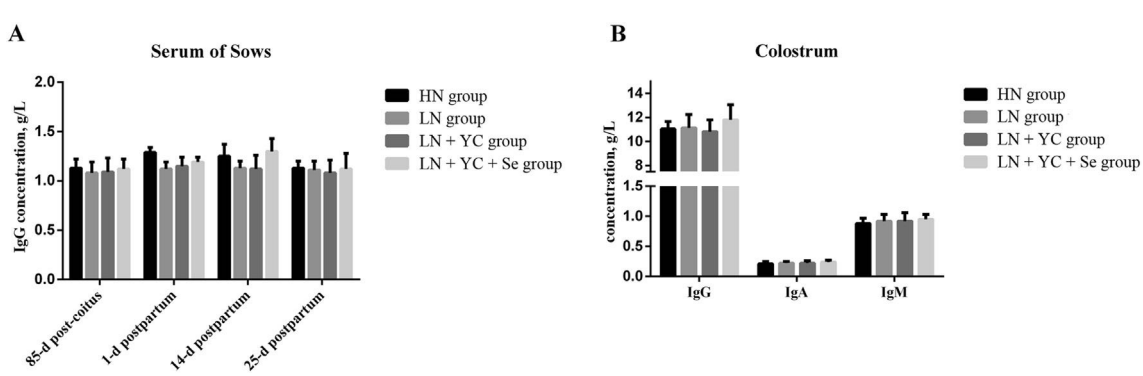

Fig. 4. Effects of dietary supplementation of yeast culture (YC) and supranutritional organic selenium (Se) on serum IgG concentrations of sows (A) and concentrations of IgG, IgA and IgM in colostrum (B). IgG = immunoglobulin G; IgA = immunoglobulin A; IgM = immunoglobulin M. High nutrient (HN) group: 3,420 kcal/kg digestible energy (DE) and 18.0% crude protein (CP); low nutrient (LN) group: 3,240 kcal/kg DE and 16.0% CP; LN + YC group: LN diet + 10 g/kg YC; LN + YC + Se group: LN diet + 10 g/kg YC + 1 mg/kg Se.

impair normal cell functions during late gestation and lactation. In recent years, more attention has been focused on the potential influence of maternal nutrition on the nutritional status and antioxidant system of their offspring. Total antioxidant capability, SOD, GSH, GSH-Px, and MDA were used for determination of redox status of serum and milk in sows in this experiment. Total antioxidant capability is a crucial integrative index to reflect the total antioxidant capacity of the animal, and SOD enzymes play an important role in catalyzing the disproportionation of superoxide to H_2O_2 and water (Fridovich, 1986). The major role of GSH is to act as a reductant in oxidation-reduction processes, and GSH-Px is considered as an antioxidant enzyme to catalyze the reduction of hydrogen peroxide in the presence of GSH. Furthermore, MDA is a product of lipid peroxidation caused by the reaction of lipid oxidation induced by oxygen-free radicals.

In this research, YC supplementation increased the activity of SOD (1 d postpartum serum and colostrum) and GSH-Px (25-d milk), and decreased the MDA content (colostrum) of sows. Similarly, effects of YC supplementation on antioxidant capacity are also observed in finishing pigs (Sauerwein et al., 2007) and weaning pigs (Yang et al., 2016). Currently, the underlying mechanism of the effects of YC supplementation is still poorly understood. It is possible that various metabolites in YC, including minerals like Se, Cu, and Zn, may be involved. Eukaryotes have 2 intracellular SOD as follows: a copper- and zinc-containing enzyme (SOD1) located in the cytosol and a manganese-containing enzyme (SOD2) located in the mitochondrial matrix (Crapo et al., 1992). Selenium is a vital component of selenoprotein enzyme GSH-Px in animals (Arthur, 2001). Therefore, supplementation of YC provides mineral substrates for antioxidant enzyme synthesis, which might increase the activity of those enzymes.

Supranutritional organic Se supplementation has been reported to improve the antioxidant status and immune response, which is attributed to the selenoproteins that are involved in oxidative stress and inflammation regulation (Mattmiller et al., 2014). Chauhan et al. (2014a) demonstrated that supplementation of sheep diets with supranutritional doses of vitamin E and Se successfully scavenged heat stress-induced reactive oxygen species generation, resulting in a decrease in oxidative stress. Hall et al. (2014) conducted an experiment in cows and observed similar results, which are supranutritional doses of Se improved antioxidant status in early lactation. In this study, we observed similar results, i.e., a high dose of Se supplementation increased the activity of GSH-Px (25 d postpartum) and SOD (1 and 14 d postpartum), with a higher concentration of GSH (1 d postpartum) and T-AOC (1 and 25 d postpartum) in sow serum. Also, MDA content (colostrum and 14-d milk) was less, and the activity of GSH-Px (colostrum and 25-d milk) greater in sow milk.

5. Conclusion

Combined of YC and organic Se supplementation in nutrient-restricted sows during late gestation and lactation appears beneficial in improving piglet preweaning performance by enhancing antioxidant capacity in serum and milk and improving milk fat content.

Conflict of interest

We declare that we have no financial and personal relationships with other people or organizations that can inappropriately influence our work, there is no professional or other personal interest of any nature or kind in any product, service and/or company that could be construed as influencing the content of this paper.

Acknowledgements

This work was supported by National Key R&D Program of China (No. 2018YFD0500600). National Natural Science Foundation of the P. R. of China (No. 31872364 and No. 31802067) and the Natural Science Foundation of Guangdong Province (No. 2018A030310201). The author thanks for Diamond V Mills, Inc. for providing Diamond V XP YC and SelenoSource AFTM2000.

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ORIGINAL RESEARCH
published: 07 May 2020
doi: 10.3389/fmicb.2020.00859

Different Sources of High Fat Diet Induces Marked Changes in Gut Microbiota of Nursery Pigs

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Specialty section:
This article was submitted to Food Microbiology, a section of the journal Frontiers in Microbiology

Received: 23 February 2020
Accepted: 09 April 2020
Published: 07 May 2020

Citation:
Yang F, Zhang S, Tian M, Chen J, Chen F and Guan W (2020) Different Sources of High Fat Diet Induces Marked Changes in Gut Microbiota of Nursery Pigs. Front. Microbiol. 11:859. doi: 10.3389/fmicb.2020.00859

Fat is one of the most important nutrients which provides concentrated energy and essential fatty acids. High fat diet markedly changes the gut microbial composition in mammals, whereas little is known about the impact of fat type on gut microbiome. This study was to evaluate the effects of fat sources on intestinal microbiota of nursery pigs. Eighteen pigs (28 days of age, 8.13 ± 0.10 kg BW) were housed individually (n = 6 per treatment) and allotted to three treatments based on a randomized complete block design. Pigs were fed basal diets with three different fat sources: 6.0% soybean oil (SBO), 6.0% palm oil (PO), and 7.5% encapsulated palm oil (EPO, contains 80% palm oil) respectively. Pigs were euthanized after 28 days of *ad libitum* feeding, and the digesta in the distal duodenum, jejunum, ileum, cecum and colon of each pig were obtained for microbial composition analysis. Correlation analyses were also performed between microbial composition with nutrients digestibility or growth performance. The results showed that pigs fed PO had marked changes in the bacteria community composition with increasing the richness and diversity in duodenum and jejunum ($P < 0.05$). Increased abundances of *Proteobacteria* in duodenum, jejunum and cecum, and decreased abundance of *Firmicutes* in jejunum were observed in pigs fed PO compared to SBO and EPO. Pigs fed EPO decreased abundances of *Proteobacteria* in duodenum and jejunum, and increased abundance of *Firmicutes* compared to pigs fed PO, and was similar to pigs fed SBO. The microbial changes (genus) had significant negative correlation with the fat digestibility. These results indicate that palm oil supplementation in nursery pig diet alters the gut microbial composition, with the most significant changes observed in small intestine. Encapsulation of palm oil, which helps increase the digestibility of palm oil, have beneficial effect on the microbial disturbance caused by palm oil supplementation. Our findings provide a better understanding of how different

fat types influence microbial composition in different parts of the intestinal tract and the correlation between bacteria composition and nutrients digestibility, which may provide a new perspective for the rational application of fat in diet.

Keywords: fat type, gut, microbial community, digestibility, nursery pigs

INTRODUCTION

Dietary composition not only affects the available nutrients for hosts, but also regulates gut microbial community (Ley et al., 2006). Fat is a critical dietary nutrient, which is mainly digested and absorbed in the small intestine, such as duodenum and jejunum, rather than the large intestine (Tancharoenrat et al., 2014). The digestibility of long-chain saturated fats (palm oil, lard, tallow) is lower than that of long-chain unsaturated fats (soybean oil, corn oil) and medium-chain saturated fats (coconut oil) (Cera et al., 1988, 1989, 1990). Those indigestible fats might act as a nutritional source for bacteria and therefore have the potential to regulate gut bacterial community.

The gut microbiota is an important part of the intestinal ecosystem, which is closely related to the intestinal epithelial cell integrity, nutrient digestion and metabolism, immune response and diseases (Nicholson et al., 2005; Hooper et al., 2012). High-fat diet interferes with the balance of energy intake and expenditure by changing the gut microbial composition (KałuŻna-Czaplińska et al., 2017), and has a long-term impact on the structure of gut microbiota (Liu et al., 2018). High-fat diet increases *Firmicutes* and decreases *Bacteroidetes* in gut (De Wit et al., 2012). In addition, dietary fat composition may also have the potential to reshape the microbiota in the gut (Huang et al., 2013). For instance, variance of Bacteroides spp. is associated with the ratio of dietary saturated and unsaturated fatty acids (Lappi et al., 2013; Simoes et al., 2013). Different type and quantity of hydrolyzate of fat may have varying impacts on the microbial composition in the intestine (Fava et al., 2013). Some hydrolyzates (medium-chain fatty acids) have antibacterial activity, which could insert into the bacteria cell membrane, change the permeability of sensitive strain, and subsequently lead to leakage of intracellular protein and ion (Altieri et al., 2009). However, some hydrolyzates (glycerol, fatty acids or triglycerides) are utilized by lipolytic bacteria to produce short-chain fatty acids, which provide energy for bacteria and intestinal epithelial cells (De et al., 2018). The genus *Phascolarctobacterium* and *Anaerovibrio* are typical lipolytic bacteria that secreted extracellular esterase to break down triglycerides and its hydrolyzates (Yohei et al., 2012; De et al., 2018). Traditionally, the conventional or cultural standard methods are widely used to study the intestinal bacterial composition. However, this is a less efficient way and has potential method bias. Many of the phylotypes found in the intestine tract are detected only through the use of molecularly based methods (Rajilć Stojanović et al., 2007). Recently, the methods of 16S rRNA gene amplification and high-throughput pyrosequencing were widely used in the microbial composition analysis (Liu et al., 2018). More phylotypes of *Phascolarctobacterium* and *Anaerovibrio* were deciphered with the advance of 16S rRNA gene sequence similarities (Watanabe et al., 2012; Florence et al., 2013).

Different segments of the intestine have different physicochemical and nutritional conditions for different microbial communities (Pereira and Berry, 2017). However, it is unclear about the effect of fat types and the digestibility of different fat on microbial community in the foregut and hindgut. Previously, we reported the digestibility of fat is largely dependent on the types and sources (Yang et al., 2018). This might change existing bacterial community and introduce new bacteria. Therefore, this study was conducted to investigate the effects of different fat types on gut microbiota in the duodenum, jejunum, ileum, cecum and colon of nursery pigs using 16S rRNA gene amplification and high-throughput pyrosequencing methods, and to analyze the correlations between gut microbial composition and nutrient digestibility or growth performance.

MATERIALS AND METHODS

Ethics Statement

All animal care and handling procedures were approved by the Institutional Animal Care and Use Committee of South China Agricultural University (SCAU-AEC-2010-0416), and consistent with the Guide for the Care and Use of Agricultural Animals in Research and Teaching (Federation of Animal Science Societies [FASS], 2010).

Animal, Diet and Experiment Design

This study was a part of series studies designed to evaluate the effects of fat type and encapsulation on nutrient utilization and gut microbes of pigs. The detailed description of the experimental setup and encapsulation process was reported in our previous study (Yang et al., 2018). Eighteen pigs [Duroc × (Landrace × Yorkshire)] weaned at 21 days of age were allotted to three treatments based on BW and sex at 28 days of age (8.13 ± 0.10 kg BW) in a randomized complete block design (six pigs per treatment: three males and three females). Treatments were: (1) basal diet with 6.0% soybean oil (SBO, Cargill Grain & Oilseeds Ltd., Dongguan, China), (2) basal diet with 6.0% palm oil (PO, Yihai Kerry Grain & Oil Ltd., Guangzhou, China), (3) basal diet with 6.0% palm oil from encapsulated palm oil (EPO, contains 80% palm oil, 8.5% dried casein, and 11.5% whey powder). All diets were formulated to meet the nutrient requirements of 7–11 kg pigs (**Supplementary Table S1**; NRC,

Abbreviations: ADFI, average daily feed intake; ADG, average daily gain; AMOVA, a molecular variance analysis; ATTD, apparent total tract digestibility; BW, body weight; CP, crude protein; DI, diarrhea incidence; DM, dry matter; EE, ether extract; EPO, encapsulated palm oil; F/G, feed conversion ratio, ADFI/ADG; OTUs, operational taxonomic units; PCoA, principal coordinates analysis; PO, palm oil; SBO, soybean oil; SEM, standard error mean.

2012). Pigs were housed individually for 4 weeks in stainless steel cages in a temperature-controlled room and allowed *ad libitum* access to feed and water throughout the experiment. Three female pigs, one from SBO, PO and EPO respectively, were removed from our experiment because of diarrhea at the end of the experiment. Pigs were euthanized by intramuscular injection of sodium pentobarbital (50 mg/kg BW, Sigma, United States) after 28 days of feeding. The digesta in the distal duodenum, jejunum, ileum, cecum and colon of each pig were collected, and then stored at −80°C until further DNA extraction.

DNA Extraction, 16S rRNA Gene Amplification and High-Throughput Pyrosequencing

Total genomic DNA in digesta (Duodenum, Jejunum, Ileum, Cecum and Colon) was extracted from 0.25 g of sample using a PowerFecal® DNA Isolation kit (MO BIO Laboratories Inc., Carlsbad, CA, United States) according to the manufacturer's instructions. The concentration of DNA was quantified using NanoDrop spectrophotometer (Thermo Fisher Scientific Inc., Wilmington, DE, United States). The V4 region of 16S rRNA gene (from 515 to 806) was amplified with universal bacterial primers (forward 5′-GTG CCA GCM GCC GCG G-3′ and reverse 5′-GGA CTA CHV GGG TWT CTA AT-3′). PCR was performed in triplicate using a 20-μL reaction system containing 0.8 μL of each primer, 10 ng template DNA, 4 μL 5 × FastPfu buffer (TransGen Biotech, China), 2 μL 2.5 mM dNTPs, and 0.4 μL FastPfu polymerase (TransGen Biotech, China). PCR was performed on ABI GeneAmp® PCR system 9700 (Applied Biosystems, Foster City, CA, United States) set as follows: 95°C for 5 min, followed by 27 cycles at 95°C for 30 s, 55°C for 30 s, and 72°C for 45 s, and a final extension at 72°C for 10 min. The PCR products from the three replicate amplifications were mixed, and electrophoresed on 2% agarose gels. Amplicons were purified using the AxyPrep DNA Gel Extraction Kit (Axygen Biosciences, Union City, CA, United States) according to the manufacturer's instructions, and were quantified with a QuantiFluor TM-ST fluorometer (Promega, Madison, WI, United States). Equal molar ratios of purified amplicons were pooled from each sample and paired-end sequenced (2 × 250) on an Illumina Hiseq PE250 platform according to standard protocols (Caporaso et al., 2012).

Bioinformatics Analysis

Raw sequence data were trimmed, filtered, aligned, and classified using the QIIME (Ver. 1.7.0) software package (Campbell et al., 2010). The base pair reads were truncated at any site receiving an average quality score <20 over a 10 bp sliding window, discarding the truncated reads that were shorter than 50 bp. The reads with primer mismatch numbers larger than 2 and with the barcode mismatches larger than 0 were removed, the mismatch ratio in the overlapping regions was not more than 0.2. The tags sequences obtained after the above processing were compared with the Gold database using UCHIME[1], and the chimeras were removed to get the effective tags (Edgar et al., 2011). Operational taxonomic units (OTUs) were clustered using the average neighbor algorithm with a cutoff of 97% similarity using UPARSE (Ver. 7.0.1001)[2]. Representative sequences from each OTU were then assigned to taxa using QIIME[3] and the Ribosomal Database Project (RDA) classifier algorithm (Cole et al., 2009) based on Silva[4] database at an 80% confidence level. Alpha diversity such as species richness estimator (Chao1 and ACE) and diversity indices (Shannon and Simpson) were calculated using MOTHUR (Schloss et al., 2009). Unweighted Unifrac distance-based principal coordinate analysis (PCoA) based on OTUs, together with a molecular variance analysis (AMOVA), were carried out to provide an overview of the microbial response to treatments.

The 16S sequence information in this paper has been deposited in the GenBank Sequence Read Archive database under accession number SRP226668.

Correlation Analysis

In our previous study, fat types significantly affected the growth performance and nutrient utilization (Yang et al., 2018). Pigs fed EPO had increased ($P < 0.05$) average daily gain (ADG) compared to pigs fed PO, whereas no difference was observed between pigs fed PO and SBO (268, 241 vs. 300 g/d; SBO, PO vs. EPO). The average daily feed intake (ADFI) was similar among treatments (427, 417 vs. 437 g/d; SBO, PO vs. EPO). Pigs fed PO had increased ($P < 0.05$) feed conversion ratio (ADFI: ADG, F/G) compared to pigs fed EPO, and pigs fed SBO was similar to PO and EPO (1.60, 1.74 vs. 1.50; SBO, PO vs. EPO). Pigs fed PO had increased ($P < 0.05$) diarrhea incidence (DI) compared to pigs fed SBO and EPO (10.68, 14.44 vs. 7.46; SBO, PO vs. EPO). Pigs fed EPO had increased ($P < 0.05$) apparent total tract digestibility (ATTD) of crude protein (CP) compared to pigs fed SBO, and pigs fed PO was similar to SBO and EPO (77.0%, 80.8% vs. 83.3%; SBO, PO vs. EPO). Pigs fed PO had decreased ($P < 0.05$) ATTD of crude fat (ether extract, EE) compared to pigs fed SBO and EPO (87.5%, 41.7% vs. 86.4%; SBO, PO vs. EPO).

To understand the effect of fat type on the relationship between microbial composition with growth performance or nutrient utilization, a Pearson's correlation analysis was performed.

Statistical Analysis

Graphing and statistical analysis were performed using GraphPad Prism (Ver. 8.0.1, La Jolla, CA, United States) and IBM SPSS Statistics software (Ver. 22.0, IBM, Armonk, NY, United States). The microbial diversity data and data of taxa richness, which had a normal distribution were analyzed by the one-way ANOVA procedures. The relative abundance of bacterial communities, which had a non-normal distribution was analyzed by the Kruskal–Wallis sum-rank test. Data were expressed as the means ± SEM. Significant differences were considered at $P < 0.05$. Correlations between bacterial abundance (genus proportion from pyrosequencing analysis) with growth

[1]http://www.drive5.com/usearch/manual/uchime_algo.html

[2]http://drive5.com/uparse/

[3]http://qiime.org/scripts/assign_taxonomy.html

[4]http://www.arb-silva.de

performance or digestibility related indicators were analyzed by Pearson's correlation test using SPSS. Correlation was considered significant when the absolute value of Pearson correlation coefficient was >0.5 and $P < 0.05$.

RESULTS

Effects of Fat Types on Bacterial Community in Digesta Revealed by Pyrosequencing

A total of 2,819,119 V4 16S rRNA sequences were obtained from the 75 samples from 15 pigs, with an average of 37,588 sequence reads (average length of 255 bp) for each sample, which were used for subsequent analysis. The rarefaction curves tended to approach a plateau, indicating that sequencing depth was sufficient to detect the vast majority of bacteria present (**Supplementary Figure S1**).

The bacterial richness and diversity at the 97% similarity level in each sample are presented in **Figure 1**. In the duodenum, pigs fed PO increased ($P < 0.05$) species richness and diversity indices compared with pigs fed SBO, as reflected by the ACE, Chao1 and Shannon indices. Pigs fed SBO and PO performed significant different species richness and diversity, but EPO was similar to SBO and PO (**Figure 1A**). In the jejunum, pigs fed PO increased ($P < 0.05$) species richness indices (ACE and Chao 1) compared with pigs fed SBO, whereas pigs fed EPO had similar ACE and Chao 1 indices compared with pigs fed SBO and PO. Species diversity indices (Shannon and Simpson) in the jejunum were not affected by different fat supplementation (**Figure 1B**). In the ileum and cecum, species richness and diversity indices were not affected by the dietary treatments (**Figures 1C,D**). In the colon, species diversity indices (Shannon and Simpson) of pigs fed PO were different from pigs fed SBO and EPO, whereas species richness indices were not affected by the dietary treatments (**Figure 1E**).

Principal coordinate analysis was performed based on unweighted Unifrac distance to reveal the impact of different fat supplementation on the microbiota composition (**Figure 2**), which indicated a distinct separation in microbiota composition in the small intestine (AMOVA, $P < 0.001$). Samples were clustered primarily by the gut segment (small intestine/large intestine). AMOVA analysis was carried out to further evaluate the statistical significance of the spatial separation among different groups in PCoA plots (**Supplementary Table S2**). There was a significant difference in the microbiota composition of pigs fed SBO, PO, and EPO in duodenum ($P = 0.003$, between SBO and PO; $P = 0.038$, between SBO and EPO; $P = 0.013$, between PO and EPO; **Supplementary Table S2**). Pigs fed SBO had significant dissimilarities compared to pigs fed PO in jejunum ($P = 0.013$) and ileum ($P = 0.034$).

At phylum level, *Firmicutes*, *Proteobacteria,* and *Cyanobacteria* were the dominant phyla in duodenum, *Firmicutes* and *Proteobacteria* were the dominant phyla in ileum and jejunum, *Firmicutes* and *Bacteroidetes* were the most

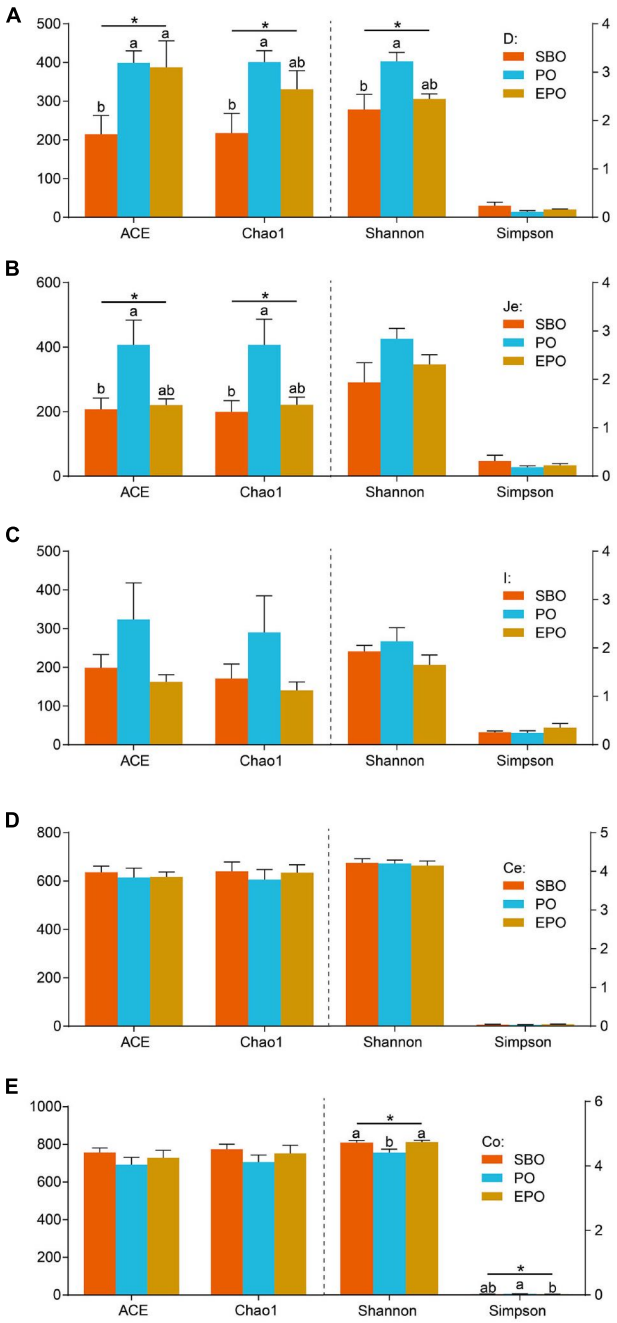

FIGURE 1 | Alpha diversity including ACE, Chao1, Shannon, and Simpson of duodenum **(A)**, jejunum **(B)**, ileum **(C)**, cecum **(D),** and colon **(E)** bacterial community at the 97% similarity level in the SBO, PO, and EPO groups. The values are expressed as the means ± SEM., bars with different letters are different from each other, $P < 0.05$.

detected phyla in cecum and colon (**Figure 3A**). In duodenum and jejunum (**Figure 3B**), pigs fed PO had the greatest relative abundance of bacteria belonging to the phyla *Proteobacteria*, and pigs fed SBO had increased ($P < 0.05$) abundance of bacteria from the phyla *Cyanobacteria* compared with pigs fed EPO. Pigs

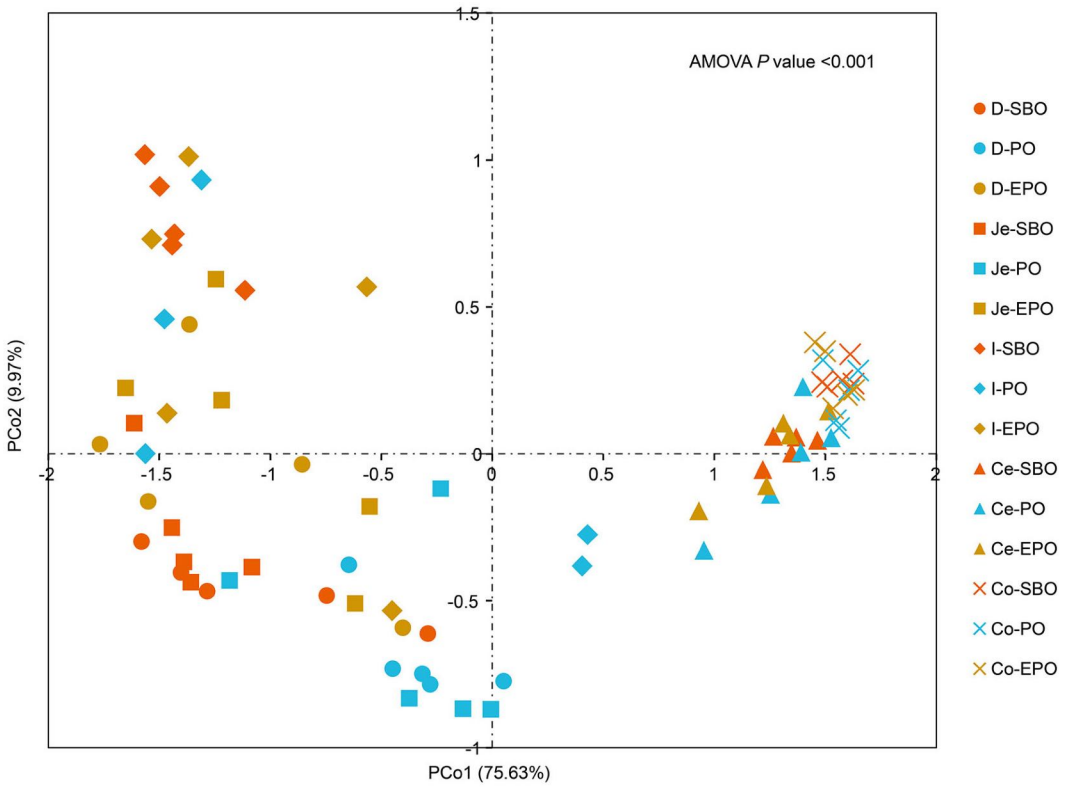

FIGURE 2 | Principle coordinate analysis (PCoA) of gut microbiota in the SBO, PO, and EPO groups by unweighted UniFrac distance (n = 5 per group). The percentage of variation explained by PCo1 and PCo2 are indicated in the axis. Abbreviation: D-, duodenum-; Je-, jejunum-; I-, ileum; Ce-, cecum-; Co-, colon; SBO, soybean oil; PO, palm oil; EPO, encapsulated palm oil.

fed EPO increased the abundance of bacteria from the phyla *Firmicutes* compared with pigs fed PO ($P < 0.05$). However, no significant changes in the abundance of bacteria in phylum were observed in ileum, cecum and colon, except that pigs fed PO had increased ($P < 0.05$) abundance of the bacteria belonging to the phyla *Proteobacteria* compared with pigs fed SBO and EPO in cecum.

The 44 most abundant genera (the mean abundance more than 2% at least one group) are listed in **Supplementary Figure S2**. In duodenum, pigs fed PO and EPO increased ($P < 0.05$) the abundance of *Halomonas*, *Enterobacter*, unclassified peptostreptococcaceae and *Clostridium sensu stricto 1* compared with pigs fed SBO (**Figure 4**). In duodenum and jejunum, pigs fed SBO had increased ($P < 0.05$) abundance of unclassified cyanobacteria compared to pigs fed EPO, and pigs fed EPO had decreased abundance ($P < 0.05$) of *Actinobacillus* compared to pigs fed PO. In cecum, pigs fed PO had increased ($P < 0.05$) abundance of *Leeia* compared to pigs fed SBO, and pigs fed SBO had increased ($P < 0.05$) abundance of *Faecalibacterium* compared to PO and EPO. In colon, pigs fed PO had decreased ($P < 0.05$) abundance of *RC9 gut group*, and increased ($P < 0.05$) abundance of *Phascolarctobacterium* and *Anaerovibrio* compared to pigs fed SBO and EPO (**Figure 4**). However, no significant changes in the abundance of genera were observed among treatment groups in ileum.

Correlations Between Microbial Composition With Growth Performance or Nutrient Digestibility

Pearson's correlation analyses were performed between microbial composition (genus) with growth performance indicators or digestibility of CP and EE, in order to understand the relationships between the microbial composition changes with the change in growth performance or nutrient utilization among different fat sources (**Figure 5**). In the duodenum (**Figure 5A**), there were significantly correlations between the digestibility and bacteria, the EE digestibility was negatively correlated with the abundance of *Actinobacillus*, *Halomonas*, *Roseburia*, *Lactobacillus*, *Prevotella, Enterobacter*, *Alloprevotella*, *Phascolarctobacterium*, *Anaerovibrio*, *Subdoligranulum*, *Megasphaera* and *Streptococcus*, and CP digestibility was negatively correlated with the abundance of unclassified Cyanobacteria and unclassified mitochondria. For growth performance, the feed conversion ratio (F/G) was positively correlated with the abundance of *Roseburia*, *Lactobacillus*, *Prevotella,* unclassified Prevotellaceae, *Phascolarctobacterium* and unclassified Ruminococcaceae.

In the jejunum (**Figure 5B**), the EE digestibility was negatively correlated with the abundance of *Actinobacillus*, *Roseburia*, *Lactobacillus*, *Prevotella,* Unclassified S24-7,

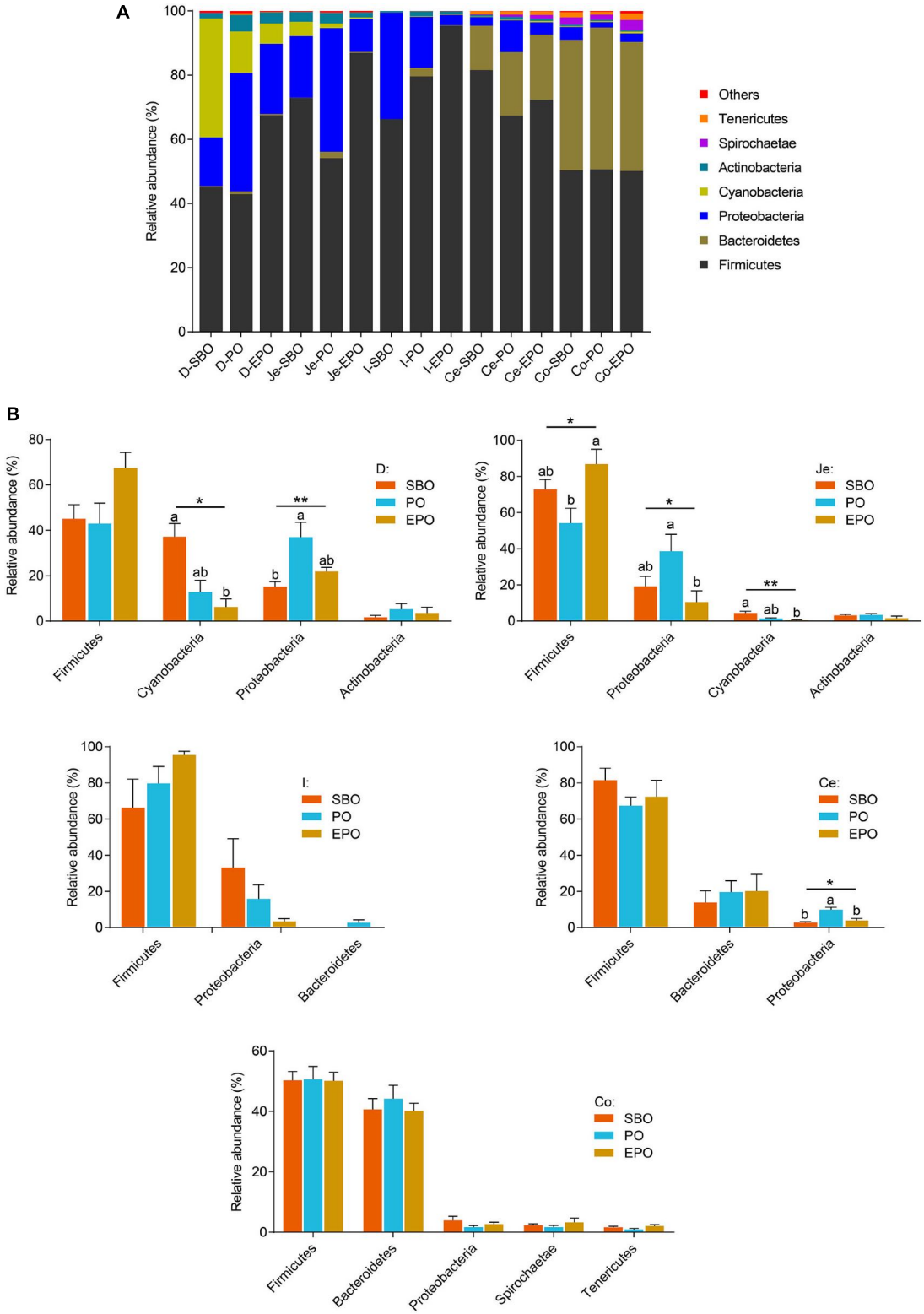

FIGURE 3 | Phylum-level composition. **(A)** The phylum -level taxonomic composition of average relative abundance of bacterial communities in the SBO, PO, and EPO groups. **(B)** The changed bacterial phyla found in each segment of the intestine of piglets (the mean abundance more than 2% at least one group, analyzed using Kruskal–Wallis sum-rank test). $^{*}P < 0.05$; $^{**}P < 0.01$. D-, duodenum-; Je-, jejunum-; I-, ileum; Ce-, cecum-; Co-, colon; SBO, soybean oil; PO, palm oil; EPO, encapsulated palm oil.

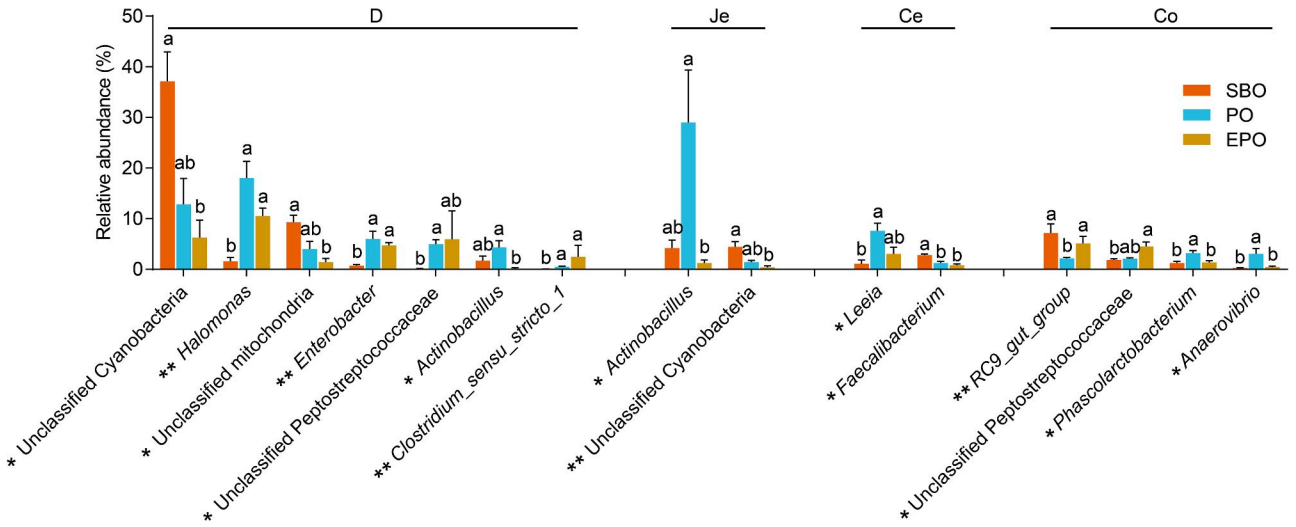

FIGURE 4 | The significantly changed genera found in each segment of intestine of piglets (the mean abundance more than 2% at least one group, analyzed using Kruskal Wallis sum-rank test). *$P < 0.05$; **$P < 0.01$. D-, duodenum-; Je-, jejunum-; Ce-, cecum-; Co-, colon; SBO, soybean oil; PO, palm oil; EPO, encapsulated palm oil.

Phascolarctobacterium, *Anaerovibrio*, *Subdoligranulum*, and *Megasphaera*. The CP digestibility was negatively correlated with the abundance of unclassified Cyanobacteria, and positively correlated with the abundance of unclassified Peptostreptococcaceae and *Clostridium_sensu_stricto_1*. For growth performance, the diarrhea incidence (DI) was negatively correlated with the abundance of unclassified Peptostreptococcaceae and *Clostridium_sensu_stricto_1*, the F/G was positively correlated with the abundance of *Lactobacillus*, *Prevotella,* Unclassified S24-7, *Alloprevotella*, *Subdoligranulum*, and *Megasphaera*, and the ADG was negatively correlated with the abundance of *Lactobacillus*, Unclassified S24-7, *Alloprevotella*, *Subdoligranulum* and *Megasphaera*.

In the ileum (**Figure 5C**), the EE digestibility was negatively correlated with the abundance of *Prevotella, Lactobacillus*, Unclassified S24-7, *Alloprevotella*, unclassified Prevotellaceae, *Phascolarctobacterium*, *Anaerovibrio*, *Subdoligranulum*, *Megasphaera*, *RC9 gut group*, and *Helicobacter*. The CP digestibility was negatively correlated with the abundance of *Actinobacillus*. For growth performance, the F/G was positively correlated with the abundance of *Actinobacillus*, *Roseburia*, *Lactobacillus*, *Prevotella, Leeia*, Unclassified S24-7, *Alloprevotella*, unclassified Prevotellaceae, *Phascolarctobacterium*, *Anaerovibrio*, *Subdoligranulum*, *Megasphaera*, *RC9 gut group*, and *Helicobacter*. The ADG was negatively correlated with the abundance of *Actinobacillus*, *Roseburia*, *Lactobacillus*, *Prevotella, Leeia*, Unclassified S24-7, *Alloprevotella*, unclassified Prevotellaceae, *Phascolarctobacterium*, *Anaerovibrio*, *Subdoligranulum*, *Megasphaera*, *RC9 gut group*, and *Helicobacter*, and was positively correlated with the abundance of unclassified Cyanobacteria.

In the cecum (**Figure 5D**), the EE digestibility was negatively correlated with the abundance of *Halomonas*, *Turicibacter*, *Lactobacillus*, *Leeia*, Unclassified S24-7, and *Anaerovibrio*, and was positively correlated with the abundance of *Roseburia* and unclassified Lachnospiraceae. The CP digestibility was negatively correlated with the abundance of unclassified Ruminococcaccae, and was positively correlated with the abundance of *Veillonella*. For growth performance, the DI was negatively correlated with the abundance of unclassified Cynobacteria. The F/G positively correlated with the abundance of *Lactobacillus,* Unclassified S24-7, *Anaerovibrio*, and *Kitasatospora*. The ADG was negatively correlated with the abundance of *Actinobacillus*, *Lactobacillus*, *Anaerovibrio* and *Clostridium_sensu_stricto_1*.

In the colon (**Figure 5E**), the EE digestibility was negatively correlated with the abundance of *Lactobacillus*, *Prevotella, Phascolarctobacterium*, *Anaerovibrio* and *Subdoligranulum*. The CP digestibility was negatively correlated with the abundance of *Halomonas* and unclassified Ruminococcaceae, and was positively correlated with the abundance of unclassified Peptostreptococcaceae, *Kitasatospora*, *Treponema* and unclassified Prevotellaceae. For growth performance, the DI was negatively correlated with the abundance of unclassified Prevotellaceae, and was positively correlated with the abundance of unclassified Lachnospiraceae and *Subdoligranulum*. The F/G was positively correlated with the abundance of unclassified Cyanobacteria, *Lactobacillus*, *Prevotella, Alloprevotella*, *Anaerovibrio,* and *Subdoligranulum*, and was negatively correlated with the abundance of *Kitasatospora*. The ADG was negatively correlated with the abundance of *Lactobacillus*, *Prevotella, Alloprevotella* and *Anaerovibrio*.

DISCUSSION

Fat is one of the three most important nutrients in human and animal foods. Different types of dietary fat have great impact on gut microbial composition in mammals (Fava et al., 2013). In this study, we investigated the changes of microbial community in the

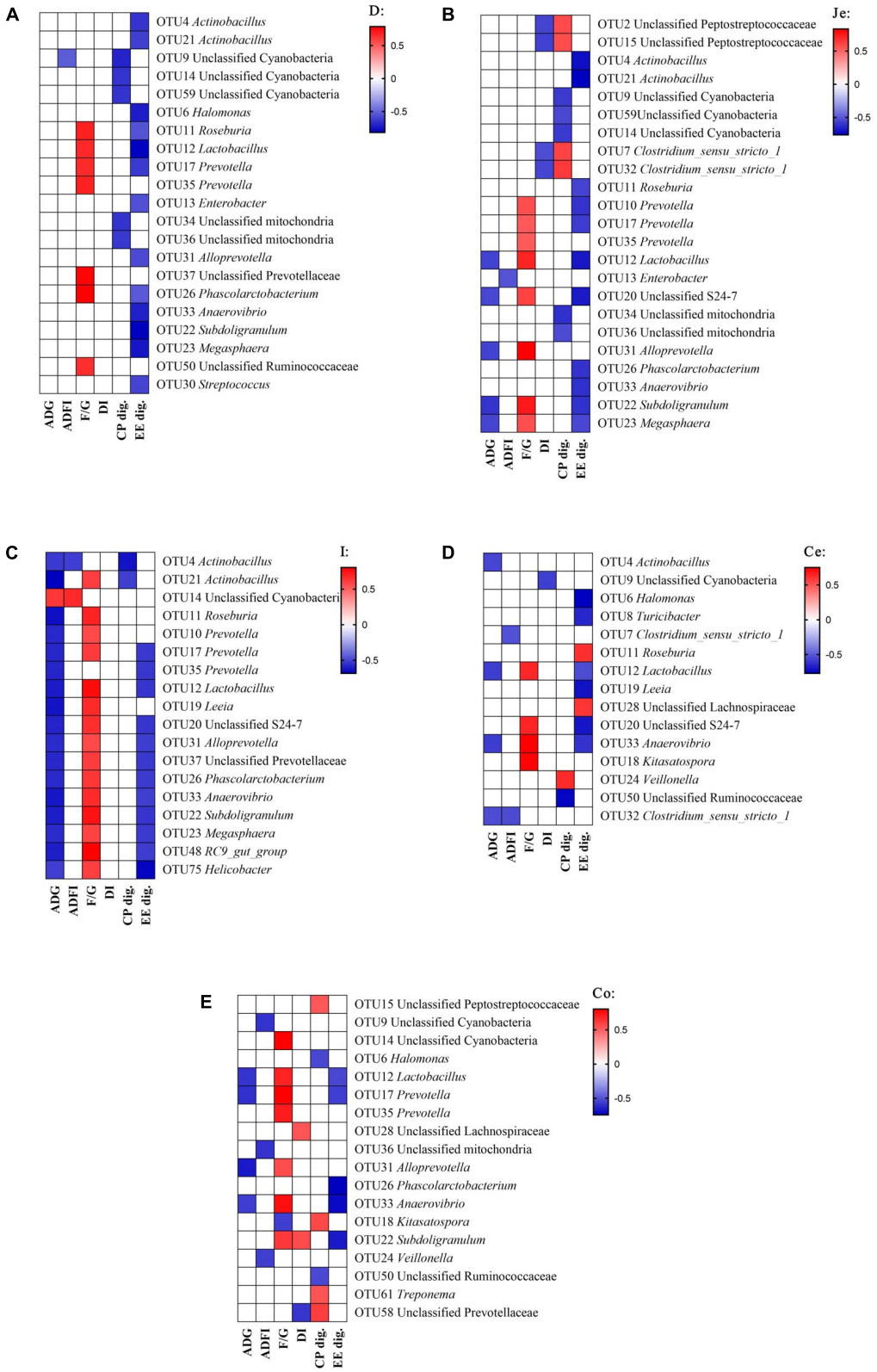

FIGURE 5 | Correlation analysis between the abundance of microbiota (genus) and phenotypic data in the gut. Correlation was considered significant when the absolute value of Pearson correlation coefficient was > 0.5 and $P < 0.05$. **(A)** duodenum, **(B)** jejunum, **(C)** ileum, **(D)** cecum, **(E)** colon; ADG, average daily gain; ADFI, average daily feed intake; F/G, Feed conversion ratio; DI, diarrhea incidence; CP dig., crude protein digestibility; EE dig., crude fat digestibility.

duodenum, jejunum, ileum, cecum and colon digesta of nursery pigs in response to different fat sources using high-throughput sequencing. Fat types markedly affected microbial composition in duodenum and jejunum, with less effect in ileum, cecum, and colon. The microbial changes were significantly correlated with the fat digestibility. These findings indicate that the differences in fat digestibility caused by fat sources significant influence intestinal microbial community.

Fat Types Induced the Microbial Changes in Nursery Pigs, With More Evident Effect Observed in the Small Intestine

Different segments of the intestine have different physicochemical and nutrient conditions for different microbial communities (Pereira and Berry, 2017). In this study, the impacts of fat types on the microbial composition in the small intestine and large intestine were varying, with marked changes in duodenum and jejunum, and less effect in cecum and colon, which was confirmed by the Alpha diversity, PCoA and AMOVA analyses of microbiota (**Figures 1**, **2** and **Supplementary Table S2**). The digestion and absorption of fat occurs in the small intestine with the majority of fat and fatty acids digested and absorbed in duodenum and jejunum, the large intestine does not participate in the digestion of fat (Tancharoenrat et al., 2014). Therefore, the different fat digestibility along the gut may account for the difference of the microbiota in the small and large intestine. In our previous study, different fat sources had varying digestibility, in which pigs fed PO decreased digestibility of crude fat (ether extract, EE) compared with pigs fed SBO and EPO (87.5%, 41.7% vs. 86.4%; SBO, PO vs. EPO). The type and quantity of hydrolyzate of fat may have varying impacts on the microbial composition in the same segment of intestine (Fava et al., 2013), and parts of the fatty acids and monoglycerides hydrolyzed from fat (triglyceride) have antibacterial activity (Altieri et al., 2009). The different hydrolyzates of soybean oil and palm oil, which have different fatty acid profile, may induce varying responses of microbial composition. In our study, the different microbial composition in PO and EPO may be caused by the varying quantities of hydrolyzates.

At phylum level, pigs fed PO markedly increased the phyla *Proteobacteria* in duodenum, jejunum and cecum, and reduced the phyla *Firmicutes* in jejunum in the present study. The phyla *Proteobacteria* is the dominant phyla in small intestine, including many kinds of pathogenic bacteria, which are intricately involved in the luminal dysbiosis caused by inflammatory bowel disease (IBD) (Mukhopadhya et al., 2012). On the contrary, *Firmicutes,* which is the most predominant phyla in the intestine, contains the vast majority of potentially beneficial species. It is reported that the depletion of *Faecalibacterium*, belonging to the *Firmicutes* phylum, has been associated with IBD (Marchesi et al., 2016). Therefore, supplementation of PO in nursery pig diet may induce a breakdown in the balance of putative pathogenic bacteria and protective commensal bacteria, and EPO supplementation increased the digestibility of palm oil, which may have beneficial effect on the balance of those intestinal microbes (**Figure 3B**).

Fat Types Induced Specific Changes in Microbial Genera and Its Correlations With Nutrients Digestibility or Growth Performance

With specific genera, pigs fed PO increased the genus *Actinobacillus* in duodenum and jejunum, and the abundance of *Actinobacillus* in duodenum and jejunum was negatively correlated with the EE digestibility. Some species within the genera *Actinobacillus* are putative pathogenic bacteria in association with clinical condition of the respiratory system (Teshima et al., 2017; To et al., 2017). In the present study, the low digestibility of palm oil significantly increased the abundance of *Actinobacillus,* whereas increased digestibility of palm oil by encapsulation reduced the abundance of *Actinobacillus* in the small intestine. The phyla *Cyanobacteria* is well known as oxygenic photosynthetic bacteria in a wide variety of ecological environments that enhances nitrogen fixation (Liberton et al., 2019). The survival of *Cyanobacteria* during gut passage may enhance nutrient availability (Friedland et al., 2005). In our study, pigs fed palm oil reduced the abundance of unclassified cyanobacteria in duodenum and jejunum, which was negatively correlated with the CP digestibility. Therefore, the protein in digesta may promote the growth of *Cyanobacteria*. The abundance of *Clostridium sensu stricto 1* and unclassified peptostreptococcaceae (belongs to *Firmicutes*) were increased in duodenum of pigs fed PO and EPO, with the same tendency in jejunum. It has been reported that the proportion of *Clostridium sensu stricto 1* was significantly decreased when pigs fed low protein diet (Fan et al., 2017), and similar results was also observed in rats (Zhu et al., 2017). In the present study, the abundance of *Clostridium sensu stricto 1* and unclassified peptostreptococcaceae were positively correlated with the CP digestibility. The abundance of bacteria may be affected by the amount of protein hydrolyzate.

In addition, the abundance of *Phascolarctobacterium* and *Anaerovibrio* were negatively correlated with the EE digestibility in duodenum, jejunum, ileum and colon (**Figure 5**). The genus *Anaerovibrio* is a typical lipolytic bacteria that hydrolyzes triglycerides to produce glycerol and fatty acids, and ferments glycerol to produce propionic and succinic acids (Schauder and Schink, 1989; De et al., 2018). The genus *Phascolarctobacterium* has the ability to ferment glycerol into acetate and propionate in the human gut (Yohei et al., 2012; Wu et al., 2017). The increased residual fat in digesta of nursery pigs may promote the proliferation of *Phascolarctobacterium* and *Anaerovibrio*. Likewise, the abundance of some genera had negative correlation with the digestibility of crude fat, such as *Halomonas*, *Enterobacter, Prevotella, Alloprevotella, Subdoligranulum, Leeia*, which all belong to putative pathogenic bacteria that induce mortality and intestinal microbiota disturbance.

The correlation between the abundance of bacteria and growth performance mainly reflected in the influence of feed conversion ratio (F/G) in this study. The proliferation of potentially

pathogenic bacteria in the gut may lead to increased levels of endotoxin secreted by the bacteria (Marchesi et al., 2016), and then affect the health and function of intestinal epithelial cells (Mani et al., 2013), consequently, result in disrupted digestion and absorption of nutrients and poor feed conversion efficiency.

CONCLUSION

The present study demonstrated that palm oil supplementation in nursery pig diet altered the gut microbial composition by increasing the abundance of *Proteobacteria* while reducing the abundance of *Firmicutes*, with more evident effect observed in the small intestine. The microbial changes were negatively correlated with fat digestibility, whereas encapsulation of palm oil (EPO) increased the digestibility of palm oil, which may have beneficial effect on the intestinal microbes. The findings provide evidence that different fat types may affect fat digestibility and further influence composition of gut microbiome.

DATA AVAILABILITY STATEMENT

The datasets generated for this study can be found in the GenBank Sequence Read Archive database, accession number SRP226668.

ETHICS STATEMENT

The animal study was reviewed and approved by Institutional Animal Care and Use Committee of South China Agricultural University.

AUTHOR CONTRIBUTIONS

WG, SZ, and FC conceived and designed the whole trial. FY and MT conducted the pig trial. FY and JC conducted the laboratory analyses. FY, SZ, and WG wrote the manuscript. All authors agreed to be accountable for all aspects of the work.

FUNDING

This research was supported by the National Key R&D Program of China (No. 2017YFD0500500).

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2020.00859/full#supplementary-material

FIGURE S1 | Rarefaction curves of duodenum **(A)**, jejunum **(B)**, ileum **(C)**, cecum **(D)** and colon **(E)** samples comparing the number of sequences with the number of OTUs from the microbiota in the digesta of piglets in each treatment group.

FIGURE S2 | The relative abundance of the most abundant genera 44 OTUs with a significant difference (the mean abundance more than 2% at least one group) in the SBO, PO, and EPO groups. (analyzed using Kruskal–Wallis sum-rank test) $^{*}P < 0.05$; $^{**}P < 0.01$. D-, duodenum-; Je-, jejunum-; I-, ileum; Ce-, cecum-; Co-, colon; SBO, soybean oil; PO, palm oil; EPO, encapsulated palm oil.

TABLE S1 | Composition and nutrient content of experimental diets (as-fed basis).

TABLE S2 | Further evaluation of the statistical significance of the spatial separation among the different groups in PCoA plots using AMOVA analysis (*P*-value).

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Received: 1 February 2020 | Revised: 4 April 2020 | Accepted: 6 April 2020

DOI: 10.1002/fsn3.1607

ORIGINAL RESEARCH

Food Science & Nutrition Open Access WILEY

L-carnitine increases cell proliferation and amino acid transporter expression via the activation of insulin-like growth factor I signaling pathway in rat trophoblast cells

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Funding information
This study was supported by National Natural Science Foundation of the P. R. of China (Nos. 31802067 and 31872364) and the Natural Science Foundation of Guangdong Province (No. 2018A030310201) and the National Key R&D Program of China (Nos. 2018YFD0500600 and 2018YFD0501000).

Abstract

Early embryo implantation and development is primarily determined by the homeostasis between cellular apoptosis and proliferation as well as placental nutrient transporters. Recent studies showed that L-carnitine enhances female reproductive performance. However, the potential function of L-carnitine on placenta is largely unknown. In our study, primary rat trophoblast cells were separated and cultured for 12 hr in medium containing various concentrations of L-carnitine (0, 1, 10, and 50 mM). Placenta trophoblast cells treated with 50 mM L-carnitine increased the proportion of cells in S phase of the cell cycle ($p < .05$). In addition, live cell percentage was increased when treated with either 10 mM or 50 mM L-carnitine, which was accompanied with decreased necrotic cells, late apoptotic cells, and early apoptotic cells ($p < .05$). Compared with the control treatment, the mRNA expression of insulin-like growth factor I (IGF-1) and insulin-like growth factor I receptor (IGF-1R) was higher in rat placenta trophoblasts treated with either 10 mM or 50 mM L-carnitine ($p < .05$). Similarly, sodium-dependent neutral amino acid transporter (SNAT)-1 and SNAT2 were up-regulated in both mRNA and protein levels when trophoblast cells were treated with 50 mM L-carnitine ($p < .05$). Inhibiting downstream targets (Akt or ERK signaling pathways) of IGF-1 signaling pathway partially blocked the effect the L-carnitine-induced increase in protein abundances of SNAT1 and SNAT2. Collectively, our data showed protective role of L-carnitine on placenta trophoblast cells through the involvement of IGF-1 signaling pathway.

KEYWORDS

IGF-1 signaling, L-carnitine, nutrient transporter, reproduction

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1 | INTRODUCTION

Dietary nutrient component is a critical factor in the regulation of reproductive performance (Chen et al., 2019; Zeng et al., 2019). The canonical function of L-carnitine is to regulate the transportation of medium-chain and long-chain fatty acids across the inner mitochondrial membrane for β-oxidation (Sada, Kato, Sumimoto, Ohmori, & Ohdan, 2015). Recent studies have shown that L-carnitine also participates in the regulation of reproduction. During lactation period, L-carnitine increases piglets weaning weight through the enhancement of milk production of the sows (Ramanau, Kluge, Spilke, & Eder, 2004b). Sows fed L-carnitine-supplemented diets during pregnancy increase the number of piglets born alive and decrease the number of stillborn and mummified pigs (Musser et al., 1999; Ramanau, Kluge, Spilke, & Eder, 2002a, 2004a). Similar results are also observed in models like mice and rat (Agarwal, Sengupta, & Durairajanayagam, 2018). These findings indicate that L-carnitine might regulate embryo quality and viability, decrease implantation failure and pregnancy loss. However, the underlying mechanism of the beneficial effects of L-carnitine during this period is still unclear. In this research, we focused on the effects of L-carnitine on cultured rat trophoblast cells and its underlying mechanism.

Successful implantation and maintenance of placenta during gestation are dependent on balanced cell proliferation and apoptosis (Sharp, Heazell, Crocker, & Mor, 2010). In the placenta, fast proliferation is observed during the early pregnancy and the proliferation diminishes gradually as pregnancy proceeds (Ishihara et al., 2000). Conversely, apoptosis ratio is low after conception and is increased temporarily before the parturition (Smith, Baker, & Symonds, 1997). The imbalance between apoptosis and proliferation might result in various pathological changes, such as spontaneous abortion and intrauterine growth retardation (IUGR; Longtine, Chen, Odibo, Zhong, & Nelson, 2012; Luo et al., 2010; Sun et al., 2013). Regulating the homeostasis between proliferation and apoptosis in the placenta plays an important role in the fetus development. Therefore, L-carnitine might be a key element to maintain the homeostasis between apoptosis and proliferation in trophoblast cells.

Placental nutrient transportation is also of fundamental importance for fetal growth, as fetal development is largely dependent on nutrients from maternal circulation. Amino acids have various potential functions in fetoplacental development (Regnault, Friedman, Wilkening, Anthony, & Hay, 2005). Placenta trophoblast cell is a useful model to study placental function, for example, nutrient transportation (Hu & Cross, 2011). In the placenta, neutral amino acids are mainly transported by the system A sodium-dependent neutral amino acid transporter (SNAT), which consists of three isoforms (SNAT 1, SNAT 2 and SNAT 4; Desforges et al., 2009; Glazier & Sibley, 2006; Jones, Powell, & Jansson, 2007), whereas cationic amino acids (lysine, arginine, and histidine) are mainly transported by the cationic amino acid transporter-1 (CAT-1), which is a major cationic transport system in the placenta (Ayuk, Sibley, Donnai, D'Souza, & Glazier, 2000). Glucose is the principal carbohydrate transported from maternal circulation to fetus (Langdown & Sugden, 2001). In the mammalian placenta, GLUT1 and GLUT3 are the two major glucose transporter isoforms (Lager & Powell, 2012). GLUT1 is predominantly expressed in the placenta during early pregnancy, which is gradually replaced by GLUT3 during late gestation (Baumann, Deborde, & Illsley, 2002; Janzen et al., 2013). A key question is whether L-carnitine could modulate fetal growth through regulating placental glucose and amino acid transporter expression.

Activation of insulin-like growth factor 1 (IGF-1) signaling pathway is a possible mechanism of how L-carnitine regulates reproduction. Previously, L-carmine was reported to increase maternal IGF-1 concentration and induce the development of fetus (Eder, 2009). In addition, IGF-1 increases blastocyst formation and have anti-apoptotic effect during early embryo development (Lin, Yen, Gong, Hsu, & Chen, 2003; Mandl, Haas, Bischof, Nöhammer, & Desoye, 2002). Although liver is the major organ for IGF-1 secretion, placenta is also considered as one of dominant sources of IGF-1 during gestation (Martín-Estal, De La Garza, & Castilla-Cortazar, 2016). Furthermore, trophoblast cells were also found to produce IGF-1 locally (Adams, 2002). Therefore, the primary objective of this study was to evaluate the effects of L-carnitine on the proliferation and apoptosis, nutrient transporter systems and activation of IGF-1 signaling pathway in rat placental trophoblast cells.

2 | MATERIALS AND METHODS

2.1 | Isolation and purification of placenta trophoblast cell

Placenta tissues were collected under sterile conditions from gestational day 11 *SD* rats and were cut into small cubic masses (1 mm^3). Dissected tissues were digested with 0.25% trypsin (Gibco) and 0.2% collagenase (Sigma Chemical Co.) for 5 min at 37°C, and subsequently treated with 200 U/ml DNase I (Sigma Chemical Co.) for 5 min at 37°C. Any undigested fragments were removed and the cells were then collected by centrifugation (300 *g*) and washed with DMEM. To further purify the cells, trophoblasts were isolated by Percoll gradient centrifugation. The Percoll gradients were made from 45% to 15% Percoll (V/V) in every 5% steps of 5.0 ml each. Trophoblasts were collected in the density of the gradient between 1.048 and 1.062 g/ml. To eliminate the contamination of red blood cells, 5.0 ml of lysis buffer (155 mM NH_4Cl, 10 mM $KHCO_3$, and 0.14 mM EDTA (pH 7.2)) was added to the cell pellets. Isolated cells were verified by using immunohistochemical staining (stained for cytokeratin and vimentin; Figure S1).

2.2 | Cell culture and treatment

Verified trophoblast cells were seeded in six-well plates. After reaching 90% confluency, cells were starved for 4 hr in a fetal calf serum (FCS)-free DMEM medium (GIBCO, Thermo Fisher Scientific). After starvation, cells were cultured in DMEM medium containing 5% FCS with 0, 1, 10, or 50 mM L-carnitine for 12 hr. For experiment using inhibitors, trophoblast cells were pretreated with 10 μM PD098059 (Sigma) or LY294002 (Sigma) for 30 min.

2.3 | Flow cytometric analysis of cell cycle and cell apoptosis

After treated with L-carnitine, cells were washed twice with PBS and then stained according to Annexin V-FITC/PI kit protocol (BD PharMingen). Briefly, cells were incubated with Annexin V-FITC and propidium iodide (PI; Sigma-Aldrich) and incubated for 15 min in the dark. Samples were read on a FACScan flow cytometer (Becton Dickinson).

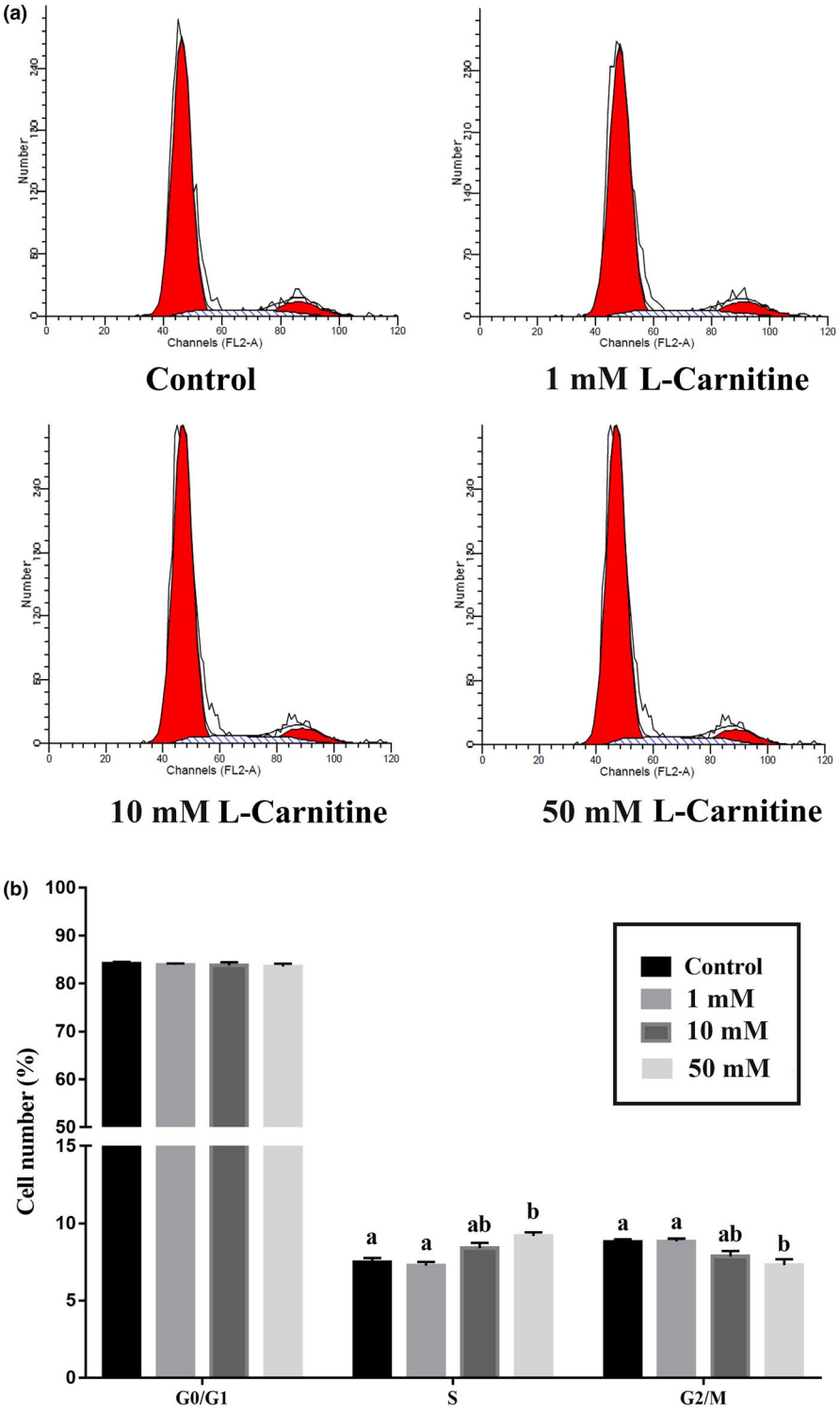

FIGURE 1 Effect of L-carnitine on cell cycle distribution in rat placenta trophoblast cells. (a) Flow cytometric analysis of rat placenta trophoblast cells treated with 0, 1, 10, or 50 mM L-carnitine. (b) Data from the cell cycle distribution were summarized and presented as the means ± SE from three independent experiments. Bars with different letters are significant different ($p < .05$)

After treated with L-carnitine, cells were treated with 0.25% trypsin (Sigma-Aldrich) and detached from the plate. Cells were stained with propidium oxide (BD Biosciences). The distribution of the cells in G0/G1, S, and G2/M phases were determined with FACScan cytometry (Becton Dickinson).

2.4 | RNA isolation and RT-PCR analysis

Total RNA was isolated using TRIzol (Life Technologies) according to the manufacturer's instruction. Total RNA was reverse-transcribed to complementary DNA (cDNA) using a PrimeScript 1st Strand cDNA Synthesis Kit (Takara) according to the manufacturer's protocol. Primers for the selected genes were designed using Primer Premier 5.0. Real-time PCR was performed using an ABI PRISM®7500 with SanTaq Plus PCR Master Mix (Sangon, Biotech) containing 3 mM $MgCl_2$, 0.2 mM dNTP, and 0.2 U/μl Pfu DNA polymerase. The PCR system consisted of 10.0 μl of SanTaq Plus PCR Master Mix, 5.0 μl of cDNA (1:20), 4 μl of double distilled water, and 1 μl of primer pairs (25 μM forward and 25 μM reverse) in a total volume of 20 μl. The protocols for all genes included a denaturation program (3 min at 95°C), amplification and quantification program repeated for 35 cycles (15 s at 95°C, 15 s at 60°C, 40 s at 72°C), followed by the melting curve program at 60–95°C with a heating rate of 0.1°C per second and continuous fluorescence measurement. Quantification of target mRNA was determined by the delta–delta CT method ($^{2-\Delta\Delta}$CT), using GAPDH as the housekeeper to normalize each sample. The primers are listed in Table S1.

2.5 | Western blot

All samples were homogenized in RIPA Lysis Buffer (Beyotime). Protein concentration was measured using a BCA Protein Assay Kit (Beyotime). About 30 μg of protein from each sample was separated on SDS-PAGE gels and then transferred to nitrocellulose membranes. After incubation with 5% skimmed milk for 1 hr at room temperature, membranes were incubated with primary antibody overnight at 4°C. The antibodies used in this experiment were listed as follows: SNAT1 (1:1,000, Santa Cruz Biotechnology), SNAT2 (1:1,000, Santa Cruz Biotechnology), p-mTOR (1:1,000, Cell Signaling Technology), mTOR (1:1,000, Cell Signaling Technology, USA), p-ERK (1:1,000, Cell Signaling Technology), ERK (1:1,000, Cell Signaling Technology), p-Akt (1:1,000, Cell Signaling Technology), Akt (1:1,000, Cell Signaling Technology), and Actin (1:1,000, Cell Signaling Technology).

2.6 | Statistical analysis

Statistical analyses were performed using the statistical software SAS version 9.2. Data were analyzed by ANOVA according to the GLM procedure of SAS. Differences among treatment were determined using Duncan's multiple range test. p value less than .05 was considered significant.

3 | RESULTS

3.1 | Effects of L-carnitine on the cell cycle in rat placenta trophoblasts

The effect of L-carnitine on the cell cycle of the rat placenta trophoblasts was determined by flow cytometry. As shown in Figure 1, more cells were in the S phase of cell cycle when treated with 50 mM L-carnitine for 12 hr, with a concomitant decrease in the proportion of cells in the G2/M phase ($p < .05$).

3.2 | Effects of L-carnitine on apoptosis in rat placenta trophoblasts

The effect of L-carnitine on apoptosis of placental trophoblasts is presented in Figure 2. The percentage of necrotic cells, late apoptotic cells, and early apoptotic cells were significantly lower in trophoblasts treated with either 10 mM or 50 mM L-carnitine ($p < .05$). Furthermore, the percentage of live cells was higher when cells were treated with L-carnitine ($p < .05$).

3.3 | Effects of L-carnitine on gene expression in rat placenta trophoblasts

Compared with control group, the mRNA expression of IGF-1 and IGF-1R was higher in rat placenta trophoblast cells treated with either 10 mM or 50 mM L-carnitine ($p < .05$; Figure 3a). L-carnitine did not affect GLUT1 or GLUT3 mRNA expression (Figure 3b). SNAT1 mRNA expression was up-regulated in cells treated with either 10 or 50 mM L-carnitine, whereas SNAT2 was only increased when cells were treated with 50 mM L-carnitine (Figure 3c).

3.4 | Effects of L-carnitine on protein abundances in rat placenta trophoblasts

Consistent with the mRNA results, the protein abundances of SNAT1 and SNAT2 were up-regulated in cells treated with either 10 mM or 50 mM L-carnitine ($p < .05$; Figure 4). As the mRNA expression of IGF1 and IGF1-R is increased when cells were treated with L-carnitine, the IGF-1 related signaling pathways were detected in our experiments. Trophoblasts treated with either 10 mM or 50 mM L-carnitine increased phosphorylation of Akt, mTOR and ERK1/2 ($p < .05$; Figure 5).

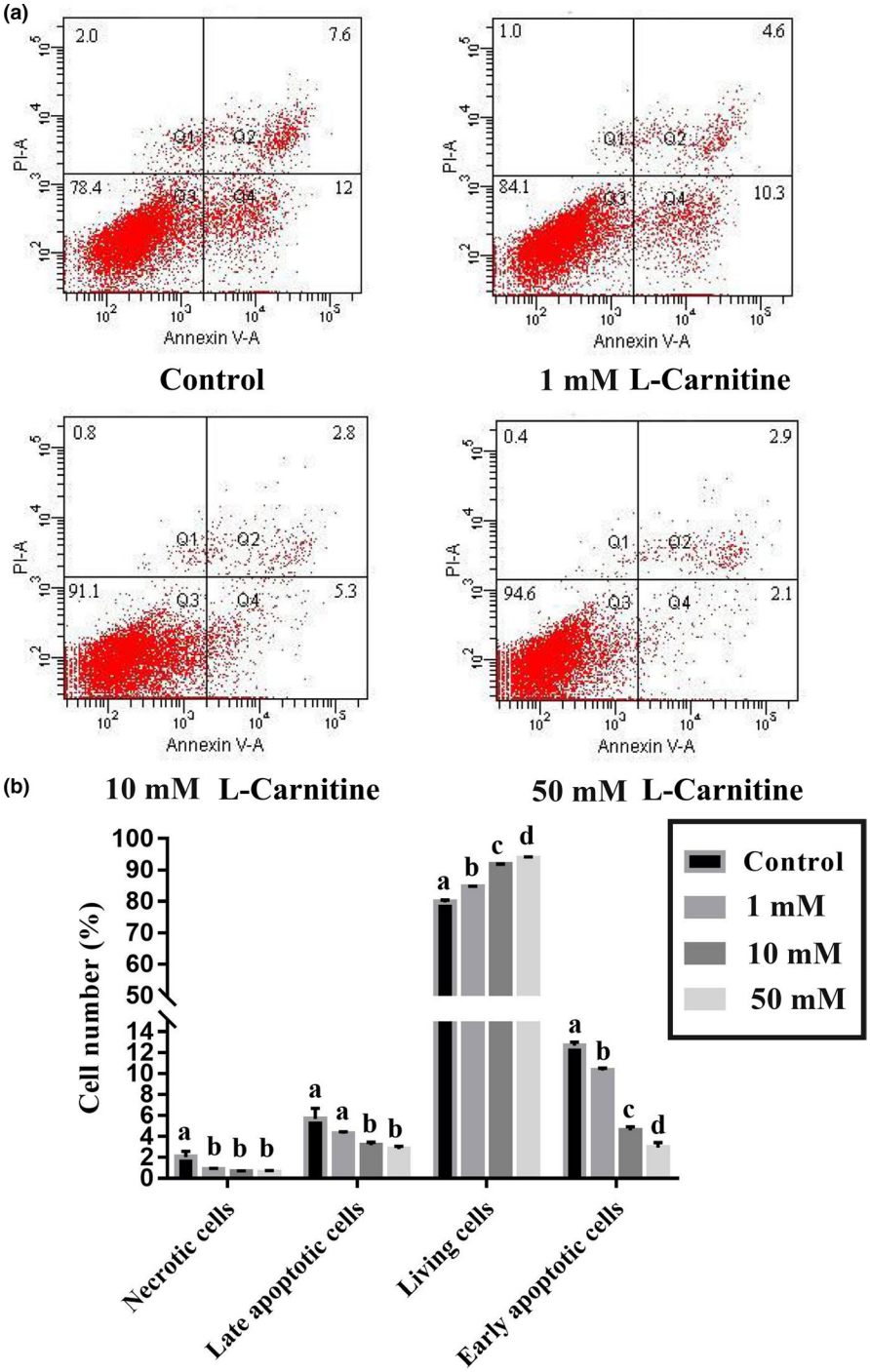

FIGURE 2 Scatter plots of Annexin V-FITC/PI staining in control or cells treated with various concentrations of L-carnitine (0, 1, 10 or 50 mM) in a quadrant analysis. Note: Q1-necrotic or dead cells (Annexin-FITC−/PI+), Q2-late apoptotic or dead cells (Annexin-FITC+/PI+), Q3-live cells (Annexin-FITC−/PI−), Q4-early apoptotic cells (Annexin-FITC+/PI−). Bars with different letters are significant different ($p < .05$)

3.5 | Effects of L-carnitine on protein abundances in rat placenta trophoblasts pretreated with IGF-1 signaling pathway inhibitors

PI3K and ERK are two critical downstream signaling pathways of IGF-1 signaling. PD098059 and LY294002 are the inhibitors of ERK and PI3K, respectively. Cells pretreated with PD098059 or LY294002 both partially rescued the effect of L-carnitine on the protein abundances of SNAT1 and SNAT2 ($p < .05$, Figure 6).

4 | DISCUSSION

The anti-apoptotic function of L-carnitine has been widely studied in different cell lines. In mouse osteoblastic cells (MC3T3-E1 cells), L-carnitine protects cells from apoptosis by inhibiting cytochrome c release and caspase-3 and caspase-9 activation (Xie et al., 2008). In neuronal cells, L-carnitine has similar anti-apoptotic actions and thus has neuroprotective effect (Ishii, Shimpo, Matsuoka, & Kinoshita, 2000). In skeletal muscle cells, L-carnitine prevents

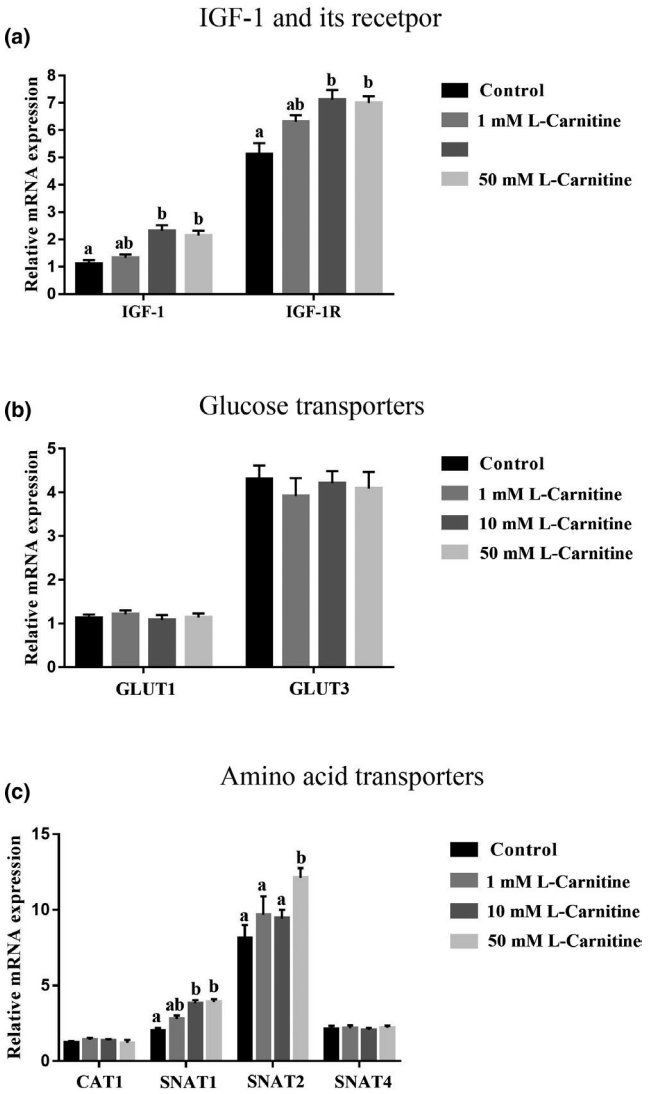

FIGURE 3 Effects of various concentrations of L-carnitine (0, 1, 10, or 50 mM) on mRNA expression of IGF-1 and IGF-1R (a), glucose transporters (b) and amino acid transporters (c) in rat placenta trophoblast cells. Bars with different letters are significant different ($p < .05$)

apoptosis and protects muscle from metabolic injuries (Vescovo et al., 2002). Similar anti-apoptotic function of L-carnitine is also observed in lymphocytes (Moretti et al., 2002; Wang et al., 2007). In the present study, we showed that L-carnitine protected rat placenta trophoblast cells against serum deprivation-induced apoptosis. Dietary supplementation of L-carnitine has been shown to increase the birth weight of piglets at weaning (Ramanau, Kluge, Spilke, & Eder, 2002b) and decrease the number of stillborn and mummified pigs (Musser et al., 1999). The trophoblasts from placentas of pregnancies with IUGR exhibit increased apoptosis compared with those from control pregnancies (Levy et al., 2002). Excessive injury of the villous trophoblast layer caused by apoptosis reduces the functional mass of syncytiotrophoblast in IUGR and limits the capacity of nutrient transportation in the villi (Scifres & Nelson, 2009). Therefore, L-carnitine supplementation during pregnancy might decrease the number of IUGR piglets by protecting placenta trophoblast cells against apoptosis.

The proliferation of trophoblast cells has been considered to be involved in embryo implantation and trophoblast invasion, which is one of the limiting factors for the establishment of pregnancy (Magariños, Sánchez-Margalet, Kotler, Calvo, & Varone, 2007). The molecular mechanisms underlying the effect of L-carnitine on placenta trophoblast cell proliferation remain unknown. Our results showed that L-carnitine supplementation increased the percentage of cells in S phase, which was accompanied with the decrease of cells in G2/M phase. These results indicate that L-carnitine can increase cell proliferation, which is consistent with previous findings by Colucci et al. (2005) and Santoro et al. (2005) in osteoblasts and ovary cells.

Besides alterations in apoptosis and proliferation, intrauterine growth restriction is associated with a variety of changes in amino acid and glucose contents from maternal circulation (Jansson, 2005). Therefore, glucose and amino acid transporters were measured in the current study. GLUT1 and GLUT3 are two primary transporters mediating facilitated glucose transfer across the placental barrier in early pregnancy (Jansson, Ylvén, Wennergren, & Powell, 2002). However, in the current study, neither GLUT1 nor GLUT3 expression was changed in trophoblast cells treated with L-carnitine. Similarly, previous study also found that fetal hypoglycemia in IUGR might not due to changes in placental glucose transporters as GLUTs protein abundances were unaltered (Jansson, Wennergren, & Illsley, 1993). Unlike glucose transporters, changes in amino acid transporters were observed when trophoblast cells were treated with L-carnitine. Transportation of amino acids from maternal to the fetus is an important process resulting in the increase of amino acid concentrations in the fetal circulation. In IUGR, placental amino acid uptake is largely decreased in both human and experimental animal models (Regnault et al., 2005). In the placenta, neutral amino acids are mainly transported by SNAT1, SNAT2, and SNAT4 (Desforges et al., 2009; Glazier & Sibley, 2006; Jones et al., 2007). In our study, trophoblast cells treated with L-carnitine significant increased SNAT1 and SNAT2. Thus, the up-regulation of amino acid transporters might partially help to increase the reproductive function when sows are treated with L-carnitine-supplemented diets.

Liver-derived IGF-1 is the principal source of IGF-1 in the blood (Sjögren et al., 1999). IGF-1 exerts its function in both endocrine and autocrine/paracrine manners (Adams, 2002). Trophoblasts not only express IGF-1 receptor, but also produce IGF-1 locally. Thus, the effective concentration of this hormone is probably greater in the placenta (Han, Bassett, Walton, & Challis, 1996). Until now, it is still not clear about the physiological role of IGF-1 during pregnancy, which could be a key player in the regulation of the embryo implantation as well as its maintenance. In this research, we demonstrated that the expression of IGF-1 and IGF1-R was up-regulated in trophoblast cells treated with L-carnitine. Activation of IGF-1 signaling pathway stimulates phosphoinositide-3 kinase (PI3-K)/Akt and Ras/Raf/MEK/ERK pathways (Denduluri et al., 2015; Hakuno & Takahashi, 2018;

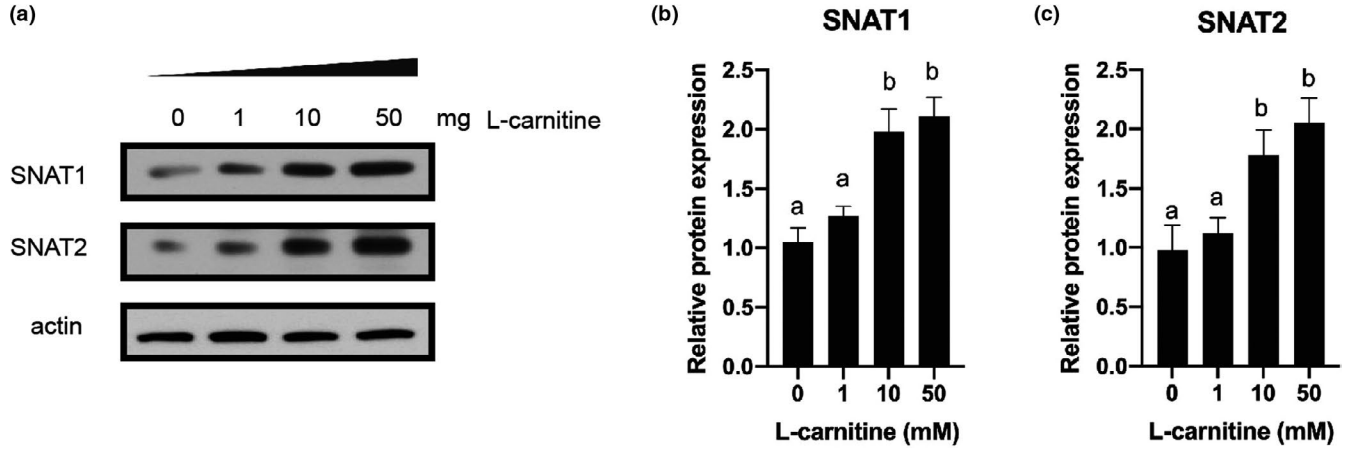

FIGURE 4 Effect of various concentrations of L-carnitine (0, 1, 10, or 50 mM) on protein abundances of SNAT1 and SNAT2 in rat placenta trophoblast cells. Bars with different letters are significant different ($p < .05$)

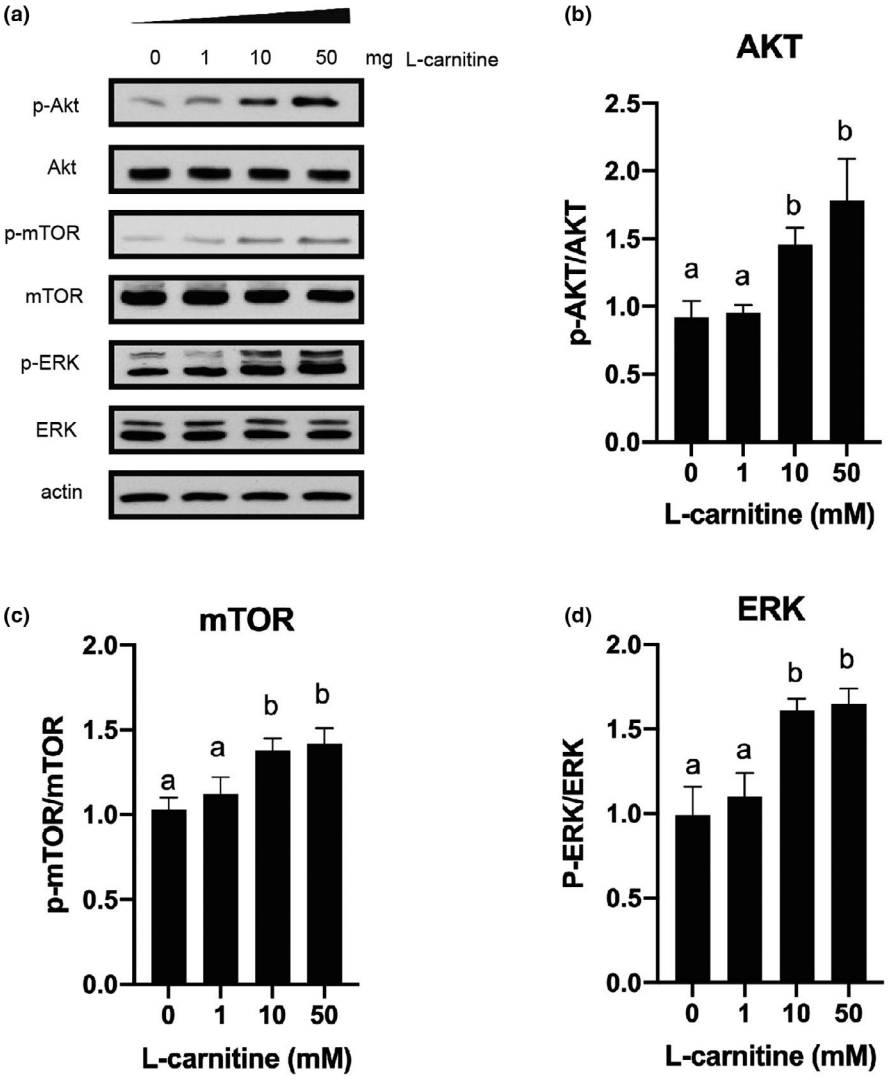

FIGURE 5 Effect of various concentration of L-carnitine (0, 1, 10 or 50 mM) on PI3K/Akt/mTOR and ERK signaling pathways in rat placenta trophoblast cells. Bars with different letters are significant different ($p < .05$)

Higashi, Sukhanov, Anwar, Shai, & Delafontaine, 2012). It has been well established that phosphorylation of Akt triggers the activation of mTORC1 signaling pathway. Interestingly, recent study also found that ERK phosphates and inhibits tuberous sclerosis (TSC), and further activates mTOR signaling pathway (Ma, Chen, Erdjument-Bromage, Tempst, & Pandolfi, 2005; Mendoza, Er, & Blenis, 2011), which indicates a potential cross talk between these two pathways. mTOR is a master regulator of a number of biological processes

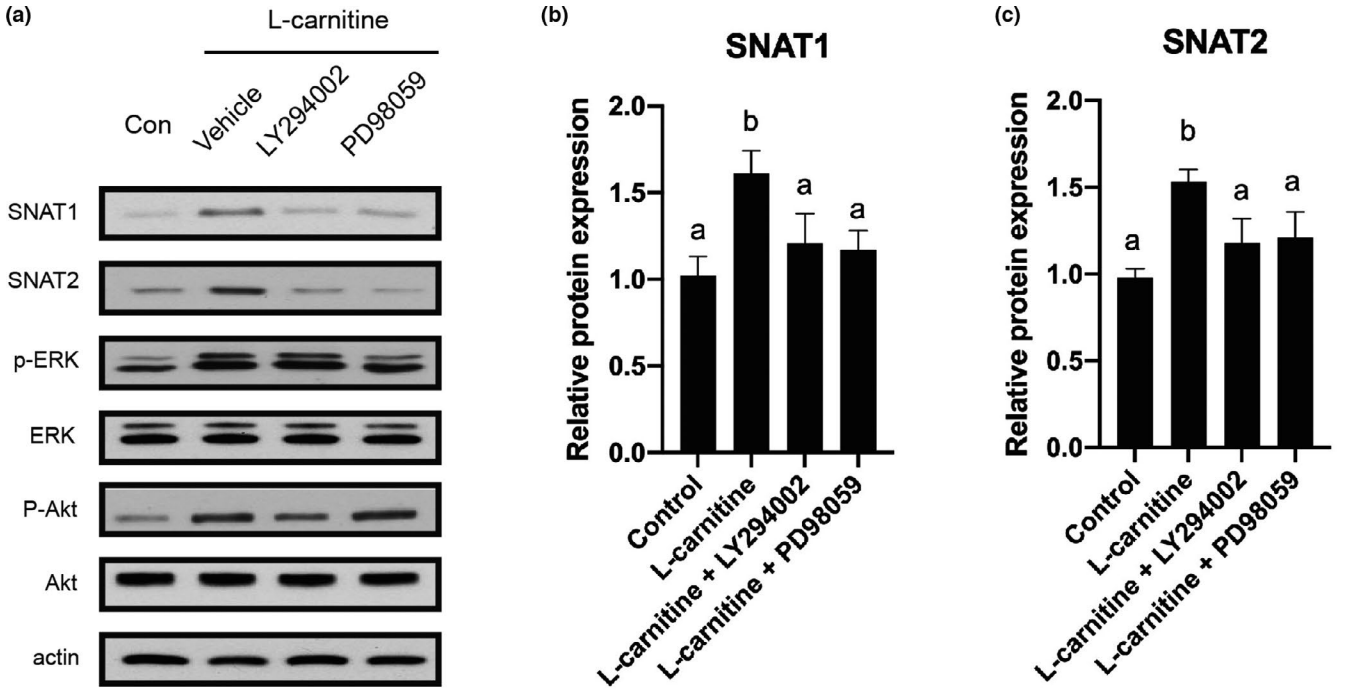

FIGURE 6 Effect of L-carnitine on protein abundances of SNAT1 and SNAT2 in rat placenta trophoblast cells pretreated with ERK or PI3K inhibitors. Bars with different letters are significant different ($p < .05$)

such as protein synthesis (via S6K1; Wang & Proud, 2006; Zhang et al., 2014), translation (via 4EBP1; Fingar et al., 2004; Proud, 2004) and autophagy (via ULK1; Jung, Ro, Cao, Otto, & Kim, 2010; Kim, Kundu, Viollet, & Guan, 2011). Thus, L-carnitine might regulate SNAT1 and SNAT2 expression through the activation of mTOR signaling pathway. As expected, both Akt and ERK signaling pathways were activated in trophoblast cells supplemented with L-carnitine. In addition, inhibition of Akt or ERK signaling pathway partially blocked the protein abundances of SNAT1 and SNAT2. Coincidently, IGF-1 has been reported to stimulate amino acid uptake in placental trophoblasts (Karl, 1995). Our results indicate that L-carnitine-induced up-regulation of SNAT1 and SNAT2 might be partially due to the activation of IGF-1 signaling pathway. Future studies are needed to clarify other potential signaling pathways that could participate in L-carnitine regulation.

5 | CONCLUSIONS

Collectively, our results showed that L-carnitine protected placenta trophoblast cells against apoptosis and increased their proliferation. The improvement of amino acid transporters (SNAT1 and SNAT2) in trophoblast cells may be partially induced by the activation of IGF-1 signaling pathway.

CONFLICT OF INTEREST

All authors declare that they have no competing interest in the present work.

AUTHORS' CONTRIBUTIONS

SH Zhang and WT Guan designed the experiment and supervised the project. ZH Wu, JH Heng, M Tian and JM Chen, F Chen performed the experiments and conducted the lab work. JH Heng and ZH Wu conducted the statistical analysis. All authors read and approved the final manuscript.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

How to cite this article: Zhang S, Wu Z, Heng J, et al. L-carnitine increases cell proliferation and amino acid transporter expression via the activation of insulin-like growth factor I signaling pathway in rat trophoblast cells. *Food Sci Nutr*. 2020;8:3298–3307. https://doi.org/10.1002/fsn3.1607

SCAULIB202518826

检索证明

根据委托人提供的论文材料，委托人**华南农业大学动物科学学院 张世海** 31 篇论文收录情况如下表。

序号	论文名称	发表刊物及发表的年月卷期/页码等	作者排名	论文等级	作者文中单位	收录情况	影响因子	中科院大类分区
1	Nutritional strategies to alleviate oxidative stress in sows	ANIMAL NUTRITION 出版年：2022 出版日期：JUN 卷期：9 页码：60-73 文献类型：Review	共同通讯作者（倒数第一）	T2 类	华南农业大学动物科学学院	SCI	IF2-year=6.3 IF5-year=6.9 （2022）	农林科学 1 区 Top 期刊：是 （2022）
2	Glyceryl butyrate attenuates enterotoxigenic Escherichia coli-induced intestinal inflammation in piglets by inhibiting the NF-κB/MAPK pathways and modulating the gut microbiota	FOOD & FUNCTION 出版年：2022 出版日期：JUN 6 卷期：13 11 页码：6282-6292 文献类型：Article	共同通讯作者（倒数第一）	T2 类	华南农业大学动物科学学院	SCI	IF2-year=6.1 IF5-year=6.5 （2022）	农林科学 1 区 Top 期刊：是 （2022）
3	Improved thermostability of lipase Lip2 from Yarrowia lipolytica through disulfide bond design for preparation of medium-long-medium structured lipids	LWT-FOOD SCIENCE AND TECHNOLOGY 出版年：2022 出版日期：AUG 15 卷期：166 页码：- 文献号：113786 文献类型：Article	共同通讯作者	T2 类	华南农业大学动物科学学院	SCI	IF2-year=6.0 IF5-year=6.0 （2022）	农林科学 1 区 Top 期刊：是 （2022）

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4	Maternal sodium acetate supplementation promotes lactation performance of sows and their offspring growth performance	ANIMAL NUTRITION 出版年：2023 出版日期：SEP 卷期：14 页码：213-224 文献类型：Article	通讯作者	T2 类	华南农业大学动物科学学院	SCI	IF2-year=6.1 IF5-year=6.6 （2023）	农林科学 1 区 Top 期刊：是 （2023）
5	Niacin/β-hydroxybutyrate regulates milk fat and milk protein synthesis via the GPR109A/Gi/mTORC1 pathway	FOOD & FUNCTION 出版年：2023 出版日期：MAR 20 卷期：14 6 页码：2642-2656 文献类型：Article	通讯作者	T2 类	华南农业大学动物科学学院	SCI	IF2-year=5.1 IF5-year=5.6 （2023）	农林科学 1 区 Top 期刊：是 （2023）
6	Maternal microbe-specific modulation of the offspring microbiome and development during pregnancy and lactation	GUT MICROBES 出版年：2023 出版日期：DEC 31 卷期：15 1 页码：- 文献号：2206505 文献类型：Review	通讯作者	T2 类	华南农业大学动物科学学院	SCI	IF2-year=12.2 IF5-year=12.3 （2023）	医学 1 区 Top 期刊：是 （2023）
7	Maternal supplementation with glycerol monolaurate improves the intestinal health of suckling piglets by inhibiting the NF-κB/MAPK pathways and improving oxidative stability	FOOD & FUNCTION 出版年：2023 出版日期：APR 3 卷期：14 7 页码：3290-3303 文献类型：Article	共同通讯作者	T2 类	华南农业大学动物科学学院	SCI	IF2-year=5.1 IF5-year=5.6 （2023）	农林科学 1 区 Top 期刊：是 （2023）

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8	Amino acid transportation, sensing and signal transduction in the mammary gland: key molecular signalling pathways in the regulation of milk synthesis	NUTRITION RESEARCH REVIEWS 出版年：2020 出版日期：DEC 卷期：33 2 页码：287-297 文献号：PII S0954422420000074 文献类型：Review	共同通讯作者（倒数第一）	T2 类	华南农业大学动物科学学院	SCI	IF2-year=7.8 IF5-year=9.621 （2020）	医学 1 区 Top 期刊：是 （2020）
9	Dietary fiber and microbiota interaction regulates sow metabolism and reproductive performance	ANIMAL NUTRITION 出版年：2020 出版日期：DEC 卷期：6 4 页码：397-403 文献类型：Review	共同通讯作者（倒数第一）	T2 类	华南农业大学动物科学学院	SCI	IF2-year=6.383 IF5-year=5.497 （2020）	农林科学 1 区 Top 期刊：是 （2020）
10	A Combination of Formic Acid and Monolaurin Attenuates Enterotoxigenic Escherichia coli Induced Intestinal Inflammation in Piglets by Inhibiting the NF-κB/MAPK Pathways with Modulation of Gut Microbiota	JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY 出版年：2020 出版日期：APR 8 卷期：68 14 页码：4155-4165 文献类型：Article	共同通讯作者	T2 类	华南农业大学动物科学学院	SCI	IF2-year=5.279 IF5-year=5.269 （2020）	农林科学 1 区 Top 期刊：是 （2020）
11	Maternal Nutrition During Late Gestation and Lactation: Association With Immunity and the Inflammatory Response in the Offspring	FRONTIERS IN IMMUNOLOGY 出版年：2022 出版日期：JAN 21	共同通讯作者（倒数第一）	A 类	华南农业大学动物科学学院	SCI	IF2-year=7.3 IF5-year=8.0 （2022）	医学 2 区 Top 期刊：是 （2022）

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		卷期：12 页码：- 文献号：758525 文献类型：Review						
12	Dietary Supplementation of Enzymatically Treated Artemisia annua L. Improves Lactation Performance, Alleviates Inflammatory Response of Sows Reared Under Heat Stress, and Promotes Gut Development in Preweaning Offspring	FRONTIERS IN VETERINARY SCIENCE 出版年：2022 出版日期：MAR 25 卷期：9 页码：- 文献号：843673 文献类型：Article	共同通讯作者（倒数第一）	A 类	华南农业大学动物科学学院	SCI	IF2-year=3.2 IF5-year=3.5 （2022）	农林科学 2 区 Top 期刊：否 （2022）
13	A critical role of AMP-activated protein kinase in regulating intestinal nutrient absorption, barrier function, and intestinal diseases	JOURNAL OF CELLULAR PHYSIOLOGY 出版年：2022 出版日期：OCT 卷期：237 10 页码：3705-3716 文献类型：Review	通讯作者	A 类	华南农业大学动物科学学院	SCI	IF2-year=5.6 IF5-year=5.9 （2022）	生物学 2 区 Top 期刊：是 （2022）
14	Effects of Dietary Monoglyceride and Diglyceride Supplementation on the Performance, Milk Composition, and Immune Status of Sows During Late Gestation and Lactation	FRONTIERS IN VETERINARY SCIENCE 出版年：2021 出版日期：AUG 16 卷期：8 页码：- 文献号：714068	共同通讯作者（倒数第一）	A 类	华南农业大学动物科学学院	SCI	IF2-year=3.471 IF5-year=3.821 （2021）	农林科学 2 区 Top 期刊：否 （2021）

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		文献类型：Article						
15	Exploring the Benefits of Probiotics in Gut Inflammation and Diarrhea-From an Antioxidant Perspective	ANTIOXIDANTS 出版年：2023 出版日期：JUL 卷期：12 7 页码：- 文献号：1342 文献类型：Review	共同通讯作者	A 类	华南农业大学动物科学学院	SCI	IF2-year=6.0 IF5-year=6.7 （2023）	医学 2 区 Top 期刊：否 （2023）
16	Energy deprivation-induced AMPK activation inhibits milk synthesis by targeting PrIR and PGC-1α	CELL COMMUNICATION AND SIGNALING 出版年：2022 出版日期：MAR 5 卷期：20 1 页码：- 文献号：25 文献类型：Article	共同通讯作者（倒数第一）	A 类	华南农业大学动物科学学院	SCI	IF2-year=8.4 IF5-year=8.0 （2022）	生物学 2 区 Top 期刊：否 （2022）
17	Novel advances in understanding fatty acid-binding G protein-coupled receptors and their roles in controlling energy balance	NUTRITION REVIEWS 出版年：2022 出版日期：FEB 卷期：80 2 页码：187-199 文献类型：Review	通讯作者	A 类	华南农业大学动物科学学院	SCI	IF2-year=6.1 IF5-year=7.8 （2022）	医学 2 区 Top 期刊：否 （2022）

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18	Sodium acetate regulates milk fat synthesis through the activation of GPR41/GPR43 signaling pathway	FRONTIERS IN NUTRITION 出版年：2023 出版日期：FEB 16 卷期：10 页码：- 文献号：1098715 文献类型：Article	通讯作者	A 类	华南农业大学动物科学学院	SCI	IF2-year=4.0 IF5-year=4.4 （2023）	农林科学 2 区 Top 期刊：否 （2023）
19	Dietary Supplementation with Lysozyme-Cinnamaldehyde Conjugates Enhances Feed Conversion Efficiency by Improving Intestinal Health and Modulating the Gut Microbiota in Weaned Piglets Infected with Enterotoxigenic Escherichia coli	ANIMALS 出版年：2023 出版日期：NOV 卷期：13 22 页码：- 文献号：3497 文献类型：Article	共同通讯作者	A 类	华南农业大学动物科学学院	SCI	IF2-year=2.7 IF5-year=3.0 （2023）	农林科学 2 区 Top 期刊：否 （2023）
20	Leucine and arginine enhance milk fat and milk protein synthesis via the CaSR/Gi/mTORC1 and CaSR/Gq/mTORC1 pathways	EUROPEAN JOURNAL OF NUTRITION 出版年：2023 出版日期：OCT 卷期：62 7 页码：2873-2890 文献类型：Article	通讯作者	A 类	华南农业大学动物科学学院	SCI	IF2-year=4.1 IF5-year=4.6 （2023）	医学 2 区 Top 期刊：否 （2023）
21	Maternal Selenium-Enriched Yeast Supplementation in Sows Enhances Offspring Growth and Antioxidant Status through the Nrf2/Keap1	ANTIOXIDANTS 出版年：2023 出版日期：DEC 卷期：12 12 页码：-	共同通讯作者	A 类	华南农业大学动物科学学院	SCI	IF2-year=6.0 IF5-year=6.7 （2023）	医学 2 区 Top 期刊：否 （2023）

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	Pathway	文献号：2064 文献类型：Article						
22	Regulation of the JAK2-STAT5 Pathway by Signaling Molecules in the Mammary Gland	FRONTIERS IN CELL AND DEVELOPMENTAL BIOLOGY 出版年：2020 出版日期：NOV 17 卷期：8 页码：- 文献号：604896 文献类型：Review	共同通讯作者（倒数第一）	A 类	华南农业大学动物科学学院	SCI	IF2-year=6.684 IF5-year=7.219 （2020）	生物学 2 区 Top 期刊：否 （2020）
23	Fat Encapsulation Reduces Diarrhea in Piglets Partially by Repairing the Intestinal Barrier and Improving Fatty Acid Transport	ANIMALS 出版年：2021 出版日期：JAN 卷期：11 1 页码：- 文献号：28 文献类型：Article	共同通讯作者	A 类	华南农业大学动物科学学院	SCI	IF2-year=3.231 IF5-year=3.312 （2021）	农林科学 2 区 Top 期刊：否 （2021）
24	Anethole Attenuates Enterotoxigenic Escherichia coli-Induced Intestinal Barrier Disruption and Intestinal Inflammation via Modification of TLR Signaling and Intestinal Microbiota	FRONTIERS IN MICROBIOLOGY 出版年：2021 出版日期：MAR 25 卷期：12 页码：- 文献号：647242 文献类型：Article	共同通讯作者	A 类	华南农业大学动物科学学院	SCI	IF2-year=6.064 IF5-year=6.843 （2021）	生物学 2 区 Top 期刊：是 （2021）

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25	Artemisinin Protects Porcine Mammary Epithelial Cells against Lipopolysaccharide-Induced Inflammatory Injury by Regulating the NF-κB and MAPK Signaling Pathways	ANIMALS 出版年：2021 出版日期：JUN 卷期：11 6 页码：- 文献号：1528 文献类型：Article	共同通讯作者（倒数第一）	A 类	华南农业大学动物科学学院	SCI	IF2-year=3.231 IF5-year=3.312 （2021）	农林科学 2 区 Top 期刊：否 （2021）
26	Dietary enzymatically-treated Artemisia annua L. supplementation could alleviate oxidative injury and improve reproductive performance of sows reared under high ambient temperature	JOURNAL OF THERMAL BIOLOGY 出版年：2020 出版日期：DEC 卷期：94 页码：- 文献号：102751 文献类型：Article	共同通讯作者	A 类	华南农业大学动物科学学院	SCI	IF2-year=2.902 IF5-year=3.102 （2020）	生物学 2 区 Top 期刊：否 （2020）
27	Ferroptosis Regulation by Nutrient Signalling	NUTRITION RESEARCH REVIEWS 出版年：2022 出版日期：DEC 卷期：35 2 页码：282-294 文献号：PII S0954422421000226 文献类型：Review	共同通讯作者（倒数第一）	A 类	华南农业大学动物科学学院	SCI	IF2-year=5.7 IF5-year=8.9 （2022）	医学 2 区 Top 期刊：否 （2022）
28	Current Evidences and Future Perspectives for AMPK in the Regulation of Milk Production and	FRONTIERS IN CELL AND DEVELOPMENTAL BIOLOGY	共同通讯作者（倒数第一）	A 类	华南农业大学动物科学学院	SCI	IF2-year=6.684 IF5-year=7.219 （2020）	生物学 2 区 Top 期刊：否 （2020）

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	Mammary Gland Biology	出版年：2020 出版日期：JUN 26 卷期：8 页码：- 文献号：530 文献类型：Review						
29	氧化应激对母畜乳腺功能的影响及其营养调控策略	动物营养学报 出版年：2020 卷期：32 12 页码：5587-5595 文献号： 文献类型：	通讯作者	B 类	华南农业大学动物科学学院	北大核心	无	无
30	Excessive BCAA regulates fat metabolism partially through the modification of m6A RNA methylation in weanling piglets	NUTRITION & METABOLISM 出版年：2020 出版日期：JAN 23 卷期：17 1 页码：- 文献号：10 文献类型：Article	共同通讯作者（倒数第一）	B 类	华南农业大学动物科学学院	SCI	IF2-year=4.169 IF5-year=5.022 （2020）	医学 3 区 Top 期刊：否 （2020）

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Animal Nutrition 9 (2022) 60–73

Contents lists available at ScienceDirect

Animal Nutrition

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Review Article

Nutritional strategies to alleviate oxidative stress in sows

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ARTICLE INFO

Article history:
Received 16 May 2021
Received in revised form
8 October 2021
Accepted 14 October 2021
Available online 3 November 2021

Keywords:
Sow
Oxidative stress
Nutritional strategy
Plant extract
Selenium
Vitamin E

ABSTRACT

The performance of high-yielding sows is directly related to the productivity of pig farming. Fetal development mainly occurs during the last month of pregnancy, and the aggressive metabolic burden of sows during this stage eventually leads to systemic oxidative stress. When affected by oxidative stress, sows exhibit adverse symptoms such as reduced feed intake, hindered fetal development, and even abortion. In addition, milk synthesis during the lactation period causes a severe metabolic burden. The biological response to oxidative stress during this period is associated with a decrease in milk production, which further affects the growth of piglets. Understanding the nutritional strategies to alleviate oxidative stress in sows is crucial to maintain their reproduction and lactation performance. Recently, advances have been made in the field of nutrition to relieve oxidative stress in sows during late pregnancy and lactation. This review highlights the nutritional strategies to relieve oxidative stress in sows reported within the last 20 years.
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1. Introduction

During the late pregnancy and lactation stages, sows start suffering oxidative stress induced by severe metabolic burden and do not fully recover until the weaning period (Berchieri-Ronchi et al., 2011; Toy et al., 2009). This process is characterized by the accumulation of active oxygen, including superoxide (O^{2-}) and hydrogen peroxide (H_2O_2), produced by the placenta and mammary glands (Esther et al., 2003), which negatively regulates reproductive performance and lactation performance. Excessive reactive oxygen species (ROS) prevents the oocyte maturation, inhibits fertilization of sperm and egg cells, and increases the risk of prenatal death (Karowicz-Bilińska et al., 2002; Toy et al., 2009). Oxidative stress in sows decreased reproductive performance such as total litter size, live litter size and litter weight gain (Zhang et al., 2020). Furthermore, oxidative stress caused decreased feed intake of sows during lactation, which leads to prolonged negative energy balance and greater loss of body condition and reduced milk production (Black et al., 1993; Dima et al., 2009).

Oxidative stress is due to the amount of ROS produced exceeding the neutralization ability of antioxidants. Multiple external or environmental factors are proposed to trigger the generation of ROS, such as ultraviolet radiation, ionizing radiation, toxins, chemicals, extreme hot environments (the key factor causing heat stress in actual production) and other adverse conditions. Excessive ROS leads to oxidative damage to proteins, lipids and DNA and eventually destroys cell function. Cellular metabolism is accompanied by the production of ROS. These metabolic byproducts include O^{2-}, H_2O_2, hydroxyl radicals (·OH) and alkoxy radicals (RO). Under the process of aerobic respiration, cells oxidize and decompose nutrients to produce substances with high reducing forces that are transmitted along the mitochondrial electron transport chain. The production of mitochondrial ROS mainly occurs at two sites in the electron transport chain, namely,

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Peer review under responsibility of Chinese Association of Animal Science and Veterinary Medicine.

Production and Hosting by Elsevier on behalf of KeAi

https://doi.org/10.1016/j.aninu.2021.10.006
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complex I (NADH dehydrogenase) and complex III (ubiquinone-cytochrome reductase). When electrons from complex I or II are transferred to coenzyme Q (ubiquinone), unstable intermediates in the coenzyme Q cycle lead to the formation of superoxide by transferring electrons directly to molecular oxygen (Finkel et al., 2000). Nitric oxide (NO) is a free radical molecule formed by a variety of cells, including airway and vascular smooth muscle cells, endothelial cells and epithelial cells (Ricciardolo et al., 2004). Reactive nitrogen species (RNS) are produced by the reaction of nitric oxide with reactive oxygen species (Fig. 1). These RNS include nitroxyl (HNO), nitrogen dioxide (NO_2) and peroxynitrite ($ONOO^-$) (Bove et al., 2006).

The detrimental or beneficial effects of ROS and RNS depend on their cellular concentrations (Valko et al., 2006). The beneficial effects of ROS occur at low or medium concentrations and are involved in resistance to pathogens and the regulation of many cellular signaling systems (Pizzino et al., 2017). Furthermore, ROS also play a physiological role in the cell's response to hypoxia (Valko et al., 2007). However, at high concentrations, the amounts of ROS/RNS exceed the neutralization abilities of enzymatic and nonenzymatic antioxidants. Excessive reactive oxygen species destroy cellular lipids, proteins or DNA and inhibit their corresponding biological functions, which could lead to decreases in lactation and reproductive performance, ultimately shortening sow life (Fig. 2).

To eliminate excessive free radicals, cells develop a series of defense mechanisms during the process of evolution (Cadenas, 1997). These defense mechanisms are mainly composed of antioxidant enzymes and nonenzymatic antioxidants. Antioxidant enzymes mainly include superoxide dismutase (SOD), glutathione peroxidase (GSH-Px), and catalase (CAT). Nonenzymatic antioxidants are represented by ascorbic acid (vitamin C), α-tocopherol (vitamin E), glutathione (GSH), carotenoids, flavonoids, etc (Fig. 3). Under normal conditions, there is a dynamic balance between the antioxidant system and free radicals, which is essential for the survival and health of organisms (Valko et al., 2006).

In actual production, as heat stress can induce oxidative stress, using wet curtain ventilation system and installing spray device to control the temperature of the pigsty is an effective way to alleviate the oxidative stress in sows. In addition, genetic selection for a breed with stronger tolerance to oxidative stress is an alternative method for alleviating the detrimental conditions. The application of nutritional strategies to reduce the effects of oxidative stress is also an effective and economical way to reduce oxidative stress and is also more suitable for the current intensive farming model (Cottrell et al., 2015). Recently, a number of antioxidants such as vitamins, minerals and plant extracts have been shown to be effective in alleviating oxidative stress in sows in the late pregnancy and lactation stages. In this review, these antioxidants are categorized in reference to their origins and active ingredients, such as plants and extracts, microbial additives, vitamins, and other additives.

2. Plants and plant extracts

Plant-derived feed is a natural feed additive that includes herbs, spices, or food flavoring agents. Plant feed additives are usually added as whole beads or as extracts of plant-derived substances. Compared to antibiotics, they are reported to be natural, less toxic, residue-free, and desirable animal feed additives. It has been clarified that plant feed is rich in crude fiber and substances with reductive activity, such as terpenes, phenols, glycosides, saccharides, aldehydes, esters, and alcohols (Reyes-Camacho et al., 2020).

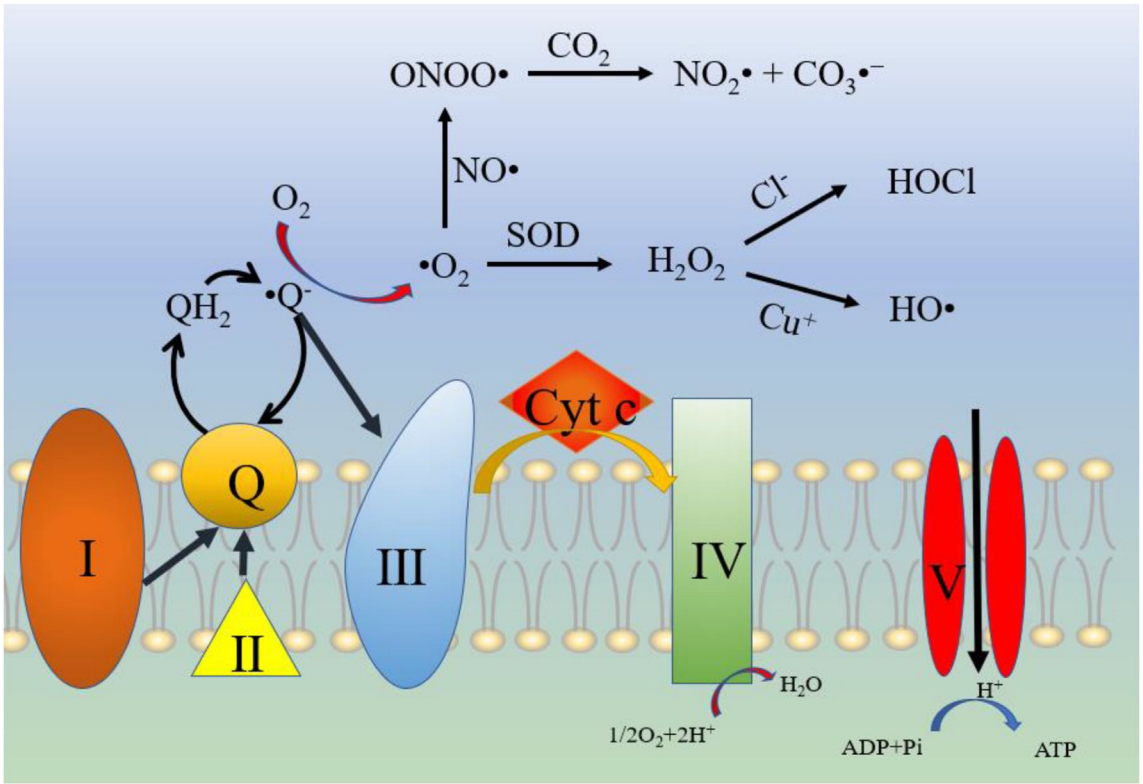

Fig. 1. Main production process of ROS/RNS. Complex III is the main source of mitochondrial ROS production. The electrons are transferred to coenzyme Q. Unstable coenzyme Q intermediates will directly transfer electrons to O_2 to generate $\cdot O_2$, which in turn will generate lots of ROS and RNS. Excessive ROS/RNS will have a variety of damage to the cells. SOD = superoxide dismutase; ROS = reactive oxygen species; RNS = reactive nitrogen species.

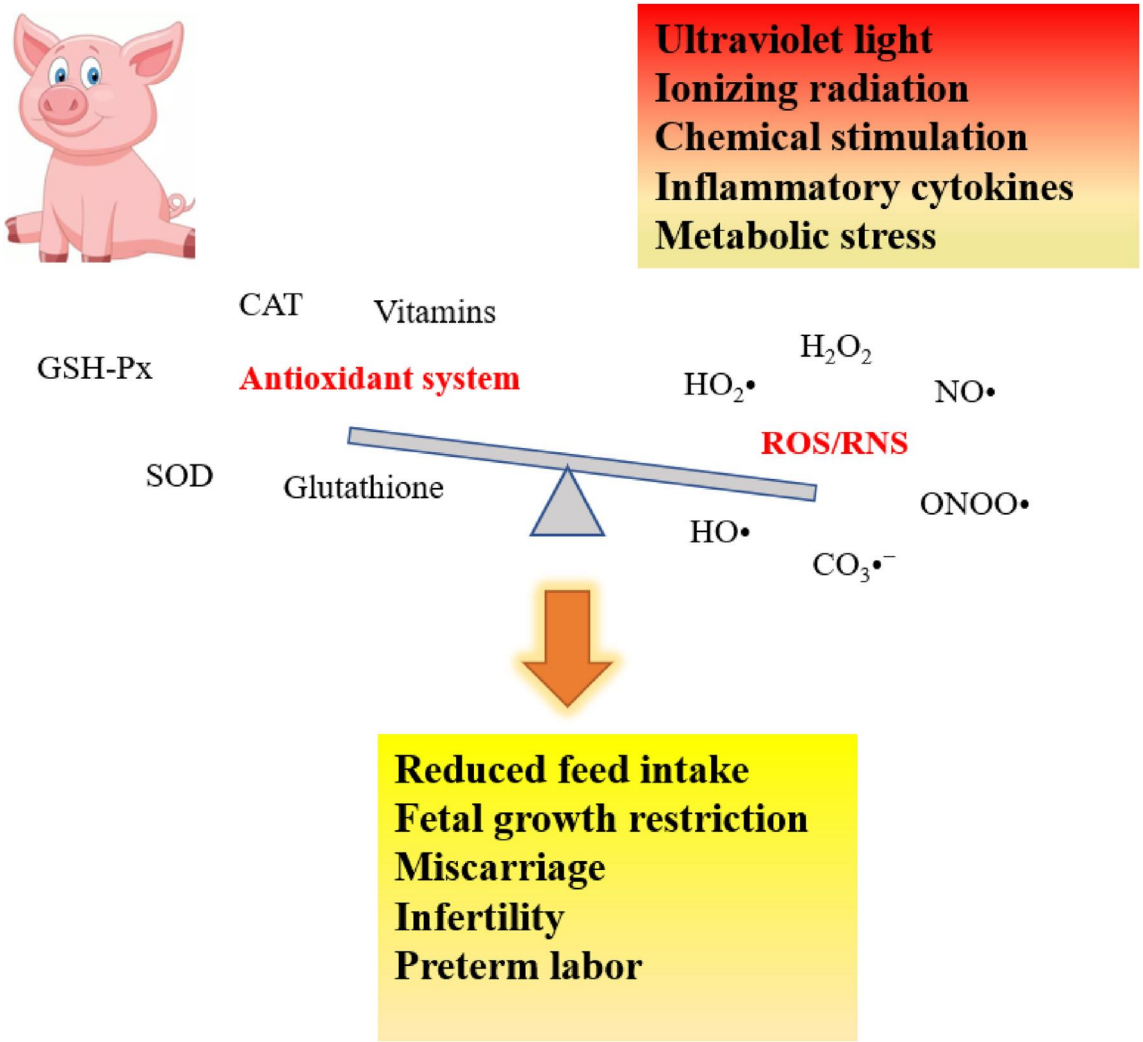

Fig. 2. Causes of oxidative stress. Oxidative stress is caused by an imbalance between production and accumulation of ROS/RNS in cells and the ability of a biological system to detoxify these reactive products. Oxidative stress is reported to be involved in a variety of pregnancy complications, such as preterm labor, infertility fetal growth restriction and miscarriage. CAT = catalase; SOD = superoxide dismutase; GSH-Px = glutathione peroxidase; ROS = reactive oxygen species; RNS = reactive nitrogen species.

These active ingredients are proposed to activate the immune system, stimulate the secretion of digestive juice, enhance antioxidant activity, and regulate the intestinal flora (Durmic et al., 2012). In addition, herbs are also reported to improve fetal development by increasing fetal blood glucose and growth hormone levels (Takei et al., 2007). Importantly, many reports have shown that the addition of plant components to sow feed can alleviate oxidative stress and has beneficial effects on the growth of piglets during the late pregnancy and lactation stages (Table 1).

2.1. Plant-derived fiber

The composition and structure of intestinal microorganisms undergo profound changes during pregnancy (Koren et al., 2012). Plant-derived fiber affects the diversity of the intestinal microbial community and plays an active role in maintaining intestinal health (Fig. 4). Adding *Pennisetum* (5% to 10%) increases the concentration of serum equol and elevates the antioxidant capacity and immune function of sows in late pregnancy by modifying the gut microbiota (increasing the abundance of *Coriobacteriaceae*) (Huang et al., 2021). In addition, higher activities of GSH-Px and insulin secretion index sensitivity in the serum of sows and lower concentrations of ROS and 8-hydroxy-2 deoxyguanosine were observed when sows were fed 2.2% konjac flour compared with the control (Tan et al., 2016). Konjac flour mainly contains konjac glucomannan (Chen et al., 2006), which has been shown to alter the fecal microbiota composition of sows (Tan et al., 2015) and to increase the plasma concentration of total short-chain fatty acids (SCFA). SCFA regulate insulin sensitivity by reducing fatty acid flow (Fernandes et al., 2011) and increasing fetal birth weight (Anderson et al., 2009). In addition, SCFA play an important role in the fermentation of equol (Decroos et al., 2005). These findings may partially explain why fiber regulates oxidative stress. Consistent with these findings, direct supplementation with inulin (a soluble fiber) also regulates the antioxidant capacity. Adding 1.6% inulin to sow feed from 80 to 109 days of pregnancy can increase the serum total antioxidant capacity (T-AOC), total superoxide dismutase (T-SOD), and GSH-Px activities of sows, leading to an optimal sow oxidative status. The concentrations of free fatty acid and total cholesterol in the serum of the sows fed inulin were also higher than those of the control group, indicating that the sow fed inulin had enhanced fat mobilization ability during delivery (Li et al., 2020), which could provide energy for sow delivery, thus shortening the delivery time and improving the birth survival rate of

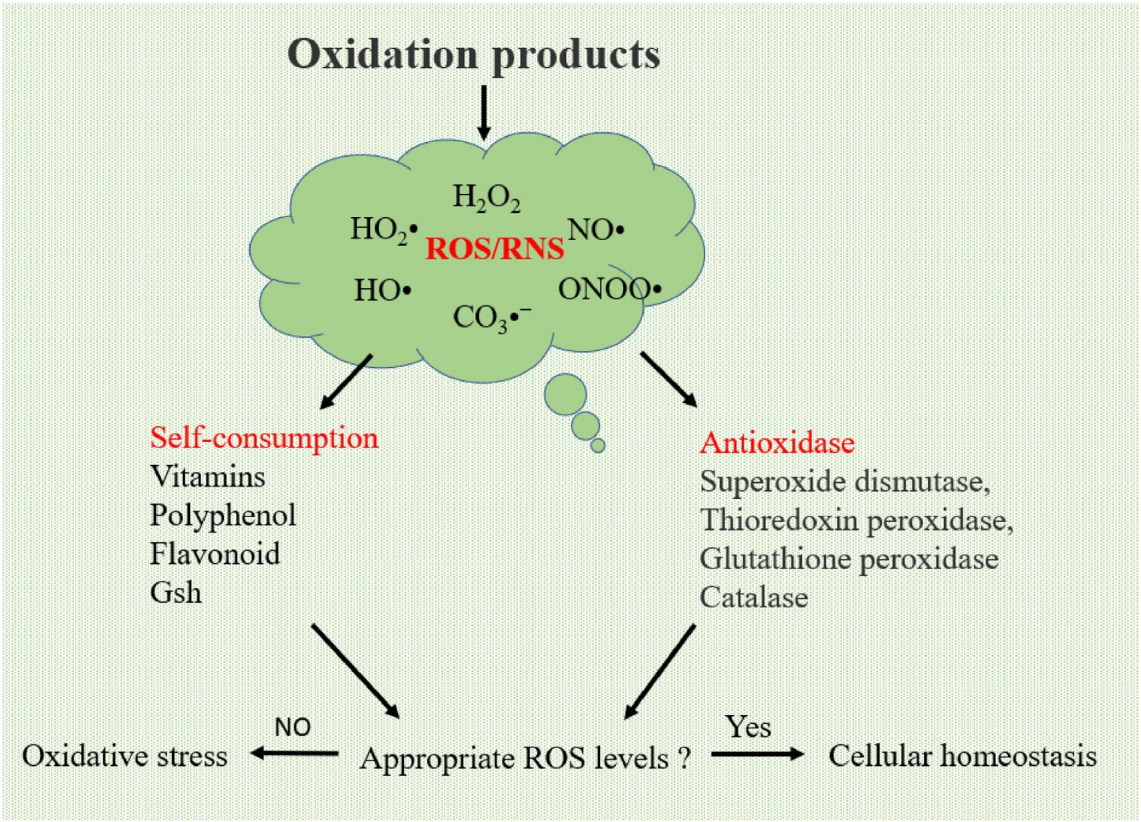

Fig. 3. Metabolic pathways of ROS/RNS. ROS = reactive oxygen species; RNS = reactive nitrogen species.

piglets. Chitosan oligosaccharide is another natural polysaccharide with good water solubility and can be used as an immunostimulant, anti-inflammatory and antioxidant (Xiong et al., 2014). Dietary chitosan oligosaccharide supplementation (30 mg/kg) in sows may promote placental nutrient transport by activating the mammalian target of rapamycin (mTOR) signaling pathway, protect sows from oxidative stress and reduce inflammatory factors in serum (Xie et al., 2016).

It worth noting that soluble fiber seems to deliver more antioxidant capacity than insoluble fiber. A large part of soluble fiber belongs to functional oligosaccharide. On one hand, soluble fiber adsorbs harmful microorganisms to reduce their colonization in the intestinal mucosa (Sweeney et al., 2012). On the other hand, soluble fiber could also be fermented by microorganisms in the hindgut to generate SCFA (acetate, propionate, and butyrate), which not only directly provide nutrition for intestinal cells, but also improve the intestinal morphology and integrity. More importantly, SCFA (especially butyrate) can alleviate oxidative stress. Butyrate protects against H_2O_2-induced DNA damage in rat or human colitis cells (Toden et al., 2007; Rosignoli et al., 2001). Besides, acetic acid and butyric acid esters were proved to inhibit the oxidative stress of mesangial cells induced by high glucose and lipopolysaccharide (Huang et al., 2017). Mechanistically, SCFA could exert antioxidant functions by regulating oxidoreductases (Knapp et al., 2013; Ebert et al., 2003; Yano and Tierney, 1989). For example, butyric acid enhanced glutathione-s-transferase activity in human colon cancer HT-29 cells (Ebert et al., 2003) and CAT activity in rat arterial smooth muscle cells (Yano and Tierney, 1989). Moreover, nuclear factor (erythroid-derived 2)-like 2 (Nrf2), a critical transcription factor to protect against oxidative stress, is also a target for butyric acid (Wang et al., 2020).

2.2. Plant-derived antioxidants

Some plants are rich in antioxidants, including phenols, flavonoids, proanthocyanidins, flavonols, vitamin C, vitamin E, beta-carotene, zinc, and selenium, which have been shown to have antioxidant potential (Jain et al., 2008). Feeding sows diets containing plant-derived natural bioactive compounds (1 g/kg) during gestation and lactation improved the protein concentration in colostrum and increased the resistance of milk to *Bacillus subtilis* and *Staphylococcus aureus*. In addition, the antioxidant status of sows is enhanced, with effects such as significant elevations of GSH-Px and SOD in the serum (Reyes-Camacho et al., 2020). Among these antioxidants, polyphenol is the most common substances and a well-known natural antioxidant in plants. Polyphenols are derived from flowers, vegetables, fruits, essential oils and tea (Zhang et al., 2016). Their antioxidant effect is comparable to that of vitamin E (Iqbal et al., 2015). A previous study showed that adding glucose polyphenols (200 to 300 mg/kg) from the 85th day of gestation to delivery improved reproductive performance, increased serum progesterone and estradiol levels and elevated the contents of IgM and IgG in the colostrum of sows, which could lead to an increase in the survival rate of piglets before weaning. In addition, malondialdehyde (MDA) levels were decreased and GSH-Px and T-AOC levels were increased when sows were treated with glucose polyphenols (Wang et al., 2019). Similarly, catechin, a plant polyphenol component, was also reported as an antioxidant plant extract (Uzun et al., 2010). Supplementation of the feed of pregnant sows with catechin (200 mg/kg) enhanced their antioxidant capacity and reproductive performance (Fan et al., 2015). Oregano essential oil, which is separated from oregano, is mainly composed of carvacrol and thymol (Sivropoulou et al., 1996). On the one hand,

Table 1
Effects of plants and plant extracts on antioxidant state, reproductive and lactation performance of sows.

Breed, feeding time, replicate number, condition of experiment and products	Antioxidant state of sows and piglets	Reproductive and lactation performance	Other effects	References
Breed: Landrace × Yorkshire Period: G90 to G114 Product: Pennisetum purpureum (5% to 10%) Replicate number: 150	Sow serum (G100): ↓ MDA; ↑ T-AOC, T-SOD, CAT, serum equol Sow serum (G114): ↑ T- AOC, T-SOD, CAT, serum equol; ↓ MDA		Sow serum (G100): ↑IgA, IgG, IgM Microbes: modify gut microbiota of sows on d 100 of gestation especially increase the abundance of Coriobacteriaceae	Huang et al. (2021)
Breed: (♀ Large White × Hybrid (Large White × Pietrain) × ♂ Talent, mainly Duroc) Period: G104 to L21 Product: hemp seed (G104 to 114, 2%), (L1 to L21, 5%) Replicate number: 5 sows/group, 8 piglets/group	Sow serum (farrowing): ↑TAC; ↓TBARS Sow serum (L7): ↓ROS, TBARS; ↑TAC Sow serum (L21): ↑CAT, SOD, GSH-Px Piglet serum (farrowing): ↑TAC, NO, CAT, SOD Piglet serum (L7): ↓TBARS; ↑TAC, SOD Piglet serum (L21): ↓TBARS, GSH-Px			Mihai et al. (2019)
Breed: Landrace backup sows Period: G100 to G114 Product: *Moringa oleifera* (8%) Replicate number: 15 sows/group	Sow serum (G60): ↑T-AOC, CAT Sow serum (G90): ↑T-AOC; ↓MDA Sow serum (L10): ↑T-AOC, GSH-Px; ↓MDA Piglet serum (weaning): ↑CAT	Reproduction: ↓stillborn, farrowing length; ↑colostrum protein		Sun et al. (2020)
Breed: Landrace × large white Period: G85 to L21 Product: enzymatically-treated *Artemisia annua* L. (EA) at 1.0 g/kg Replicate number: 45 sows/group Experimental condition: Sows in the control group were housed at control rooms with temperature 27.12 ± 0.18 °C and temperature-humidity index (THI) 70.90 ± 0.80. Sows in the heat stress + EA groups fed 1.0 g/kg EA, and reared at heat stress rooms (temperature: 30.11 ± 0.16 °C, THI: 72.70 ± 0.60).	Sow serum (G114): ↓MDA; ↑T-AOC, SOD Sow serum (L14): ↓MDA; ↑T-AOC Sow serum (L21): ↑T-AOC Piglet serum (L0): ↑T-AOC, T-SOD; ↓MDA Piglet serum (L21): ↑T-AOC	Reproduction: ↑litter weight, individual piglet weight Colostrum: ↑yield, T-AOC, T-SOD; ↓MDA Milk (L14): ↑T- AOC, SOD		Wenfei et al. (2020)
Breed: Large White Period: G1 to G114 Product: Konjac flour (2.2%) Replicate number: 25 sows/group	Sow serum (L1): ↓8-OHdG, ROS; ↑GSH-Px Sow serum (L3): ↓ROS		Sow (G109): ↓HOMA-IR; ↑HOMA-IS Sow (L3): ↓HOMA-IR, TNF-α; ↑HOMA-IS	Tan et al. (2016)
Breed: Landrace × Yorkshire sows Period: G108 to weaning Product: Silymarin (40 g/day) Replicate number: 55 sows/group	Sow serum (L18): ↑CAT Sow serum (L7): ↑GSH-Px	Reproduction: ↓farrowing duration; ↑ADFI during lactation, average piglet birth weight Colostrum: ↑yield, Lactose Milk (L18): ↑protein, urea	Sow serum (L7): ↓TNF-α, TBA; ↑PRL	Xiaojun et al. (2020)
Breed: Landrace × Yorkshire Period: G80 to G109 Product: Inulin (1.6%) Replicate number: 44 sows/group	Sow serum (farrowing): ↑T-AOC, T-SOD, GSH-Px	Reproduction: ↑sow ADFI, piglet BW at birth, piglet ADG, piglet BW at weaning, weaning survival rate; ↓farrowing duration, the rate of dead fetuses	Sow serum (farrowing): ↑TC, FFA	Li et al. (2020)
Breed: Yorkshire (average parity 4.4) Period: 20 d after breeding to weaning Product: Resveratrol (300 mg/kg) Replicate number: 20 sows/group	Finishing pigs (longissimus thoracis): ↑SOD, mRNA expression of SOD2; ↓MDA		Finishing pigs: ↑Backfat thickness	Meng et al. (2020)
Breed: Landrace × Yorkshire Period: during gestation and lactation Product: Phytogenic actives (1 g/kg) Replicate number: 27 sows/group	Sow serum (G35): ↑CAT, TBARS, NO, TAC; ↓SOD Piglet plasma (at birth): ↑CAT, TBARS Piglet plasma (postweaning d 7): ↑SOD, GSH-Px Piglet plasma (L20): ↑CAT, SOD	Colostrum: ↑protein Milk (L20): ↑Fat	Suckling pigs (on the 20th day) Expression of ↓CLDN4, ANPEP; ↑IL-10, IDO	David et al. (2020)
Breed: Yorkshire × Landrace Period: G107 to weaning Product: soybean isoflavone (SI) and astragalus polysaccharide mixture (APS) (200 mg/kg) (SI:APS = 1:5) Replicate number: 13 sows/group	Sow serum (L1): ↑IGF-1, PRL, GH, SOD, T-AOC, GSH-Px; ↓MDA Sow serum (L10): ↑IGF-1, PRL, GH, CAT, SOD, T-AOC, GSH-Px; ↓MDA	↑Sow ADFI, total lactation yield	Sow serum (L10): ↑IL-2, TNF-α, IgG, IgA Sow serum (L21): ↑IL-2, TNF-α, IgG, IgA	Hongzhi et al. (2021)

Table 1 (*continued*)

Breed, feeding time, replicate number, condition of experiment and products	Antioxidant state of sows and piglets	Reproductive and lactation performance	Other effects	References
Breed: Large White × Landrace Period: G80 to L21 Product: grape seed polyphenols (200 to 300 mg/kg) Replicate number: 16 sows/group	Sow serum (G110): ↑SOD, T-AOC, GSH-Px; ↓MDA	Reproduction: ↓fetal death rate; ↑farrowing survival, preweaning survivability	Sow serum (G110): ↑IgG, IgM, progesterone, estradiol	Wang et al. (2019)
Breed: Large White × Landrace Period: G85 to parturition Product: chitosan oligosaccharide (30 mg/kg) Replicate number: 8 sows/group	Sow serum (G110): ↑SOD, GSH-Px; ↓MDA Sow placenta: ↑expression of Cuznsod, CAT (1 to 2)		Sow placenta: expression of ↓IL-6, IL-8; ↑VEGF-A	Xie et al. (2016)
Breed: Landrace × Yorkshire Period: G1 to weaning Product: inulin (1.5%) Replicate number: 15 sows/group	Sow serum (G90): ↑SOD; ↓MDA Sow serum (L0): ↑GSH-Px; ↓MDA Sow serum (L21): ↑SOD, GSH-Px; ↓MDA Piglet plasma (at birth): ↑GSH-Px; ↓MDA Piglet plasma (at weaning): ↑SOD; ↓MDA	↓Within-litter birth weight coefficient of variation	↓Sow BW gain during gestation, lactation BW loss	Wang et al. (2016)
Breed: Landrace × Large White Period: mating to G40 Product: Catechins (200 mg/kg) Replicate number: 12 sows/group	Sow serum (Farrowing): ↑CAT, SOD; ↓MDA, H_2O_2	Reproduction: ↑the number of piglets born alive, piglet born healthy; ↓stillborn		Fan et al. (2015)
Breed: Yorkshire × Landrace Period: G85 to L18 Product: glycitein (15, 30, or 45 mg/kg; 45 mg/kg is the best) Replicate number: 56 sows/group Experimental condition: The experiment was conducted between July and October 2012, when monthly averages for maximum and minimum temperature (°C) were (33, 25), (33, 25), (31, 23), and (29, 21).	Sow serum (L1): ↑SOD, T-AOC; ↓MDA	Reproduction: ↑weaned BW per litter, ADG of piglets at lactation period Colostrum: ↑SOD, GSH-Px; ↓MDA Milk(D7): ↑SOD, GSH-Px, fat, protein; ↓MDA		Hu et al. (2015)
Breed: Large white Period: gestation and lactation Product: oregano essential oil (15 mg/kg) Replicate number: 30 sows/group	Sow serum: ↑GSH-Px on G60; ↓8-OHdG on G90 Sow serum (L1): ↑GSH-Px; ↓TBARS, 8-OHdG, ROS	Reproduction: ↑piglet mean BW at d 21, ↑piglet ADG during lactation	Sow faeces bacterial counts (G109): ↓*Escherichia coli*, *Enterococcus*; ↑*Lactobacillus*	Tan et al. (2015)

G = gestation day; L = lactation day; MDA = malondialdehyde; T-AOC = total antioxidant capacity; T-SOD = total superoxide dismutase; CAT = catalase; GSH-Px = glutathione peroxidase; 8-OHdG = 8-hydroxydeoxyguanosine; HOMA-IR = insulin resistance; HOMA-IS = insulin secretion index; TNF-α = tumor necrosis factor-α; TC = total cholesterol; FFA = free fatty acid; CLDN4 = recombinant claudin 4; ANPEP = alanyl aminopeptidase; IL-10 = interleukin 10; IDO = indoleamine 2,3-dioxygenase 1; IGF-1 = insulin like growth factor 1; PRL = prolactin; GH = growth hormone; IL-2 = interleukin 2; IgG = immunoglobulin G; IgA = immunoglobulin A; GSH-Px 3 = glutathione peroxidase 3; VEGF-A = vascular endothelial growth factor A; TBARS = thiobarbituric acid reactive substances; BW = body weight.

the structure of sow intestinal microbes was found to be altered with the oregano essential oil diet (OEO) (15 mg/kg), with an increased number of *Lactobacilli* in sow feces and decreased the numbers of *Enterococcus* and *Escherichia coli*. On the other hand, feeding OEO was shown to increase the insulin sensitivity of sows, thus increasing the feed intake of sows during lactation. Besides OEO diet significantly reduced sow serum thiobarbituric acid reactive substances and 8-hydroxydeoxyguanosine concentrations after delivery (Tan et al., 2015). Soybean isoflavone is a type of polyphenol that is also a natural active phytoestrogen (Moon et al., 2006). It has a similar structure to mammalian estrogen (Yuan et al., 2012), which promotes the testosterone and prolactin secretion of lactating sows and increases lactation yield. The addition of the mixture of soybean isoflavone and astragalus polysaccharide (200 mg/kg) to sow feed improved the serum prolactin, growth hormone and Insulin-like growth factor 1 contents of lactating sows, resulting in increased sow lactation. At the same time, this mixture increased the serum antioxidant levels of sows with increasing serum SOD and GSH-Px contents during lactation (Wu et al., 2021). Silymarin extracted from *Silybum marianum* plants contains various flavonoids (silybin as the main component), which have antioxidation, liver protection, blood lipid reduction, blood pressure reduction, anti-diabetes and anti-obesity functions (Surai, 2015). Silymarin supplementation (40 g/day) during late pregnancy and lactation in sows was found to increase circulating concentrations of serum prolactin, reduce oxidative stress (elevated serum composition of CAT and GSH-Px during lactation) and reduce insulin resistance and inflammatory responses (decreases in tumor necrosis factor-α and interleukin-1 β, as well as increase feed intake in sows, resulting in the improved growth performance of their offspring (Jiang et al., 2020). This might be because silymarin prevents the formation of free radicals by activating some nonenzymatic antioxidants and antioxidant enzymes through the Nrf2 and nuclear factor-kB (NF-kB) pathways (Surai, 2015).

In addition to directly using polyphenols, feeding sows with plant-enriched polyphenols also alleviates their oxidative stress. A recent study showed that the addition of *Moringa oleifera* (8%) improved the reproductive performance of sows and had positive impacts on the serum antioxidant indexes of sows and piglets, such as significant increases in serum T-AOC at 60 and 90 days in

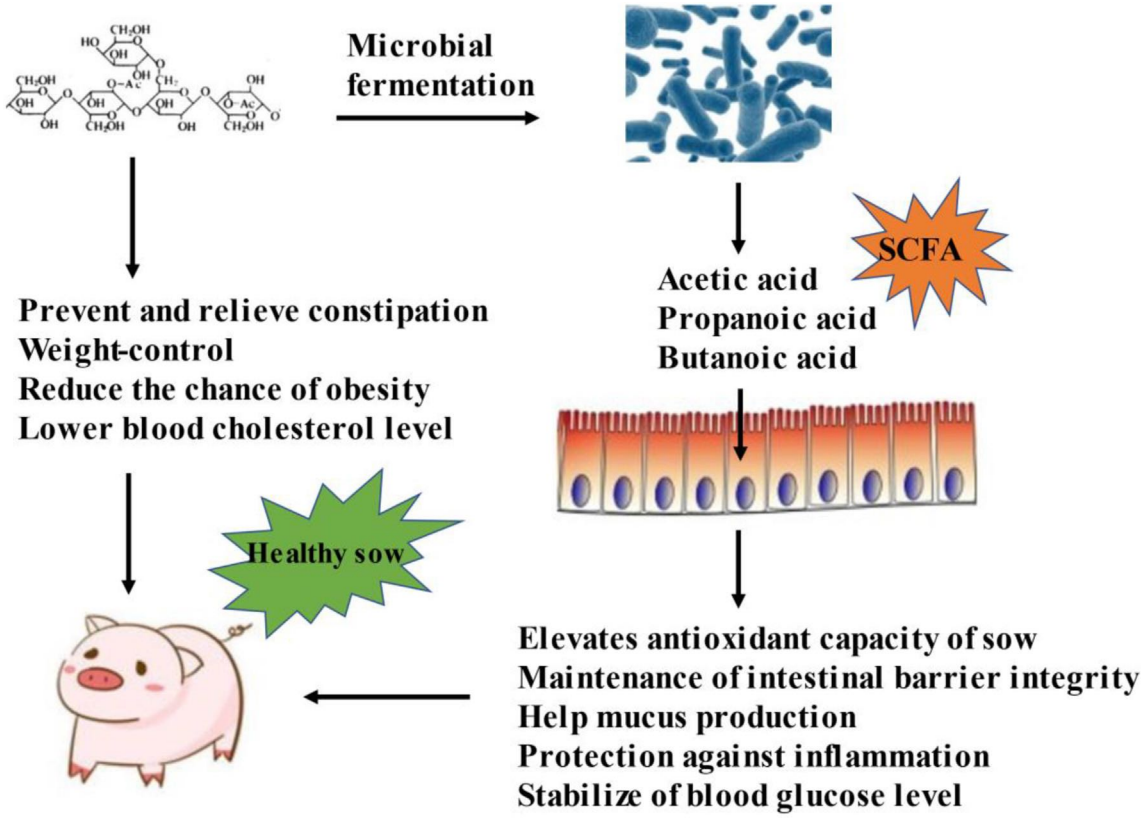

Fig. 4. The crude fiber is beneficial to health of sows. The fee rich in crude fiber can improve that body condition of the sow, improve the reproductive performance of the sow, and effectively solve the problem of constipation of pregnant sows and less lactation after delivery. Furthermore, short-chain fatty acids (SCFA) are the main metabolites produced by the microbiota in the large intestine through the anaerobic fermentation of dietary fiber and resistant starch, which help maintain that integrity of the intestinal barrier mucus production, prevent inflammation, etc.

pregnant sows. In addition, the addition of *M. oleifera* was shown to significantly increase the protein level in colostrum (Sun et al., 2020). *M. oleifera* contains phenol and other effective antioxidant substances, which could alleviate oxidative stress in animals (Osman et al., 2012). Therefore, we speculated that the antioxidant effect of *M. oleifera* is partially derived from its antioxidant substances. Similarly, dietary supplementation of enzymatically treated *Artemisia annua* L. at 1.0 g/kg was shown to result in significant elevations in the serum T-AOC levels of sows and piglets during pregnancy and lactation, and the serum MDA level was found to be decreased. In addition, enzymatically treated *Artemisia annua* L. was found to promote the intestinal barrier integrity of piglets (Zhang et al., 2020). The beneficial effect of *Artemisia annua* L supplementation might be due to its enrichment in sesquiterpenes (artemisinin), volatile oils, alkaloid phenols and flavonoids (Zhang et al., 2020), which can resist oxidation and enhance immunity (Ferreira et al., 2010). Hemp seeds are rich in polyphenols, polyunsaturated fatty acids, and other bioactive compounds (Callaway, 2004). Supplementing hemp seeds (2% to 5%) in the late pregnancy and lactation stages of sows was found to effectively reduce the levels of serum thiobarbituric acid reactive substances of sows and piglets and increase the levels of antioxidant enzymes such as total antioxidant capacity (TAC) and SOD (Palade et al., 2019).

In summary, plant-derived feed rich in polyphenols (such as *M. oleifera*, *Artemia annua* L. and hemp seeds) have been shown to be effective in improving the antioxidant capacity of sows. However, the direct addition of plants usually has low active ingredients. Direct addition of high-dose plant active ingredients (such as grape polyphenols, catechin, silymarin, oregano essential oil and soybean isoflavones) might produce better results. Compared with administration with an extract of polyphenols, feeding sows additives enriched in fiber or polysaccharides (such as *pennisetum*, konjac flour, chitosan oligosaccharide and inulin) might play a more prominent role in regulating the structure of the intestinal flora of sows, maintaining intestinal health, and reducing constipation.

3. Selenium and vitamin E

Selenium is a necessary micronutrient for animals, which is proposed to maintain growth, support muscle activity, promote reproductive organ development, and regulate the immune system (Schwarz et al., 2009; Tóth et al., 2018). Both inorganic selenium, such as selenite or selenate, and organic selenium, such as yeast selenium or DL-selenomethionine (Se-Met), are used as selenium supplements in animal husbandry. With the advancement of research, it has been gradually recognized that there are some limitations for the use of inorganic selenium. Firstly, compared with organic selenium, inorganic selenium is less efficiently retained, and most of which are excreted via the urine. Pharmacokinetic studies showed that the absorption rate of Se-Met (98%) was superior to that of sodium selenite (84%). In addition, excretion of Se-Met (15%) was lower than that of sodium selenite (35%) (Duntas et al., 2014). Blodgett et al. (1989) reported a sevenfold increase in sodium selenite levels in sow feed only resulted in modest increases in selenium concentrations in blood and colostrum. In addition, inorganic selenium easily interacts with other minerals to reduce their absorption efficiency (Surai, 2006).

The addition of organic selenium has beneficial effects on pregnant sows and their offspring. For example, addition of selenium (1 mg/kg organic Se) was found to increase the concentrations of serum GSH-Px, SOD, GSH and T-AOC in sows during lactation, reflecting that the sow is in a good antioxidant state at this stage. In addition, the antioxidant properties and nutrient content of milk were also shown to be improved (Zhang et al., 2020). Similarly, sow fed Se-enriched yeast (0.3 Se mg/kg diet) were shown to have improved antioxidant capacity in the serum and milk of sows compared with sows fed inorganic selenium (Chen et al., 2016). Collectively, there were two main benefits of organic selenium for pregnant sows (Table 2). First, organic selenium can build up a reserve of selenium in the form of Se-Met in tissues, mainly muscles, that can be used under stress conditions to improve antioxidant defense (Surai, 2006). Second, organic selenium in the form of Se-Met can efficiently transport selenium from sows to fetuses and newborn piglets via placenta, colostrum, and milk (Stewart et al., 2013).

It worth noting that even though Pig Nutrition Requirement (National Research Council, 2012) recommends 0.15 mg/kg selenium in feed for pregnant and lactating sows, the optimal level of selenium in sow feed is still controversial. Some scientists found selenium supplementation beyond the prescribed dose can interfere with insulin homeostasis and thus negatively affect pig performance (Pinto et al., 2012). However, high dose selenium supplementation (above the upper limit of 0.5 mg/kg of selenium according to Chinese standards) has also been reported to have beneficial effects on animals (Meyer et al., 2011; Chauhan et al., 2014; Stewart et al., 2013). We speculated that this might be due to the partial transfer of selenium to piglets through the placenta, which not only reduced the accumulation of selenium in sows, but also had a beneficial effect on piglets. The optimum level of selenium addition still needs to be further studied.

At the molecular level, according to previous studies, selenium is metabolized into various small molecular weight selenocompounds, such as hydrogen selenide and methylated selenium compounds, that affect cellular processes such as DNA repair and epigenetics (Grealish et al., 2000; Bera et al., 2013). Among them, hydrogen selenide ultimately synthesizes various selenoproteins through a series of complex biochemical reactions. The common selenoproteins are glutathione peroxidase and thioredoxin reductase, which are involved in alleviating oxidative damage and synthesizing thyroid hormone metabolism, respectively (Hoffmann, 2007) (Fig. 5). In view of these results, the addition of selenium to sow feed is beneficial and could improve the antioxidant capacity of the sow. The biological value of the organic form of selenium is much higher, and 1 mg/kg organic selenium is recommended for sows.

Table 2
Effects of selenium, vitamin E, vitamin C and yeast culture on antioxidant state, reproductive and lactation performance of sows.

Breed, feeding time, replicate number, condition of experiment and products	Antioxidant state of sows and piglets	Reproductive and lactation performance	Other effects	References
Period: Piglets: birth to 38 d of age Sow: (G84 to weaning) Product: Se-Met 0.26 and 0.43 mg Se/kg Or Na–Se 0.40 and 0.60 mg Se/kg Replicate number: 31 sows/group	Piglet plasma (Se-Met groups) ↑GSH-Px 3 at birth and d 5 after parturition	Reproduction: ↑piglet body weight (Se-Met groups at d 24)	Se-Met-groups at d 38 ↑Se-concentrations in plasma and had a dose–response effect	Falk et al. (2020)
Breed: Landrace × Yorkshire Period: G85 to L35 Product: yeast culture (YC) and organic selenium (Se), 10 g/kg YC + organic Se (1 mg/kg Se) Replicate number: 40 sows/group	Sow serum (L1): ↑SOD, T-AOC, GSH Sow serum (L25): ↑GSH-PX	Colostrum: ↑GSH-Px, T-SOD; ↓MDA Milk (d 4): ↑fat		Zhang et al. (2020)
Period: G90 to weaning Product: only vitamin E or vitamin E and Proviox polyphenols Gestation diets: Proviox polyphenols (50 mg/kg), vitamin E (50 mg/kg) Lactation diets: Proviox polyphenols (75 mg/kg), vitamin E (75 mg/kg) Replicate number: 13 sows/group	Sow serum (Farrowing): ↑GSH-Px, T-SOD, TAS Sow serum (Weaning): ↑GSH-Px, T-SOD, TAS Piglet serum (d 21 of age): ↑GSH-Px, T-SOD, TAS	Reproduction: ↑litter weight at birth and weaning	Piglet serum (d 21 of age): ↑α-Tocopherol, retinol	Lipiński et al. (2019)
Breed: Yorkshire mixed-parity sows Period: G85 to L21 Product: N-carbamylglutamate and vitamin C (both 0.05%) Replicate number: 13 sows/group	Sow serum (weaning): ↓MDA	Reproduction: ↑initial litter weight, average birth weight, weaning litter weight, average weaning weight	Sow: ↓Respiration; ↑teed intake Sow serum (weaning): ↑blood IgG	Feng et al. (2017)
Breed: Large White × Landrace Period: G107 to L21 Product: vitamin E (250 IU/kg) Replicate number: 24 sows/group	Piglet serum (L21): ↑T-AOC, CAT	Reproduction: ↑piglet weight at weaning, d 0 to21 ADG Colostrum: ↑tocopherol, fat, IgG, IgA Milk: ↑tocopherol, fat, IgG, IgA	Piglet serum (L21): ↑tocopherol, IgG, IgA Sow serum (At parturition): ↑tocopherol, IgG, IgA Sow serum (L21): ↑tocopherol, IgG, IgA	Lin et al. (2017)
Period: G21 to weaning Product: selenium (0.30 mg/kg) and vitamin E (90 IU/kg) Replicate number: 60 sows/group	Sow serum (60-d post-coitus): ↑T-AOC, SOD Sow serum (11-d postpartum): ↑T-AOC, SOD, GSH-Px, GSH; ↓MDA	Colostrum: ↑T-AOC, SOD, GSH-Px, GSH; ↓MDA Milk (d 11): ↑T-AOC, SOD, GSH-Px, GSH; ↓MDA		Chen et al. (2016)
Breed: Landrace × Yorkshire Period: G85 to L35 Product: yeast culture (10 g/kg) + organic Se (1 mg/kg). Replicate number: 40 sows/group	Sow serum (d 1 postpartum): ↑T- AOC, T-SOD, GSH Sow serum (d 25 postpartum): ↑GSH-Px	Colostrum: ↑T-SOD, GSH-Px; ↓MDA Milk (d 4): ↑fat		Zhang et al. (2020)

G = gestation day; L = lactation day; MDA = malondialdehyde; T- AOC = total antioxidant capacity; T-SOD = total superoxide dismutase; CAT = catalase; GSH-Px 3 = glutathione peroxidase 3; GSH = glutathione; IgA = immunoglobulin A.

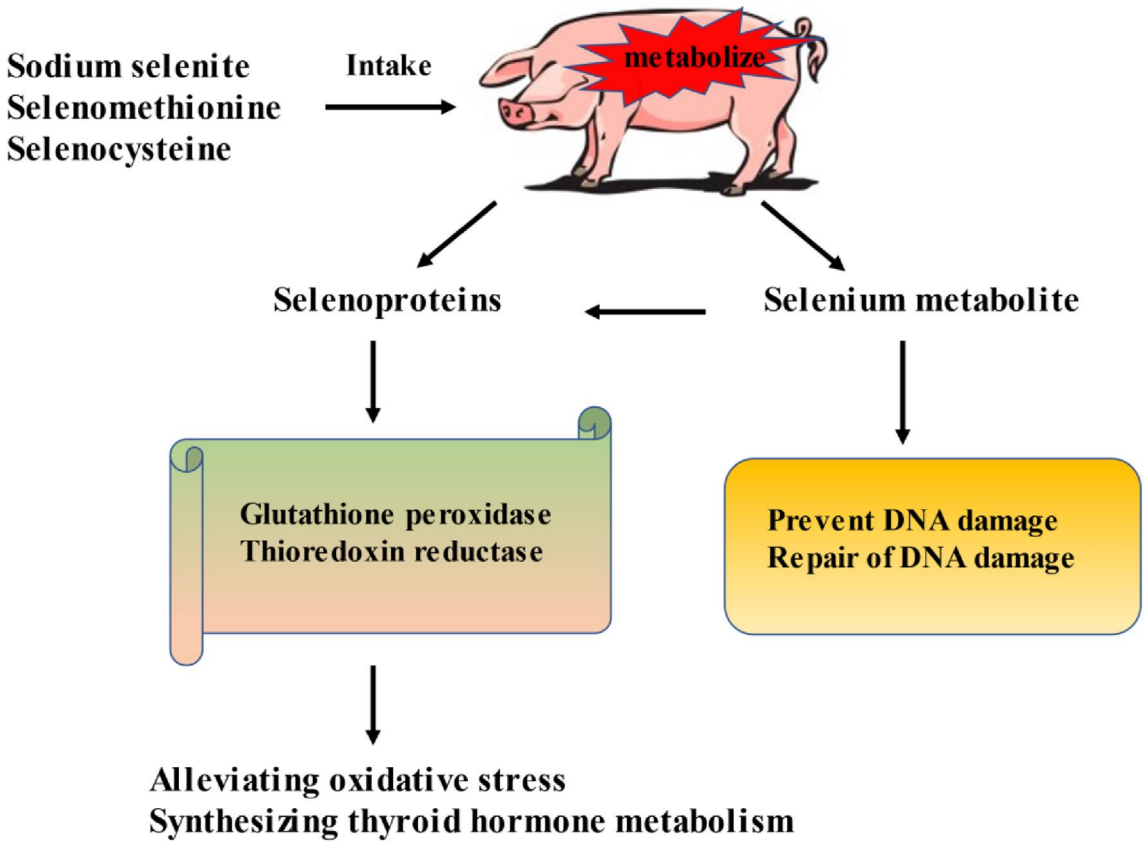

Fig. 5. Nutrition function of selenium. The selenium in the feed is absorbed by the sows and metabolized to produce small molecular weight seleno-compounds and some others participate in the synthesis of selenoprotein, thereby improving the antioxidant performance of the sows, regulating the energy metabolism of the sows, and reducing cell DNA damage.

Vitamin E is an effective antioxidant present in the cell membrane that maintains the normal function of cells by capturing free radicals and their oxidizing substances (Halliwell, 2012), counteracting lipid peroxidation in cells. From the 107 d of gestation to 21 d of lactation, adding 250 IU/kg vitamin E to sows' diets was found to significantly improve the weaning weight of piglets, stimulate the activity of antioxidant enzymes in the red blood cells of sows, and significantly affect the nutrient composition and immunoglobulin level of colostrum (Wang et al., 2017). Similarly, sows fed vitamin E and polyphenols were shown to have improved antioxidant status, higher GSH-Px and SOD activities, and upregulated serum retinol levels (Lipinski et al., 2019). It is worth noting that when sows were fed a low dose of vitamin E (90 or 30 IU/kg), their antioxidant status and reproductive performance did not appear to be improved (Chen et al., 2016). Therefore, this evidence indicates that adding sufficient vitamin E to sows' diets during late pregnancy can alleviate the oxidative stress state and that its effect is closely related to the dosage. The recommended amount of vitamin E for sows is (150 to 250 IU/kg). Similar to vitamin E, as an important antioxidant, vitamin C helps remove excess ROS from the body. The combined addition of *N*-carbamylglutamate and vitamin C reduces the levels of malondialdehyde and cortisol in sow serum and increases the level of IgG (Feng et al., 2017). In terms of reproduction, previous researchers have reported that the reproductive performance of sows fed vitamin E or vitamin C was significantly affected by the duration of supplementation. For example, short-term vitamin E or C supplementation to peripartum sows does not affect reproductive performance such as litter size, birth weight and number of stillbirths (Sosnowska et al., 2011; Mahan et al., 2000). In contrast, vitamin E and vitamin C supplementation significantly improved sow reproductive performance when tested during extended periods of gestation (Mavromatis et al., 1999; Lechowski et al., 2016). Mechanistically, vitamin C supplementation resulted in elevated plasma levels of β-estradiol that favored reproductive organ development (Coffey et al., 1993). In addition, decreased body fat consumption in lactating sows after vitamin C supplementation indicated that vitamin C helped maintain a stable level of body weight and backfat thickness, thereby reducing metabolic stress in lactating sows (Kolb et al., 2001; Zhao et al., 2002).

4. Microbial additives

Probiotics produce a favorable response in the host by improving the microbial balance in the intestine (Afrc, 1989). They promote or inhibit the growth of certain intestinal microorganisms and thus have a beneficial effect on the host (Table 3). Recent studies have reported that the perinatal intestinal flora of sows is associated with oxidative stress (Hayakawa et al., 2016). *Clostridium butyricum* regulates the balance of intestinal microecology, promoting the reproduction of *bifidobacteria* and inhibiting the proliferation of *E. coli*. It is worth noting that probiotics have been reported to improve the antioxidant capacity of sows and piglets (Xie et al., 2016).

Yeast and yeast extracts have been widely used in animal husbandry. Yeast extract contains nucleotides, vitamins, and β-glucans (Vieira et al., 2016), which might be beneficial to pig growth, metabolism, and health. Previous studies have shown that 5′-inosinate disodium enhances the flavor and palatability of feed. Yeast

Table 3
Effects of microbial additive on antioxidant state, reproductive and lactation performance of sows.

Breed, feeding time, replicate number, condition of experiment and products	Antioxidant state of sows and piglets	Reproductive and lactation performance	Other effects	References
Breed: Large White sows Period: G85 to L21 Product: dietary yeast extract (10 g/kg) Replicate number: 20 sows/group	Decrease oxidative stress of weaned piglets	Reproduction: ↑ADFI of sows on L1, the number of weaned piglets per litter	The concentration of 5′-monophosphate nucleotides and total nucleotides in milk increased linearly with the increase of yeast extract in diet	Cqta et al. (2021)
Breed: Landrace × Yorkshire Period: G90 to L21 Product: *Bacillus subtilis* PB6 (4×10^8 CFU/kg) Replicate number: 16 sows/group	Sow serum (parturition): ↑T-AOC; ↓MDA Sow serum (L21): ↑CAT Piglet serum: ↓Cortisol concentrations	Reproduction: During lactation: ↑born alive rate, litter weight gain, piglet survival rate Farrowing: ↓duration of farrowing, piglet birth interval Colostrum: ↑fat	Sow serum: ↓Endotoxin on L14 and L21	Zhang et al. (2020)
Breed: Large White × Landrace Period: G1 to Weaning Product: isomaltooligosaccharide and *Bacillus* Replicate number: 26 sows/group	Sow placental: ↓MDA, GSH-Px, CAT; ↑T-AOC	Reproduction: ↑average piglet BW, placental efficiency	Umbilical venous serum: ↑INS, IgG, GH; ↓IgM	Gu et al. (2019)
Breed: Landrace × York Period: G90 to L21 Product: *Clostridium butyricum* (0.2% to 0.4% and 0.4% is better) Replicate number: 45 sows/group	Sow serum (at parturition): ↓MDA Sow serum (L14): ↓MDA; ↑T-AOC, T-SOD Sow serum (L21): ↑CAT; ↓GSH-Px, Endotoxin Piglet serum (L14): ↓MDA, Endotoxin, Cortisol; ↑T- AOC Piglet serum (L21): ↑T- AOC; ↓Endotoxin, Cortisol	Reproduction: ↑litter weight gain; ↓duration of farrowing, estrous interval Colostrum: ↑protein, IgM Milk: ↑protein, IgM	*Clostridium butyricum* 0.2% increased the relative abundance of Bacteroides, but decreased the relative abundance of Proteus, Bifidobacterium, Actinomycetes, Acid Bacteria, Verrucous Microorganism and Mycelia/Bacteroides.	Cao et al. (2019)

G = gestation day; L = lactation day; MDA = malondialdehyde; T- AOC = total antioxidant capacity; T-SOD = total superoxide dismutase; CAT = catalase; GSH-Px = glutathione peroxidase; IgM = immunoglobulin M; IgG = immunoglobulin G; INS = serum insulin.

cell wall components (e.g., mannan and β-glucan) inhibit the growth of porcine intestinal pathogenic microorganisms to improve their growth performance by improving intestinal health (Shen et al., 2009). The sows provided with *yeast* derivatives (2 g/kg) is found to have more beneficial microbes (*Roseburia, Parprevotella, Eubacterium*), and less opportunistic pathogens (*Proteus*, *Vibrio desulfurization*, *E. coli*, and *Helicobacter*) in their feces (Shah et al., 2018). Adding 10 g/kg yeast extract to the sows' diets increases their feed intake during lactation and the number of weaned piglets per litter and reduces the oxidative stress of weaned piglets (Cqta et al., 2021). In addition, the serum T-AOC and GSH-Px of weaned piglets were higher than those of the control, while the blood thiobarbituric acid reactive substances level was decreased (Tan et al., 2021). Another study found that the addition of yeast extract (10 g/kg) and selenium to sows' diets increased the neonatal weight and the average daily gain during the lactation period of piglets. In addition, the contents of SOD and GSH-Px in colostrum were also increased, accompanied by a decrease in MDA (Zhang et al., 2020). Similarly, serum antioxidant enzyme activities, especially glutathione peroxidase, were significantly higher in weaned piglets when fed directly with 4% yeast-derived protein than in control-fed piglets (Liang et al., 2014).

B. subtilis can be used as growth promoter. It inhibits the reproduction of pathogens and promotes the degradation of nutrients in feed, thereby improving the utilization rate of feed. When *B. subtilis* (4×10^8 CFU/kg) was added to sows' daily rations, they exhibited better physical strength during delivery, and the birth interval of the piglets was shortened (Zhang et al., 2020). Concentrations of MDA, endotoxin in sow serum and cortisol in piglet serum were also found to be reduced (Zhang et al., 2020). Another study showed that the combination of *isomaltooligosaccharide* and *B. subtilis* increased the growth hormone (GH) concentration in umbilical vein serum, increased the concentration of placental T-AOC and reduced the concentration of MDA, thus improving the placental efficiency of sows (Gu et al., 2019). *B. subtilis* (2×10^9 CFU/kg) cultures have also been reported to be effective in attenuating zearalenone-induced oxidative stress (ameliorating serum SOD, and MDA levels) and organ apoptosis in pregnant sows (Zhou et al., 2020). Compared with other economic animals, the pigs are more vulnerable to the influence of deoxynivalenol (DON), and the pig fed with the DON (4 mg/kg) polluted feed showed the activity of inhibiting SOD, GSH-Px, GSH and T-AOC (Liao et al., 2020). However, these DON-induced oxidative stresses were partially counteracted by the administration of sow *B. subtilis* ASAG 216 (1×10^8, 0.1%), as evidenced by increased serum GSH-Px and SOD activities and decreased MDA and H_2O_2 contents (Ru et al., 2021). The addition of 0.2% *C. butyricum* to sow feed reduced the concentration of MDA in sow serum at delivery and increased the concentration of T-AOC, thereby reducing the time interval to litter. Furthermore, the lactose and protein contents in the milk were increased with the supplementation of 0.2% *C. butyricum* (Cao et al., 2019).

In summary, probiotic supplementation during pregnancy could regulate the intestinal microbial structure of sows, improve intestinal health, and enhance the antioxidant capacity of sows.

5. Other nutritional strategies

In addition to the nutrients mentioned above, the effects of other additives on the antioxidant performance of sows are summarized in Table 4. Glutathione, a ubiquitous thiol-containing tripeptide made from the amino acids glycine, cysteine, and glutamine acid, plays a pivotal role in reducing oxidative stress (Jefferies et al., 2003). Cysteamine (CS) is a precursor of GSH, which is an important endogenous antioxidant (Fig. 6). Mechanistically, cysteamine promotes the transport of cysteine into cells for GSH synthesis, thereby achieving cell redox homeostasis (Besouw et al., 2013). Dietary CS (100 to 500 mg/kg) supplementation could partially improve the antioxidant status of sows and their offspring,

Table 4
Effects of other nutritional strategies on antioxidant state, reproductive and lactation performance of sows.

Breed, feeding time, replicate number, and products	Antioxidant state of sows and piglets	Reproductive and lactation performance	Other effects	References
Breed: Landrace × York sow Period: G85 to L21 Product: cysteamine (100 to 500 mg/kg) of CS, but 100 mg/kg is the best. Replicate number: 21 sows/group	Sow serum (L0) ↑GSH Sow Placenta (parturition): ↑T-AOC Piglet serum (at birth): ↑GSH	Colostrum: ↑GSH Reproduction: ↓Stillbirth rates, Invalid piglet rates; ↑Placental efficiency		Huang et al. (2021)
Breed: Landrace × Large White Period: G85 to delivery Product: n-acetyl-cysteine (500 mg/kg) Replicate number: 9 sows/group	Sow serum (parturition): ↑GSH-Px, SOD, T-AOC; ↓H_2O_2, MDA Sow Placenta (parturition): ↑GSH-Px, NO; ↓SOD, MDA		Sow serum (at parturition): ↓IL-1β, IL-18 Sow Placenta (at parturition): ↑IGF-1, IGF-2, E-cadherin	Luo et al. (2019)
Breed: Landrace × Yorkshire Period: G90 to parturition Product: β-carotene (30 or 90 mg/kg but 90 mg/kg is better) Replicate number: 16 sows/group	Sow serum (parturition): ↑GSH-Px, NO		β-carotene may increase NO production by up-regulating the relative abundance of Corynebacterium	Xupeng et al. (2020)
Breed: Landrace × Large white Period: G1 to L21 Product: L-carnitine at 100 mg/kg from G1 to G90, and 200 mg/kg from G91 to L21. Replicate number: 30 sows/group	Sow serum (G110): ↑GSH-Px, GSH; ↓MDA Sow serum (L1): ↑T-SOD, GSH-Px, GSH; ↓MDA Sow serum (L21): ↑T-SOD, GSH-Px, GSH; ↓MDA Piglet serum (at birth): ↑T-SOD, GSH-Px, GSH; ↓MDA Piglet serum (Weaning): ↑T-SOD, GSH-Px, GSH; ↓MDA Piglet Liver (Weaning): ↑T-SOD, GSH-Px, GSH; ↓MDA	Reproduction: ↑Initial litter weight, Average birth weight, Weaning litter weight, Average weaning weight		Wei et al. (2019)
Breed: Landrace × Yorkshire Period: G1 to weaning Product: methyl donor (3 g/kg) Replicate number: 14 sows/group	Umbilical cord blood (at birth): ↑SOD, CAT, GSH-Px Piglet serum (at birth): ↑SOD, GSH-Px, T-AOC; ↓MDA Piglet Liver (at birth): ↑CAT, GSH-Px, T-AOC		In chorioallantois (at birth): ↑CAT, GSH-Px, SOD, ASA, NOS	Mou et al. (2018)

G = gestation day; L = lactation day; MDA = malondialdehyde; T- AOC = total antioxidant capacity; T-SOD = total Superoxide dismutase; CAT = catalase; GSH = glutathione; GSH-Px = glutathione peroxidase; IL-1 β = interleukin-1 β; IL-2 = interleukin 2; IGF-1 = insulin like growth factor 1; IGF-2 = insulin like growth factor 2.

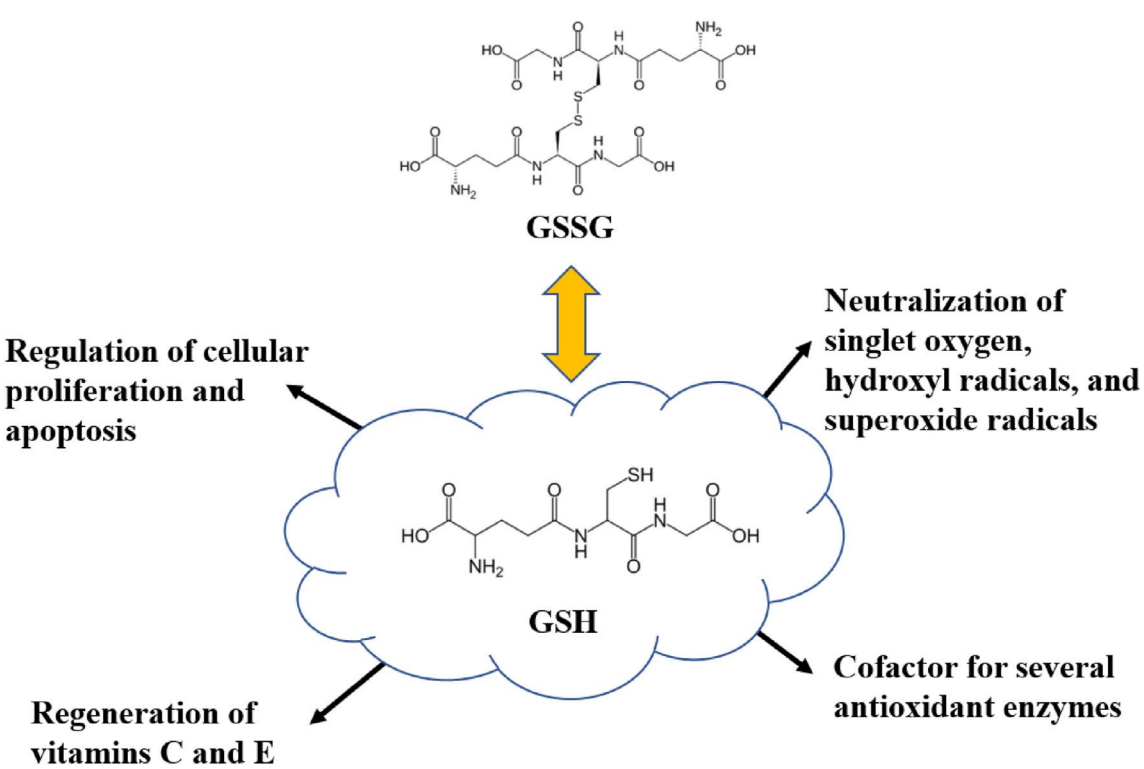

Fig. 6. Glutathione and its role in cellular functions. GSH = glutathione; GSSG = glutathione oxidized.

increase the mean birth weight of piglets (from 1.4 to 1.6 kg), and reduce stillbirth (from 12% to 6%) (Huang et al., 2021). It is also possible to increase GSH levels in the sow's placenta and increase the placental vascular density, which is beneficial to the growth and survival of the embryo (Huang et al., 2021). The potential mechanisms by which CS protects cells against oxidative stress are listed as follows. First, cysteamine undergoes a sulfhydryl–dithio exchange reaction to eliminate oxidized substances in the cells (Ozaki et al., 2007). Second, cysteamine inhibits the nucleotide-containing pyridine domain in the γ-ribosome to improve the redox state of the maternal placenta (Luo et al., 2019). It is worth noting that high doses of CS could attenuate glutathione peroxidase activity. *N*-acetylcysteine (NAC) is a similar product to CS. The addition of NAC (500 mg/kg) to the sow diet was also found to significantly reduce serum and placental inflammatory cytokine levels and increase placental NO production and the gene expression levels of vascular endothelial growth factor and hypoxia inducible factor-1α by inhibiting the NOD-like receptor protein 3 inflammasome and to increase the numbers of lactic acid bacteria and *bifidobacteria* in the sow hindgut (Luo et al., 2019).

Beta-Carotene, a common plant pigment, is generally considered to be the precursor of vitamin A. One study showed that β-carotene (30 or 90 mg/kg) significantly increased serum GSH-Px in sows. In addition, β-carotene may increase NO production by upregulating the relative abundance of Corynebacterium (Yuan et al., 2020). According to previous studies, an increase in homocysteine levels leaded to an increase in ROS levels (Agarwal et al., 2005). Prenatal supplementation with maternal methyl donors (3 g/kg) in sow feed significantly reduced serum homocysteine concentrations in sows at d 35 and 110 of pregnancy. The supplementation increased the activity of antioxidant enzymes such as SOD, CAT and GSH-Px in chorioallantoic acid, piglet plasma, and liver, thereby enhancing maternal, placental, and offspring antioxidant capacity (Mou et al., 2018). The main function of L-carnitine is to help long-chain fatty acids cross the mitochondrial inner membrane for β-oxidative degradation. The results determined in vitro indicated that L-carnitine was an effective antioxidant (Wei et al., 2019). L-Carnitine supplementation (100 to 200 mg/kg) in sow diets increased the survival number and weaning body weight of weaned piglets, decreased plasma MDA levels in sows, and alleviated DDGS-induced oxidative stress in sows (Wei et al., 2019).

6. Conclusions

Further in-depth study on the mechanism of oxidative stress in sows will help us develop more effective additives to reduce losses due to oxidative stress. Based on the current research, we have reached the following conclusions: plant additives rich in polyphenols can effectively alleviate the oxidative stress of sows during the late pregnancy and lactation stages. These additives are probably the most effective antioxidant products available because they are more effective in improving oxidative stress indicators in sows (Table 1). And products with high crude fiber and additives rich in soluble sugar can effectively regulate the structure of the gut microbiota of sows and affect microbial diversity to maintain the intestinal health of sows. For vitamin products (such as vitamin E and vitamin C), a high concentration (>100 IU/kg) is required to exert an efficient antioxidant effect. Microbial preparations tend to promote the growth of beneficial flora. The addition of *B. subtilis* and *C. butyricum* has been proven to be effective. It has also been proven to be effective to add some substances involved in body metabolism to the sows' feed, such as L-carnitine and methyl donors. In addition, the types of additives, the addition stage, and the dosage affect the expected antioxidant function. Therefore, reasonably considering the use of products with different combinations and repeating experiments in production practice are required to identify the most suitable product for specific livestock farms.

7. Outlook

At present, most studies only focus on evaluating the effects of individual additives, and the effects of products with different active components remain to be determined in future studies. In addition, the specific scavenging mechanism of active components in various additives on free radicals is still unclear and remains to be studied. Furthermore, it worth noting that oxidative stress could reduce the lactational feed intake. However, it is still unclear whether nutritional strategies such as increasing nutrient density, and changing the Lys-to-energy ratio could eliminate the negative effects from oxidative stress. As described in this review, MDA is widely used as a marker of lipid oxidation. However, polyphenols and vitamin products could directly react with MDA, which might affect the oxidative stress status of sows. Thus, a better biomarker of oxidative stress might need to be identified in the future. It is interesting to note that most of the additives stimulate the synthesis of antioxidant enzymes in sows, suggesting that their pathway of activating antioxidant enzymes might be similar to that of ROS, which is also worth studying. Currently, the alleviation of sow oxidative stress by nutritional means is mainly focused on improving the concentration of antioxidant substances or the activity of antioxidant enzymes in the sow. However, there are few studies on how to reduce ROS production from the source and repair injuries caused by oxidative stress. Finally, a growing number of studies have shown that appropriate concentrations of ROS play an important role in maintaining animal health. The priority is now to determine the ranges in which ROS levels in sows in the late pregnancy and lactation stages are most beneficial for their productive performance.

Conflict of interest

We declare that we have no financial and personal relationships with other people or organizations that can inappropriately influence our work, there is no professional or other personal interest of any nature or kind in any product, service and/or company that could be construed as influencing the content of this paper.

Acknowledgment

This study was financially supported by National Natural Science Foundation of the P. R. of China (No. 31872364 and No. 31802067) and Guangdong Basic and Applied Basic Research Foundation (No. 2021A1515010440).

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Cite this: *Food Funct.*, 2022, **13**, 6282

Glyceryl butyrate attenuates enterotoxigenic *Escherichia coli*-induced intestinal inflammation in piglets by inhibiting the NF-κB/MAPK pathways and modulating the gut microbiota†

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Received 20th April 2022,
Accepted 17th May 2022

DOI: 10.1039/d2fo01056a

rsc.li/food-function

The aims of this study were to evaluate whether a diet supplemented with glyceryl butyrate could attenuate the immune-inflammatory response in piglets challenged with enterotoxigenic *Escherichia coli* (ETEC), and to explore the mechanisms of its regulation. Eighteen weaning piglets were assigned to three diets: basal diet (CON), antibiotics diet (ATB), and 0.5% glyceryl butyrate diet (GB group). Significantly lower concentrations of IL-1β, IL-6 and TNF-α in the jejunum and IL-6 in the ileum were observed in the GB group than that in the CON group ($P < 0.05$). Moreover, a decreasing trend of IL-1β ($P = 0.075$) and TNF-α ($P = 0.070$) was observed in the ileum in the GB group. Correspondingly, the GB group had significantly increased mRNA expression of porcine beta defensins (pBDs) in the jejunum (pBD1, pBD2, pBD114 and pBD129) and ileum (pBD2, pBD3, pBD114 and pBD129) ($P < 0.05$), and protein abundance of Claudin 1, Occludin, and ZO-1 in the jejunum and ileum ($P < 0.05$). Further research results showed that the improvement of beta defensins and tight junctions in the GB group was related to the decreased phosphorylation of the NFκB/MAPK pathway. In addition, the results of 16S rDNA sequencing showed that glycerol butyrate supplementation altered the ileal microbiota composition of piglets, increasing the relative abundance of *Lactobacillus reuteri*, *Lactobacillus salivarius*, and *Lactobacillus agrilis*. In summary, glyceryl butyrate attenuated the immune-inflammatory response in piglets challenged with ETEC by inhibiting the NF-κB/MAPK pathways and modulating the gut microbiota, and thus improved piglet intestinal health.

1 Introduction

Early weaned piglets lack effective resistance to diseases and stress due to incomplete intestinal development and low immunity.[1,2] Thus, a dysregulated or perturbed state of the gut microbiota increases the susceptibility of piglets to enteric pathogens.[3] Enterotoxigenic *Escherichia coli* (ETEC) is a major cause of postweaning diarrhoea, leading to high mortality and significant economic losses.[4] In the past, antibiotics have been widely used in the pig industry because of their low price and good disease prevention effect.[5] However, the misuse of antibiotics in animal production, residues in foods, and the emergence of ultra-resistant bacteria have raised awareness of the distinct possibility that their overuse will eventually threaten human safety.[6] Therefore, alternatives to antibiotics that are friendly to humans, animals, and the environment are urgently needed.

Based on methods such as high-throughput sequencing and metabolomics, intestinal diseases have been linked to aberrant gut microbial community composition.[7] Therefore, research is increasingly focused on exploring effective approaches for microbial therapy (*e.g.*, probiotic and prebiotic interventions) and even microbial metabolite therapy (*e.g.*, short-chain fatty acids, antimicrobial peptides).[8,9] Short-chain fatty acids (SCFAs), the most abundant metabolites produced by microorganisms in the gut, including formic acid, acetic acid, propionic acid, and butyric acid, have received considerable attention for their effective antimicrobial properties.

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† Electronic supplementary information (ESI) available: Table S1: compositions of the Experimental Diet and Nutrient Profile (As-Fed Basis); Table S2: antibody information used in this experiment. The raw sequence data reported in this paper have been deposited in the Genome Sequence Archive (Genomics, Proteomics & Bioinformatics 2021) in National Genomics Data Center, China National Center for Bioinformation (GSA: CRA006142) that are publicly accessible at **https://bigd.big.ac.cn/gsa/browse/CRA006142**. See DOI: **https://doi.org/10.1039/d2fo01056a**

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Importantly, SCFAs are beneficial to both humans and animals. In prior work, to better understand gut health of piglets challenged with ETEC, we tested the effects of adding dietary formic acid, a mixture of formic acid and propionic acid, formic acid with propionic acid supplemented with either capric acid, or monolaurin, and a mixture of formic acid and monolaurate.[10,11] In these studies, the additives listed above were observed to improve local intestinal immunity and enhance barrier function.[10,11]

Butyric acid, one of the major SCFAs, not only acts as a substrate for energy production but also regulates cell proliferation and apoptosis, thereby promoting the functional maturation of intestinal epithelial cells.[12,13] Moreover, butyrate helps to improve the composition of intestinal microflora, reduce the expression of proinflammatory cytokines, and promote gut health and development.[14–16] In feed production, butyric acid is usually used as its sodium salt rather than the free acid. However, in practical applications, sodium butyrate faces difficulties in use due to its highly unpleasant odour and powdery, hygroscopic character, which is easily absorbed in the upper gastrointestinal tract. In contrast, glyceryl butyrate is a triglyceride that is broken down by lipases to release copious amounts of butyrate into the gut lumen. For the present study, we hypothesized that glyceryl butyrate supplementation would improve gut barrier function by modulating the gut microbial community structure and inhibiting gut inflammation. Therefore, this study aimed to investigate the effect of dietary glyceryl butyrate on the gut microbiota and barrier function in weaned piglets and to explore whether this treatment has potential value as an alternative to antibiotics in maintaining good gut health in weaned piglets.

2 Materials and methods

2.1 Animals and experimental design

All the procedures performed in animal use and care during this study were approved by the South China Agricultural University Animal Care and Use Committee (no. 20110107-1, Guangzhou, China). Eighteen individually housed male piglets (25 ± 1 d, Duroc × Landrace × Yorkshire) with an average weight of 7.5 ± 0.5 kg, were divided into three groups and each group was fed either: a basal diet (CON group, Table S1†), a diet supplemented with antibiotics (50 mg kg^{-1} quinocetone, 75 mg kg^{-1} chlortetracycline, and 50 mg kg^{-1} kitasamycin; ATB group), or a diet supplemented with glyceryl butyrate (GB group, 0.5% glyceryl butyrate). Glyceryl butyrate used in this study was a mixture of glycerol monobutyrate (main ingredient, more than 50%), glyceryl dibutyrate and glycerin (Health 104 L, SILO SpA, Italy). The nutritional level of the basal diet meets the nutritional requirements of 6–10 kg pigs in the National Research Council (NRC) (2012). During the initial adaptation for the experiment, all the piglets were provided *ad libitum* access to basal diet feed and fresh water and housed in a room at 30 ± 2 °C.

After a 3-day adaptation period, piglets were fed one of the three experimental diets. ETEC (CVCC225, serotype O149: K91: K88ac; toxins LT and ST) in nutrient broth was purchased from the Chinese Veterinary Collection Center. The preparation of the nutrient broth and the piglet feeding procedure were based on previous studies.[10,11] On Day 8 of the experiment, all piglets were gavaged with 10 mL of nutrient broth (5×10^9 CFU per mL of ETEC *E. coli*). The rectal temperature of all piglets was measured with a thermometer (Omron MC-347) at 0, 9, 24 and 48 hours after challenge, and the fecal morphology of the piglets was also observed to determine whether the intestinal inflammation occurred. Forty-eight hours after gavage with ETEC, all piglets were slaughtered by humane euthanasia with sodium pentobarbital. Jejunal and ileal mucosa and ileal content samples were collected and stored at −80 °C.

2.2 ELISA analysis

Concentrations of interleukin (IL)-1β, IL-6 and tumour necrosis factor (TNF)-α in the jejunum and ileum mucosa were analysed by a Porcine IL-1β ELISA Kit (ml002302-C), Porcine IL-6 ELISA Kit (ml002311-C) and Porcine TNF-α ELISA Kit (ml002306-C), respectively, purchased from Mlbio company (Shanghai, China). The chopped intestinal mucosa and the corresponding volume of PBS (1 : 9 weight-to-volume ratio) were added to a homogenisation tube and homogenised at high speed at low temperature to break the cells. Then, the instructions of the ELISA kit were followed for detection. Optical density was analysed by a Thermo Scientific Multiskan GO Microplate Spectrophotometer (Thermo Fisher Scientific, Rockford, IL, USA) at 450 nm. The concentration of the target samples was calculated according to the regression equation established by the standard curve and the absorbance value of the sample.

2.3 RNA extraction and RT-qPCR analysis

Total RNA from the mucosa of the jejunum and ileum was extracted using a Tissue RNA Purification Kit (EZB-RN001, EZBioscience, USA). RNA concentration and quality were detected by a NanoDrop ND-1000 spectrophotometer. Subsequently, first-strand cDNA was synthesized using a Colour Reverse Transcription Kit (with gDNA Remover) (A0010CGQ, EZBioscience, USA). Real-time quantitative PCR (RT-qPCR) analysis was performed using a 7500 Fast Real-time PCR system (Applied Biosystems and Life Technology, Inc.) in a total 20 μL volume composed of 10 μL of 2× Colour SYBR Green qPCR Master Mix (A0012-R2, EZBioscience, USA), 0.4 μL of each PCR primer (10 μM), 2 μL of cDNA, and 7.2 μL of dd water. The RT-qPCR procedures were set for denaturation at 95 °C for 5 min, followed by 40 cycles of 95 °C for 10 s, 60 °C for 30 s, and 72 °C for 30 s. Relative gene expression was calculated using the relative quantification method ($2^{-\Delta\Delta Ct}$). The primers used in this study are shown in Table 1.

2.4 Immunoblotting analysis

Total protein from the mucosa of the jejunum and ileum was extracted using RIPA lysis buffer (Beyotime, Shanghai, China), which contained 1% protease inhibitors PMSF and 1% phosphatase inhibitors. The concentration of total protein was measured using a BCA protein assay kit (Beyotime, Shanghai,

Table 1 RT-qPCR primer sequences

Genes	Accession	Direction	Sequences (5′–3′)	Size
pBD-1	NM_213838.1	Forward	ACCGCCTCCTCCTTGTATTCCTC	147
		Reverse	AGGTGCCGATCTGTTTCATCTTTGG	
pBD-2	NM_214442.2	Forward	ATTAACCTGCTTACGGGTCTTGGC	122
		Reverse	CCACTGTAACAGGTCCCTTCAATCC	
pBD-3	XM_021074698.1	Forward	TCTTCTTGTTCCTGATGCCTCTTCC	146
		Reverse	GCCACTCACAGAACAGCTACCTATC	
pBD-114	NM_001129973.1	Forward	GTACCTTGGTGGATCCTGAACGATG	127
		Reverse	AACGCCCTCTGAATGCAGCATATC	
pBD-129	NM_001129975.1	Forward	AATGATCCGCACACTTGAAGAGGTC	137
		Reverse	TGGTGTTGATGCTGGCGAAAGG	
β-Actin	XM_021086047.1	Forward	GATCTGGCACCACACCTTCTACAAC	107
		Reverse	TCATCTTCTCACGGTTGGCTTTGG	

pBD-1, porcine β-defensin 1; pBD-2, porcine β-defensin 2; pBD-3, porcine β-defensin 3; pBD-114, porcine β-defensin 114; pBD-129, porcine β-defensin 129.

China). Then, 25 μg of total protein from each sample was resolved by electrophoresis on a 10% polyacrylamide gel, transferred to polyvinylidene difluoride membranes (Millipore, Bedford, MA), and blocked with 5% BLOT-QuickBlocker (Sangon, Shanghai, China) for 40 min at room temperature. After rinsing with TBST buffer five times, the membranes were incubated with primary antibody at 4 °C overnight. Antibodies against beta actin, Claudin 1, Occludin, Zonula Occludens (ZO)-1, NFκB, p-NFκB, JNK, p-JNK, Erk, p-Erk, P38 and p-P38 were purchased from commercial companies (Table S2†). Subsequently, the membrane was washed 5 times with TBST buffer for 5 min each and incubated with corresponding secondary antibodies at room temperature for 1.5 h. After five rinses, band signals were detected using the ECL Plus chemiluminescence detection kit (Applygen Technologies Inc., Beijing, China) and a Chemiluminescence Image Analysis System (Tanon, Shanghai, China). Finally, the bands were analysed with Image Processing Software (Image-Pro Plus 6.0) (Rockville, MD), and the relative protein abundance was normalized to total β-actin or total target protein levels.

2.5 Bacterial data analysis

The piglet ileum microbiome was analyzed by high throughput 16S rDNA sequencing technology and was performed by Gene Denovo (Guangzhou, China). After genomic DNA was extracted from the ileum content samples, the V3–V4 region of 16S rDNA was amplified with barcode-specific primers. The primer sequences were 341F: CCTACGGGNGGCWGCAG and 806R: GGACTACHVGGGTATCTAAT. The purified amplification products (*i.e.*, amplicons) were connected to sequencing adapters to construct a sequencing library, which was sequenced on a NovaSeq 6000 (PE250, Illumina Inc., CA, United States). Finally, the 16S rDNA sequencing data were analyzed with the Omicsmart (Gene Denovo, Guangzhou, China) online tools.

2.6 Statistical analysis

Data analysis was performed using one-way ANOVA according to the general linear model (GLM) procedure of IBM SPSS Statistics software (Ver. 22.0, IBM, Armonk, NY, United States). Statistical significance was considered if $P \leq 0.05$ and as tendency when $0.05 < P < 0.10$. Data are presented as the means ± standard errors of the means.

3 Results

3.1 Secretion of proinflammatory factors in the jejunum and ileum

In the piglet jejunum (Fig. 1A), the concentration of IL-1β, IL-6 and TNF-α in the ATB group and GB group was significantly lower than that in the CON group ($P < 0.05$). In the ileum (Fig. 1B), compared with the CON group, the concentration of IL-1β, IL-6 and TNF-α in the ATB group was significantly lower in the ileum ($P < 0.05$). Additionally, the concentration of IL-1β ($P = 0.075$) and TNF-α ($P = 0.070$) in the GB group showed a decreasing trend compared with that in the CON group; however, there was no significant difference compared with the ATB group. The IL-6 concentration in ileal mucosa in the GB group was lower than that in the CON group ($P < 0.05$); however, it was higher than that in the ATB group ($P < 0.05$).

3.2 Protein abundance of tight junctions in the jejunum and ileum

In the jejunum (Fig. 2A and C), the relative protein abundance of Claudin 1 and ZO-1 in the ATB and GB groups was significantly higher than that in the CON group. And, the protein abundance of Claudin 1 and ZO-1 showed no significant difference between the ATB group and GB group. In the ileum (Fig. 2B and D), the relative protein abundance of Claudin 1, Occludin and ZO-1 in the ATB group was significantly higher than that in the CON group ($P < 0.05$). A higher relative protein abundance of Claudin 1 ($P = 0.095$), Occludin ($P = 0.090$) and ZO-1 ($P < 0.05$) in the GB group than that in the CON group. Furthermore, the relative protein abundance of Claudin 1 ($P < 0.05$) and Occludin ($P = 0.081$) in the GB group was lower than that in the ATB group. However, the relative ZO-1 abundance

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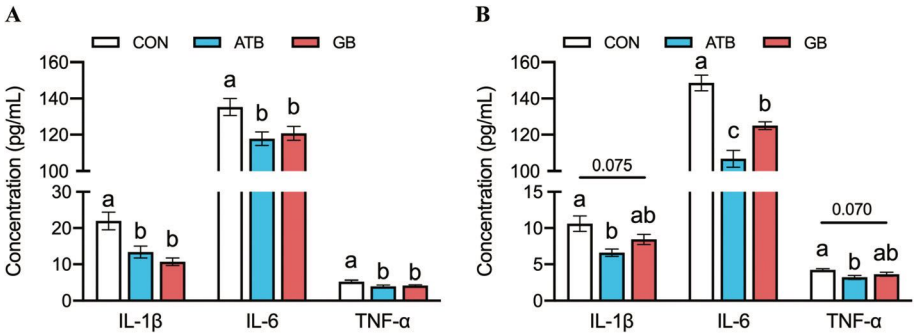

Fig. 1 Concentrations of proinflammatory factors in the piglet jejunum (A) and ileum (B). Different letters (a–c) indicate significant differences among the groups ($P < 0.05$).

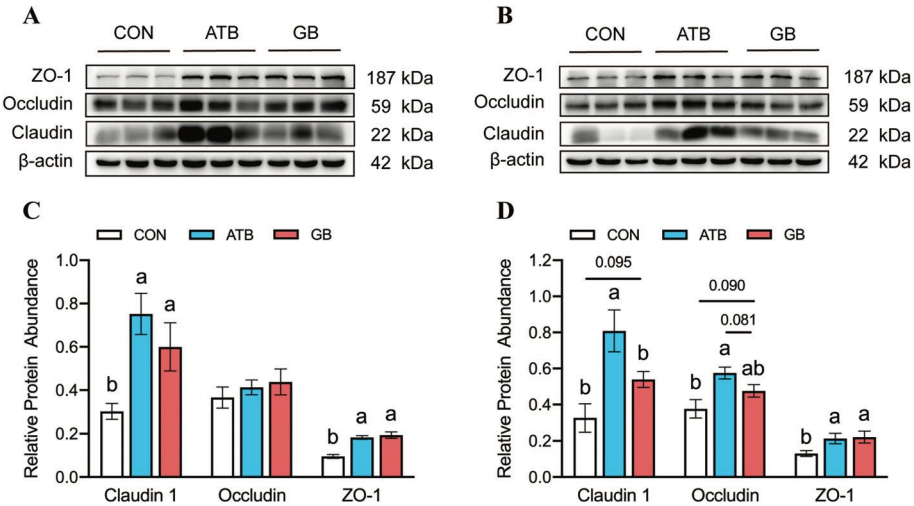

Fig. 2 The protein abundance of tight junctions in the piglet jejunum (A and C) and ileum (B and D). Different letters (a–c) indicate significant differences among the groups ($P < 0.05$).

in the GB group showed no significant difference compared with the ATB group.

3.3 mRNA expression of beta defensins in the jejunum and ileum

The mRNA expression of beta defensins in the piglet jejunum and ileum was showed in Fig. 3A and B. In the jejunum (Fig. 3A), a higher mRNA expression of pBD1, pBD2 and pBD129 in the ATB and GB groups than that in the CON group ($P < 0.05$). And, the mRNA expression of pBD1, pBD2 and pBD129 showed no significant difference between the ATB group and GB group. Furthermore, the pBD3 mRNA expression in the GB group was significantly lower than that in the ATB group ($P < 0.05$). In the ileum (Fig. 3B), a significantly higher

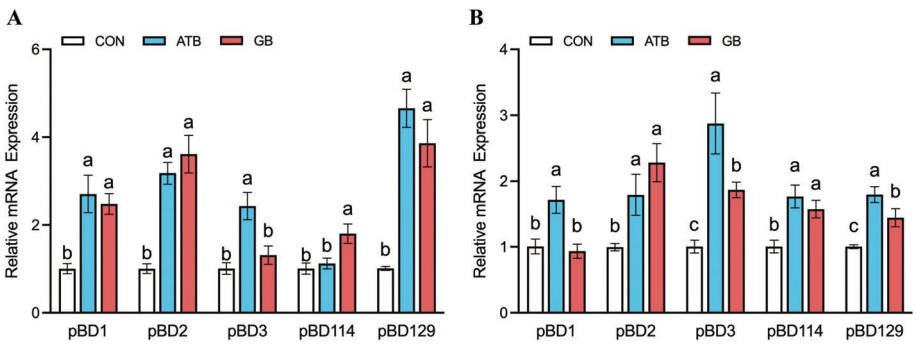

Fig. 3 mRNA expression of beta denfensins in the jejunum (A) and ileum (B). Different letters (a–c) indicate significant differences among the groups ($P < 0.05$).

mRNA expression of pBD1, pBD2, pBD3, pBD114 and pBD129 in the ATB group than that in the CON group ($P < 0.05$). And, the mRNA expression of pBD2, pBD3, pBD114 and pBD129 in the GB group were significantly higher than that in the CON group ($P < 0.05$). Moreover, the mRNA expression of pBD2 and pBD114 showed no significant difference between the ATB group and GB group. However, the pBD3 and pDB129 mRNA expressions in the GB group were significantly lower than that in the ATB group ($P < 0.05$).

3.4 Activation of the NFκB/MAPK pathway

To elucidate the underlying mechanisms by which glyceryl butyrate improves intestinal function, further study examined the activation of the nuclear factor kappa B (NFκB) and mitogen-activated protein kinase (MAPK) pathways in the piglet jejunum and ileum (Fig. 4A–D). In the jejunum (Fig. 4A and B), lower phosphorylation of Erk, P38, JNK and NFκB was observed in the ATB and GB groups than in the CON group ($P < 0.05$). However, the phosphorylation of P38 ($P = 0.090$) and JNK ($P < 0.05$) in the GB group was higher than that in the ATB group. In the ileum (Fig. 4C and D), the phosphorylation of Erk, P38, JNK and NFκB in the ATB group was significantly lower than that in the CON group ($P < 0.05$). Erk phosphorylation in the GB group was significantly lower than that in the CON group ($P < 0.05$), while it was significantly higher than that in the ATB group ($P < 0.05$). The phosphorylation of P38 and NFκB in the GB group showed no significant difference compared with that in the CON group; however, it was higher than that in the ATB group ($P < 0.05$). Furthermore, no significant difference in JNK phosphorylation was observed in the GB group compared with the CON and ATB groups.

3.5 Microbes in the piglet ileal contents

A total of 1 680 039 effective sequences were generated from 15 ileum content samples (three treatments, $n = 5$), with an average of 112 003 sequences per sample, and 2533 OTUs (97% similarity) were obtained for subsequent analysis. Data from sequencing can be accessed online at the National Genomics Data Center (Beijing, China) under accession number CRA006142. Four major bacterial phyla in the piglet ileum were Firmicutes (93.15–98.90%), Cyanobacteria (0.31–2.79%), Proteobacteria (0.54–2.08%) and Actinobacteria (0.19–1.69%), which accounted for more than 99.5% of the total bacterial community (Fig. 5A). The proportion of the ileal bacterial community showed obvious differences among the various experimental diet treatments. Compared with the CON group, the relative abundance of Firmicutes in the ATB group decreased from 98.90% to 93.15%, and the relative abundance of Cyanobacteria, Proteobacteria and Actinobacteria increased from 0.31%, 0.54%, 0.19% to 2.79%, 2.08% and 1.69%, respectively. Moreover, the ratio of Firmicutes in the GB group (98.42%) was higher than that in the ATB group (93.15%). Within the changes in Firmicutes, the relative abundance of Lactobacillus in the ATB and GB groups was 81.10% and 87.54% at the genus level, respectively, while that in the control group was 92.11% (Fig. 5B). A major source of the changes in Lactobacillus at the genus level was the species *Lactobacillus delbrueckii*, *Lactobacillus reuteri*, *Lactobacillus salivarius* and *Lactobacillus agrilis*

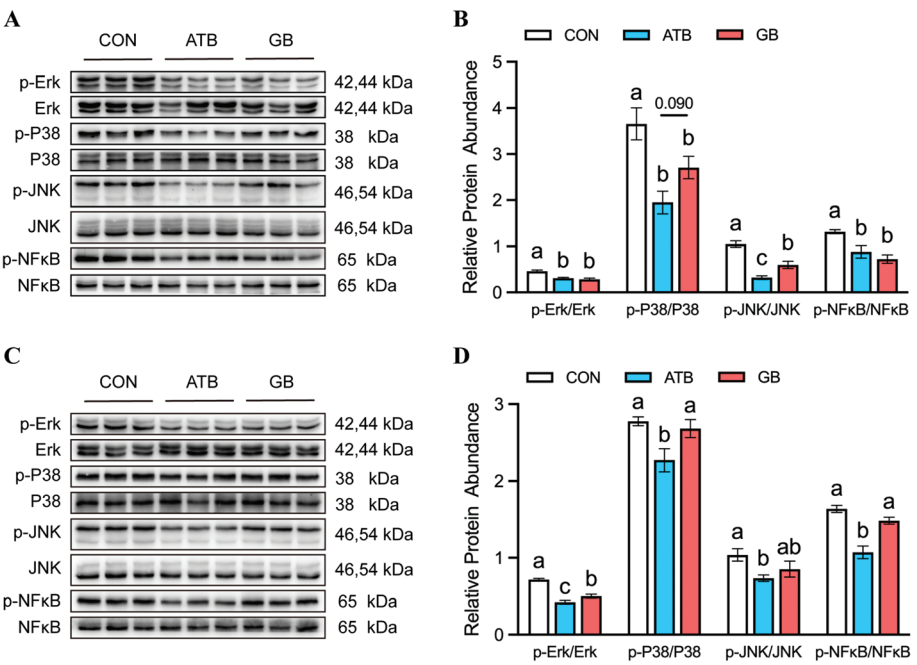

Fig. 4 The relative protein abundance of NFκB/MAPK pathway in the jejunum (A and B) and ileum (C and D). Different letters (a–c) indicate significant differences among different groups ($P < 0.05$).

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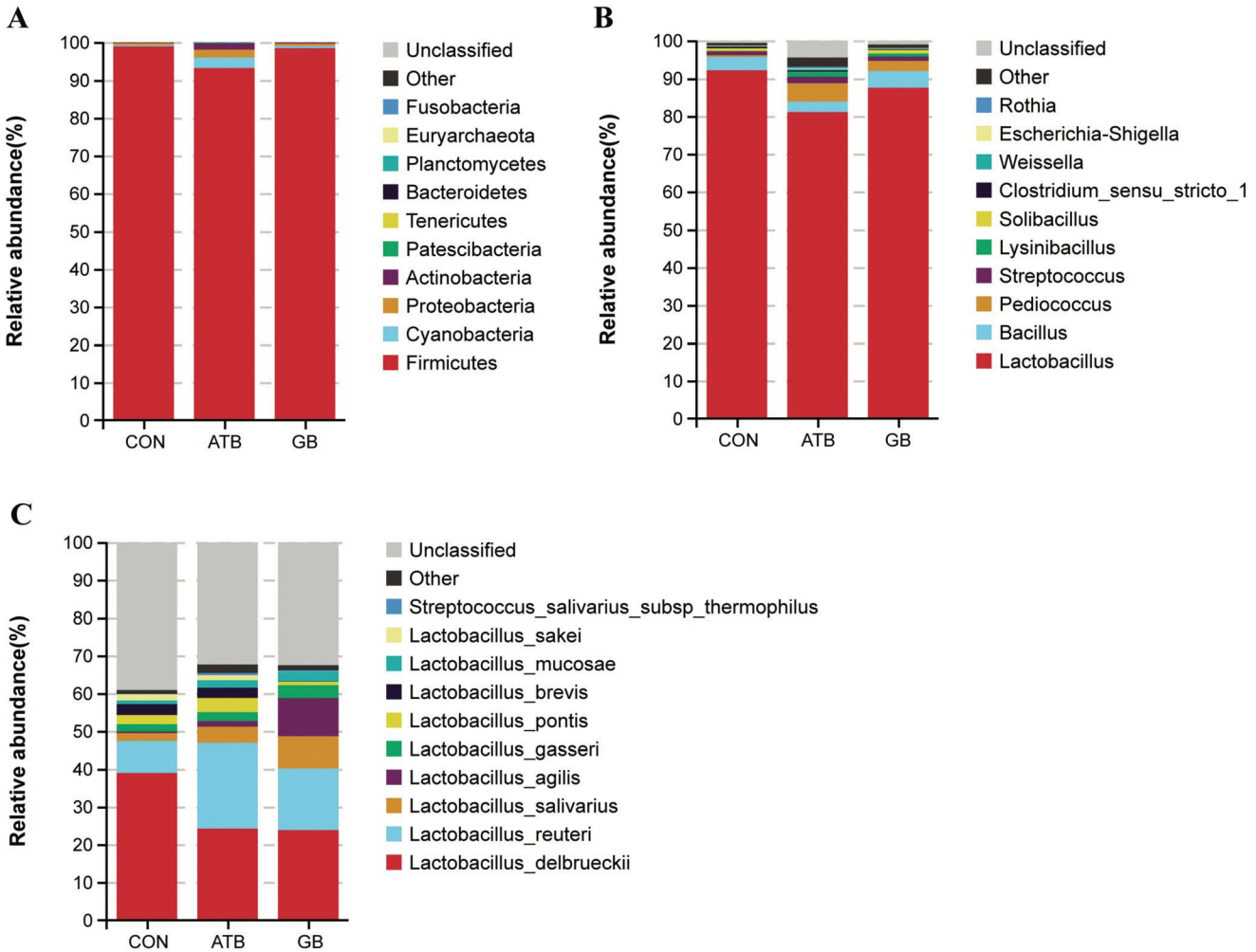

Fig. 5 Effect of glycerol butyrate on the microbial composition of the ileal microbiota of piglets challenged with ETEC (enterotoxigenic *Escherichia coli K88*) (n = 5). (A) Relative abundance at the phylum level. (B) Relative abundance at the genus level. (C) Relative abundance at the species level.

(Fig. 5C). In addition, the results at the genus level showed that the GB group had a better inhibitory effect on the growth of *Clostridium sensu stricto 1* and *Escherichia–shigella* than the ATB group (Fig. 5B).

The alpha diversity of the ileal microbiota indicated by ACE, Chao1, Simpson, Shannon, Pielou and Observed species (Fig. 6A–F). Compared with the CON group, Shannon and Pielou in the ATB group increased significantly ($P < 0.05$), while the GB group showed an increasing trend ($P = 0.096$). In addition, Shannon and Pielou indices in the GB group tended to decrease compared with those in the ATB group ($P = 0.066$ and $P = 0.087$, respectively). There were no significant differences in ACE, Chao1, Simpson and Observed_species. For beta diversity, the PCoA and NMDS analyses based on Bray–Curtis distance showed that the microbiota in the GB group obviously tended to separate from the CON and ATB groups (Fig. 6G and H).

In this study, the functional predictions of the ileal microbiota were analyzed by PICRUSt2 and are shown in Fig. 7. The top three relative microbial enrichment pathways at level_2 of the KEGG pathway were carbohydrate metabolism, amino acid metabolism and lipid metabolism in all treatments (Fig. 7A). Among the top 10 abundant pathways, lipid metabolism and xenobiotic biodegradation and metabolism in the GB group were higher than those in the CON group and ATB group (Fig. 7B). Moreover, the relative abundances of carbohydrate metabolism, amino acid metabolism, metabolism of cofactors and vitamins, metabolism of other amino acids, replication and repair, metabolism of terpenoids and polyketides, and energy metabolism in the GB group were increased compared to those in the CON group. A stronger function of secondary bile acid biosynthesis was observed in the GB group than in the CON group and ATB group at level_3 of the KEGG pathway (Fig. 7C). Furthermore, the relative abundance of biosynthesis of vancomycin group antibiotics, fatty acid biosynthesis, lysine biosynthesis, aminoacyl-tRNA biosynthesis and pentose phosphate pathway in the GB group at level_3 of the KEGG pathway were increased compared to the CON group.

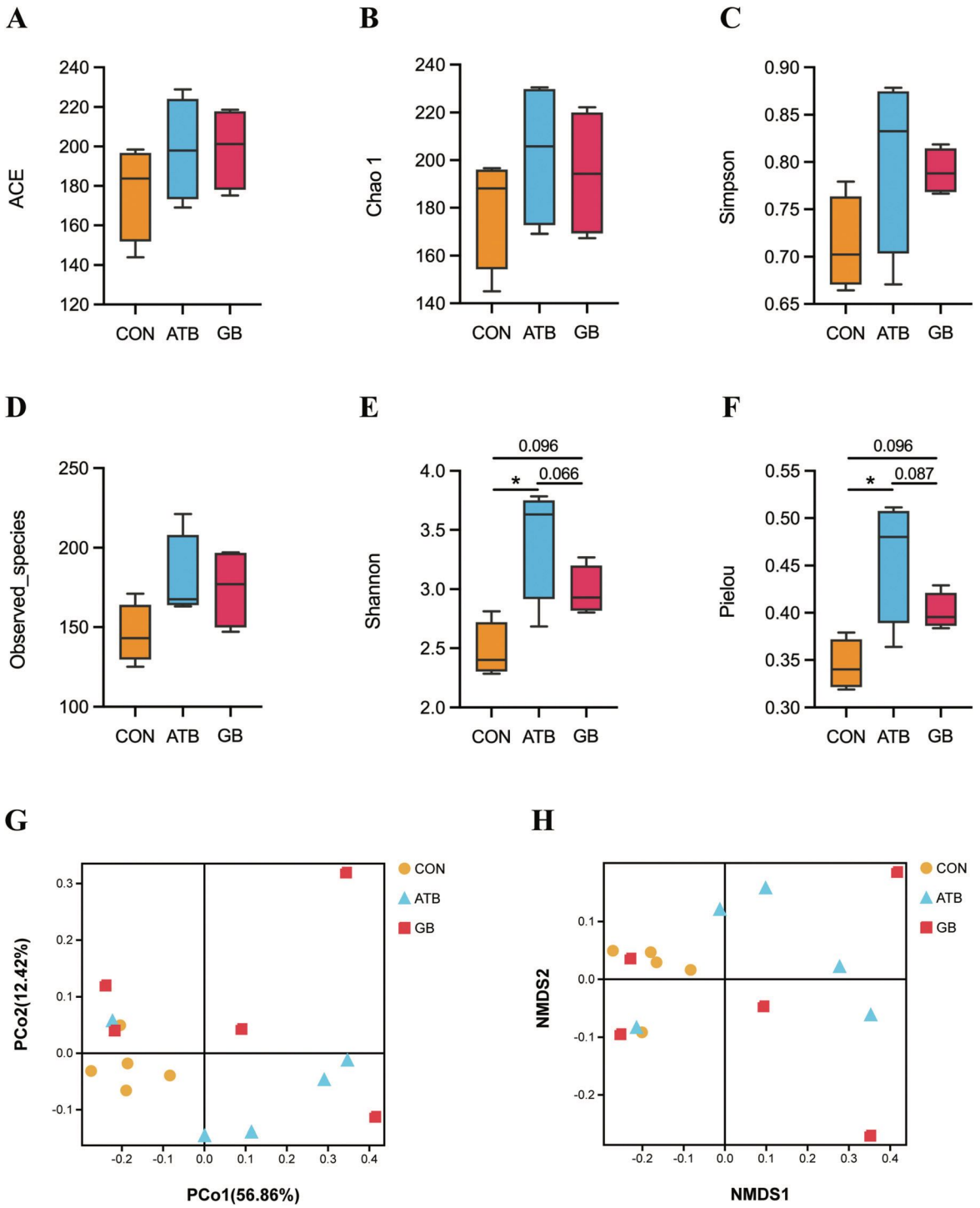

Fig. 6 Effect of glycerol butyrate on alpha diversity and beta diversity of ileal microbiota of piglets challenged with ETEC (enterotoxigenic *Escherichia coli K88*) (n = 5). (A–F), alpha diversity. (G), principal coordinate analysis-based bray. (H), NMDS Plot analysis-based bray. (*) indicates significant differences among the groups (P < 0.05).

4 Discussion

ETEC can secrete enterotoxins, which damage the intestinal barrier function of piglets and eventually lead to piglet diarrhea.[17] In our previous study, the diarrhea rate increased significantly when piglets were challenged with ETEC.[10,11,18] Correspondingly, TNF-α, IL-6 and IL-1β were significantly increased in piglet plasma and intestinal tissue. Interestingly, supplementation with formic acid, a mixture of formic and propionic acids, and glycerol monolaurate in diets downregu-

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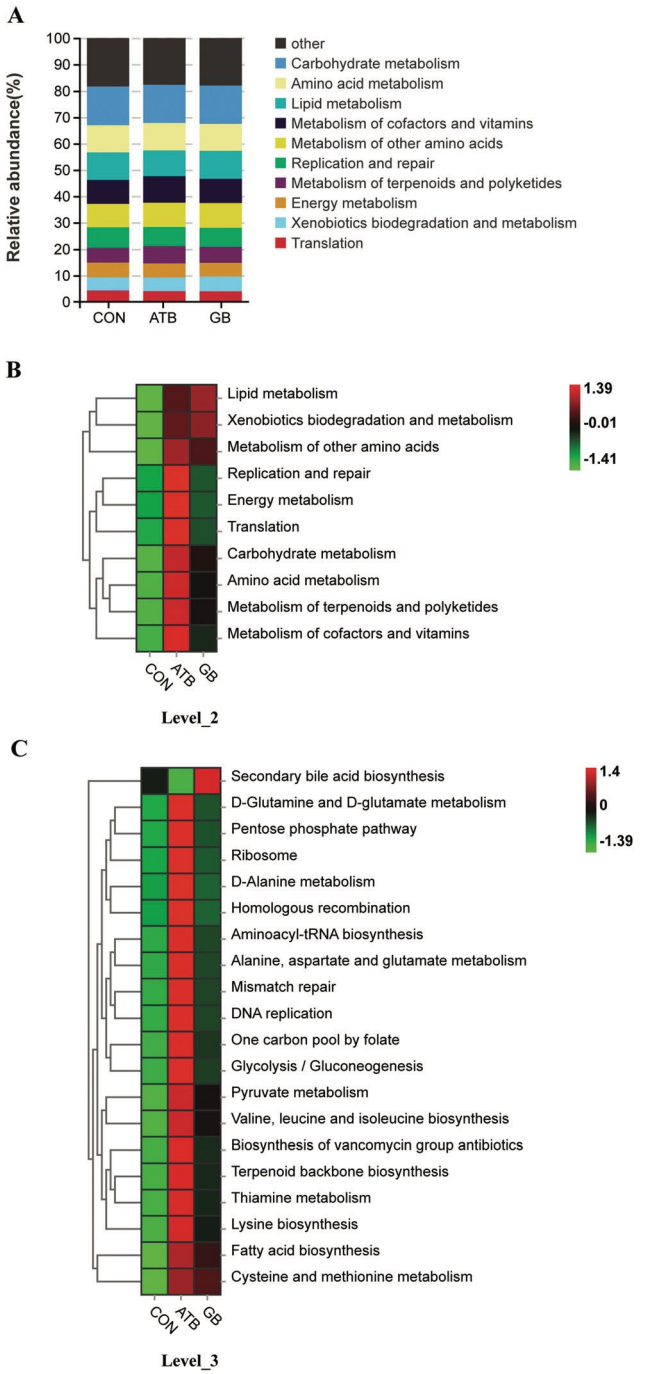

Fig. 7 Effect of glycerol butyrate on changes in the metabolic functions of ileal microbiota of piglets challenged with enterotoxigenic *Escherichia coli K88* (n = 5). (A and B) The top ten abundant microbial pathways at KEGG pathway level 2. (C) The distribution of the top 20 relative abundant microbial pathways at KEGG pathway level_3.

lated the proinflammatory factor levels of piglet plasma and intestinal tissue.[10,11] A previous study showed that a 25 mL kg^{-1} SCFA mixture (acetic, propionic, and butyric acids, 45, 15, and 11 mM, respectively) per day downregulated the mRNA abundances of IL-1β and IL-6.[19] Butyrate has been shown to modulate the levels of inflammatory factors in the small and large intestines of piglets.[15,20,21] Early intervention with sodium butyrate alone can modulate ileal inflammatory cytokines in neonatal piglets, suggesting that oral sodium butyrate may benefit the health of neonatal piglets.[20] Butyrate administration significantly reduced LPS-induced increases in piglet clinical scores and colon histology scores by reducing proinflammatory cytokines.[21] Furthermore, tributyrin relieved intestinal inflammation, as reflected by decreased mRNA expression of TNF-α, IFN-γ, and IL-6 in the intestine.[15] In the present study, glycerol butyrate was added to piglet diets and reduced the levels of IL-1β, IL-6, and TNF-α in piglet jejunum and ileum. The results suggest that glycerol butyrate can also reduce the level of ETEC-induced proinflammatory factors in the jejunum and ileum.

Usually, acute inflammation results in impaired intestinal barrier function in piglets. Mechanical barriers (*e.g.*, tight junctions), immune barriers (*e.g.*, beta defensins) and biological barriers (*e.g.*, gut microbes) are important components of the piglet gut barrier. As the first physical barrier, tight junctions are the most important connections between cells and are primarily composed of Claudin 1, Occludin, and ZO-1.[22] Tight junctions only allow the passage of soluble small molecules and block the passage of macromolecules and microorganisms.[22] Overproduction of proinflammatory cytokines in gut tissue disrupts tight junctions and increases paracellular permeability in the gut.[23–25] In the present study, the protein abundance of tight junctions was decreased in response to ETEC infection, which was related to the increase in IL-1β, IL-6 and TNF-α in the control group. It is worth noting that glycerol butyrate improved the protein abundance of tight junctions in the jejunum and ileum of piglets. Studies have shown that supplementation of tributyrin in piglet diets prevented the decrease in the protein abundance of Claudin 1, Occludin and ZO-1 in intestines caused by oxidative stress.[15] In addition, tributyrin may attenuate acetic acid-induced intestinal injury by promoting tight junction formation in piglets.[26] In addition to affecting intestinal mucosal permeability, fatty acids also regulate the secretion of intestinal defensins.[27] Defensins are produced by intestinal epithelial cells and phagocytes in the gastrointestinal tract. In addition to infection or inflammation, dietary compounds such as butyrate can also induce defensins. Glycerol butyrate was also observed in the present study to rescue the decreased defensin levels caused by ETEC infection. This result once again showed that butyrate was an effective inducer of defence peptide genes.[28] Additionally, various microbes in the gut are an important part of the intestinal barrier function of piglets, which will be discussed separately in the microbe section below.

To explore the mechanisms that cause intestinal changes in piglets, the NFκB/MAPK pathway, a classic inflammatory regulatory pathway, was selected to explain the above changes. The lipopolysaccharide of ETEC can trigger an inflammatory response by being recognized by Toll-like receptor 4 (TLR4), which regulates innate immunity by recognizing and combining various pathogen-associated molecular patterns. TLR4 activation triggers an inflammatory signalling cascade through

myeloid differentiation factor 88 (MyD88)-dependent and/or MyD88-independent pathways, leading to the nuclear proliferation of transcription factor NF-κB translocation, which subsequently leads to the production of proinflammatory factors such as TNF-α, IL-1β and IL-6.[29–33] This process is usually accompanied by inflammatory cell infiltration and epithelial damage. It was reported that sodium butyrate supplementation in piglet diets can downregulate LPS-induced inflammatory responses *via* RelA/p65 (NF-κB).[34] Similarly, dietary supplementation with sodium butyrate ameliorated weaning-related gut injury and inhibited the production of inflammatory mediators, and the JNK signalling pathway was likely involved in this process.[14] Furthermore, in IPEC-J2 cells, sodium butyrate was reported to regulate epithelial cell proliferation by regulating the phosphorylation of P38 and JNK.[35] The present study comprehensively analyzed the role of glycerol butyrate in regulating the NFκB/MAPK pathway, including NFκB, JNK, P38 and Erk. In the jejunum, glycerol butyrate effectively downregulated the increased phosphorylation of NFκB, JNK, P38 and Erk caused by ETEC infection. Interestingly, the ability of glycerol butyrate to modulate the NFκB/MAPK pathway in the ileum was not as good as that in the jejunum, which may be related to the reduced concentration of butyrin absorbed in the intestine.

Pathogenic bacterial (such as ETEC) infection can disrupt intestinal flora homeostasis, induce intestinal inflammation, damage the intestinal barrier, and lead to diarrhea in piglets. In the small intestine, food or intestinal contents are also key factors affecting the structure and abundance of gut community structure. To investigate the effect of glycerol butyrate on intestinal microbiota proliferation in piglets, this study analyzed microbes in ileal contents by high throughput 16S rDNA sequencing. The alpha diversity results showed that the bacterial diversity in the GB group showed an increasing trend relative to that in the CON group. The beta diversity results indicated that the GB group microbiota tended to be distinct from the CON group and the ATB group. Both alpha and beta diversity showed significant differences between the CON and ATB groups. Thus, there was a significant difference in the flora structure between the CON group and the ATB group. It is worth noting that the microbial flora of the GB group was similar but not identical to the CON and ATB groups. This evidence suggests that butyrin supplementation has a similar function to antibiotics in altering gut microbiota composition. Specifically, the changes in the intestinal flora of piglets were reflected in Lactobacillus species. The decrease in the relative abundance of *Lactobacillus delbrueckii* in the ATB group and GB group may be due to the restriction of its reproduction by drug or glycerol butyrate treatment. The main function of *Lactobacillus delbrueckii* in the gut is to ferment nutrients, thereby improving feed conversion. Interestingly, *Lactobacillus reuteri*, *Lactobacillus salivarius* and *Lactobacillus agrilis* were significantly increased in the ATB group and GB group, which is of great significance for inhibiting intestinal inflammation and enhancing intestinal barrier function. Compelling evidence shows that *Lactobacillus reuteri* has excellent probiotic properties,[36] including alleviation of weaning stress in piglets,[37] competitive exclusion against pathogens,[38] positive modulation of piglet redox status and immune function,[39–41] and promotion of intestinal butyrate and branched-chain fatty acid synthesis.[40,42] *Lactobacillus reuteri* induced the expression of pBD2 in the jejunum and pBD2, pBD3, pBD114 and pBD129 in the colon.[41] *Lactobacillus salivarius* is a well-characterized bacteriocin producer and probiotic organism.[43,44] In an earlier study, *Lactobacillus salivarius* improved host immunity by inducing IL-10 and some immunoglobulin levels, as well as inducing an increase in monocyte and natural killer cell numbers.[45] Interestingly, *Lactobacillus salivarius* can induce pBD2 production in the digestive tract of pigs.[46] Moreover, recent studies have shown that *Lactobacillus_agilis* has positive anti-inflammatory and antioxidant activity *in vitro*.[47] In addition, butyric acid is a natural ligand of GPR41 (free fatty acid receptor 3, FFA3) and GPR43 (FFA2), which can mediate the sensor PPAR-γ to regulate transcriptional processes and ultimately regulate intestinal tight junction and defensin synthesis.[48] In summary, the addition of glyceryl butyrate to the diet enriched the abundant beneficial flora in the piglet gut, thereby enhancing piglets' intestinal barrier function and reducing the occurrence of inflammation.

5. Conclusion

Dietary glycerol butyrate supplementation attenuated inflammatory responses and enhanced intestinal barrier function by inhibiting the NF-κB/MAPK pathway in ETEC-challenged piglets. *Lactobacillus reuteri*, *Lactobacillus salivarius* and *Lactobacillus agrilis* played a critical role in this process. This evidence suggests that glycerol butyrate is a potential replacement for antibiotics in the production of healthy and thriving swine.

Author contributions

Conceptualization, MT, LL, SZ and WG. data curation, formal analysis and methodology, MT, LL, ZT and HZ. Funding acquisition, SZ and WG. Supervision, FC and SZ. Visualization, MT. Writing – original draft, MT and LL. Writing – review & editing, SZ and WG.

Institutional review board statement

All of the procedures performed in animal using and caring during this study were approved by the South China Agricultural University Animal Care and Use Committee (no. 20110107-1, Guangzhou, China).

Data availability statement

Please contact the author if further information is required.

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Conflicts of interest

All authors declare no conflict of interest.

Acknowledgements

This study was supported by the Project of Swine Innovation Team in Guangdong Modern Agricultural Research System (2020KJ126), National Natural Science Foundation of the China (No. 31802067 and 31872364), Guangdong Basic and Applied Basic Research Foundation (No. 2021A1515010440), Science and Technology Program of Guangzhou (No. 202102020056).

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LWT - Food Science and Technology 166 (2022) 113786

Contents lists available at ScienceDirect

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Improved thermostability of lipase Lip2 from *Yarrowia lipolytica* through disulfide bond design for preparation of medium-long-medium structured lipids

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ARTICLE INFO

Keywords:
Lipase Lip2
Thermostability
Disulfide bond
Molecular dynamics simulation
Medium-long-medium structured triacylglycerols

ABSTRACT

Lipase Lip2 from *Yarrowia lipolytica* with great potential value in the enzymatic preparation of medium-long-medium structured lipids (MLM-SLs), but the synthetic efficiency might be limited by its poor thermostability. We identified flexible regions of lipase Lip2 by molecular dynamics (MD) simulation, and then disulfide bonds were engineered into these regions to enhance its thermostability. Compared with the wild type, mutant 4sN with four disulfide bonds showed a 19.22 and 27.75 °C increase in the melting temperature (T_m) and the half-loss temperature at 15 min (*T15 50*), respectively. The half-life of the wild type at 50 °C was 1.66 min, while that of mutant 4sN at 60 °C was 40.52 min. MD simulation showed that the thermal stability improvement in the mutant was attributed to enhanced structural rigidity. Finally, the MLM-SLs were synthesized from olive oil and caprylic acid with immobilized wild-type and mutant 4sN. Mutant 4sN displayed a 5 °C higher optimum temperature and a higher reaction rate than the wild type. Meanwhile, residual activity of mutant 4sN after 7 consecutive cycles increased by 20.84% compared to the wild type. The increased reaction rate and recyclability of lipase Lip2 provide greater potential for industrial production of MLM-SLs.

1. Introduction

Medium-long-medium structured lipids (MLM-SLs) typically contain medium-chain fatty acids at *sn*-1,3 positions and long-chain fatty acids at *sn*-2 position (Osborn & Akoh, 2002). Compared with conventional fats and oils, MLM-SLs with health benefits display lower caloric values (Lee et al., 2021). Thus, MLM-SLs were attracted considerable attention in the food industry. Numerous studies have investigated the potential applications of MLM-SLs in food products to replace conventional fats and oils. Osborn, Shewfelt, and Akoh (2003) reported that compared with unmodified canola oil, MLM-SLs synthesized from caprylic acid and canola oil significantly increased the perception of sweetness and reduced bubble formation for the formulation of chocolate-flavoured nutritional beverages. Besides, other attributes of beverages were not much difference by using MLM-SLs. Kim, Shewfelt, Lee, and Akoh (2005) incorporated MLM-SLs prepared from canola oil and caprylic acid into cold spreadable butterfat. The results showed butterfat displayed both counterbalanced hypercholesterolemic attributes and enhanced cold-spreadability. Moore and Akoh (2017) employed MLM-SLs that synthesized from coconut oil and sunflower oil with oleic acid to develop edible films for sport nutrition products. Cozentino et al. (2022) used MLM-SLs produced from grape oil and capric acid to formulate new functional hazelnut and chocolate spreads. Besides, MLM-SLs could be used as cooking oil for frying sweet potato chips and applied as ingredients for coating lipid, energy bars and milk drinks (Jennings, Shewfelt, & Akoh, 2010; Sellappan & Akoh, 2000; Timm, Xu, Skall, & Jacobsen, 2003). In addition, since hydrolysis ability and absorption rates of MLM-SLs are faster than those for long-chain TAGs,

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https://doi.org/10.1016/j.lwt.2022.113786
Received 30 March 2022; Received in revised form 20 May 2022; Accepted 17 July 2022
Available online 20 July 2022
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MLM-SLs are considered as easily available energy sources for patients with absorption problems (Reis, Holmberg, Watzke, Leser, & Miller, 2009). Thus, MLM-SLs have shown great potential in both food and pharmaceutical industries.

Lipases (triacylglycerol hydrolases, E.C. 3.1.1.3) are one of the most important types of industrial biocatalysts, which can be used for the production of SLs because they can catalyze esterification, acidolysis, alcoholysis and interesterification reactions in non-aqueous media (Hasan, Shah, & Hameed, 2006). Compare with chemical method, enzyme catalysis has several advantages, such as performing at the mild temperature and the atmospheric pressure (Holm & Cowan, 2008). However, the variety of available commercial immobilized lipases for the production of MLM-SLs is limited and the demand for more efficient enzymes at lower prices is greatly critical. The lipase Lip2 from *Yarrowia lipolytica* is a typical lipase. In the previous study, Casas-Godoy, Marty, Sandoval, and Ferreira-Dias (2013) reported that MLM-SLs have been successfully produced by acidolysis between olive oil and fatty acids with immobilized lipase Lip2 (Casas-Godoy et al., 2013). The result showed that the incorporation of olive oil with caprylic acid when using immobilized lipase Lip2 was similar to the result of using the commercial lipases Lipozyme RM IM and Lipozyme TL IM in the similar reaction. It implied that lipase Lip2 had great potentials for the industrial production of MLM-SLs. In addition, the optimal reaction temperature in this study was 40 °C. However, lipase Lip2 is unstable at above 40 °C (Aloulou, Puccinelli, De Caro, Leblond, & Carrière, 2007). The poor thermal stability of lipase Lip2 might cause its inactivation and limit its reaction rate in the reaction process. Thus, it is highly desirable to enhance the thermal stability of lipase Lip2, which might provide an important basis for applications of MLM-SLs in the food industry.

Over the last decade, the use of computational design for generating functional changes in enzymes has grown significantly because of the availability of many crystal structures and the improvement of computer performance and algorithms (Meng et al., 2020). The rapid discovery of mutations that enhance the thermostability of enzymes has been successfully achieved by various computational design methods. In recent years, some flexible molecules and protein regions have been successfully identified via molecular dynamics (MD) simulations. Importantly, the fluctuation of these flexible regions can be reduced by the addition of extra intermolecular forces such as hydrogen bonds, salt bridges, aromatic interactions and disulfide bonds (Kumar, Tsai, & Nussinov, 2000), which provide a pronouncedly effective way to enhance the thermostability of enzymes. Among these intermolecular forces, disulfide bonds, which are covalent links between the sulfur atoms of the thiol groups (–SH) in two cysteine residues, not only stabilize proteins against undergoing reversible unfolding by reducing the main-chain entropy of the unfolded state but also stabilize proteins against irreversible unfolding by reducing the rate of protein unfolding (Sanchez-Romero et al., 2013). A number of studies have reported that the thermostability of proteins was improved by the introduction of disulfide bonds into lipases. An additional disulfide bond A162C–K308C was introduced into lipase B from *Candida antarctica*, and the results showed that the mutant displayed an 8.5 °C increase in the half-loss temperature at 60 min (*T60 50*) compared with the wild type (Le, Joo, Yoo, & Kim, 2012). The nonnative disulfide bond F223C/G247C in the *Rhizomucor chinensis* lipase improved its thermostability, yielding a half-life ($t_{1/2}$) approximately 18-fold longer than that of the wild type at 60 °C (Wang, Wang, Xu, & Yu, 2020). The $t_{1/2}$ value of lipase from *Rhizopus oryzae* was only approximately 11.7 min at 55 °C, whereas the activity of its variant E190C/E238C remained at approximately 58.2% after a 720 min incubation (Zhao et al., 2018). However, few reports have described the effects of disulfide bonds on lipase application.

In this study, our goal was to enhance the thermostability of lipase Lip2 through disulfide bond design. MD simulations were initially applied to identify the flexible regions of lipase Lip2. Then, additional disulfide bonds were introduced into flexible regions for experimental verification. Additionally, the biophysical effects of the introduced disulfide bonds on the thermostability of lipase Lip2 were investigated by MD simulations. Finally, the acidolysis reaction between olive oil and caprylic acid was catalyzed by using the immobilized Lip2 variant to produce MLM-SLs.

2. Materials and methods

2.1. Wild-type Lip2 plasmid and expression strains

The gene encoding the wild-type lipase Lip2 (GenBank accession no. AJ012632) with an extra 6 × His-tag in the N-terminal was synthesized by GenScript Company (GenScript, Nanjing, China) as reported previously (Wang et al., 2011). The gene was cloned into the pPICZα vector with the restriction sites *Eco*RI and *Not*I, and the expression vector pPICZα-lip2H was obtained. The *E. coli* strain Top 10 was obtained from WEIDI (Shanghai, China), and the *P. pastoris* strain X33 was obtained from Invitrogen (Carlsbad, CA, USA). All materials and reagents were of analytical reagent grade.

2.2. MD simulation and disulfide bond design

MD simulations were carried out to identify the flexible regions of lipase Lip2. The X-ray structure of lipase Lip2 (PDB ID 3O0D) was obtained from the Protein Data Bank, and missing atoms were repaired with Swiss-PdbViewer 4.1.0 software. MD simulations were performed using GROMACS 5.15 software with the Amberff99SBnmr1-ILDN forcefield (Long, Li, Walter, Griesinger, & Brüschweiler, 2011). The protein was placed in a cubic box containing TIP3P water, and the distance between the surface of the protein and the cubic box was at least 1.2 nm. Sufficient sodium ions were added to balance the charges in the system. The MD simulations started with energy minimization, and then the system was equilibrated by heating from 0 to the desired value over 500 ps. Then, 100 ns of MD simulation was carried out with a 2-fs time step at the desired temperature. The root mean square fluctuation (RMSF), root mean square deviation (RMSD) and hydrophobic solvent-accessible surface area (SASA) were analyzed with Gromacs tools. Disulfide by Design 2 (DbD2) was applied to predict residue pairs for potential disulfide formation (Craig & Dombkowski, 2013). Disulfide bonds were designed in the flexible regions with high RMSF values.

2.3. Site-directed mutagenesis

Site-directed mutants were generated using the KOD-Plus DNA polymerase mutagenesis kit (Toyobo, Osaka, Japan) according to the manufacturer's instructions. The reaction product was transformed into *E. coli* TOP10, and the mutations were confirmed by DNA sequencing analysis.

2.4. Expression and protein purification

The wild type and all mutants were expressed in *P. pastoris* X33 according to instructions of the *Pichia* expression kit manual (Invitrogen, Carlsbad, CA, USA). The constructed plasmids were linearized with *Pme*I and transformed into *P. pastoris* strain X33 via electroporation. Positive transformants were screened by using yeast extract peptone dextrose (YPD) plates containing 100 μg/ml Zeocin. The cells were inoculated into 10 mL YPD medium and incubated at 30 °C until the OD600 value reached 0.8–1.0, and then the cells were transferred into 100 mL buffered methanol complex (BMMY) medium and incubated at 29 °C for 96 h. Methanol was added to the medium every 24 h, and the final concentration was 1%.

The fermentation supernatant was harvested by centrifugation and concentrated by ultrafiltration with a molecular weight cutoff of 10 kDa (Amicon Ultra, Millipore, MA, USA). Crude enzyme samples were purified with a 1 mL HisTrap FF crude column (GE Healthcare, Uppsala, Sweden) according to the manufacturer's instructions. The washing

buffer was 20 mM sodium phosphate buffer at pH 6.5 containing 1000 mM NaCl and 120 mM imidazole, and the elution buffer was 20 mM sodium phosphate buffer at pH 6.5 containing 500 mM NaCl and 275 mM imidazole. Then, the sample enzyme was desalted using a desalting column (GE Healthcare, Uppsala, Sweden) with 20 mM sodium phosphate buffer at pH 6.5. The purity of the sample was evaluated by 12% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE).

2.5. Differential scanning fluorimetry

The melting temperature (T_m) was determined by the differential scanning fluorimetry (DSF) method with SYPRO orange dye (Sigma-Aldrich; St. Louis, MO, USA) as an indicator. Twenty microliters of purified protein (0.2 mg/mL) was mixed with 5 μL 50-fold SYPRO orange dye, and fluorescence measurement was performed by scanning from 20 to 99 °C with a 1% thermal gradient via a 7500 Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). The data were fitted to the Boltzmann equation with GraphPad Prism 7.0 to calculate the T_m value.

2.6. Thiol titration

The introduction of additional disulfide bonds was verified by using 5,5′-dithiobis (2-nitrobenzoic acid) (DTNB) to titrate the free cysteines in both wild-type Lip2 and mutants (Ellman, 1959). The purified enzyme was diluted with 100 mM Tris-HCl buffer at pH 7.0 containing 8 M urea, and the final enzyme concentration was 0.3 mg/mL under the non-reduced condition. Under the reduced condition, 25 μL β-mercaptoethanol was added to the sample. The two samples were incubated at at 37 °C for 1 h. The reduced sample was precipitated with 2 mL 30% (w/v) trichloroacetic acid to remove the reducing agent. The precipitate was harvested by centrifugation and redissolved in 2 mL of Tris-HCl-urea buffer. Then, 1 mL reduced or nonreduced sample was mixed with 15 μL 10 mM DTNB or 100 mM Tris-HCl buffer at pH 7.0, and the absorbance was determined at 412 nm. The number of disulfide bonds was determined based on the following equation: $N_{disulfide} = N_{cys} * \frac{A_{412}{}^{total} - A_{412}{}^{free}}{A_{412}{}^{total} * 2}$, where $N_{disulfide}$ is the number of disulfide bonds, the N_{cys} is the total number of cysteine residues, and $A_{412}{}^{total}$ and A_{412}^{free} are the absorbances of the reduced and nonreduced samples, respectively.

2.7. Enzymatic characteristics

The activity of the purified enzymes was determined with *p*-nitrophenyl-palmitate (*p*NP-palmitate) as a substrate (Wang, Liu, Xu, Zhang, & Yan, 2015). The reaction substrate mixture contained 80 μl 5 mM *p*NP-palmitate dissolved in acetonitrile/alcohol (1:1) and 915 μl 50 mM Tris-HCl buffer (pH 7.0). Five microliters of purified enzyme was added to the mixture for a 10-min reaction at 30 °C. The reaction was terminated with 1 mL 20% (w/v) TCA, and then 500 μL 20% (w/v) sodium carbonate was added to the mixture, followed by measurement of the absorbance at 405 nm.

The purified lipases were diluted with 50 mM Tris-HCl (pH 7.0), and the final concentration was 0.1 mg/mL. The $t_{1/2}$ (half-life) values were examined by measuring the residual activity of the samples after incubation at 50 °C. To evaluate the *T15 50* (half-loss temperature at 15 min) values, the samples were incubated at temperatures ranging from 30 to 75 °C for 15 min, and the residual activity was measured. The T_{opt} (optimum temperature) values were investigated by measuring the activity of the samples at various temperatures ranging from 30 to 55 °C. The pH_{opt} (optimum pH) was determined by measuring the enzyme activity at different pH values from 2.0 to 8.0 (Na_2HPO_4-citric acid buffer). To determine pH stability, the samples were incubated in different buffers, including Na_2HPO_4-citric acid buffer (pH 2.0–8.0) and Gly-NaOH buffer (pH 9.0–11.0), at 25 °C for 24 h, and then the residual activity was measured. Kinetic parameters, including the catalytic constant (k_{cat}), Michaelis constant (K_m) and specificity constant (k_{cat}/K_m), were measured with different concentrations of *p*NP-palmitate at 40 °C. Kinetic parameter values were obtained by using the nonlinear regression function in GraphPad Prism 7.0.[22]

2.8. Immobilization of enzymes and acidolysis reaction

The wild type and mutant 4sN were produced from 5 L of fermentation broth. All methods used for the preparation of MLM-SLs and analysis were performed according to a previous description (Casas-Godoy et al., 2013). The supernatant samples were immobilized with Accurel support MP 1000, and then MLM-SLs were produced by the acidolysis of virgin olive oil and caprylic acid with immobilized enzymes, which was followed by analysis of the reaction products. The substrate consisted of 3 g of virgin olive oil and caprylic acid, corresponding to a molar ratio of 2:1 of olive oil to capric acid, which was the optimal ratio determined by a previous study (Casas-Godoy et al., 2013). A fixed amount of immobilized lipase (5% by the total weight of substrates) was added to the substrate mixture at different temperatures (35, 40, 45 and 50 °C) for 48 h of reaction. The reaction products were analyzed with a HPLC (Waters, Milford, MA, USA) equipped with an evaporative light scattering detector (Alltech, Deerfield, IL, USA) and a reverse-phase analytical ProntoSIL C30 column (250 mm × 4.6 mm, 5 μm, Bischoff, Leonberg, Germany). The results are presented as the incorporation degree of the desired fatty acid into TAG. The incorporation degree (I) was analyzed according to the equation $I = \frac{MFA}{MT} * 100\%$, where MFA is the moles of the medium-chain fatty acid (capric acid) in the TAG, while MT is the total moles of fatty acids in the triacylglycerols.

2.9. Reusability study of immobilized lipases

After the acidolysis reaction at 40 °C, the immobilized lipases were recovered and washed with n-hexane (30 mL) for three times. Then the recovered lipases were applied for the next cycle. The activity of the first batch was considered as 100%.

2.10. Statistical analysis

All assays were independently performed at least 3 times, and data were expressed as means±standard deviations. Statistical analyses were performed with the Student *t*-test. *P* value less than 0.05 was considered significant.

3. Results and discussion

3.1. MD simulations for identifying flexible regions of Lip2

Previous reports have revealed that the flexible regions of proteins can be stabilized by intermolecular forces such as disulfide bonds, aromatic interactions, hydrogen bonds and salt bridges to enhance the thermostability of proteins. Covalent disulfide bonds have been considered the strong intermolecular force for stabilizing proteins (Kumar et al., 2000; Dombkowski, Sultana, & Craig, 2014). To identify the flexible regions of lipase Lip2, MD simulations were performed at different temperatures (303, 323, 343 and 473 K) for 100 ns. As shown in Fig. 1, the higher the temperature was, the faster the Cα RMSD values increased. The Cα RMSD value increased slowly at 303, 323 and 343 K, with values below 0.20 nm at 100 ns, while it further increased at 473 K, with a value of 0.56 nm at 100 ns. This result suggests that the overall structure of lipase Lip2 is unstable at high temperatures, as expected.

According to visual analysis of the 100 ns MD simulation, the results showed that lipase Lip2 maintained most of its tertiary structure at 303, 323 and 343 K and gradually lost its tertiary structure at 473 K (data not shown), which indicated that lipase Lip2 underwent an unfolding

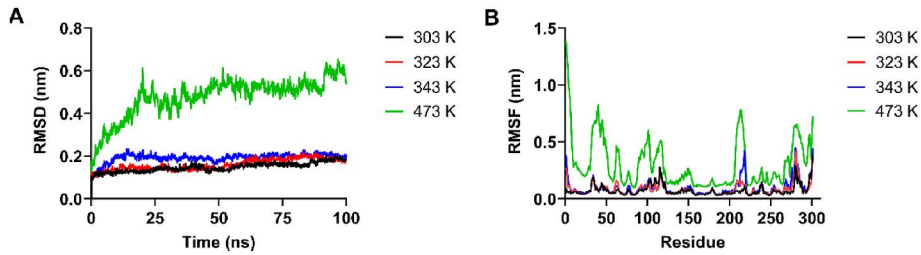

Fig. 1. Molecular dynamics (MD) analysis of wild-type at different temperatures (303, 323,343 and 473 K). (A), (B) and (C) represent the Cα root mean square deviation (RMSD) and the root mean square fluctuation (RMSF), respectively.

process. RMSF values can be applied to denote the positional fluctuations of amino acid residues during MD simulations, as unstable protein regions display higher RMSF values. As shown in Figs. 1 and 2, 5 regions (region A: V1 to Q12, region B: V31 to Y71, region C: G87 to H126, region D: F206 to L222, and region E: G267 to V298) were unstable and showed higher RMSF values at 303, 323, 343 and 473 K.

Disulfide by Design 2 is a program for disulfide bond design in proteins (Craig & Dombkowski, 2013). The χ3 torsion angles and Cα-Cβ-Sγ angles of all residue pairs in Lip2 were analyzed, and potential residue pairs suitable for the formation of disulfide bonds that met the geometric criteria were identified. Disulfide bonds were designed in these 5 regions to rigidify the flexible regions, and residue pairs close to the activity center were filtered. Finally, 17 potential residue pairs were selected for experimental assessment.

3.2. Construction, expression and purification of enzymes

The introduction of mutations was performed by PCR-based site-directed mutagenesis. Recombinant plasmids containing the wild type and mutant genes were transformed into *P. pastoris* strain X33 for expression. As a result, 16 disulfide bond mutants were expressed successfully, excluding the mutant V125C-G193C. Lipase samples were purified with Ni^{2+}-chelating affinity columns, and the purities were evaluated by 12% SDS–PAGE (Fig. 3).

3.3. Identification of single stabilizing disulfide bonds

The T_m values of the wild type and mutants were measured by the DSF method to screen stabilizing disulfide bonds. DSF is a rapid and sensitive method for evaluating the thermostability of proteins. Each experimental replication requires only a small amount (20 μL) of protein at a low concentration (0.10–0.30 mg/ml). The entire measurement process using the real-time PCR machine lasts less than 2 h. These characteristics make this method more appropriate for rapidly identifying effective mutants. T_m values can be used to evaluate the thermodynamic stability of enzymes, and the results are shown in Table 1. Ten stabilizing disulfide bonds with $\Delta T_m > 1.0$ °C that effectively improved the thermostability of lipase Lip2 were identified (Table 1). To determine whether the increase in thermostability was due to disulfide bond formation, the number of disulfide bonds was measured. The thiol titration test showed that the additional disulfide bond was successfully introduced into all stabilizing mutants (Fig. 4). The T_m value of the wild type was 48.32 °C. The best disulfide bond, V1C-Q242C, was located in region A and contributed a 16.79 °C increase in the T_m value, but it was not used in the follow-up experiments because of its greatly reduced catalytic performance. This might result from the decreased flexibility of the Q234-E247 loop, which is probably critical for the process of enzyme catalysis. The T_m values of mutants Y2C–N210C, S8C–S214C and S14C–V216C increased by 10.61, 7.50 and 5.62 °C, respectively, and the additional disulfide bonds in these three mutants were located in regions A and E. Other beneficial disulfide bonds were discretely located in other regions, including regions B, C and D. Mutants A103C–N127C and L105C-L124C lost the most catalytic performance and were not used in the follow-up experiments. Disulfide bonds A103C–N127C and L105C-L124C are located in the lid hinge region and might be unfavorable for lid opening, which is essential for allowing the substrate to enter the catalytic pocket. Finally, we identified seven beneficial disulfide bonds, including Y2C–N210C, S8C–S214C, S14C–V216C, H60C–S69C, A118C–V177C, D122C-G196C and S269C-A275C (Fig. 2).

To investigate the kinetic stability of the wild type and mutants, the half-loss temperature at 15 min (*T15 50*) and the half-life ($t_{1/2}$) at 50 °C were determined. As shown in Table 1, most mutants displayed a 10 °C increase in the *T15 50* value. The *T15 50* values of mutants Y2C–N210 and S8C–S214C increased by 13.40 and 7.50 °C, respectively, compared with that of the wild type. The wild type was deactivated rapidly at 50 °C, and its $t_{1/2}$ value was 1.66 min. Compared with that of the wild type, the $t_{1/2}$ values of mutants Y2C–N210C and S8C–S214C increased by 118-fold (198.05 min) and 25-fold (43.87 min), respectively. These results showed that the mutants with an additional disulfide bond were more stable than the wild type at the high temperature, denoting the great contribution of disulfide bonds to the thermostability of lipase Lip2. According to the optimum temperature assays, the T_{opt} values of all

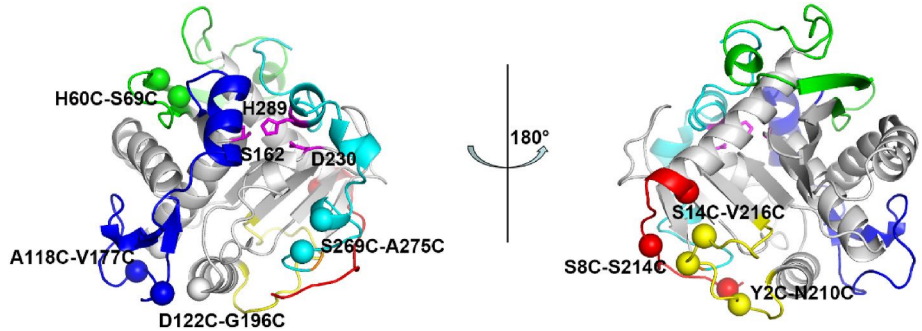

Fig. 2. Beneficial disulfide bond mutation sites of Lip2. Region A is shown in red, region B is shown in green, region C is shown in blue, region D is shown in yellow and region E is shown in cyan. Mutation sites are shown as spheres. The catalytic triad is consisted of S162, D230 and H289 shown as sticks. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

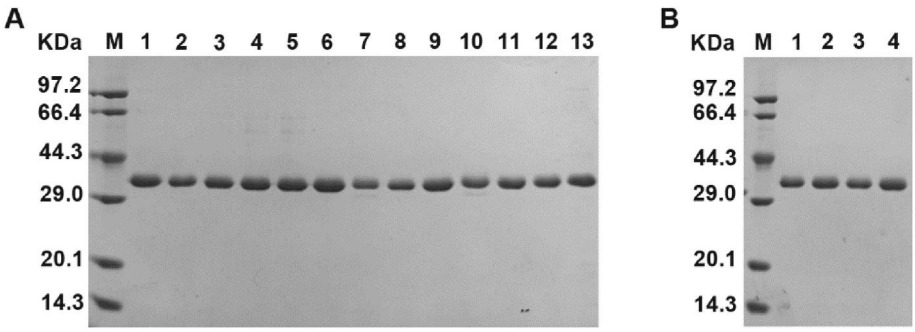

Fig. 3. The SDS-PAGE analysis of purified wild-type and mutant enzymes. M is molecular mass marker. (A) Lane 1 to 13 are Wild-type, V1C-Q242C, V1C-Q268C, Y2C–N210C, S4C-Q266C, S8C–S214C, S8C-M264C, S14C–V216C, N52C-A77C, H60C–S69C, D61C-D67C, A103C–N127C, L105C-L124C, A112C–Y137C and A118C–V177C, respectively. (B) Lane 1 to 4 are D122C-G196C, V125C-G193C, S269C-A275C and 4sN, respectively.

Table 1
Thermostability and optimum temperature of wild-type and mutant enzymes.

Enzyme	Location	T_m (°C)	$\triangle T_m$ (°C)	*T15 50* (°C)	$\triangle$*T15 50* (°C)	*t 1/2* (min)	T_{opt} (°C)
Wild-type		48.32 ± 0.25		40.86 ± 0.03		1.66 ± 0.31	35
V1C-Q242C	region A	65.11 ± 0.03	16.79	\	\	\	\
V1C-Q268C	region A, E	48.81 ± 0.15	0.49	\	\	\	\
Y2C–N210C	region A, D	58.93 ± 0.13	10.61	54.26 ± 0.11	13.4	198.05 ± 12.57	40
S4C-Q266C	region A	48.65 ± 0.14	0.33	\	\	\	\
S8C–S214C	region A, D	55.82 ± 0.14	7.50	51.01 ± 0.39	10.15	43.87 ± 3.55	40
S8C-M264C	region A	47.52 ± 0.13	0.80	\	\	\	\
S14C–V216C	region A, D	53.94 ± 0.05	5.62	49.91 ± 0.40	9.05	17.24 ± 1.42	40
N52C-A77C	region B	49.14 ± 0.12	0.82	\	\	\	\
H60C–S69C	region B	54.29 ± 0.12	5.97	52.36 ± 0.22	11.5	42.78 ± 3.19	40
D61C-D67C	region B	46.93 ± 0.08	−1.39	\		\	\
A103C–N127C	region C	49.38 ± 0.17	1.06	\	\	\	\
L105C-L124C	region C	52.19 ± 0.09	3.87	\	\	\	\
A112C–Y137C	region C	46.77 ± 0.16	−1.56	\	\	\	\
A118C–V177C	region C	51.65 ± 0.13	3.33	50.77 ± 0.33	9.91	13.62 ± 1.12	40
D122C-G196C	region C	52.87 ± 0.15	4.55	50.50 ± 0.10	9.64	12.57 ± 1.09	40
V125C-G193C	region C	\		\	\	\	\
S269C-A275C	region E	50.31 ± 0.05	1.99	45.43 ± 0.24	4.57	6.07 ± 0.88	40
4sN		68.24 ± 0.18	19.22	68.61 ± 0.35	27.75	40.52 ± 2.39	40

Mutant V125C-G193C was not expressed successfully in *P. pastoris* strains X33.
t 1/2 of the wild type and single disulfide bond mutants were measured under the temperatures of 50 °C, and that of mutant 4sN was measured under the temperatures of 60 °C.
\ presents no determination. ± means standard deviation for three independent experiments is shown.

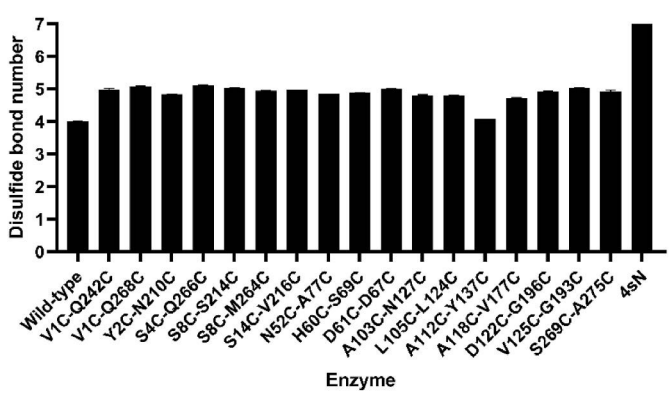

Fig. 4. Disulfide bond number of the wild type and mutants. Error bars represent standard deviations between replicates.

beneficial mutants were increased from 35 to 40 °C, which indicated that higher temperatures and more energy were required to activate the full catalytic performance of the mutants. These results suggest that the introduction of additional disulfide bonds into lipase Lip2 could effectively improve its thermostability.

Disulfide bonds stabilize proteins against undergoing reversible unfolding due to reducing the main-chain entropy of unfolded states and stabilize proteins against irreversible unfolding by slowing the rate of unfolding (Sanchez-Romero et al., 2013). The thermostability of many proteins has been enhanced via the design of disulfide bonds since the concept of stabilizing proteins with nonnative disulfide bonds was first evaluated by Villafranca and coworkers (Villafranca, Howell, Oatley, Xuong, & Kraut, 1987). However, the effects of disulfide bonds on protein thermostability are always unpredictable. For example, thirteen disulfide bonds were separately introduced into the haloalkane dehalogenase from *Sphingomonas paucimobilis* UT26, and the results showed only one effective disulfide bond, A5C-A185C, which contributed a 5.0 °C increase in apparent unfolding temperature, whereas mutants with other disulfide bonds displayed similar or even significantly decreased apparent unfolding temperatures compared with that of wild type (Floor et al., 2014). In addition, three single-disulfide bond mutants (D129C-A142C, L173C–W179C and A127C-A182C) out of five designed mutants of the chitosanase from *Bacillus ehimensis* displayed reduced thermostability (Sheng, Ji, Zheng, Wang, & Sun, 2016), and compared with the wild-type pullulanase from *Anoxybacillus* sp. WB42, except for mutants T245C-A326C and W651C–V707C, other disulfide bond mutants exhibited lower thermostability (Pang, Zhou, Cui, Liu, & Zhou, 2020). In this study, six disulfide bond mutants showed similar or decreased T_m values ($\triangle T_m < +1.0$). The thermostability effects of disulfide bonds on proteins may be attributed to the comprehensive effects

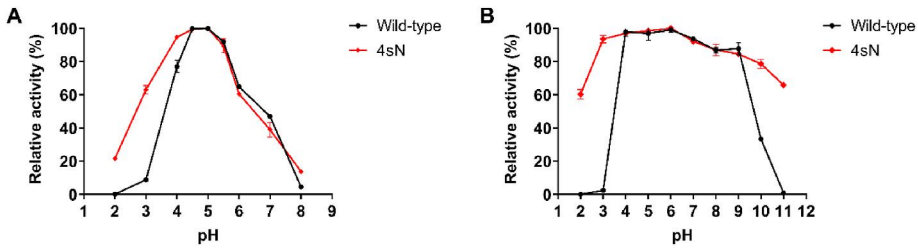

Fig. 5. Effects of pH on the activity and stability of the wild type and mutant 4sN. (A) Effect of pH on the activity of wild-type lipase and mutants 4sN. (B) Effect of pH on the stability of the wild type and mutant 4sN. Error bars represented standard deviations between replicates.

of the addition of a disulfide bond and the extension of side-chain interaction networks, which are cooperative results of the balance of multiple factors and are called local conformation synergism (Boonyaputthikul et al., 2019). These results might be due to these nonnative disulfide bonds causing the loss of existing favorable interactions or the formation of unfavorable contacts (Sheng et al., 2016). Therefore, the design of disulfide bonds should consider synergistic effects to optimize the thermostability of proteins.

Disulfide bonds V1C-Q242C, Y2C–N210C, S8C–S214C and S14C–V216C are located at the N-terminus, which implies that the N-terminus is a key region for enhancing the thermostability of lipase Lip2. Many studies have reported that additional disulfide bonds located at the termini play important roles in the thermostability of proteins. The xylanase from *Trichoderma reesei* with the disulfide bond F14C-Q52C introduced into its N-terminus showed a half-life at 60 °C improved by 2.5 times compared to that of the wild type (Tang et al., 2017). Compared with the wildtype phytase from *Bacillus licheniformis* WHU, the half-life at 60 °C of the mutant with the disulfide bond G197C/A358C in the C- terminus was increased by 3.8-fold (Zhang et al., 2020). In addition, engineering the extra disulfide bond P35C-G426C into the N-terminus of the alkaline amylase from *Alkalimonas amylolytica* resulted in a 1.8 °C increase in the T_m value (Liu et al., 2014). These results suggest that the termini of proteins should be considered potential regions for the introduction of extra disulfide bonds to stabilize protein structures.

3.4. Combination of disulfide bonds

3.4.1. Thermostability of the combined mutant 4sN

Beneficial disulfide bonds were stepwise combined in lipase Lip2 based on their locations. Multiple disulfide bonds reduced protein secretion in the *P. pastoris* strain X33 (Navone et al., 2021); thus, only four beneficial disulfide bonds were simultaneously introduced into lipase Lip2, considering both thermostability and protein secretion. Finally, the combined mutant 4sN containing four stabilizing disulfide bonds (Y2C–N210C, S8C–S214C, H60C–S69C and D122C-G196C) was obtained. As shown in Table 1, the optimal temperature of mutant 4sN was 40 °C, which was the same as that of the single-stabilizing disulfide bond mutants and was increased by 5 °C compared to that of the wild type. The T_m and *T15 50* values of mutant 4sN were 68.24 and 68.61 °C, respectively, which were 19.22 °C and 27.75 °C higher than those of the wild type. Notably, the half-life of the wild type at 50 °C was 1.66 min, while that of the mutant 4sN at 60 °C was 40.52 min. The greatly enhanced thermostability of lipase Lip2 indicates that the enzyme might have more potentials for the industrial synthesis of MLM-SLs.

3.4.2. Optimal pH and stability of the wild-type Lip2 and the mutant 4sN

The effects of disulfide bonds on the enzyme at various pH values were investigated. As shown in Fig. 5, the optimum pH values of the wild-type Lip2 and mutant 4sN were between 4.0 and 5.0. The relative activity of the wild type was 8.8%, while that of mutant 4sN increased to 63.1% at pH 3.0. This result suggests that multiple disulfide bonds increase the relative activity in acidic environments. At pH 4.0 to 9.0, the wild-type enzyme retained at least 75% relative activity after incubation at 25 °C for 24 h. However, the wild-type enzyme lost most of its activity in other pH environments. The stable pH range of mutant 4sN was extended, and the mutant exhibited at least 50% relative activity at pH 2.0 to 11.0. This result indicates that multiple disulfide bonds greatly enhanced the enzyme pH stability in acidic and alkaline environments. The introduction of additional disulfide bonds to enhance pH stability has also been demonstrated for other enzymes (He et al., 2019; Liu et al., 2014; Tang et al., 2017).

3.4.3. Kinetic parameters of the wild type Lip2 and the mutant 4sN

The kinetic parameters were further investigated by Michaelis–Menten calculations. As shown in Table 2, mutant 4sN displayed a similar K_m value to that of the wild type, and its k_{cat} increased by 15.5%. Thus, the k_{cat}/K_m value of 4sN increased by 24.9%. The k_{cat}/K_m value can intuitively represent the catalytic efficiency of an enzyme. This result implied that mutant 4sN had a higher catalytic efficiency than the wild type for the substrate *p*NP-palmitate under the measured conditions.

3.4.4. MD simulations

MD simulations were carried out at 473 K to investigate the thermal stabilization effect in mutant 4sN, as the wild-type enzyme displayed unfolding at 473 K in visual analysis (data not shown). The Cα RMSD value of the wild type was 0.56 nm, while that of mutant 4sN was 0.45 nm at 100 ns (Fig. 6). The results indicated that the global structural fluctuation of mutant 4sN was enhanced by multiple disulfide bonds. In addition, the hydrophobic SASA of mutant 4sN increased more slowly than that of the wild type. It is generally accepted that protein denaturation occurs in a two-stage process. The first step involves the partial unfolding of peripheral protein chains, which is reversible. The second step involves the exposure of the protein interior, and then hydrophobic regions irreversibly aggregate via inter- and intramolecular interactions (Ladero et al., 2006). Disulfide bonds enhanced the free-energy barrier of protein unfolding via the enhancement of the structural rigidity, as indicated by the Cα RMSD values, which led to a reduction in the rate of enzyme unfolding and decreased exposure of the hydrophobic SASA; thus, the thermostability of the enzyme was enhanced.

3.5. Preparation of MLM-SLs

MLM-SLs could reduce calories and are suitable for the control of obesity, lipid malabsorption and other metabolic disorders, which have great potential in both food and pharmaceutical industries. (Utama,

Table 2
Kinetic parameters of the wild type and mutant 4sN.

Enzyme	K_m (mM)	k_{cat} (s^{-1})	K_{cat}/K_m ($s^{-1}mM^{-1}$)
Wild-type	0.41 ± 0.03	11.78 ± 0.17	28.73
4sN	0.38 ± 0.03	13.63 ± 0.12*	35.88

Kinetic parameters were determined at pH 5.0 and 40 °C.
± means standard deviation for three independent experiments is shown. * indicates significant differences at $P < 0.05$.

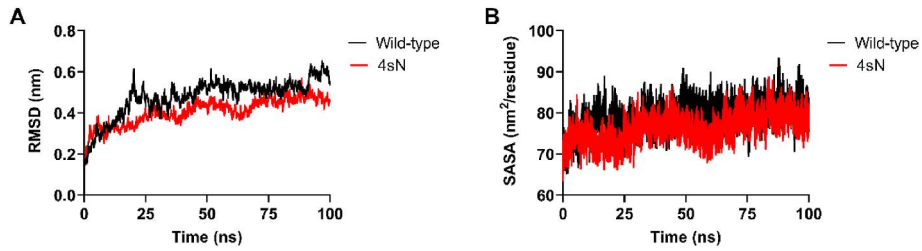

Fig. 6. Molecular dynamics (MD) analysis of the wild type and mutant 4sN at 473 K. (A) Cα RMSD (B) hydrophobic solvent-accessible surface area (SASA).

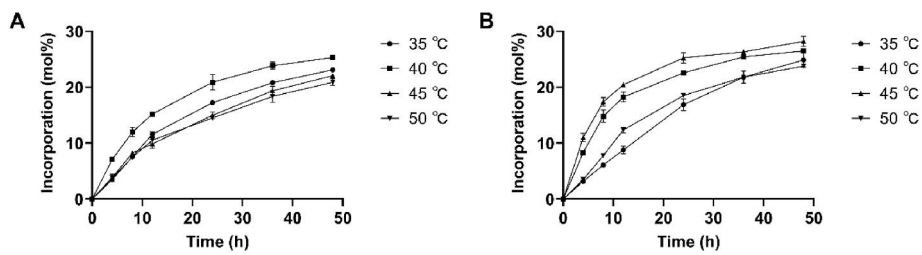

Fig. 7. Effect of reaction time on caprylic acid incorporation into olive oil with immobilized enzymes wild-type (A) and mutant 4sN (B) at different temperatures. Error bars represented standard deviations between replicates.

Sitanggang, Adawiyah, & Hariyadi, 2019). There are many successfully cases of replacing conventional fats and oils with MLM-SLs (Cozentino et al., 2022; Jennings et al., 2010; Kim et al., 2005; Moore & Akoh, 2017; Osborn et al., 2003; Sellappan & Akoh, 2000; Timm, Xu, Skall Nielsen, & Jacobsen, 2003). To verify the practical utility of mutant 4sN, we further evaluated the synthesis of MLM-SLs by using the immobilized mutant 4sN. Wild-type and mutant Lip2 were produced from fermentation broth and immobilized with Accurel support MP 1000, and then MLM-SLs were synthesized by the acidolysis of olive oil and caprylic acid by using the immobilized enzymes (Casas-Godoy et al., 2013). The synthesis of MLM-SLs was carried out at different temperatures. As shown in Fig. 7, at 48 h, the incorporation achieved by mutant 4sN were 26.50 and 28.21 mol% at 40 and 45 °C, respectively, while those of the wild type were 23.21 and 25.33 mol%. The optimum temperature of mutant 4sN was shifted from 40 to 45 °C. In addition, at 12 h, the incorporation achieved by mutant 4sN were 18.24 and 20.43 mol% at 40 and 45 °C, respectively, while those of the wild type were below 15%. These results suggested that the reaction rate of mutant 4sN was higher than that of the wild type at 40 and 45 °C. Lip2 is unstable at high temperatures and is easily inactivated above 40 °C (Aloulou et al., 2007). The enzyme might gradually deactivate during the reaction process. Mutant 4sN showed a faster reaction rate than the wild-type at 40 °C, which might attribute to more stability during the reaction process. In addition, the reaction rate of mutant 4sN was further increased at 45 °C, which might result from the activation of maximal catalytic activity required more energy. Meanwhile, the high temperature would be helpful to increase substrate solubility, decrease the viscosity of the reaction medium, which might accelerate the reaction rate (Xu, Cen, Zou, Xue, & Zheng, 2020). The faster reaction rate might decrease the time of MLM-SL synthesis in the industrial process. Notably, the incorporation of caprylic acid in this work was better than that obtained with immobilized r-ROL (15.5 mol%) (Nunes, Pires-Cabral, Guillén, Valero, & Ferreira-Dias, 2012). Furthermore, the incorporation of caprylic acid in similar reactions using the commercial immobilized lipases Lipozyme RM IM and Lipozyme TL IM were 26.0–30 and 27.0 mol%, respectively (Lee & Akoh, 1998; Liu, Liu, Li, & Sun, 2008; Nunes, Pires-Cabral, & Ferreira-Dias, 2011).

The recyclability of the wild-type and 4sN were evaluated for 7 cycles of synthesis of MLM-SLs. After 7 consecutive cycles, the residual activity of the wild type was 71.43%, while that of mutant 4sN was 92.27% (Fig. 8). The increased recyclability might due to the enhanced

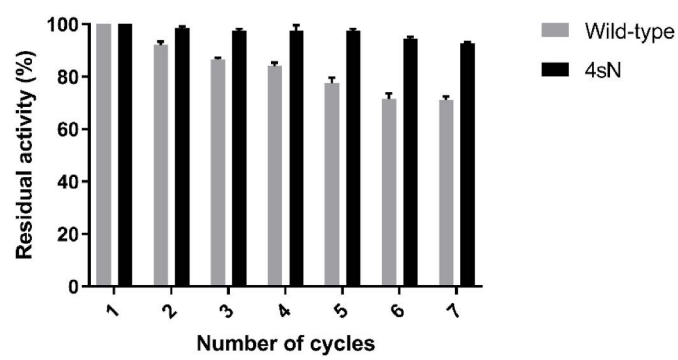

Fig. 8. Recyclability of immobilized wild-type and mutant 4sN during acidolysis of caprylic acid and olive oil. Error bars represented standard deviations between replicates.

thermostability of mutant 4sN, which allowed the enzyme to maintain its activity over a longer operational period. Compared with free enzymes, immobilized enzymes with long-term operational stability can be easily recovered and reutilized, and thus they are more suitable for continuous industrial production (Li et al., 2020). The both increased reaction rate and recyclability of the enzyme might bring economic benefits during the continuous industrial synthesis of MLM-SLs. These results indicate that mutant 4sN has more potential for the industrial preparation of MLM-SLs, and could provide an important basis for applications of MLM-SLs in the food industry.

4. Conclusions

In this work, the thermostability of lipase Lip2 was improved through disulfide bond design based on the use of MD simulations to identify the flexible regions, and seven beneficial disulfide bonds were obtained, which was followed by the subsequent combination of effective disulfide bonds. Compared with the wild type, the combined mutant 4sN containing four additional disulfide bonds showed 19.22 and 27.75 °C increases in the melting temperature (T_m) and half-loss temperature at 15 min (*T15 50*), respectively. It suggested that disulfide bond design with MD simulation was an effective approach for enhancing the thermostability of Lip2. For preparation of MLM-SLs, the

acidolysis reaction of olive oil with caprylic acid catalyzed by using the immobilized enzyme. Immobilized mutant 4sN displayed a faster reaction rate and greater recyclability than immobilized wild type. It indicated the mutant 4sN with better enzymatic characteristics might reduce the production cost for the continuous industrial synthesis of MLM-SLs, which could provide an important basis for applications of MLM-SLs in the food industry.

Compliance with ethical statement

This article does not contain any studies with human participants or animals performed by any of the authors.

CRediT authorship contribution statement

Lilang Li: Conceptualization, the study, Research question, Designed, Writing – original draft, Conceptualization, Writing – review & editing. **Weikun Wu:** Conceptualization, Methodology, Software. **Zixiao Deng:** Data curation, Writing – original draft. **Shihai Zhang:** Provided critical feedback, Funding acquisition, Writing – review & editing, Supervised the study. **Wutai Guan:** Provided critical feedback, Funding acquisition, Writing – review & editing, Supervised the study.

Declaration of competing interest

The authors declare that they have no conflict of interest.

Acknowledgements

We are greatly grateful to Professors Hon Cheung Lee and Yong Juan Zhao (School of Chemical Biology and Biotechnology, Peking University Shenzhen Graduate School) for presenting the *P. pastoris* X-33 strain.

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Animal Nutrition 14 (2023) 213–224

Contents lists available at ScienceDirect

Animal Nutrition

journal homepage: http://www.keaipublishing.com/en/journals/aninu/

Original Research Article

Maternal sodium acetate supplementation promotes lactation performance of sows and their offspring growth performance

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ARTICLE INFO

Article history:
Received 26 September 2022
Received in revised form
18 April 2023
Accepted 19 April 2023
Available online 25 April 2023

Keywords:
Sow
Sodium acetate
Lactation performance
Milk fat
Gut health

ABSTRACT

Milk yield and composition are critical determining factors for the early growth and development of neonates. The objective of this experiment was to comprehensively evaluate the effects of dietary sodium acetate (SA) supplementation on the milk yield and composition of sows and the growth performance of their offspring. A total of 80 sows (Landrace × Yorkshire, 3 to 6 parity) were randomly assigned to 2 groups (with or without 0.1% SA) from d 85 of gestation to d 21 of lactation. The result shows that maternal 0.1% SA supplementation significantly increased sows milk yield, milk fat, immunoglobulin A (IgA) and IgG content in milk ($P < 0.05$), with the up-regulation of short-chain fatty acids receptors (GPR41 and GPR43) expression and the activation of mammalian target of rapamycin complex C1 (mTORC1) signaling pathway. Consistently, in our in vitro experiment, SA also activated mTORC1 signaling in porcine mammary epithelial cells ($P < 0.05$). Furthermore, the improvement of milk quality and quantity caused by maternal SA supplementation led to the increase in body weight (BW) and average daily weight gain (ADG) of weaning piglets, with the improvement of gut health and colonization of the beneficial bacteria ($P < 0.05$). In conclusion, maternal supplementation of 0.1% SA improved the lactation performance (milk yield and milk fat) of sows, possibly with the activation of GPR41/GPR43-mTORC1 signaling. Furthermore, enhanced milk quality improved growth performance, gut health and the colonization of beneficial microbial flora of their piglets.
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1. Introduction

Over the past decade, advances in breeding technology have resulted in a dramatic increase in sows' litter size, which led to a great challenge for milk yield. The lactation performance of sows is critical for early growth and development of their piglets (Harrell et al., 1993). Insufficient milk intake leads to the reduction of weaning body weight (BW) and the decrease in weaning survival rate of piglets during lactation (Farmer, 2018).

Maternal nutrient intake directly affects milk yield and milk composition in lactating sows. Insufficient maternal energy intake could induce AMP-activated protein kinase (AMPK) activation,

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Peer review under responsibility of Chinese Association of Animal Science and Veterinary Medicine.

Production and Hosting by Elsevier on behalf of KeAi

https://doi.org/10.1016/j.aninu.2023.04.003
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which further inhibits mammary gland milk synthesis by targeting prolactin receptor (PrlR) and proliferator-activated receptor gamma coactivator-1 alpha (PGC-1α) (Wu et al., 2022). Dietary supplementation of functional amino acids (such as branched-chain amino acids, methionine, arginine, and lysine) has been widely demonstrated to regulate milk synthesis (Wu et al., 2020). However, it is still unknown whether other nutrients participate in the regulation of milk synthesis.

Rumen microbiota-derived short-chain fatty acids (SCFA) (especially acetate and butyrate) have been recognized as substrates for milk fat synthesis for a long time. In monogastric animals, serum SCFA is much lower, which is mainly derived from the fermentation of dietary fiber in the hindgut. Intriguingly, feeding high-fiber diets during gestation also increases serum acetic acid level with the upregulation of colostrum and milk fat content in sows (Jensen et al., 2012). In addition, studies have shown that acetic acid is more effective in promoting milk fat synthesis in bovine mammary epithelial cells than other SCFA (Sheng et al., 2016). However, the specific mechanism remains unclear. One possible mechanism is that SCFA induce the mobilization of fatty acids in mammary adipose tissue, and promote fatty acid transport from adipose tissue to mammary acinar (Feyera et al., 2019; Jensen et al., 2012). Sterol regulatory element binding proteins (SREBP1) is a critical regulator to promote milk fat synthesis (Li et al., 2014). Mechanistically, SCFA might modulate the expression of SREBP1 through the activation of mTORC1 pathway in the mammary gland (Düvel et al., 2010; Li et al., 2014; Park et al., 2015; Wang et al., 2014).

The main purpose of this experiment was to study the effect and underlying mechanism of maternal sodium acetate (SA) supplementation on milk yield and composition of sows. Furthermore, growth performance, gut health and intestinal colonization of microbial flora of their piglets were also determined.

2. Materials and methods

2.1. Animal ethics statement

All of the procedures performed in animal feeding and sample harvesting during this study were approved by the South China Agricultural University Animal Care and Use Committee (Guangzhou, China).

2.2. In vivo animal experiment

2.2.1. Animals and experimental design

As shown in Fig. S1, a total of 80 Landrace × Yorkshire, 3 to 6 parity) sows were divided into 2 treatments (control treatment and 0.1% SA, 40 replicates per treatment) by parity, backfat thickness and historical reproductive performance. The feeding experiment started from d 85 of gestation to d 21 of lactation, which was conducted on a modern commercial swine farm in Qingyuan City, Guangdong Province, China.

2.2.2. Diets and management

According to NRC (2012), diets were formulated to meet or exceed the nutritional needs of sows during gestation and lactation. The ingredient and chemical composition are shown in Table S1. During gestation, all sows were housed in individual gestation stalls (2.1 m × 0.6 m), and fed twice a day (08:00 and 14:00). Then they were transferred to the farrowing crates at d 110 of gestation. According to the backfat thickness, sows were fed 3 to 3.5 kg/d diets from the d 85 to 114 of gestation. After farrowing, the feed supply for the sows was increased by 1 kg/d until d 3 and then ad libitum feeding. The litter size was standardized to approximately 12 ± 1 piglets by cross-fostering within 24 h of farrowing.

2.2.3. Sow and litter performance

At farrowing, the number of litter size, litter weight, live births, stillbirths, mummy and weak (BW < 0.8 kg) piglets were recorded. Litter weight were recorded again at 24 h after farrowing of sows and cross-fostering of piglets. While, sows' litter size and litter weight were recorded on d 7, 14 and 21 of lactation. Piglets' BW, survival rate, average daily gain (ADG) and sows' average daily feed intake (ADFI) during lactation were recorded and calculated, $n = 40$. On d 85 of gestation, day of farrowing and d 21 of lactation, the backfat thickness of the sow was measured at P2 point (left side of the 10th rib and 6 cm away from the spine) according to the previous method (Mateo et al., 2007).

2.2.4. Sow lactation performance

Colostrum yield and colostrum intake were calculated using the method provided by Theil et al. (2014):

$$\text{Colostrum intake (g)} = -106 + 2.26\ \text{WG} + 200\ \text{BWB} + 0.111\ \text{D} - 1,414\ \text{WG/D} + 0.0182\ \text{WG/BWB},$$

where WG = weight gain of individual piglets (g) from the first piglet 24 h after birth, BWB = the birth weight of piglets (kg), D = colostrum intake time per piglet (min).

The total milk yield of each sow is calculated based on the ADG and litter size of the piglets using the following formula (Miao et al., 2019):

$$\text{Total milk yield (kg)} = 4 \times \text{ADG} \times \text{Litter size} \times \text{Lactation days}.$$

2.2.5. Milk sampling and analysis

Samples were collected from 6 representative sows per group, according to the average BW and parity of sows in each treatment group. Colostrum was collected within 12 h after farrowing the first piglet without oxytocin injection, and milk was collected on d 21 of lactation after the intramuscular injection of 20 IU oxytocin per sow. Milk samples were rapidly placed in liquid nitrogen, then transferred to −80 °C refrigerator preservation.

The composition of milk (lipids, crude protein, lactose, solids-not-fat) were determined by automatic milk composition analyzer (Milko-Scan 134 A/B, Foss Company, Denmark). And the concentrations of immunoglobulin A (IgA), IgG and IgM in milk were determined using ELISA kit (Wuhan Huamei), according to a method described in literature (Zanello et al., 2013).

2.2.6. Plasma sampling and analysis

On the day of farrowing and the d 21 of lactation, 20 mL blood was collected from ear veins of sows and anterior vena cava of piglets (select 1 piglet, near the average BW in each treatment group, from each of the 6 representative sows) using vacuum blood collection vessel containing EDTA, $n = 6$. After centrifugation at 3000 × g at 25 °C for 15 min, the plasma was transferred to a 1.5-mL cryovial, then quickly placed in liquid nitrogen and transferred to −80 °C refrigerator for storage. The concentrations of prolactin and immunoglobulin in plasma of sows and piglets were determined using Wuhan Huamei ELISA kit.

2.2.7. Tissue sample and intestinal morphology

After blood collection on d 21 of lactation, these piglets were sacrificed by electrocution for subsequent experimental sample collection. Duodenum, jejunum and ileum were collected according

to the method provided by Ren et al. (2020). Formalin-fixed, paraffin-embedded intestinal samples were stained with hematoxylin and eosin, then made into 4 to 6 μm thick sections. Villous height and crypt depth were measured for 6 well-oriented crypt-villus units per sample, and the ratio of villous height to crypt depth (VCR) was calculated.

2.2.8. Sows milk-derived cells isolation

Sows milk-derived cells were isolated from milk using the method provided by Twigger et al. (2022). Briefly, milk samples were diluted in an equal volume of sterile phosphate-buffered saline (PBS) and centrifuged at 870 × g at 20 °C for 20 min to isolate milk cells. Then the supernatant was removed and the pellet was resuspended in 5 to 10 mL of pre-chilled PBS. Finally, the sample was transferred to a new 10 mL tube and centrifuged at 490 × g at 4 °C for 5 min to collect the milk-derived cells after removing the supernatant.

2.3. In vitro cell culture experiment

The porcine mammary epithelial cells (PMEC) used in this study were isolated and characterized in our laboratory from the mammary glands of sows (G 90). PMEC used in this study were cultured in the complete medium consisted of DMEM/F12 (Thermo), containing 10% fetal bovine serum (FBS), 5 μg/mL insulin-like growth factor-1 (IGF-1), 10 ng/mL epidermal growth factor (EGF), 5 μg/mL insulin-Transferrin-Selenium (ITS) and 10 μg/mL penicillin-streptomycin (PS) in a cell incubator at 37 °C and 5% CO_2 concentration. When the cells reached to around 50% confluence, they were seeded in culture plates and incubated with in DMEM/F12 containing 10% FBS, 1 μg/mL hydrocortisone, 10 μg/mL PS, 0.5 μg/mL insulin and 2 μg/mL prolactin (Ma et al., 2018). The cell differentiation process was carried out for about 2 d until the cells reach 80% confluency. Then cells were used for the following experiments. PMEC were cultured with different concentrations (0, 0.25, 0.5, 0.75, 1.0 and 1.5 mM) of SA, $n = 3$. After 24 h incubation, cells were harvested using EZ-press RNA Purification Kit (EZBioscience, China) and RIPA lysis buffer for real-time PCR and western-blotting respectively.

2.4. RNA isolation and real-time PCR

Total RNA was isolated from cell samples using EZ-press RNA Purification Kit (EZBioscience, China) according to the manufacturer's instructions. The mRNA concentration was measured by NanoDrop spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). Each component's 260:280 ratio is between 1.6 and 1.8. The cDNA was prepared using an RNA reverse transcription kit (EZBioscience, China) according to instructions. Real-time PCR uses ABI StepOnePlus real-time PCR Systems. The reaction system was 10 μL Real-Time PCR Master Mix, 2 μL cDNA, 0.8 μL of each PCR primer and 7.2 μL DEPC water. The following thermal profile was used for RT-PCR, 95 °C for 1 min, followed by 40 cycles of denaturation at 95 °C for 15 s, annealing at 59 °C for 15 s, and extension at 72 °C for 40 s. The primers used in the experiment were synthesized by Sangon Biotech Co. Ltd. (Shanghai, China). Primer sequences are shown in Table S2.

2.5. Western-blotting

Milk-derived cells and PMEC were homogenized in RIPA lysis buffer containing 1% PMSF and 1% phosphatase inhibitor. Subsequently, 5 × protein loading buffer was added, fully shaken and mixed. Finally, the protein samples were boiled and denatured, and stored at −20 °C. The proteins were separated by electrophoresis on a 10% polyacrylamide gel and transferred to polyvinylidene fluoride membranes. The membranes were blocked with 5% skim milk in Tris-buffered saline with Tween (TBST), followed by overnight probing with primary antibodies. After washing, membranes were incubated with secondary antibodies. The chemiluminescent signal was detected by using ECL reagents (P1020), and bands were quantified by ImageJ Software (ImageJ 1.52a). The antibodies used in this experiment were as follows. Primary antibodies: fatty acid synthase (FASN) (1:2000, ab99359), acetyl-CoA carboxylα (ACACA) (1:1000, ab72046), fatty acid binding protein 3 (FABP3), (1:1000, ab231568), diacylglycerol O-Acyltransferase 1 (DGAT1) (1:500, ab100982), sterol-regulatory element binding protein1 (SREBP1) (1:1000, ab28481), 4E-binding protein 1 (4EBP1) (1:1000, ab2606), ribosomal protein S6 kinase 1 (S6K1) (1:1000, ab9366), GPR41 (1:1000, ab236654) and GPR43 (1:1000, ab131003) all purchased from Abcam, USA; Mammalian target of rapamycin (mTOR) (1:1000, #2983), P-mTOR (1:1000, #5356), P-4EBP1 (1:1000, 9451S) and P-S6K1 (1:1000, #9234) all purchased from Cell Signaling Technology; beta-actin (1:2000, bs-0061R) purchased from Beijing Bo Osen Biotechnology Co., Ltd.; Secondary antibodies: goat anti-rabbit IgG (1:5000, 511203) and goat Anti-mouse IgG (1:5000, 511103) purchased from ZenBio.

2.6. Oil red O staining of lipid droplets and triglyceride content

In this experiment, the oil red O staining method was used to detect the effect of different concentrations of SA on the synthesis of lipid droplets in cells. Specifically, cells were seeded into 24-well plates with a pipette and cultured as described above. The collected cells were rinsed 3 times with PBS and fixed with 4% paraformaldehyde for 30 min at room temperature, then oil red O solution was carefully added to the cells (150 μL per well). Samples were stained for 3 h under room temperature. Afterward, the staining solution was discarded and cells were washed once with 65% isopropanol and 5 times with PBS. Finally, 250 μL of distilled water was added, and pictures of samples were observed and taken under a 400× inverted microscope. Triglyceride Assay Kit (Jiancheng, Nanjing, China) was used to detect the content of triglycerides (TAG) in PMEC.

2.7. 16S rDNA sequencing

Microbiome analysis using 16S rDNA high-throughput sequencing technology after collecting ileal digesta from piglets on d 21 of lactation (performed by Gene Denovo [Guangzhou, China]). After genomic DNA was extracted from the sample, the V3 + V4 region of 16S rDNA was amplified with barcode-specific primers. The primer sequence is 341F: CCTACGGGNGGCWGCAG and 806R: GGACTACHVGGGTATCTAAT. Connect the purified amplification products (i.e., amplicons) to sequencing adapters to construct a sequencing library, which was sequenced on a NovaSeq 60000 (PE250, Illumina Inc., CA, United States). Finally, the 16S rDNA sequencing data were analyzed using the Omicsmart (Gene Denovo, Guangzhou, China) online tool.

2.8. Statistical analysis

Data from this study were analyzed based on a randomized complete block design using the MIXED procedure of SAS (Cary, NC). Treatment (control group and SA group) was the fixed effect and the parity was the block. The sows and their litters were used as the experimental units for the analysis of data of reproductive performance. The pre-weaning survival was analyzed using the Chi-square test. Other in vivo experimental data were analyzed by independent samples *t*-test. Data from in vitro experiments were processed using one-way ANOVA analysis. Composition and

diversity of microbial communities expressed as standardized operational taxonomic unit (OTU) reads were analyzed using R software (version 3.2.2; R Software Inc., Auckland, New Zealand). Analysis of relative abundance of phyla and order at different levels using the Kruskal–Wallis Test. All analysis results were expressed in the form of mean ± SEM, and $P < 0.05$ was used as the criterion of the significance of difference.

3. Results

3.1. Sow reproductive performance

As shown in Table 1, ADFI and backfat thickness of sows were not affected by maternal diets during the experiment ($P > 0.05$). Besides, dietary SA supplementation during gestation had no effect on total litter size, number of mummies, birth weight, litter weight, and the estrus interval after weaning ($P > 0.05$). Maternal SA numerically reduced the number of weak offspring by 51% compared to the control group.

3.2. Sodium acetate supplementation regulated yield and composition of colostrum and milk

As shown in Table 2, SA supplementation significantly increased colostrum ($P = 0.028$) and total milk yield ($P = 0.044$) of sows during lactation. Furthermore, we measured the colostrum and d 21 milk composition. The results showed that SA supplementation significantly increased the contents of colostrum fat ($P = 0.039$), colostrum crude protein (CP) ($P < 0.001$), colostrum lactose ($P = 0.012$) and colostrum solids-not-fat (SNF) ($P < 0.001$) and milk fat content on day 21 of lactation ($P = 0.015$).

3.3. Sodium acetate supplementation regulated Ig composition of plasma, colostrum and milk

As shown in Table 3, SA supplementation significantly increased the plasma IgA and IgG content of sows on the day of farrowing and the d 21 after farrowing ($P < 0.05$), but had no effect on IgM. Consistently, SA supplementation significantly increased IgA and IgG levels in colostrum and d 21 of milk ($P < 0.05$; Table 4). It is worth noting that IgG in the plasma of the offspring piglets was numerically increased both at born ($P = 0.086$) and weaning ($P = 0.054$) (Table S3).

Table 1
Effect of dietary sodium acetate (SA) supplementation on reproductive performance of sows.

Item	Treatment group[1]		SEM	*P*-value
	CON	SA		
Parity	3.43	3.40	0.16	0.912
Total litter size	13.88	13.65	0.61	0.797
Weak offspring	1.03	0.53	0.19	0.065
Stillbirths	0.80	0.55	0.14	0.222
Mummy	0.20	0.25	0.08	0.674
Live births	13.03	13.40	0.53	0.375
Litter weight, kg	18.82	19.55	0.77	0.506
Individual birth weight, kg	1.47	1.47	0.04	0.993
Weaning to estrus interval, d	4.08	4.23	0.11	0.325
ADFI, kg				
Week 1 of lactation	3.48	3.51	0.12	0.876
Week 2 of lactation	4.72	4.72	0.17	0.989
Week 3 of lactation	5.60	5.58	0.13	0.889
Lactation	4.60	4.60	0.10	0.991
Sow backfat thickness, mm				
Day 85 of gestation	18.60	18.98	0.60	0.663
Day 114 of gestation	17.73	18.18	0.66	0.629
Day 21 of lactation	16.33	17.00	0.62	0.445
Late pregnancy loss, mm	0.88	0.80	0.26	0.839
Lactation loss, mm	1.40	1.18	0.31	0.605

ADFI = average daily feed intake; SEM = standard error of the mean.
[1] CON, sows were fed a base diet; SA, sows were fed a basal diet supplemented with 0.1% sodium acetate ($n = 40$).

Table 2
Effect of dietary sodium acetate (SA) supplementation on yield and composition of colostrum and milk in sows.

Item	Treatment group[1]		SEM	*P*-value
	CON	SA		
Average piglet colostrum intake on d 1, g	444.00[b]	497.77[a]	12.34	0.025
Colostrum yield for 24 h, kg/d	5.46[b]	6.68[a]	0.28	0.028
Total milk yield, kg	200.72[b]	216.52[a]	3.94	0.044
Colostrum composition, %				
Fat	4.04[b]	4.77[a]	0.24	0.039
CP	6.98[b]	7.86[a]	0.15	<0.001
Lactose	3.34[b]	3.66[a]	0.07	0.012
SNF	18.56[b]	20.94[a]	0.39	<0.001
Milk on d 21 composition, %				
Fat	6.96[b]	7.46[a]	0.14	0.015
CP	4.60	4.58	0.02	0.539
Lactose	6.51	6.48	0.04	0.665
SNF	11.98	11.93	0.07	0.604

CP = crude protein; SNF = solids-not-fat; SEM = standard error of the mean.
[a,b] Different letters indicate significant differences between the 2 data.
[1] CON, sows were fed a base diet; SA, sows were fed a basal diet supplemented with 0.1% sodium acetate ($n = 6$).

Table 3
Effect of dietary sodium acetate (SA) supplementation on plasma immunoglobulin and prolactin levels of sows.

Item	Treatment group[1]		SEM	*P*-value
	CON	SA		
Farrowing				
IgA, mg/mL	0.41[b]	1.09[a]	0.08	<0.001
IgG, mg/mL	1.48[b]	2.01[a]	0.15	0.032
IgM, mg/mL	0.14	0.15	0.02	0.692
Prolactin, ng/mg	4.99[b]	5.87[a]	0.22	0.021
Day 21 after farrowing				
IgA, mg/mL	0.50[b]	1.31[a]	0.07	<0.001
IgG, mg/mL	0.77[b]	1.41[a]	0.13	0.006
IgM, mg/mL	0.41	0.44	0.04	0.536
Prolactin, ng/mg	4.78[b]	6.43[a]	0.35	0.008

IgA = immunoglobulin A; IgG = immunoglobulin G; IgM = immunoglobulin M; SEM = standard error of the mean.
[a,b] Different letters indicate significant differences between the 2 data.
[1] CON, sows were fed a base diet; SA, sows were fed a basal diet supplemented with 0.1% sodium acetate ($n = 6$).

Table 4
Effect of dietary sodium acetate (SA) supplementation on immunoglobulin levels in colostrum and milk of sows.

Item	Treatment group[1]		SEM	*P*-value
	CON	SA		
Colostrum				
IgA, mg/mL	3.61[b]	5.25[a]	0.46	0.032
IgG, mg/mL	20.50[b]	27.10[a]	1.60	0.034
IgM, mg/mL	1.27	1.37	0.16	0.718
Day 21 of milk				
IgA, mg/mL	0.32[b]	0.42[a]	0.03	0.038
IgG, mg/mL	1.05[b]	1.70[a]	0.16	0.020
IgM, mg/mL	0.12	0.13	0.01	0.582

IgA = immunoglobulin A; IgG = immunoglobulin G; IgM = immunoglobulin M; SEM = standard error of the mean.
[a,b] Different letters indicate significant differences between the 2 data.
[1] CON, sows were fed a base diet; SA, sows were fed a basal diet supplemented with 0.1% sodium acetate (n = 6).

3.4. Sodium acetate regulated milk fat synthesis and activated mTORC1 pathway in milk-derived cells isolated from sow milk

As shown in Fig. 1A and B, SA supplementation significantly increased ($P < 0.05$) the expression of milk fat synthesis-related proteins (FASN, ACACA and DGAT1). Compared with the control group, the addition of SA significantly activates ($P < 0.05$) mTORC1 signaling pathway (as indicated by the increased level of P-mTOR/mTOR, P-S6K1/S6K1 and P-4EBP1/4EBP1) (Fig. 1C and D). Furthermore, SA increased the expression of SCFA receptors (GPR41 and

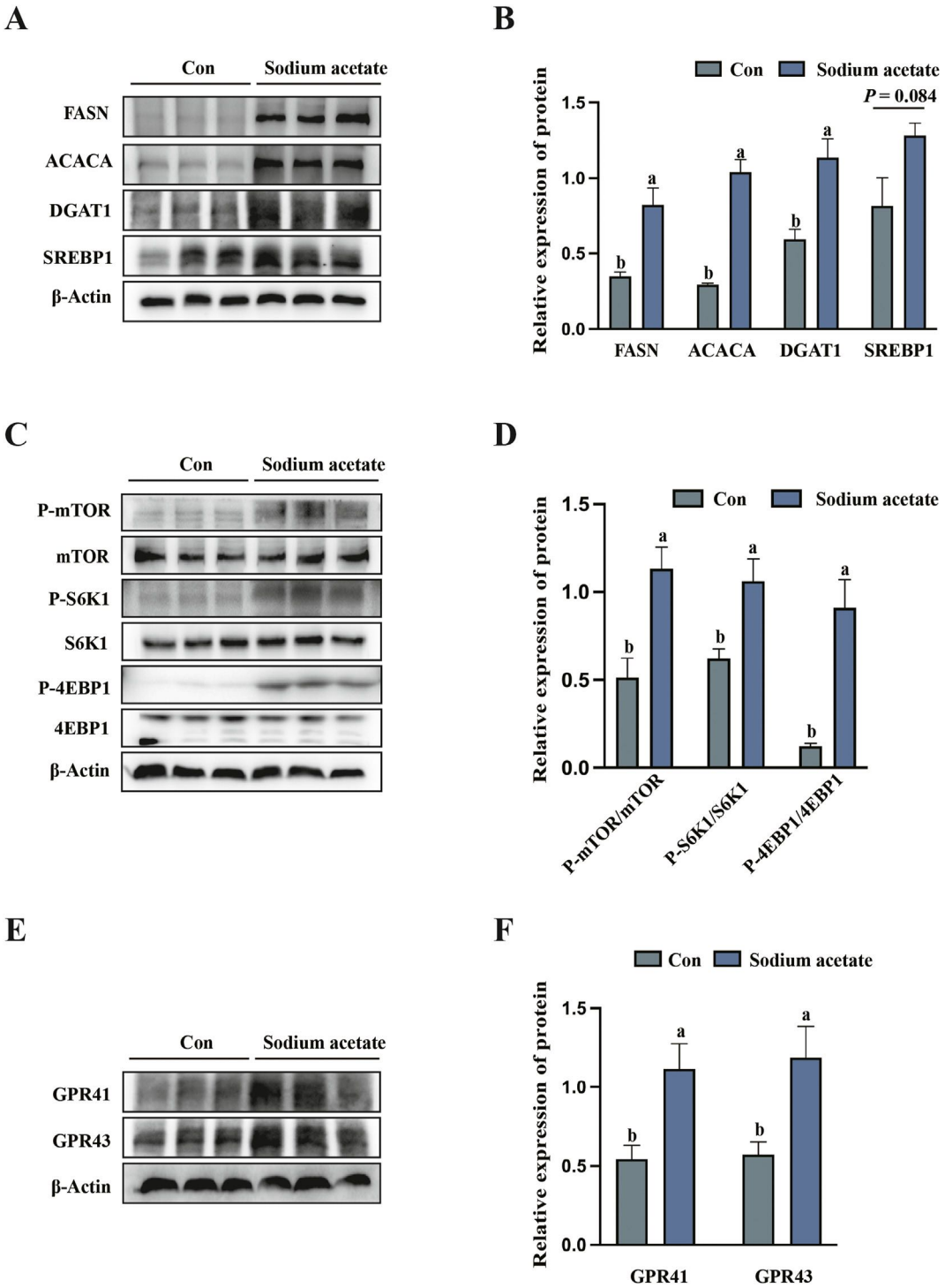

Fig. 1. Sodium acetate (SA) addition promotes the expression of milk fat synthesis-related proteins in milk-derived cells, accompanied by activation of GPR41/43-mTORC1 signaling pathway. (A and B) Relative protein expression abundance of milk fat synthesis-related genes in milk-derived cells (FASN = fatty acid synthase; ACACA = acetyl-CoA carboxylα; DGAT1 = diacylglycerol O-Acyltransferase 1; SREBP1 = sterol-regulatory element binding protein1). (C and D) Phosphorylation of mTORC1 pathway-related target proteins in milk derived cells (mTORC1 = mammalian target of rapamycin complex C1; S6K1 = ribosomal protein S6 kinase 1; 4EBP1 = 4E-binding protein 1). (E and F) Relative protein expression abundance of GPR41 and GPR43 receptors in milk-derived cells. All data with error bars are averages ± SEM ($n = 3$). In histograms, no letter or the same letter above the bar indicates no significant difference ($P > 0.05$), and different letters indicate significant difference ($P < 0.05$).

Table 5
Effect of dietary sodium acetate (SA) supplementation on lactation performance of sows and growth performance of piglets.

Item	Treatment group[1]		SEM	*P*-value
	CON	SA		
Litter size				
After cross-foster				
Day 7	11.95	11.70	0.15	0.249
Day 14	11.84	11.52	0.14	0.126
Day 21	11.66	11.29	0.16	0.070
Pre-weaning survival, %	98.7	97.7	0.004	0.235
Litter weight				
After cross-foster				
Day 7	34.28	34.08	0.96	0.885
Day 14	49.52	51.66	1.25	0.231
Day 21	70.70	73.36	1.61	0.248
Piglet BW [2], kg				
Day 7	2.87	2.91	0.07	0.713
Day 14	4.16[b]	4.56[a]	0.11	0.013
Day 21	6.04[b]	6.62[a]	0.15	0.007
Piglet ADG [2], g/d				
Week 1	163.99	170.67	5.71	0.411
Week 2	195.94[b]	224.65[a]	7.02	0.028
Week 3	268.49[b]	294.31[a]	11.25	0.036
Piglet ADG during lactation	209.47[b]	229.87[a]	6.29	0.025

BW = body weight; ADG = average daily weight gain; SEM = standard error of the mean.
[a,b] Different letters indicate significant differences between the 2 data.
[1] CON, sows were fed a base diet; SA, sows were fed a basal diet supplemented with 0.1% sodium acetate ($n = 40$).
[2] The weight per litter was divided by the number of pigs to calculate piglet BW and then piglet ADG, $n = 40$.

GPR43) in milk-derived cells (Fig. 1E and F), which indicates SA might regulate through GPR41/GPR43-mTORC1.

3.5. Maternal sodium acetate supplementation modulates offspring growth performance, gut health and intestinal microbiota

As indicated by Table 5, the addition of SA had no effect on litter size and survival rate at weaning ($P > 0.05$). However, there was a significant increase on piglets' BW at the second week of born ($P = 0.013$), weaning BW ($P = 0.007$) and ADG during lactation ($P = 0.025$).

In duodenum (Fig. 2A and B), the VCR value was significantly increased in SA group compared with the control group ($P < 0.05$). In jejunum (Fig. 2C and D), VCR was significantly increased in SA group compared with the control group ($P < 0.05$). Furthermore, in the duodenum, SA addition significantly upregulated the protein expression of tight junctions such as ZO-1, occludin and cladin-1 (Fig. 3A and B). In the jejunum (Fig. 3C and D) and ileum (Fig. 3E and F), the addition of SA significantly increased the tight junction protein expression of ZO-1 and occludin ($P < 0.05$).

A total of 1,273,031 valid sequences were generated from 10 ileal content samples (collected from piglets at d 21 of lactation) (2 treatments, $n = 5$), with an average of 127,303 sequences per sample and obtained 9,925 OTU (97% similarity) after removing noise sequences. The Venn diagram was used to analyze and compare the common and unique species of each group, so as to get a preliminary understanding of the species composition characteristics between the groups. As shown in Fig. 4A, there were 252

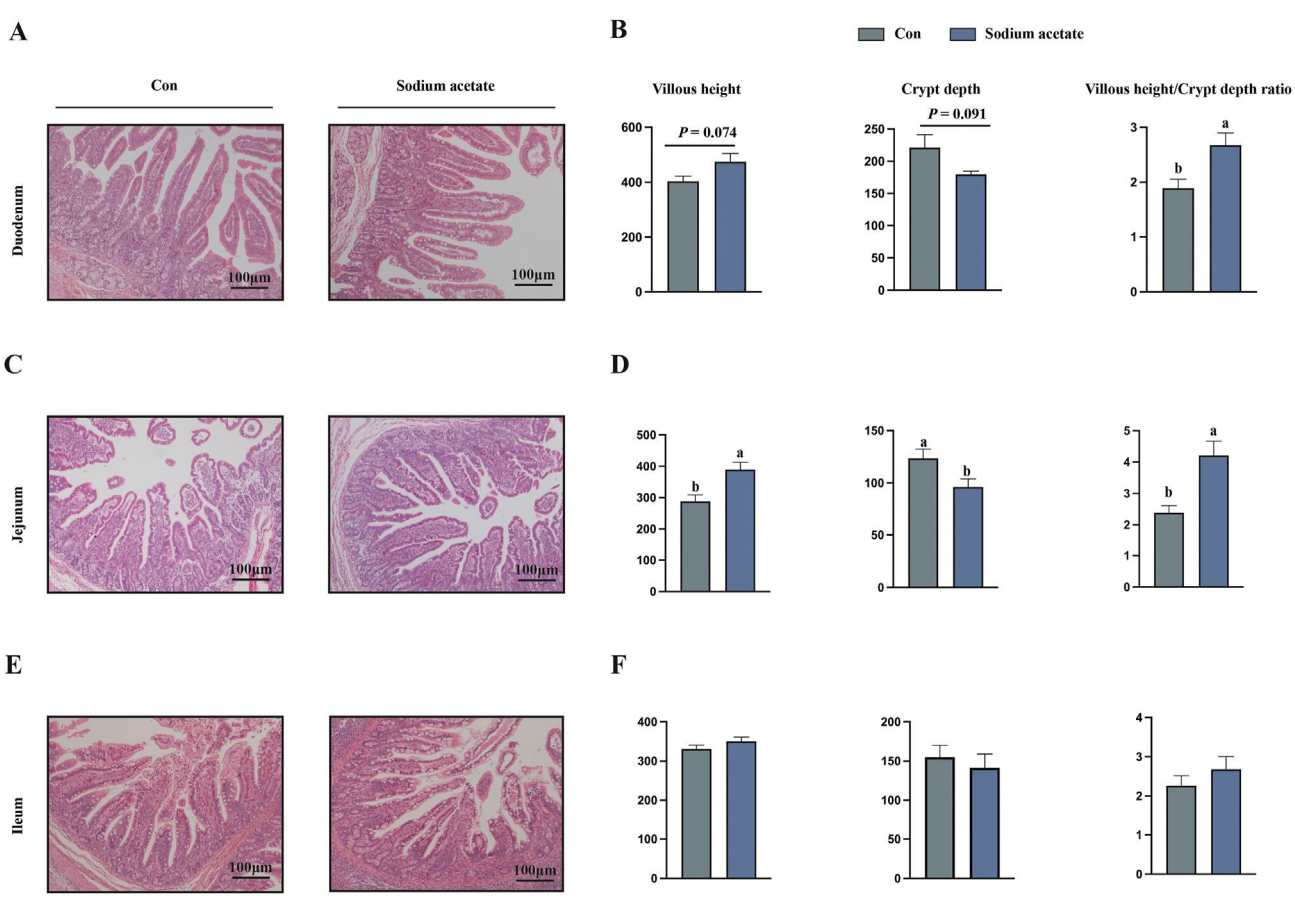

Fig. 2. Effects of maternal sodium acetate (SA) addition supplementation on intestinal morphology of piglets. (A, C and E) H&E staining of duodenum, jejunum and ileum. (B, D and F) Villous height, crypt depth, and ratio of villous height to crypt depth of the intestine. All data with error bars are averages ± SEM ($n = 6$). In histograms, no letter or the same letter above the bar indicates no significant difference ($P > 0.05$), and different letters indicate significant difference ($P < 0.05$).

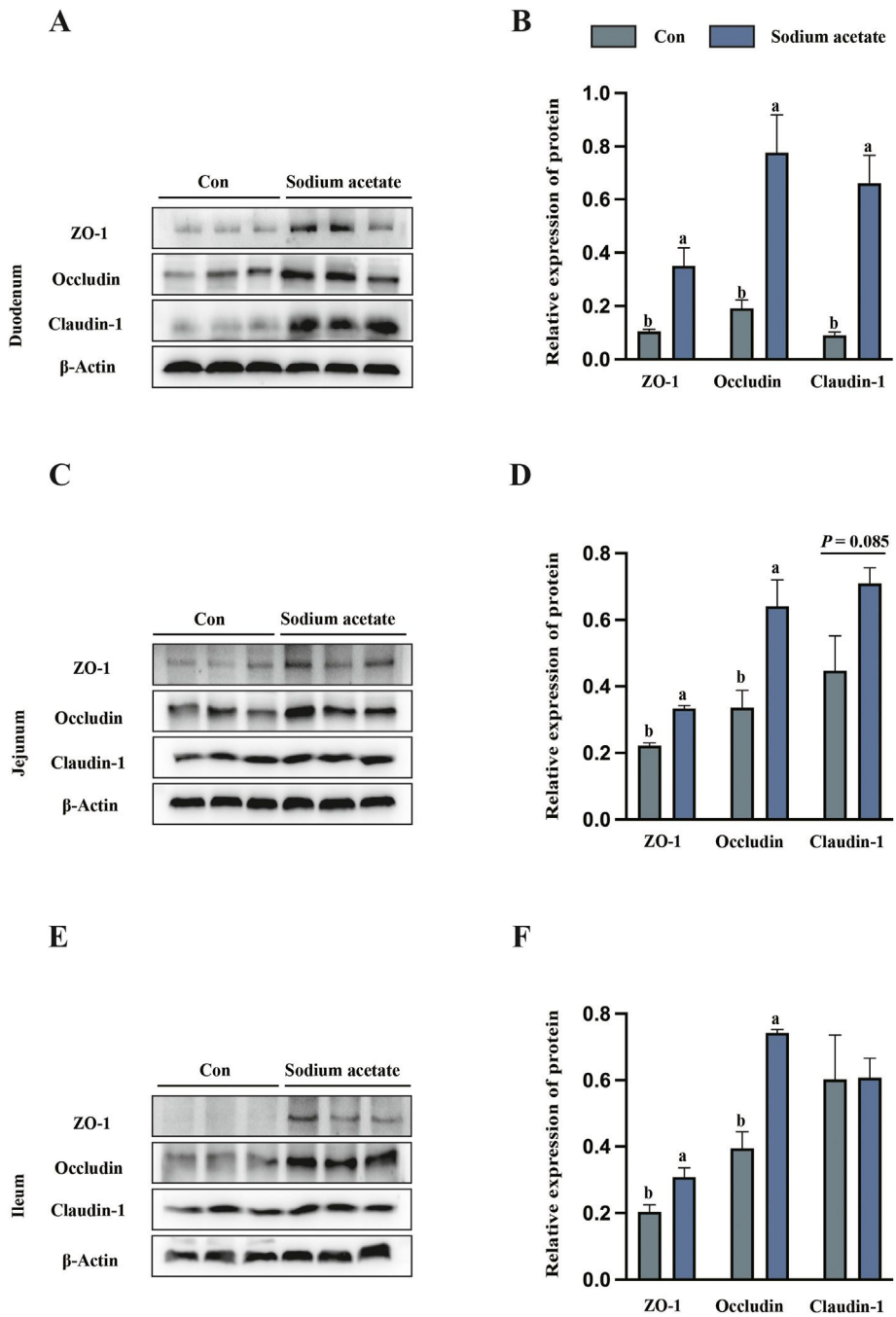

Fig. 3. Maternal sodium acetate (SA) addition supplementation promotes the expression of tight junctions in piglet intestines. (A and B) The relative expression of tight junction proteins in duodenum. (C and D) The relative expressions of tight junction proteins in jejunum. (E and F) The relative expression of tight junction proteins in ileum. All data with error bars are averages ± SEM ($n = 3$). In histograms, no letter or the same letter above the bar indicates no significant difference ($P > 0.05$), and different letters indicate significant difference ($P < 0.05$).

and 243 elements unique to the control and SA groups, respectively. The alpha diversity of the ileal microflora indicated by Shannon (Fig. 4B). Compared with the control group, there was no significant difference in alpha diversity in the SA group. As shown by the beta diversity results (Fig. 4C), there was a trend to separate between control group and SA group by PCoA analysis based on Bray–Curtis distance, which shows the similarity between samples to find the main sample difference distance in complex samples. At the phylum level (Fig. 4D), the 4 most common bacteria in the ileal contents of piglets are: Firmicutes (45.37% to 50.58%), Bacteroidetes (40.10% to 32.72%), Euryarchaeota (4.20% to 5.88%) and Proteobacteria (3.71% to 4.77%). At the order level (Fig. 4E), the 5 most common bacteria in the ileal contents of piglets are: Bacteroidales (39.76% to 32.64%), Clostridiales (33.61% to 36.95%), Selenomonadales (7.33% to 6.34%), Methanobacteriales (4.17% to 5.86%) and Lactobacillales (1.59% to 5.29%). Within the changes in Firmicutes, the most influential at the order level were Clostridiales and Lactobacillales. The proportion of genus level is shown in Fig. 4F, among them we screened 2 bacteria with the most obvious differences ($P < 0.05$) are *Anaerovibrio* and *Subdoligranulum* (Fig. 4G).

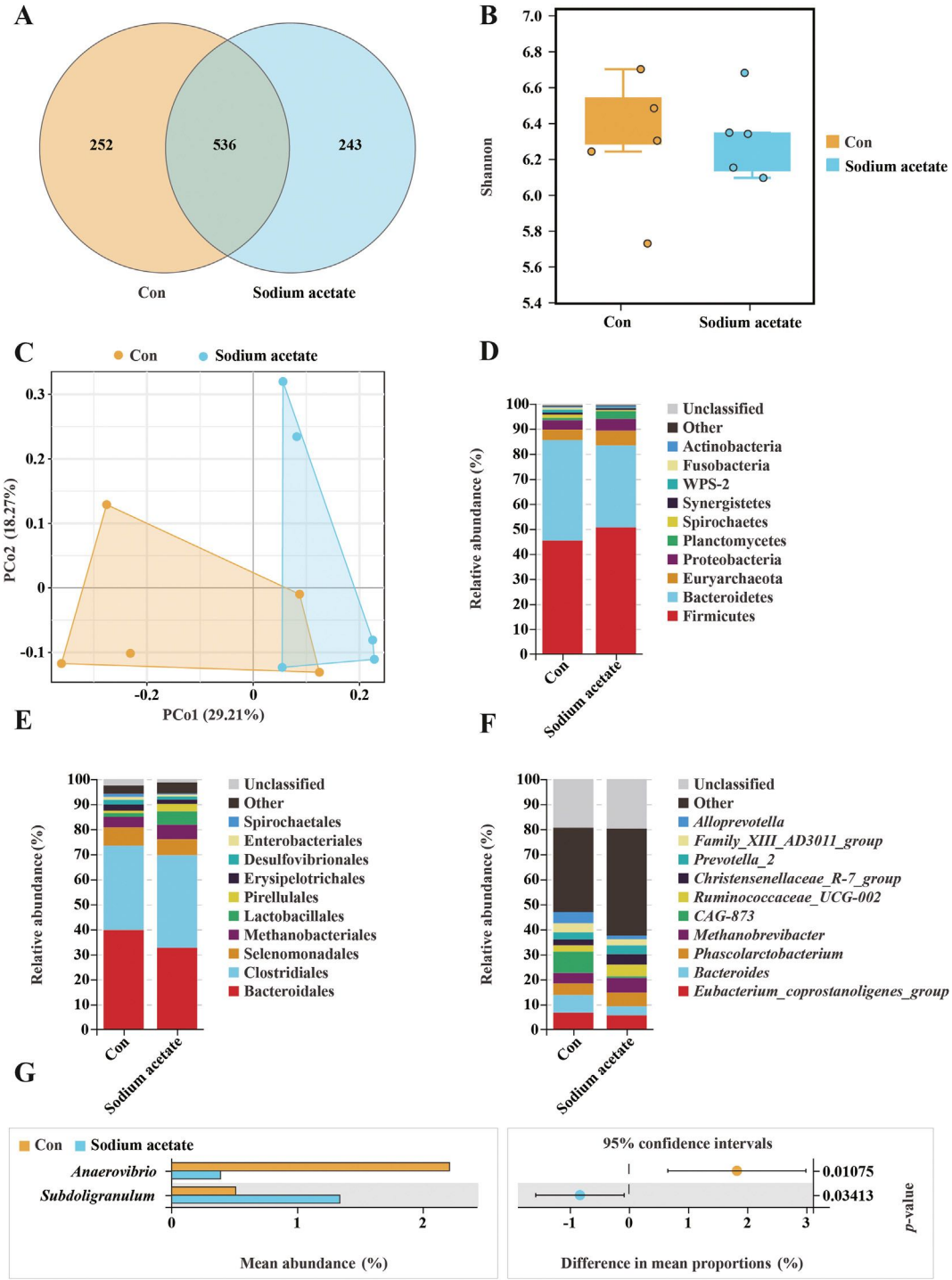

Fig. 4. Maternal sodium acetate (SA) addition supplementation affects intestinal microbes of piglets, $n = 5$. (A) Venn graph of gut microbes in the 2 groups. (B) Alpha diversity index: Shannon. (C) Principal coordinate analysis based on Bray–Curtis distance. (D) Relative abundance at the phylum level. (E) Relative abundance at the order level. (F) Relative abundance at the genus level. (G) Differential bacteria in genus.

3.6. Sodium acetate regulated milk fat synthesis and activated mTORC1 signaling pathway in PMEC in vitro

To further verify the effect of SA on milk fat synthesis, PMEC were used as an in vitro model. PMEC were treated with different concentrations of SA (0, 0.25, 0.5, 0.75, 1.0 and 1.5 mM) for 24 h. The results indicated 0.75 mM SA efficiently promoted the synthesis of lipid droplets ($P < 0.05$; Fig. 5A) and TAG content ($P < 0.05$; Fig. 5B). Furthermore, 0.75 mM SA significantly upregulated the mRNA expression abundance of *FASN, ACACA, FABP3* and *SREBP1* genes in PMEC ($P < 0.05$; Fig. 5C). Consistently, as shown in Fig. 5D and E, 0.75 mM SA also upregulated the protein expression of ACACA, FABP3 and DGAT1 ($P < 0.05$). Phosphorylation level of mTOR, S6K1 and 4EBP1 was also upregulated under the treatment of 0.75 mM SA ($P < 0.05$; Fig. 5F and G), which indicates the activation of mTORC1 signaling pathway.

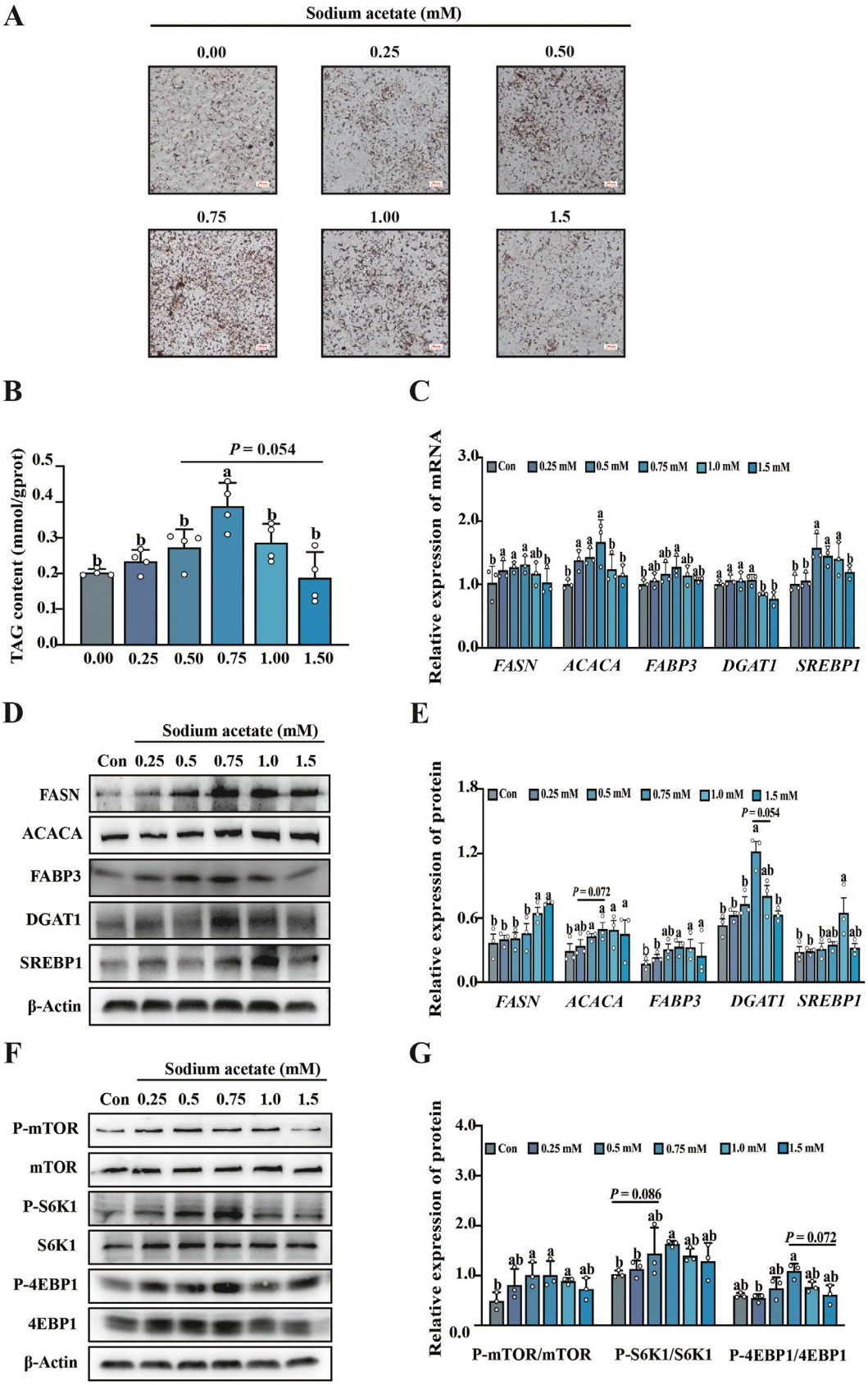

Fig. 5. Sodium acetate (SA) addition promoted milk fat synthesis in porcine mammary epithelial cells (PMEC) and was accompanied by activation of mTORC1 signaling pathway. Treatment of PMEC with different concentrations (0, 0.25, 0.5, 0.75, 1.0 and 1.5 mM) of SA. (A) Oil red O staining images of PMEC. Scale bar is 100 μm. (B) The content of TAG in PMEC after treatment with different concentrations of SA (TAG = triacylglycerol). (C) mRNA abundance of milk fat synthesis-related genes in PMEC (*FASN* = fatty acid synthase; *ACACA* = acetyl-CoA carboxylα; *FABP3* = fatty acid binding protein 3; *DGAT1* = diacylglycerol O-Acyltransferase 1; *SREBP1* = sterol-regulatory element binding protein1). (D and E)

4. Discussion

Maternal milk yield and composition directly affect early growth and development of piglets. Previously, addition of SA has been reported to significantly increase milk yield in ruminants, such as in dairy cows (Aii et al., 1990). In this study, we first reported that maternal SA supplementation in monogastric animals (sows) also promotes maternal milk yield and milk fat.

Milk fat is an important nutrient component in milk, which provides a large amount of energy for early mammalian growth and development (Zhang et al., 2018). Sodium acetate is the main product of gastrointestinal bacterial fermentation of dietary fiber (Dalile et al., 2019). As a critical substrate for milk fat synthesis, SA efficiently promotes the synthesis of TAG in bovine mammary epithelial cells (Sheng et al., 2016) with upregulation of the expression abundance of de novo fatty acid synthesis genes (*ACACA, FASN*) and transcriptional regulation genes involved in lipid synthesis (*SREBP1*) (Zhao et al., 2021). Consistently, dietary supplementation of SA also increases plasma SA content and significantly increases milk fat content mainly by increasing the production of de novo fatty acids in the mammary gland in ruminants (Matamoros et al., 2021). Similarly, in our experiments, maternal SA supplementation significantly increased the milk fat content and the expression abundance of milk fat synthesis-related proteins (ACACA, FASN, DGAT) in milk-derived cells of sows. Although SA increased the milk fat synthesis both in ruminants and monogastric animals, the underlying mechanism might not be the same. It should be noted that the concentration of SCFA in the blood of sows is much lower than that in dairy cows (Urrutia and Harvatine, 2017). Therefore, in sows, the proportion of SCFA act as substrates that are directly involved in fat synthesis is relatively small. In monogastric animals, SA can be recognized by mammary epithelial cell-specific receptors as a signaling molecule, thereby regulating process of milk synthesis.

G protein coupled receptors are the largest family of cell membrane receptors, which involved in the regulation of multiple cellular and physiological functions (Wettschureck and Offermanns, 2005). G protein coupled receptors directly binds to G proteins in the membrane, which is composed of 3 subunits, including G_{α} (bound to GTP or GDP), G_{β} and G_{γ} (Reimann et al., 2012; Tomita et al., 2014). G_{α} subunit is consisted of $G_{\alpha i}$, $G_{\alpha s}$, $G_{\alpha q}$ and $G_{\alpha 12/13}$. GPR41 and GPR43 have been identified as SCFA receptors in recent years. GPR43 is coupled to $G_{\alpha i}$ and $G_{\alpha q}$, while GPR41 only coupled to $G_{\alpha i}$ (Flodgren et al., 2007; Tian et al., 2022b). It is worth noting that, the activity of mTORC1 is inhibited after the specific activation of $G_{\alpha s}$ by AKAP13, which implies that activation of $G_{\alpha i}$ can inversely increase the activity of mTORC1 (Zhang et al., 2021). The mTORC1 signaling pathway is not only involved in the regulation of protein translation, but also an important regulator of lipid anabolism (Soliman, 2013). Furthermore, mTORC1 signal pathway has been reported to regulate mammary gland development, protein translation and milk fat synthesis of dairy cows (Düvel et al., 2010; Wang et al., 2014). Importantly, in bovine mammary epithelial cells, sodium butyrate induced the increase of milk fat synthesis and activation of mTORC1 signaling was abolished when *GPR41* was knocked out (Cheng et al., 2020). In this study, addition of SA significantly increased the expression of GPR41 and GPR43 proteins, accompanied by the activation of the mTORC1 signaling pathway. This evidence suggests that SA might promote milk fat synthesis in sows by activating the GPR41/43-mTORC1 pathway, which still requires further investigation.

Sows lactation performance is closely related to piglets growth performance (Hojgaard et al., 2020). In this study, maternal SA supplementation-induced increases of ADG and weaning BW in piglets during lactation are partially attributed to the increase in milk synthesis. Previously, sows milk production and milk IgA content have been reported to promote weaning BW, ADG and intestinal health of piglets (Rezaei et al., 2022; Shang et al., 2019). In addition, sufficient milk fat intake could prevent neonates from gastrointestinal disease (Brink and Lönnerdal, 2020; Koopman et al., 1984). Intestinal morphology directly reflects the development and health of gut (Yi et al., 2021). The intestinal barrier of piglets is mainly composed of mechanical barrier, immune barrier and biological barrier (Tian et al., 2022a). As the first physical barrier, tight junctions are the most important connections between cells, which are primarily composed of claudin 1, occludin, and ZO-1 (Suzuki, 2013). Tight junctions only allow soluble and small molecular substances to pass through, hindering the passage of macromolecular substances and microorganisms, thus ensuring homeostasis (Lee, 2015). Consistent with the growth performance of piglets, maternal SA supplementation increased the VCR values and promoted the expression abundance of tight junction proteins in small intestine of piglets.

Intestinal microorganisms play a crucial role to maintain the health and growth of piglets. Milk has been identified as a crucial factor contributing to the establishment of intestinal microbial community (Gresse et al., 2017). Previously, it has been demonstrated that milk fat content can increase the proportion of Firmicutes in the ileal microbes of piglets, which is consistent with the finding in this study (Thum et al., 2020). In our experiments, the increased abundance of Firmicutes in the piglet gut was mainly due to the increase in Clostridiales and Lactobacillales in order level. Clostridiales are negatively relative to inflammatory bowel disease (Baumgart et al., 2007), while Lactobacillales are widely considered as beneficial bacteria that contribute to the maintenance of normal intestinal function and piglet BW increase (Li et al., 2018). At the genus level, *Anaerovibrio* was significantly decreased while *Subdoligranulum* was significantly increased in the SA group. *Anaerovibrio* was associated with intestinal epithelial damage, tissue inflammation, and leaky gut (Rocafort et al., 2019). However, *Subdoligranulum* abundance was positively correlated with microbial abundance and negatively correlated with interleukin-6 level (Van Hul et al., 2020).

5. Conclusion

In conclusion, maternal supplementation of 0.1% SA from late gestation to lactation (D85 to L21) significantly increased sows' milk production, milk fat and immunoglobulins, which might attribute to the activation of GPR41/43-mTORC1 signaling pathway. An improvement in milk quality further promoted growth performance, gut health and the colonization of beneficial microbial flora of their piglets.

Author contributions

Yingao Qi, **Shihai Zhang** and **Wutai Guan**: designed the study. **Yingao Qi**, **Tenghui Zheng**, **Yongxing Zhong** and **Siwang Yang**: conducted research. **Yingao Qi** and **Qianzi Zhang**: analyzed the

Relative protein expression abundance associated with milk fat synthesis in PMEC (FASN = fatty acid synthase; ACACA = acetyl-CoA carboxylα; FABP3 = fatty acid binding protein 3; DGAT1 = diacylglycerol O-acyltransferase 1; SREBP1 = sterol-regulatory element binding protein1). (F and G) Phosphorylation levels of mTORC1 signaling pathway-related target proteins in PMEC (mTORC1 = mammalian target of rapamycin complex C1; S6K1 = ribosomal protein S6 kinase 1; 4EBP1 = 4E-binding protein 1). All data with error bars are averages ± SEM ($n = 3$). In histograms, no letter or the same letter above the bar indicates no significant difference ($P > 0.05$), and different letters indicate significant difference ($P < 0.05$).

data. **Yingao Qi** and **Baofeng Li**: wrote the manuscript. **Xiangfang Zeng** and **Fang Chen**: critically reviewed the manuscript.

Declaration of competing interest

We declare that we have no financial and personal relationships with other people or organizations that can inappropriately influence our work, and there is no professional or other personal interest of any nature or kind in any product, service and/or company that could be construed as influencing the content of this paper.

Acknowledgment

This study was financially supported by the National Key R&D Program of China (2021YFD1300700), Guangdong Basic and Applied Basic Research Foundation (2021A1515010440 and 2023A1515012098), Science and Technology Program of Guangzhou (202102020056). We thank the members of our laboratory for their assistance during the animal experiment and sample collection.

Appendix supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.aninu.2023.04.003.

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Cite this: *Food Funct.*, 2023, **14**, 2642

Niacin/β-hydroxybutyrate regulates milk fat and milk protein synthesis *via* the GPR109A/G_i/mTORC1 pathway

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Received 9th January 2023,
Accepted 8th February 2023

DOI: 10.1039/d3fo00127j

rsc.li/food-function

As a crucial receptor of BHBA and niacin, GPR109A is largely expressed in the mammary gland. However, the role of GPR109A in milk synthesis and its underlying mechanism is still largely unknown. In this study, we first investigated the effect of GPR109A agonists (niacin/BHBA) on milk fat and milk protein synthesis in a mouse mammary epithelial cell line (HC11) and PMECs (porcine mammary epithelial cells). The results showed that both niacin and BHBA promote milk fat and milk protein synthesis with the activation of mTORC1 signaling. Importantly, knockdown GPR109A attenuated the niacin-induced increase of milk fat and protein synthesis and the niacin-induced activation of mTORC1 signaling. Furthermore, we found that GPR109A downstream G protein-$G_{\alpha i}$ and -$G_{\beta\gamma}$ participated in the regulation of milk synthesis and the activation of mTORC1 signaling. Consistent with the finding *in vitro*, dietary supplementation with niacin increases milk fat and protein synthesis in mice with the activation of GPR109A-mTORC1 signaling. Collectively, GPR109A agonists promote the synthesis of milk fat and milk protein through the GPR109A/G_i/mTORC1 signaling pathway.

Introduction

Milk is a predominant nutrient source for neonates, which contains a large number of macronutrients like protein, lipid, lactose *etc.*[1,2] During lactation, maternal milk not only delivers nutrients to offspring, but also provides sufficient immunoglobulins to establish protective immunity.[3] The milk protein is mainly composed of casein and whey protein, the synthesis of which is mediated by the availability of amino acids and the activation of mTORC1 (the mechanistic target of rapamycin complex 1) signaling.[4] Triglycerides (TAGs) are the main component of milk lipids. Fatty acids attached to glycerol are either derived from *de novo* synthesis or transported from dietary fatty acids.[5] Increasing milk production by dietary nutrition is an efficient way to promote the growth of neonates.

G protein-coupled receptors (GPCRs) are classified as the members of the largest families of membrane proteins in mammals,[6,7] which are involved in the regulation of multiple physiological processes,[8,9] such as immunity, inflammation, and energy balance.[10,11] As a receptor for niacin and β-hydroxybutyrate,[12] GPR109A is widely expressed in the gut and has been reported to suppress colitis and colon cancer,[13] alleviate lipid disorders,[14] and suppress the progression of atherosclerosis.[15] Intriguingly, GPR109A is also highly expressed in the mammary gland.[16] β-Hydroxybutyrate (BHBA), a GPR109A agonist, has been reported to regulate dairy milk production. As a main component of ketone bodies,[17] BHBA can be converted from acetoacetate in the liver, which further increases milk TAG secretion through the activation of SREBP1 (sterol regulatory element binding protein 1).[18] In addition, the other GPR109A agonist niacin also increases the proportion of milk oleic acid in mid-lactation cows.[19] The GPR109A agonist has been proven to promote milk synthesis in dairy cows, whereas it is unclear whether niacin and BHBA are involved in the regulation of milk synthesis in monogastric animals.

The activation of the GPR109A receptor leads to the dissociation of $G_{\alpha i}$ and $G_{\beta\gamma}$ subunits, which further activates

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downstream effector proteins and mediates a series of biochemical responses.[20] And G_i contains $G_{\alpha i}$ and $G_{\beta\gamma}$. The $G_{\alpha i}$ subunit inhibits the adenylate cyclase activity to downregulate cAMP production.[21] Recently, cAMP has been found to be a critical regulator of the mammalian target of rapamycin C1 (mTORC1),[22] which has been reported to regulate protein and lipid biosynthesis through its downstream effectors,[23] such as S6K1 (ribosomal protein S6 kinase beta 1), 4EBP1 (eIF4E-binding protein)[24] and SREBP1.[25] In this study, we found that GPR109A agonists (niacin/BHBA) promote milk fat and milk protein synthesis in mammary epithelial cells of monogastric animals. Furthermore, we demonstrated that the GPR109A/G_i/mTORC1 signaling pathway participated in the regulation of niacin/BHBA-induced milk synthesis.

Materials and methods

Cell culture

The mouse mammary epithelial cell line (HC11) and the porcine mammary epithelial cells (PMECs) were grown to confluency in DMEM/F12 medium with 10% fetal bovine serum, 5 μg mL^{-1} insulin, 10 ng mL^{-1} epidermal growth factor, and 10 μg ml^{-1} penicillin–streptomycin in a cell incubator at 37 °C under a 5% CO_2 atmosphere. To differentiate mammary epithelial cells, confluent cells were grown for 24 h in a medium without EGF supplementation. Subsequently, the mammary epithelial cells were cultured in DIP medium (1 μM dexamethasone, 5 μg ml^{-1} insulin, and 5 μg ml^{-1} prolactin) for the indicated time under the following experiments.

Experiment I

This experiment is used to detect the effect of BHBA on milk protein and fat synthesis in HC11 cells. The HC11 cells were cultured at different concentrations (0, 0.5, 1, 2, 4, and 8 mM) of BHBA for 24 h, $n = 3$. BHBA doses were selected according to their physiological concentration. Then the cell lysate and medium were collected to measure the TAG content. In addition, the cell lysate is used to detect the cell viability, and the mRNA and protein expressions of α-casein, β-casein, WAP (whey acidic protein), GPR109A, FASN (fatty acid synthase), ACACA (acetyl-CoA carboxylase α), DGAT1 (diacylglycerol acyltransferase 1), SREBP1, PPARγ (peroxisome proliferator-activated receptor gamma), FABP4 (fatty acid binding protein 4), and β-actin.

Experiment II

This experiment is used to detect the effect of niacin on milk protein and fat synthesis in HC11 cells. The HC11 cells were cultured at different concentrations (0, 0.5, 1, 2, 4, and 8 mM) of niacin for 24 h, $n = 3$. Niacin doses were selected according to their physiological concentration. Then the cell lysate and medium were collected to measure the TAG content. In addition, the cell lysate is used to detect the cell viability, and the mRNA and protein expressions of α-casein, β-casein, WAP, GPR109A, FASN, ACACA, DGAT1, SREBP1, PPARγ, FABP4, and β-actin.

Experiment III

This experiment is used to detect the effect of BHBA or niacin on the mTOR pathway in HC11 cells. The HC11 cells were cultured in 2 mM BHBA and 1 mM niacin for 24 h, respectively, $n = 3$. Then the cell lysate was collected to measure the phosphorylation of mTOR, S6K1 4EBP1 and β-actin.

Experiment IV

To explore the potential role of mTOR in milk protein and milk fat synthesis, rapamycin (RAPA) was used to inhibit the mTOR pathway. The HC11 cells were pretreated with 50 nM RAPA for 2 h, and then the cells were cultured in a medium containing 1 mM niacin. Subsequently, the cells were collected to measure the TAG content, the RNA and protein expressions of α-casein, β-casein, WAP, GPR109A, FASN, ACACA, FABP4, and β-actin, and the phosphorylation of mTOR, S6K1 and 4EBP1.

Experiment V

To reveal the potential role of GPR109A in the synthesis of milk protein and milk fat, GPR109A siRNA was used to interfere with the expression of GPR109A. The HC11 cells were pretreated with 20 μM GPR109A siRNA for 2 h, and then the cells were cultured in a medium containing 1 mM niacin for 24 h. Subsequently, the cells were collected to measure the TAG content, the mRNA and protein expressions of α-casein, β-casein, WAP, GPR109A, FASN, ACACA, FABP4, and β-actin, and the phosphorylation level of mTOR, S6K1, and 4EBP1.

Experiment VI

The GPR109A subunits (G_i, $G_{\beta\gamma}$ or $G_{\alpha i}$) were suppressed using various inhibitors to further clarify how GPR109A regulates milk synthesis. The HC11 cells were pretreated with 10 nM PTX (Pertussis toxin) (an inhibitor of G_i), or 10 nM Gallein (an inhibitor of $G_{\beta\gamma}$), or 10 nM NF023 (an inhibitor of $G_{\alpha i}$) for 2 h, respectively. Then the cells were treated with 1 mM niacin. After treatment, the cells were collected to measure the TAG content, the mRNA and protein expressions of α-casein, β-casein, WAP, GPR109A, FASN, ACACA, FABP4, and β-actin, and the phosphorylation of mTOR, S6K1, and 4EBP1.

Experiment VII

This experiment is used to detect the effect of BHBA or niacin on milk protein and fat synthesis in PMEC cells. The PMECs were cultured at different concentrations (0, 0.5, 1, 2, 4, and 8 mM) of BHBA or niacin for 24 h, respectively, $n = 3$. Then the cell lysate was collected to measure the TAG content, the cell viability, and the mRNA and protein expressions of α-casein, β-casein, WAP, GPR109A, and β-actin.

Animals and *in vivo* study

All animal procedures were performed in accordance with the Guidelines for Care and Use of Laboratory Animals of South

China Agricultural University and the experiments were approved by the Animal Subjects Committee of South China Agricultural University. C57BL/6 female mice (6 weeks old) were purchased from the Guangdong Medical Laboratory Animal Center and housed under a 12 h light–dark cycle at 24 °C. They had *ad libitum* access to food and water. From day 7 to 11 of lactation, twelve mice were randomly divided into control and niacin groups. A pre-experiment was conducted to determine the optimal dose of niacin in mice to regulate lactation performance (25 mM niacin in drinking water). Thus, in this trial, the control group is treated with normal water, while the niacin group is treated with 25 mM niacin in drinking water.[26] The body weight records of dams and litter were collected on days 7 and 11 of lactation. Blood was collected retro-orbitally in a 1.5 ml centrifuge tube, and then the mice were sacrificed after blood collection. Parts of the mammary glands were fixed and used as an H&E staining material. The remaining mammary glands were frozen in a −80 °C freezer for future analysis.

Cell viability assay

2×10^4 HC11 cells were inoculated on a 96-well plate and cultured in DMEM/F12 medium containing 10% FBS for 48 hours. After washing the cells three times with PBS, different concentrations of niacin or BHBA were used to culture the cells for 24 hours. Cell Counting Kit-8 (HY-K0301, Medchemexpress, China) was used to detect the cell viability according to the instructions. The wavelengths of all holes were measured at 450 nm using an automatic microplate reader (Bio-Rad Laboratories Inc., Foster City, CA).

Measurement of the TAG content

The cell culture and treatment were carried out according to the method previously described.[27] After 24 hours of cell culture, the cell culture medium was collected, the HC11 cells were rinsed with precooled PBS 3 times, and 200 μl of RIPA lysate was added to each well. According to the instructions, the triglyceride assay kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) was used to determine the content of triglycerides in cells and culture medium. The wavelengths of all holes were measured at 510 nm using an automatic microplate reader (Bio-Rad Laboratories Inc., Foster City, CA).

RNA extraction and purification

The total RNA extraction reagent (EZBioscience, China) was used to extract total RNA from cells. The quality, purity, and concentration of RNA samples were evaluated by electrophoresis on 1.5% agarose gel (130 V, 18 min) and a NanoDrop spectrophotometer (A260/A280), respectively. RNA was reverse transcribed and gDNA (genomic DNA) was removed using the Color reverse transcription kit (with a gDNA remover) (EZBioscience, China) according to the manufacturer's instructions.

Subsequently, real-time PCR was performed using the 2× color SYBR GREEN qPCR master mix (ROX2 plus) (A0012-R2, EZBioscience, China) on QuantStudioTM 6 Flex (Applied Biosystems, CA, United States). The PCR was carried out as follows: heating at 95 °C for 5 min for activating the Hot-start enzyme, then 40 cycles of denaturing at 95 °C for 10 s and annealing at 60 °C for 30 s. The relative expression level of target genes was determined by analyzing the data using the $2^{-\Delta\Delta Ct}$ method. Primer sequences we used for real-time PCR are listed in Tables 1 and 2.

Western blotting

Western blotting was used to measure the level of protein expression of the milk protein, the milk fat synthesis-related genes, the G protein-coupled receptor GPR109A, and the

Table 1 Primers used for q-PCR analysis (mouse)

Gene	Accession	Sequence (5′-3′)	Size
α-Casein	NM_007784.3	F-GTGGCTGCTGCTTTTGCTATGC R-ACTGCTGCTATGCTGTTGCTGAG	81
β-Casein	NM_009972.2	F-ATGAGCAGAAACTTCAGAAGGT R-GGATTGCAAGAGATGGTTTGAG	139
WAP	XM_017314502.2	F-ACAGAGTGTATCATCTGCCAAA R-ACCAATGTTGACAGGAGTTTTG	102
FASN	NM_007988.3	F-AGCACTGCCTTCGGTTCAGTC R-AAGAGCTGTGGAGGCCACTTG	94
ACACA	XM_036156218.1	F-GAAGTCAGAGCCACGGCACA R-GGCAATCTCAGTTCAAGCCAGTC	119
DGAT1	XM_006520405.4	F-CAGCTGTGGCCTTACTGGTTGA R-CGGCACCACAGGTTGACATC	118
FABP4	NM_024406.3	F-CATCCGGTCAGAGAGTACTTTT R-TAGGGTTATGATGCTCTTCACC	109
SREBP1	XM_006532716.4	F-AGAAGCTCAAGCAGGAGAACCTGA R-ACTTCGGGTTTCATGCCCTCCATA	127
PPARγ	XM_036165927.1	F-GGAAGACCACTCGCATTCCTT R-GGAAGACCACTCGCATTCCTT	121
GPR109A	NM_030701.3	F-CGACAGGGCAGCATCATCTT R-CGATGGTGAGACCCCACAAG	139
β-Actin	NM_007393.5	F-CCACCATGTTACCCAGGCATT R-CGGACTCATCGTACTCCTGC	190

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Table 2 Primers used for q-PCR analysis (porcine)

Gene	Accession	Sequence (5′-3′)	Size
α-Casein	NM_001004030.1	F-GGATCAGACGAAGACAAGTGCCTAC R-TGACAGGTTCCTCACTGGTGGAG	86
β-Casein	NM_214434.2	F-TTACACCGAGCCCATCCCTTACC R-GATGAAGAAGAGGCACCACCACAG	86
WAP	NM_213841.2	F-CTCCAGGGACGACCAGTGTAGG R-ACTGAAGAGGGGCCTCTGTGTC	95
GPR109A	XM_021072989.1	F-CCAGCCCATCTTTCCCCAAC R-CAGTGAGCTCGAGGCTTGTG	108
β-Actin	397563	F-TGCGGGACATCAAGGAGAAG FR-AGTTGAAGGTGGTCTCGTGG	176

mTOR pathway-related protein. The total proteins of HC11 cells were isolated using RIPA lysis buffer (P0013B, Beyotime, Shanghai, China). Western blot was performed as a regular procedure using a PVDF membrane. Afterward the membrane was incubated for 12 h at 4 °C with a specific antibody. And then, the membrane was incubated for 1 h at room temperature with a secondary antibody. The protein bands were visualized using ECL chemiluminescence reagents (P1020, ApplyGen, Beijing, China) and the FluorChem M fluorescent imaging system (ProteinSimple, CA, United States) according to the manufacturer's instructions. All antibodies used in this experiment are shown in Table 3.

Statistical analysis

All experimental data were analyzed with the Student's *t*-test or the one-way ANOVA procedures of SPSS according to the experimental design (Ver. 25.0, IBM Inc., United States). Results were presented as means with their standard error of mean (SEM). Multiple comparisons between groups were made using the least significant difference (LSD) test. The experiment used $P < 0.05$ as the basis for statistical significance. Graphs were drawn and analyzed using GraphPad Prism 8.0 software. All data are shown as the mean ± SEM.

Table 3 Antibodies used for western blotting analysis

Gene	Company	Cat. no.	Dilution
β-Actin	Bioss	Bs-0061R	1 : 5000
ACACA	Abcam	ab72046	1 : 1000
FASN	Abcam	ab99359	1 : 1000
SREBP1	Abcam	ab3259	1 : 1000
PPARγ	Abcam	ab178860	1 : 1000
DGAT1	Abcam	ab181180	1 : 1000
FABP4	Abcam	ab92501	1 : 1000
mTOR	Cell Signaling Technology	2983	1 : 1000
p-mTOR	Cell Signaling Technology	5536	1 : 1000
S6K1	Abcam	ab9366	1 : 1000
p-S6K1	Cell Signaling Technology	9234	1 : 1000
4EBP1	Abcam	ab2606	1 : 1000
p-4EBP1	Cell Signaling Technology	94515	1 : 1000
GPR109A	Novus Biologicals	NBP1-92180	1 : 1000
α-Casein	Santa Cruz Biotechnology	sc-373707	1 : 100
β-Casein	Santa Cruz Biotechnology	sc-166520	1 : 100
WAP	Santa Cruz Biotechnology	sc-398276	1 : 100

Results

Niacin promotes the synthesis of milk fat and milk protein in HC11 cells

To study the effect of niacin on milk fat and milk protein synthesis, the HC11 cells were cultured with different concentrations of niacin (0, 0.5, 1, 2, 4, and 8 mM) for 24 h. As shown in Fig. 1A, cell viability was significantly improved after stimulation with 1 mM niacin ($P < 0.05$). In addition, 1 mM niacin significantly increased the concentration of TAG in the cell lysate and medium ($P < 0.05$) (Fig. 1B and C). Correspondingly, 1 mM niacin significantly increased the expression of genes and proteins involved in milk fat synthesis (such as ACACA, FASN, and FABP4) ($P < 0.05$) (Fig. 1D–F). Similarly, 1 mM niacin also significantly promoted milk protein synthesis (α-casein, β-casein and WAP) ($P < 0.05$) (Fig. 1G–I). 1 mM niacin also increased the expression of GPR109A (Fig. 1D–I). These experimental results showed that 1 mM (the optimal concentration) niacin promotes the synthesis of milk fat and milk protein in the HC11 cells.

BHBA promotes the synthesis of milk fat and milk protein in HC11 cells

Similarly, to study the effect of BHBA on milk fat and milk protein synthesis, the HC11 cells were cultured with different concentrations of BHBA (0, 0.5, 1, 2, 4, and 8 mM) for 24 h. A significant increase in cell viability was observed after stimulation with 2 mM BHBA ($P < 0.05$). In addition, the concentration of TAG in the cell lysate and medium was significantly increased by 2 mM BHBA ($P < 0.05$). Correspondingly, 2 mM BHBA significantly increased the expression of genes and proteins involved in milk fat synthesis and milk protein (such as ACACA, FASN, FABP4, GPR109A, α-casein, β-casein and WAP) ($P < 0.05$) (Fig. 2D–I). These experimental results show that 2 mM (the optimal concentration) BHBA promotes the synthesis of milk fat and milk protein in the HC11 cells.

Niacin/BHBA activates the mTORC1 signaling pathway in HC11 cells

According to previous results, 2 mM BHBA and 1 mM niacin were selected for subsequent experiments. 1 mM niacin significantly promoted the phosphorylation of mTOR, S6K1 and

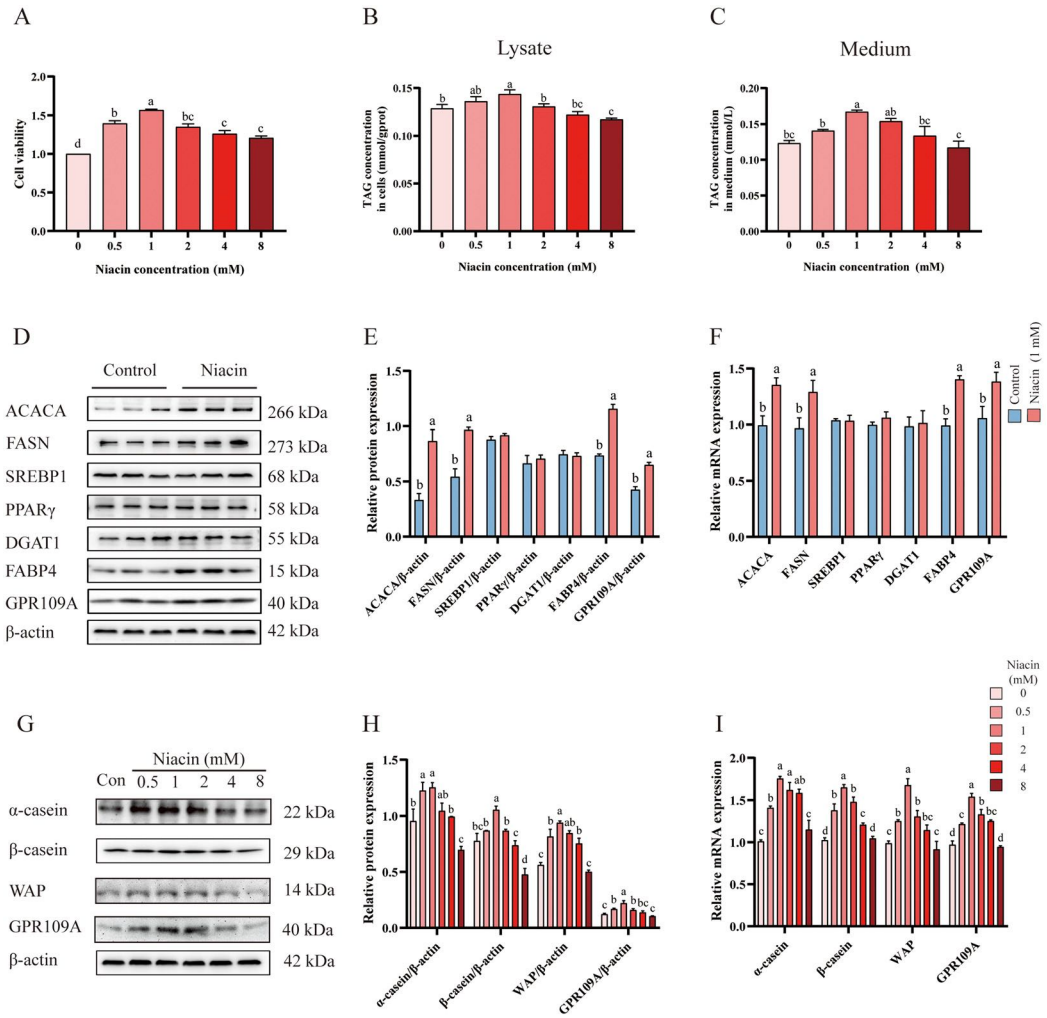

Fig. 1 Niacin promotes the synthesis of milk fat and milk protein in HC11 cells. (A) Cell viability was measured by CCK-8 following treatment with different concentrations (0, 0.5, 1, 2, 4, and 8 mM) of niacin for 24 h, $n = 3$. (B and C) TAG concentration in cells (B) and medium (C), $n = 3$. (D–F) 1 mM niacin regulated the expression and abundance of mRNAs and proteins involved in milk fat synthesis in HC11 cells. In (D), bands were incubated with target proteins including ACACA, FASN, SREBP1, PPARγ, DGAT1, FABP4, GPR109A and β-actin antibodies, $n = 3$. Protein expressions of target proteins are shown in (E), $n = 3$. (F) Relative mRNA expressions of target proteins, $n = 3$. (G–H) Effects of different concentrations of niacin (0, 0.5, 1, 2, 4, and 8 mM) on milk protein and GPR109A synthesis in HC11 cells. In (G), bands were incubated with target proteins including α-casein, β-casein, WAP, GPR109A and β-actin antibodies, $n = 3$. Protein expressions of target proteins are shown in (H), $n = 3$. (I) Relative mRNA expressions of target proteins, $n = 3$. Statistical analysis was performed by one-way ANOVA, and the different letters (a, b, and c) indicate a significant difference among different treatments ($P < 0.05$).

4EBP1 in the HC11 cells ($P < 0.05$) (Fig. 3). Similarly, 2 mM BHBA also significantly promoted the phosphorylation of mTOR, S6K1 and 4EBP1 in the HC11 cells ($P < 0.05$).

Inhibition of mTOR activity partially blocks niacin-induced milk fat and milk protein synthesis in HC11 cells

To explore the role of the mTOR pathway in niacin-induced milk fat and milk protein synthesis, the mTOR-specific inhibitor rapamycin was used to block the activity of mTOR. As expected, rapamycin reversed the increased phosphorylation level of mTOR, S6K1, and 4EBP1 induced by 1 mM niacin ($P < 0.05$) (Fig. 4A and B). Moreover, rapamycin blocked ($P < 0.05$) the upregulation of milk fat and milk protein synthesis induced by niacin ($P < 0.05$) (Fig. 4C, D, E, F and G). Similarly, rapamycin weakened niacin exerting an effect on ACACA, FASN, and FABP4 expressions in HC11 cells ($P < 0.05$) (Fig. 4H–J). These results indicate that niacin promotes the synthesis of milk fat and milk protein in the HC11 cells partially *via* the mTOR pathway.

Inhibition of GPR109A blocks the niacin-induced synthesis of milk fat and milk protein in HC11 cells

To confirm the role of GPR109A in milk fat and protein synthesis, GPR109A siRNA was used to knockdown the expression of GPR109A in HC11 cells ($P < 0.05$) (Fig. 5A–C). Niacin-induced milk fat and protein synthesis is attenuated in GPR109A-knock-

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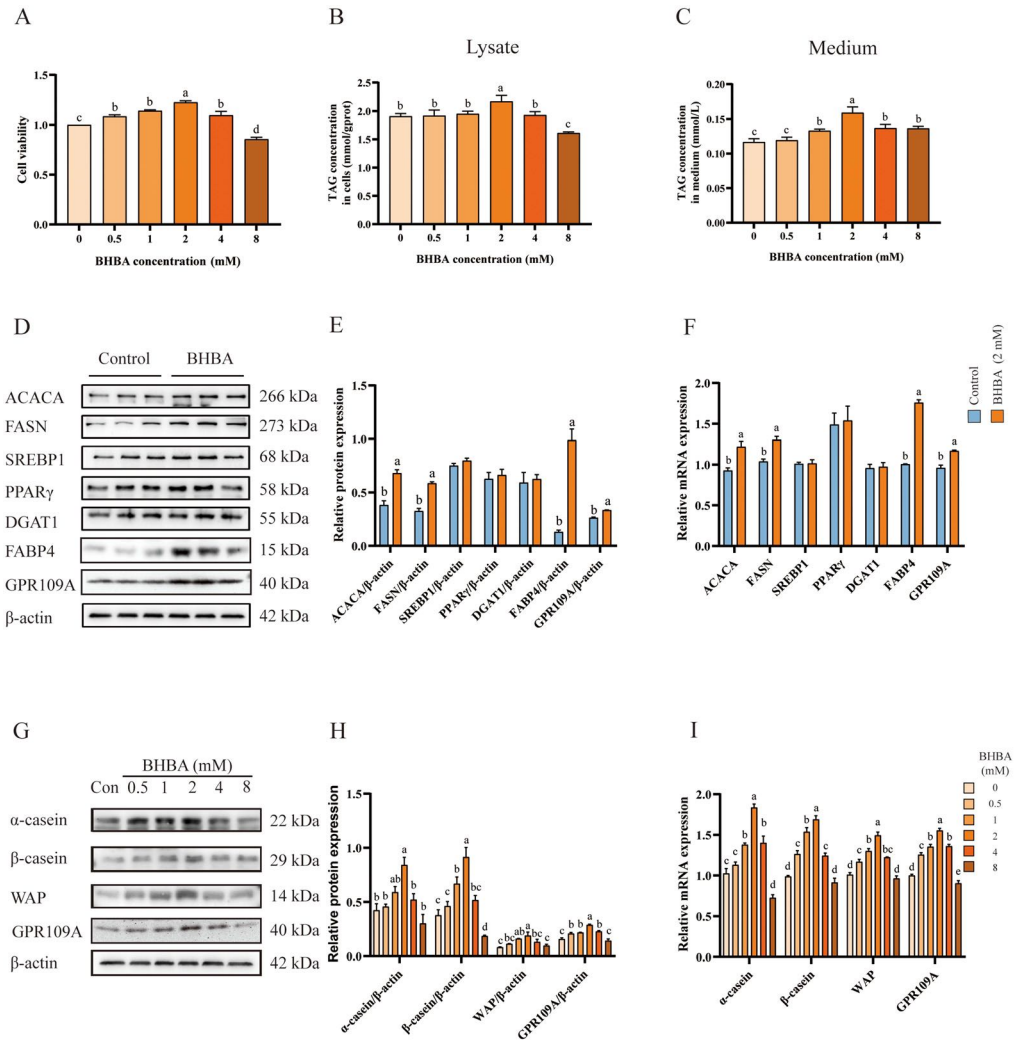

Fig. 2 BHBA promotes the synthesis of milk fat and milk protein in HC11 cells. (A) Cell viability was measured by CCK-8 following treatment with different concentrations (0, 0.5, 1, 2, 4, and 8 mM) of β-hydroxybutyric acid (BHBA) for 24 h, n = 3. (B and C) TAG concentration in cells (B) and medium (C), n = 3. (D–F) 2 mM BHBA regulates the expression and abundance of mRNAs and proteins involved in milk fat synthesis in HC11 cells. In (D), bands were incubated with target proteins including ACACA, FASN, SREBP1, PPARγ, DGAT1, FABP4, GPR109A and β-actin antibodies, n = 3. Protein expressions of target proteins are shown in (E), n = 3. (F) Relative mRNA expressions of target proteins, n = 3. (G and H) Effects of different concentrations of BHBA (0, 0.5, 1, 2, 4, and 8 mM L^{-1}) on milk protein synthesis in HC11 cells. In (G), bands were incubated with target proteins including α-casein, β-casein, WAP, GPR109A and β-actin antibodies, n = 3. Protein expressions of target proteins are shown in (H), n = 3. (I) Relative mRNA expressions of target proteins, n = 3. Statistical analysis was performed by one-way ANOVA, and the different letters (a, b, and c) indicate a significant difference among different treatments (P < 0.05).

down HC11 cells (P < 0.05) (Fig. 5A–H). Consistently, the increased phosphorylation levels of mTOR signaling induced by niacin are inhibited in GPR109A knockdown HC11 cells (P < 0.05) (Fig. 5I and J). This evidence indicates that niacin might regulate milk fat and protein synthesis through the GPR109A/mTORC1 pathway. To further decipher how GPR109A regulates mTOR signaling, we pretreated the HC11 cells with the inhibitors of the G proteins ($G_{\beta\gamma}$, $G_{\alpha i}$ and G_i) coupled with GPR109A. When the HC11 cells were pretreated with NF023, Gallein, or PTX, niacin-induced GPR109A expression is inhibited (P < 0.05) (Fig. 6A–C). In addition, NF023, Gallein, or PTX treatments significantly attenuated the niacin-mediated synthesis of milk fat and milk protein to varying degrees (P < 0.05) (Fig. 6A–H). Consistently, inhibition of $G_{\alpha i}$, G_i and $G_{\beta\gamma}$ significantly inhibited the niacin-induced phosphorylation of mTOR, S6K1 and 4EBP1 (P < 0.05) (Fig. 6I and J). This result further suggests that niacin regulates milk fat and protein synthesis through the GPR109A/Gi/mTORC1 pathway.

Niacin/BHBA promotes the synthesis of milk fat and milk protein in PMECs

Verifying the effect of niacin/BHBA on milk synthesis is not species specific. We further determine the effects of niacin/BHBA on milk fat and protein synthesis in PMECs. PMECs

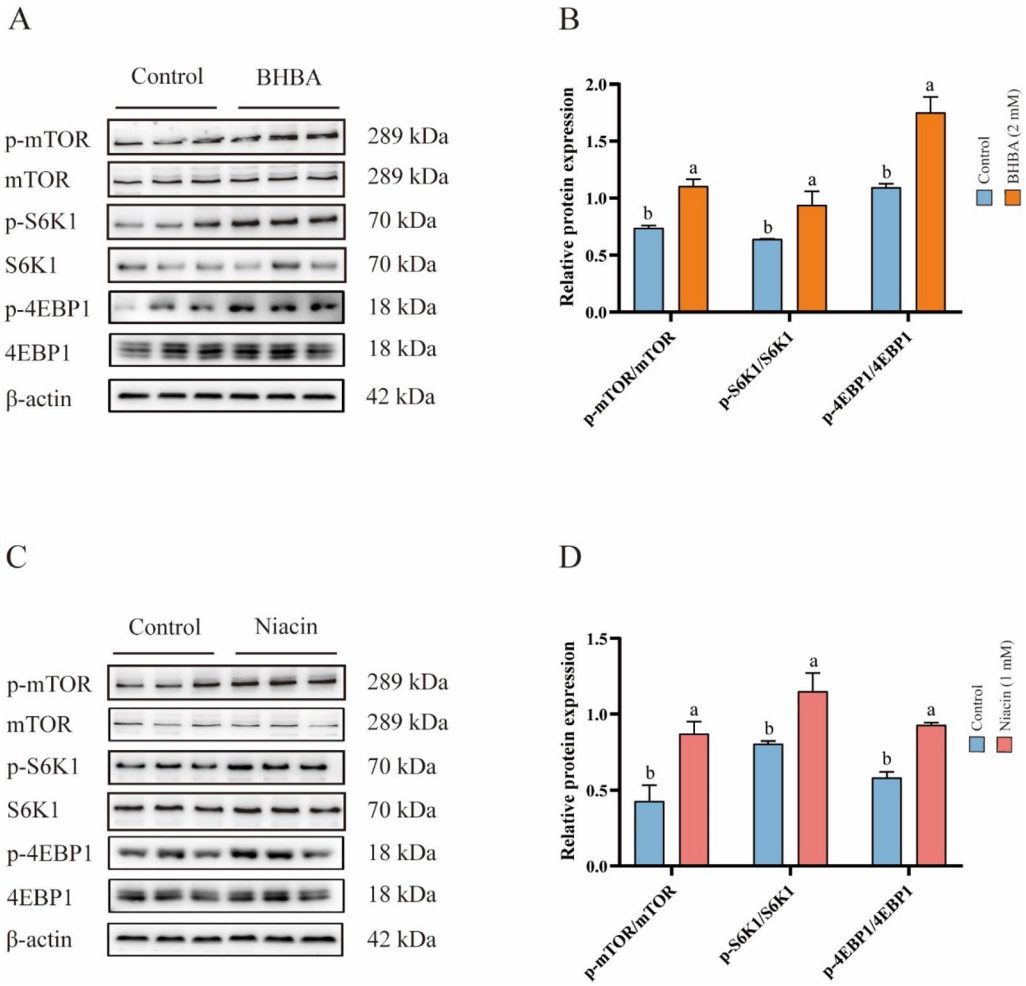

Fig. 3 Niacin/BHBA promotes the phosphorylation of the mTOR pathway. (A, B, C and D) The levels of phosphorylated proteins in the mTOR pathway in the HC11 cells induced by 2 mM BHBA and 1 mM niacin. Statistical analysis was performed using the Student's *t*-test, and the different letters (a and b) indicate a significant difference among different treatments ($P < 0.05$).

were cultured in 10% FBS DMEM/F12 with different concentrations of niacin or BHBA (0, 0.5, 1, 2, 4, and 8 mM) for 24 h. 2 mM niacin (Fig. 7A–F) or BHBA (Fig. 7G–L) induced an increase in cell viability and milk fat and protein synthesis ($P < 0.05$). Consistently, 2 mM niacin or BHBA also induced an increase in the protein abundance of GPR109A ($P < 0.05$) (Fig. 7D–F and J–L). These data show that niacin and BHBA promote milk fat and protein synthesis in PMECs with the stimulation of GPR109A.

Niacin promotes the lactation performance in mice

To investigate whether dietary niacin supplementation could regulate lactation performance *in vivo*, mice were given 25 mM niacin in drinking water during lactation. There was no effect of niacin supplementation on the body weight (Fig. 8A), body weight gain (Fig. 8B), and the feed intake (Fig. 8C) of dams. 25 mM niacin supplementation increased the body weight of offspring mice ($P < 0.05$) (Fig. 8D). Furthermore, serum prolactin (PRL) levels were increased in the niacin-treated group ($P < 0.05$) (Fig. 8E). No difference was observed in the alveolus area between the control and niacin treatment groups (Fig. 8F–H), whereas niacin increased the number of alveoli in the mammary gland ($P < 0.05$) (Fig. 8F–H). For lactation performance, niacin increased the milk fat and protein synthesis in the mammary gland ($P < 0.05$) (Fig. 8I–N). Mechanistically, niacin induced the expression of GPR109A ($P < 0.05$) (Fig. 8I–K) with the activation of mTOR signaling (promoted the phosphorylation of mTOR, S6K1 and 4EBP1) ($P < 0.05$) (Fig. 8O and P).

Discussion

G protein coupled receptors (GPCRs) are largely involved in the regulation of physiology and pathophysiology in animals. Previously, GPR109A has been reported to be highly expressed in the mammary gland epithelial tissue, whereas

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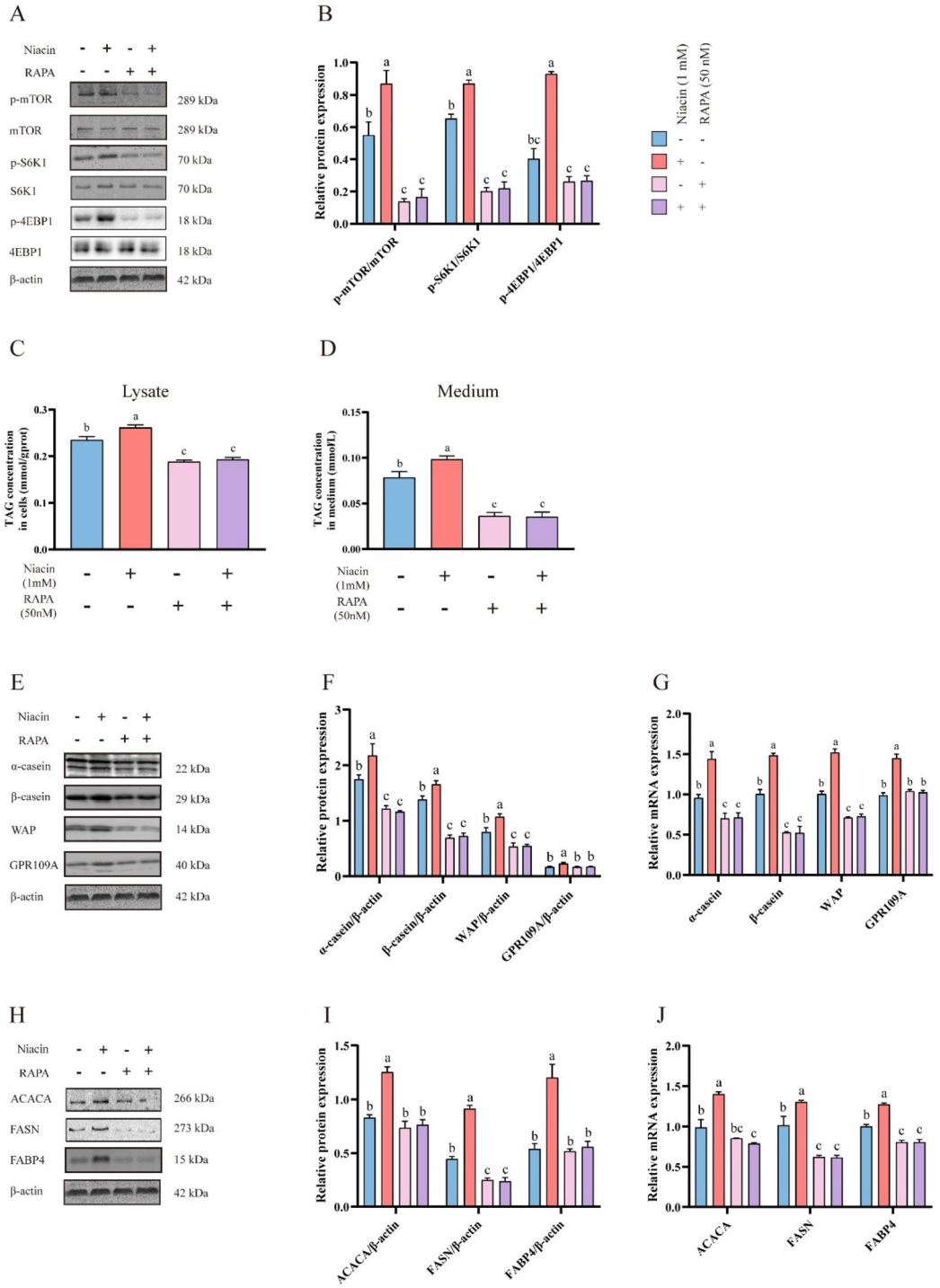

Fig. 4 Inhibiting mTOR partially blocks niacin-induced milk fat and milk protein synthesis in HC11 cells. The HC11 cells were pretreated for 2 h with RAPA (50 nM) and then incubated for 1 day with niacin (1 mM). (A and B) TAG concentration in cells (A) and medium (B), $n = 3$. (C–E) Effects of RAPA treatment on the niacin-induced milk protein gene expression and abundance of proteins and mRNAs in HC11 cells. In (C), bands were incubated with target proteins including α-casein, β-casein, WAP, GPR109A and β-actin antibodies, $n = 3$. Protein expressions of target proteins are shown in (D), $n = 3$. (E) Relative mRNA expressions of target proteins, $n = 3$. (F–H) Effects of RAPA treatment on the niacin-induced milk fat gene expression and abundance of proteins and mRNAs in HC11 cells. In (F), bands were incubated with target proteins including ACACA, FASN, FABP4 and β-actin antibodies, $n = 3$. Protein expressions of target proteins are shown in (G), $n = 3$. (H) Relative mRNA expressions of target proteins, $n = 3$. (I) The levels of phosphorylated proteins of mTOR, S6K1 and 4EBP1 were examined by western blotting, $n = 3$. (J) Immunoblot bands of p-mTOR/mTOR, p-S6K1/S6K1 and p-4EBP1/4EBP1 were digitized, $n = 3$. Statistical analysis was performed by one-way ANOVA, and the different letters (a, b, and c) indicate a significant difference among different treatments ($P < 0.05$).

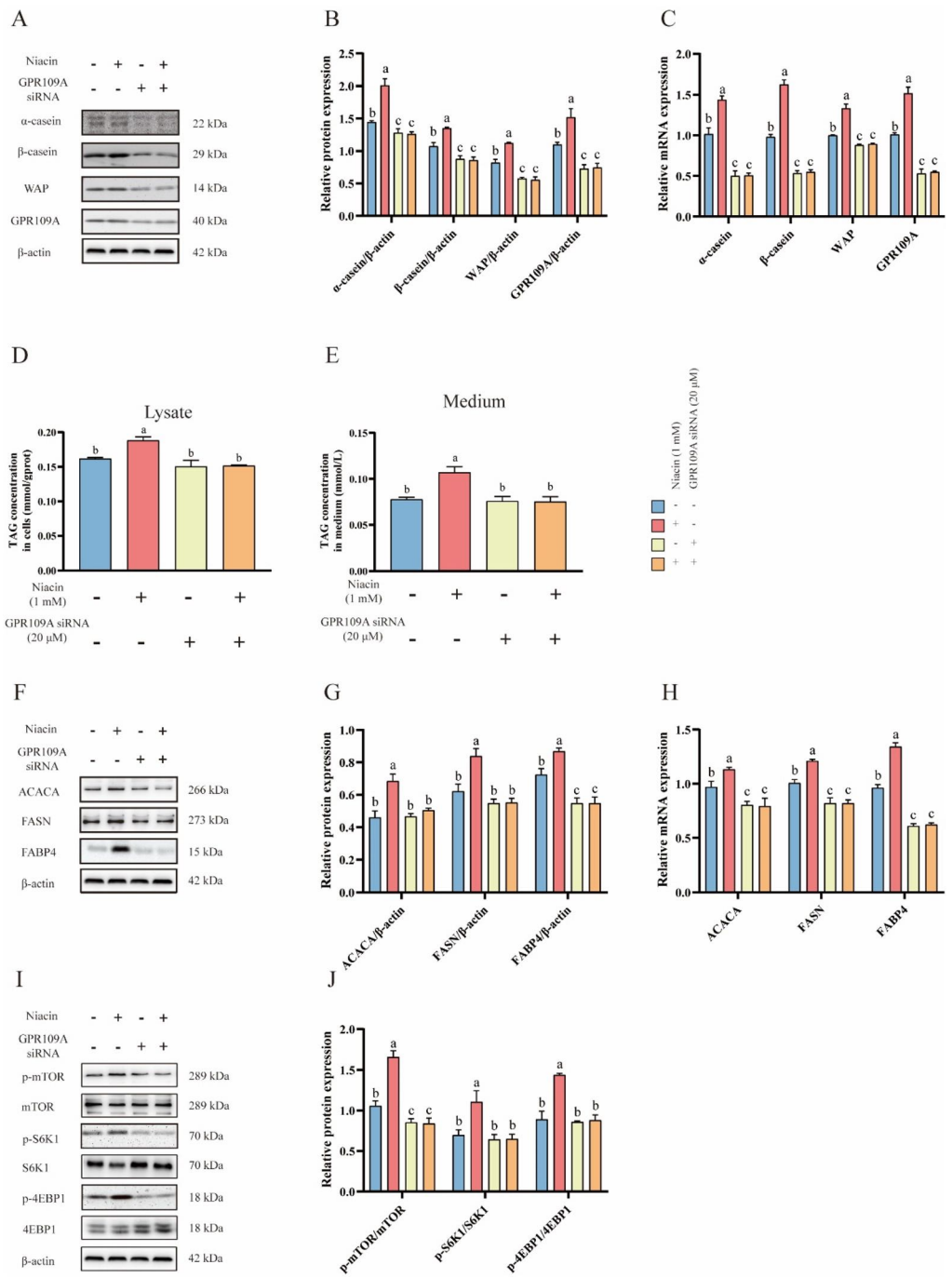

Fig. 5 Inhibition of GPR109A by siRNA blocks niacin-induced milk fat and milk protein synthesis of HC11 cells. The HC11 cells were pretreated for 2 h with siRNA (20 μM) and then incubated for 1 day with niacin (1 mM).(A-C) α-casein, β-casein, WAP, GPR109A and β-actin protein levels were detected by Western blot, n=3. (B) Immunoblot bands of α-casein, β-casein, WAP and GPR109A were digitized and are expressed as the ratios to β-actin, n=3. (C) is relative mRNA expressions of α-casein, β-casein, WAP and GPR109A proteins, n=3. (D and E) TAG concentration in cell (D) and medium (E), n=3. (F) ACACA, FASN, FABP4 and β-actin protein levels were detected by Western blot, n=3. (G) Immunoblot bands of ACACA, FASN and FABP4 were digitized and are expressed as the ratios to β-actin, n=3. (H) is relative mRNA expressions of ACACA, FASN and FABP4 proteins, n=3. (I) The levels of phosphorylated proteins of mTOR, S6K1, and 4EBP1 were examined by Western blotting, n=3. (J) Immunoblot bands of p-mTOR/mTOR, p-S6K1/S6K1 and p-4EBP1/4EBP1 were digitized, n=3. Statistical analysis was performed by one-way ANOVA, and the different letters (a, b, and c) indicate a significant difference among different treatments ($P < 0.05$).

its biological function in the mammary gland is still not clear. In lactating cows, the GPR109A agonists (niacin and BHBA) have been confirmed to promote the synthesis of milk protein and milk lipids. So far, the potential connection between GPR109A and milk synthesis remains largely unexplored. In this study, we first demonstrated that GPR109A

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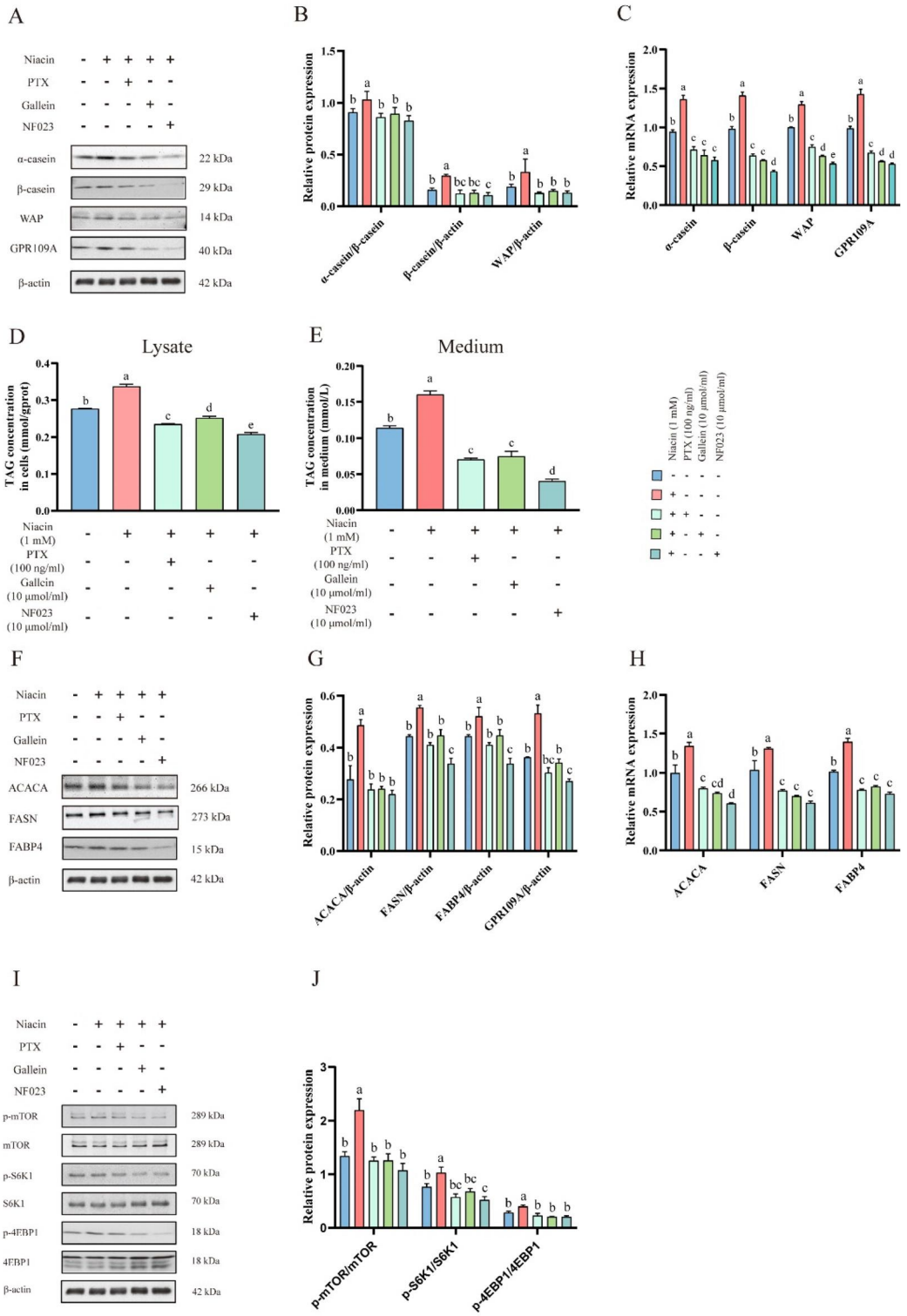

Fig. 6 Inhibition of GPR109A by NF023, Gallein and PTX blocks niacin-induced milk fat and milk protein synthesis in HC11 cells. The HC11 cells were pretreated for 2 h with NF023 (10 μmol), Gallein (10 μmol), and PTX (100 ng ml^{-1}) and then incubated for 1 day with niacin (1 mM). (A and B) TAG concentration in cells (A) and medium (B), n = 3. (C) α-Casein, β-casein, WAP, GPR109A and β-actin protein levels were detected by western blot, n = 3. (D) Immunoblot bands of α-casein, β-casein, WAP and GPR109A were digitized and are expressed as the ratios to β-actin, n = 3. (E) Relative mRNA expressions of α-casein, β-casein, WAP and GPR109A proteins, n = 3. (F) ACACA, FASN, FABP4 and β-actin protein levels were detected by western blot, n = 3. (G) Immunoblot bands of ACACA, FASN and FABP4 were digitized and are expressed as the ratios to β-actin, n = 3. (H) Relative mRNA expressions of ACACA, FASN and FABP4 proteins, n = 3. (I) The protein levels of p-mTOR, mTOR, p-S6K1, S6K1, p-4EBP1 and 4EBP1 were examined by western blotting, n = 3. (J) Immunoblot bands of p-mTOR/mTOR, p-S6K1/S6K1 and p-4EBP1/4EBP1 were digitized, n = 3. Statistical analysis was performed by one-way ANOVA, and the different letters (a, b, c, d, and e) indicate a significant difference among different treatments ($P < 0.05$).

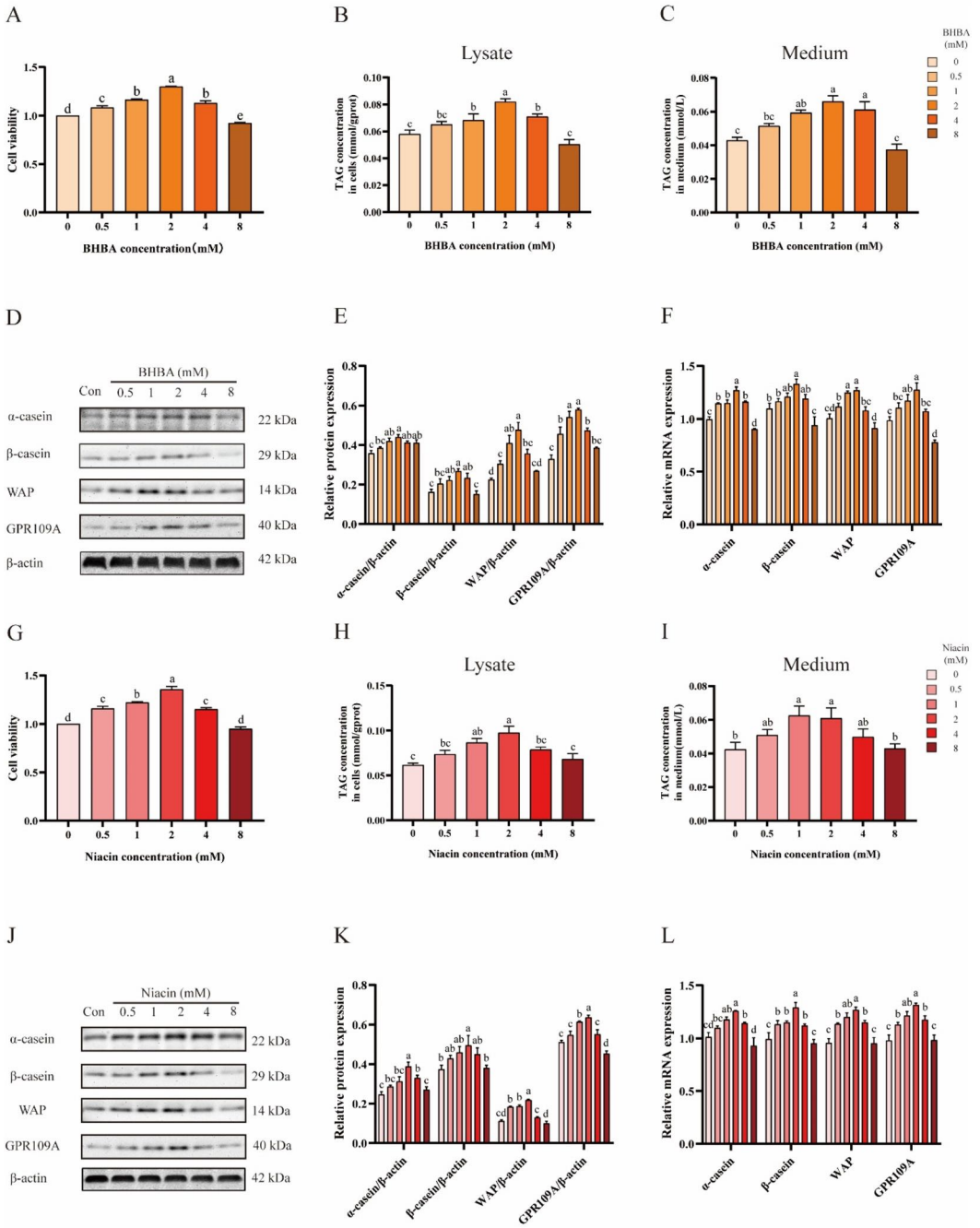

Fig. 7 Niacin/BHBA promotes the production of milk fat and milk protein in PMECs. (A–F) The effects of different concentrations of BHBA (0, 0.5, 1, 2, 4, and 8 mM) on milk fat and milk protein in PMECs. (A) Cell viability was measured by CCK-8 following treatment for 24 h, n = 3. (B and C) TAG concentration in cells (B) and medium (C), n = 3. (D–F) The expression and abundance of milk protein and GPR109A mRNAs and proteins in PMECs. In (D), bands were incubated with target proteins, n = 3. Protein expressions of target proteins are shown in (E), n = 3. (F) Relative mRNA expressions of target proteins, n = 3. (G–L) The effects of different concentrations of niacin (0, 0.5, 1, 2, 4, and 8 mM) on milk fat and milk protein in PMECs. (G) Cell viability was measured by CCK-8 following treatment for 24 h, n = 3. (B and C) TAG concentration in cells (B) and medium (C), n = 3. (D–F) The expression and abundance of milk protein and GPR109A mRNAs and proteins in PMECs. In (D), bands were incubated with target proteins, n = 3. Protein expressions of target proteins are shown in (E), n = 3. (F) Relative mRNA expressions of target proteins, n = 3. (G–L) Effects of different concentrations of niacin (0, 0.5, 1, 2, 4, and 8 mM) on milk fat and milk protein in PMECs. Statistical analysis was performed by one-way ANOVA, and the different letters (a, b, c, d, and e) indicate a significant difference among different treatments (P < 0.05).

plays a crucial role in the regulation of milk fat and milk protein synthesis. The agonists of GPR109A (niacin and BHBA) within a certain concentration range promote milk fat and milk protein synthesis both in HC11 cells and PMECs. The expressions of the genes related to the synthesis of milk fat and protein, such as ACACA, FASN, FABP4, α-casein,

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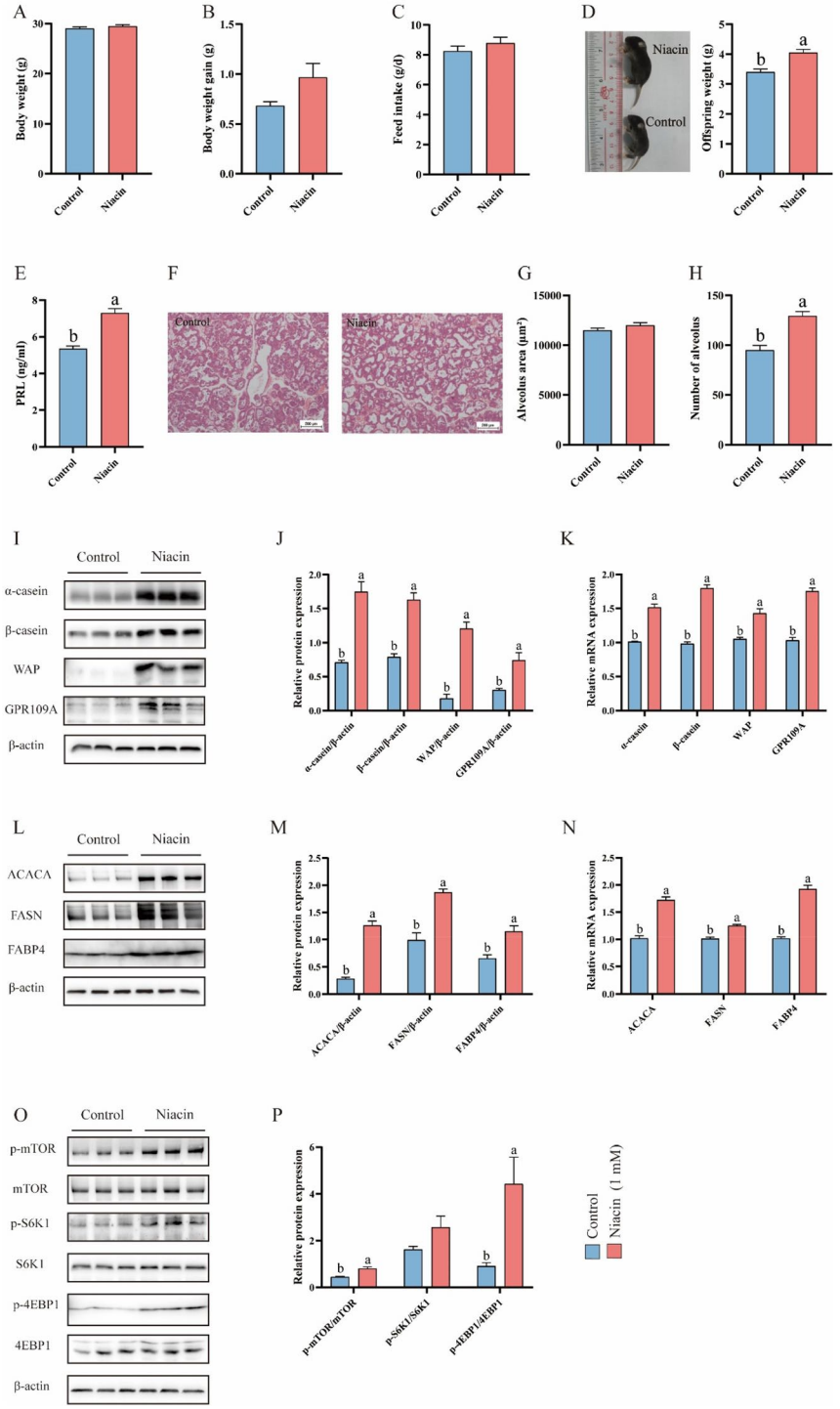

Fig. 8 Effects of dietary niacin supplementation on the lactation performance of dams. (A) The body weight of dams, n = 6. (B) The body weight gain of dams, n = 6. (C) The daily feed intake of dams, n = 6. (D) The average weight of offspring mice, n = 6. (E) The content of PRL in the serum of dams, n = 6. (F) The fourth pair of mammary glands of dams. Representative hematoxylin and eosin (H&E)-stained tissue sections are shown. The scale bar represents 200 μm. (G–H) Alveolus area and the number of alveoli in mammary glands, n = 6. (I–K) The expression and abundance of milk protein and GPR109A mRNAs and proteins in the mammary gland. In (I), bands were incubated with target proteins, n = 3. Protein expressions of target proteins are shown in (J), n = 3. (K) Relative mRNA expressions of target proteins, n = 3. (L) ACACA, FASN, FABP4 and β-actin protein levels were detected by western blot, n = 3. (M) Immunoblot bands of ACACA, FASN and FABP4 were digitized and are expressed as the ratios to β-actin, n = 3. (N) Relative mRNA expressions of ACACA, FASN and FABP4 proteins, n = 3. (O) The protein levels of p-mTOR, mTOR, p-S6K1, S6K1, p-4EBP1 and 4EBP1 were examined by western blotting, n = 3. (P) Immunoblot bands of p-mTOR/mTOR, p-S6K1/S6K1 and p-4EBP1/4EBP1 were digitized, n = 3. Statistical analysis was performed using the Student's t-test, and the different letters (a and b) indicate a significant difference among different treatments ($P < 0.05$).

β-casein and WAP, were also significantly promoted by the treatment of GPR109A agonists. At the same time, the mTOR signaling pathway was activated in HC11 cells and PMECs. In addition, we also verified this finding in C57BL/6 mice treated with niacin in drinking water. It is worth noting that high concentrations of niacin could be toxic to cells, which could further decrease the lactation ability of mammary epithelial cells by inhibiting cell growth.

The mTOR signaling pathway is the most important signaling pathway which regulates cell growth, proliferation, and survival.[28] mTOR consists of two distinct complexes, mTORC1 and mTORC2, which exert actions by regulating other kinases, such as S6 kinase and 4EBP1.[29] Recent studies have shown that mTORC1 can regulate casein synthesis.[30–32] In addition, mTORC1 is found to be a key player in milk fat synthesis.[33,34] Our experiments show that the inhibition of mTORC1 with rapamycin blocked the niacin-induced increase of genes related to milk fat and milk protein synthesis. Intriguingly, the expression of GPR109A is also reduced under the treatment of rapamycin. These results further confirmed that the GPR109A agonists require mTORC1 signaling to regulate milk fat and protein synthesis.

GPR109A is a G-protein-coupled receptor for sensing BHBA[35] and niacin.[14]The present research on BHBA mainly focuses on the regulation of ketosis,[36] and subclinical ketosis is associated with the loss of milk production.[37] Intriguingly, a certain concentration of BHBA could increase the production of milk fat[38] and milk protein[39] in lactating dairy cattle. Furthermore, BHBA treatment could increase TAG secretion in bovine mammary epithelial cells.[40] Niacin, an essential vitamin for living organisms, has been proved to suppress inflammation and stimulate adiponectin secretion. 14 g d^{-1} niacin could significantly improve the content of milk fat and milk protein in dairy Holstein cows.[41] These studies suggest that a suitable concentration of BHBA/niacin has a positive effect on the lactation performance, which is consistent with our results. Collectively, our research revealed that niacin and BHBA regulate milk fat and protein synthesis through GPR109A. It would be interesting to identify whether niacin/BHBA could affect the cell membrane localization of GPR109A when a GPR109A immunofluorescence staining antibody is available in the future.

Notably, GPR109A regulates milk synthesis mainly through the activation of mTORC1 pathways. As a classical GPCR, GPR109A is coupled to $G_{\alpha i}$ and $G_{\beta\gamma}$, and when GPR109A is activated by binding to a ligand, the GTP-bound $G_{\alpha i}$ would be active, and the $G_{\beta\gamma}$ dimer would dissociate from GPR109A to trigger downstream signaling cascades. Previously, it is demonstrated that the activation of $G_{\alpha s}$ increases cellular cAMP, which further inhibits the activation of mTORC1.[42,43] Thus, in this study, the activation of $G_{\alpha i}$ might reduce cAMP levels, which further promotes the activity of the mTOR pathway. Pretreating HC11 cells with either NF023, Gallein or PTX decreases milk fat and milk protein synthesis to a certain extent, which indicates that $G_{\beta\gamma}$ also participated in the regulation of milk synthesis. However, the potential signaling pathway by which $G_{\beta\gamma}$ induced milk synthesis is largely unclear.

Conclusion

GPR109A agonists (such as niacin) could promote the synthesis of milk lipids and milk protein through the GPR109A/G_i/mTORC1 signaling pathways in HC11 cells. Dietary niacin supplementation could be an efficient way to promote maternal milk synthesis and benefit the growth of neonates.

Author contributions

SHZ and WTG conceived and designed the experiments. JMC, TBL, SCZ, XHL and XHY performed the cell culture experiment. JMC, CCW, YYL, XFZ and FC participated in the animal experiment. JMC and TBL analyzed the data and conducted the research. JMC discussed the results and wrote the paper. SHZ and WTG conducted the final editing. All authors read and approved the final manuscript.

Data availability statement

The datasets generated for this study are available on request to the corresponding author.

Conflicts of interest

The authors declare no competing financial interest.

Acknowledgements

This study was financially supported by the National Key R&D Program of China (No. 2021YFD1300700), the Guangdong Basic and Applied Basic Research Foundation (No. 2021A1515010440 and 2023A1515012098), the Science and Technology Program of Guangzhou (No. 202102020056), the National Natural Science Foundation of China (No. 31802067 and 31872364), and the Anhui Province Key Laboratory of Animal Nutrition Regulation and Health (No. APKLANRH202105).

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GUT MICROBES
2023, VOL. 15, NO. 1, 2206505
https://doi.org/10.1080/19490976.2023.2206505

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REVIEW

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Maternal microbe-specific modulation of the offspring microbiome and development during pregnancy and lactation

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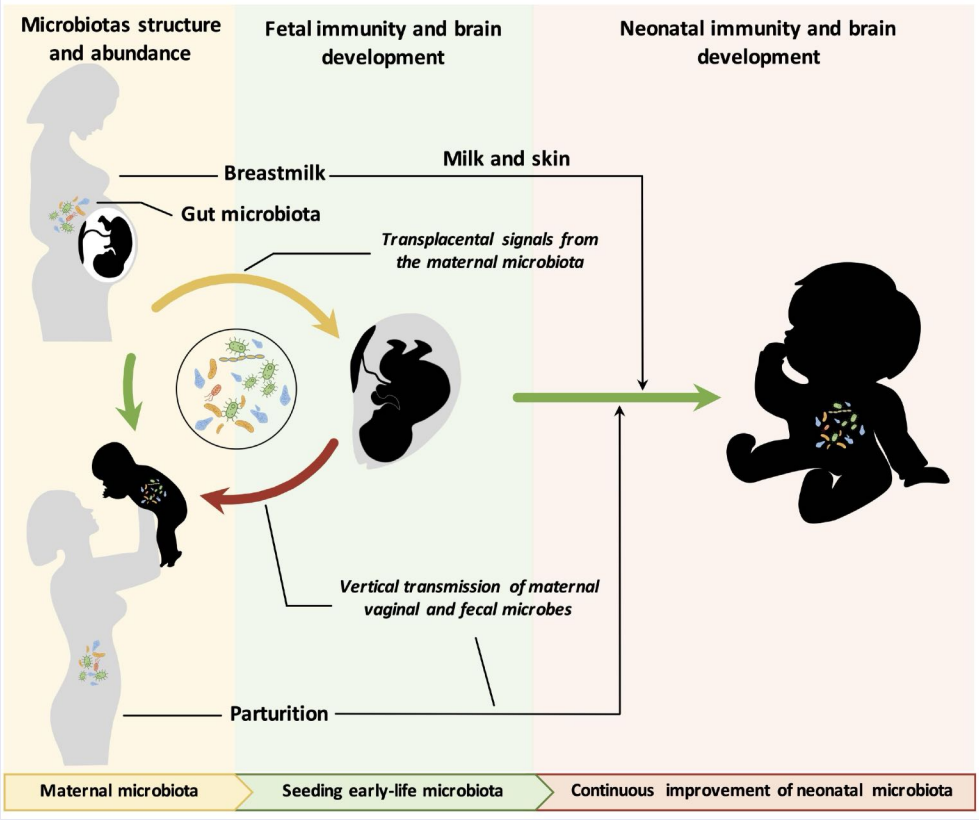
ABSTRACT

The maternal microbiome is essential for the healthy growth and development of offspring and has long-term effects later in life. Recent advances indicate that the maternal microbiome begins to regulate fetal health and development during pregnancy. Furthermore, the maternal microbiome continues to affect early microbial colonization via birth and breastfeeding. Compelling evidence indicates that the maternal microbiome is involved in the regulation of immune and brain development and affects the risk of related diseases. Modulating offspring development by maternal diet and probiotic intervention during pregnancy and breastfeeding could be a promising therapy in the future. In this review, we summarize and discuss the current understanding of maternal microbiota development, perinatal microbial metabolite transfer, mother-to-infant microbial transmission during/after birth and its association with immune and brain development as well as corresponding diseases.



ARTICLE HISTORY
Received 24 August 2022
Revised 2 March 2023
Accepted 17 April 2023

KEYWORDS
Maternal microbiota; microbial metabolite; fetus; neonate; immunity; brain development

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1. Introduction

The human body is colonized by hundreds of millions of commensal microbes, which are essential for maintaining the health of humans. Most of these microbes belong to four phyla: Firmicutes, Bacteroidetes, Actinobacteria and Proteobacteria.[1,2] In contrast to the relatively stable gut microbiota in adults, the neonatal microbiota undergoes dramatic fluctuation and exhibits high adaptability and plasticity. Recent advances in genome sequencing technologies have expanded our knowledge regarding microbiota colonization during early life and have helped decipher its critical functions in host physiological processes, such as metabolism,[3] immunity,[4] cognition[5] and intestinal homeostasis.[6] Disruption of microbiome establishment during the newborn period can lead to life-threatening diseases such as necrotizing enterocolitis (NEC)[7,8] and late-onset sepsis (LOS)[9] and might increase the risk of obesity,[10] diabetes[11] and asthma[12] in adulthood.

Similar to the genome, the microbiome is inherited by the infant from the mother. The seeding of newborn intestinal bacteria is considered to largely occur during the birthing process. After birth, neonatal gut microbes are persistently affected by maternal factors via direct contact with the mother's skin and breast milk. Full-term vaginal delivery and exclusive breastfeeding of neonates are considered the "golden rules" for optimal microbiota selection in neonates.[13] Approximately 11% of maternal-derived bacterial strains (mainly *Bacteroides* and *Bifidobacterium*) persist during the first year of life.[14] Since the neonatal microbiota is malleable in early-stage newborns, this period is a crucial time window for external intervention (such as with environmental and dietary factors) to regulate neonatal gut dysbiosis and its related diseases. This review focuses on the fluctuation of the maternal microbiome during pregnancy and lactation as well as the impact of the maternal microbiota and its metabolites on infants, including on early gut colonization, immune maturation, and brain development. Insights into the timing and mechanisms by which the maternal gut microbiota regulates the development of neonates can be used to develop gut microbiome-based therapies to prevent and/or treat related diseases in neonates.

2. Maternal microbiome and fetal health and development during gestation

2.1 Fluctuation of maternal gut microbes during pregnancy

During pregnancy and lactation, maternal gut microbes support the increased energy demand for fetal growth and milk synthesis by digesting carbohydrates that are resistant to host-secreted digestive enzymes. Pregnancy involves hormonal, psychological and immunological changes that regulate gut microbiota variation during different stages of pregnancy. Prior studies have showen that *Proteobacteria* and *Actinobacteria* in the maternal gut are enriched during the third trimester of pregnancy, with increases in the levels of proinflammatory cytokines.[15] However, recently, a comprehensive overview of the intestinal microbiota from approximately 1500 women indicated that the core microbiota of pregnant women and nonpregnant women was similar during the progression of gestation[16] and remained stable during the perinatal period.[17] Individual heterogeneity (including in body mass index (BMI), diet, antibiotic treatment, residency status and disease) is the dominant factor that shapes the intestinal microbiota during gestation (Figure 1).[16]

2.2 Influences on the maternal microbiome during pregnancy

2.2.1 Maternal BMI

Overweight mothers undergo shifts in their intestinal microbiota during gestation, especially between the first and third trimesters.[18] Decreased levels of *Bifidobacterium* spp. and *Bacteroides* spp. as well as increased levels of *Staphylococcus* and *Escherichia coli* spp. are commonly observed in overweight mothers.[19,20] In addition, changes in gut microbes regulate maternal metabolism during pregnancy through their modification of metabolic hormones (such as adipokines, insulin and gastrointestinal polypeptides).[21] Therefore, the microbial communities in overweight and obese pregnant women might induce inflammation, impair glucose and insulin tolerance, and further lead to gestational diabetes. Although maternal weight status has been found to affect the composition

of the offspring microbiome in animal models,[22] a meta-analysis of human studies found that maternal weight status had a limited predictive effect on the composition of the microbiome in infants.[23]

2.2.2 Maternal dietary patterns

Recent evidence indicates that maternal dietary patterns and nutritional compounds are crucial factors that shape the intestinal microbial community (Figure 1). A higher Firmicutes/Bacteroidetes ratio in the gut during pregnancy was observed in mothers who consumed a high-fat diet.[24] Dietary fiber is a predominant factor that regulates the richness and diversity of the gut microbiota.[25] Pregnant women who are vegetarians exhibit decreases in the abundances of *Collinsella* and *Holdemania* and increases in the abundances of *Roseburia* and *Lachnospiraceae*, which are accompanied by an increased production of short-chain fatty acids (SCFAs).[26] Conversely, deficiency in dietary fiber intake results in an increased abundance of *Collinsella* among the intestinal microbes and enhanced lactate fermentation in pregnant women.[27] In addition to dietary fiber, plant-derived foods, such as vegetable proteins and polyphenols, increase the number of *Christensenellaceae, Dehalobacterium*, and *Eubacterium* [28] and the production of butyrate and propionate in the gut.[29,30]

Recent studies have also uncovered the effects of many other nutrients that shape the gut microbial community during pregnancy. For instance, a higher level of vitamin D intake has been found to decrease alpha diversity and lead to enrichment in *Actinobacteria* and *Proteobacteria* in pregnant women, which might result in inflammatory bowel disease, obesity, autism, allergy, and asthma.[31] Lower vitamin E consumption is associated with higher levels of *Sutterella*,[31] which can cause gastrointestinal disorders by degrading IgA.[32] In addition, *Sutterella* has been found in greater abundance in infants with autism spectrum disorder.[33,34] Omega-3 fatty acid intake increases the abundances of microbes in the *Ruminococcaceae* and *Lachnospiraceae* families as well as in the *Blautia, Bifidobacterium, Coprococcus*, and *Oscillospira* genera.[28]

An unhealthy maternal diets has been demonstrated to increase the risk of preterm delivery and impair embryonic growth and development.[35–37] However, research on the effects of maternal diet composition on infant gut microbes is in the early stages. Consumption of a high-fat diet (more than 40% of total energy) during pregnancy decreases the number of *Bacteroides* species in the infant.[38] However, the correlations between *Bacteroides* and the risk of developing obesity are still under intense debate.[39,40] In addition, the influence of the mother's dietary intake during pregnancy on *Prevotella* abundance is closely related to the risk of food allergy in the infant. The higher the abundance of *Prevotella* in the maternal gut is, the lower the risk of food allergy in infants at 12 months.[41] Future studies need to clarify the optimal dietary formula for the establishment of healthy gut microbes during pregnancy.

2.2.3 Maternal antibiotic exposure

To prevent preterm birth and infection after cesarean (C) section, approximately 40% of women are administered antibiotics during pregnancy.[42–44] Broad-spectrum antibiotics, such as cephalosporins, are frequently prescribed to women in developed countries.[45,46] Few recently published human studies have evaluated the effects of antibiotic treatment during pregnancy on the maternal microbiome. Maternal exposure to antibiotics during delivery can lead to a reduction in the infant's gut microbial diversity.[23] However, differences in the composition and diversity of the infant gut microbiome due to maternal antibiotic exposure have limited impact and may last only one month.[47,48]

2.3 Maternal microbiome and fetal health and development

2.3.1 Maternal microbiome regulates fetal health during pregnancy

For over a century, the development of healthy infants was considered to occur in a sterile environment until birth through the vaginal canal.[49] Unexpected microbial invasion is commonly associated with intrauterine infections and preterm labor.[50,51] During early pregnancy, listeriosis (caused by *Listeria monocytogenes*) can

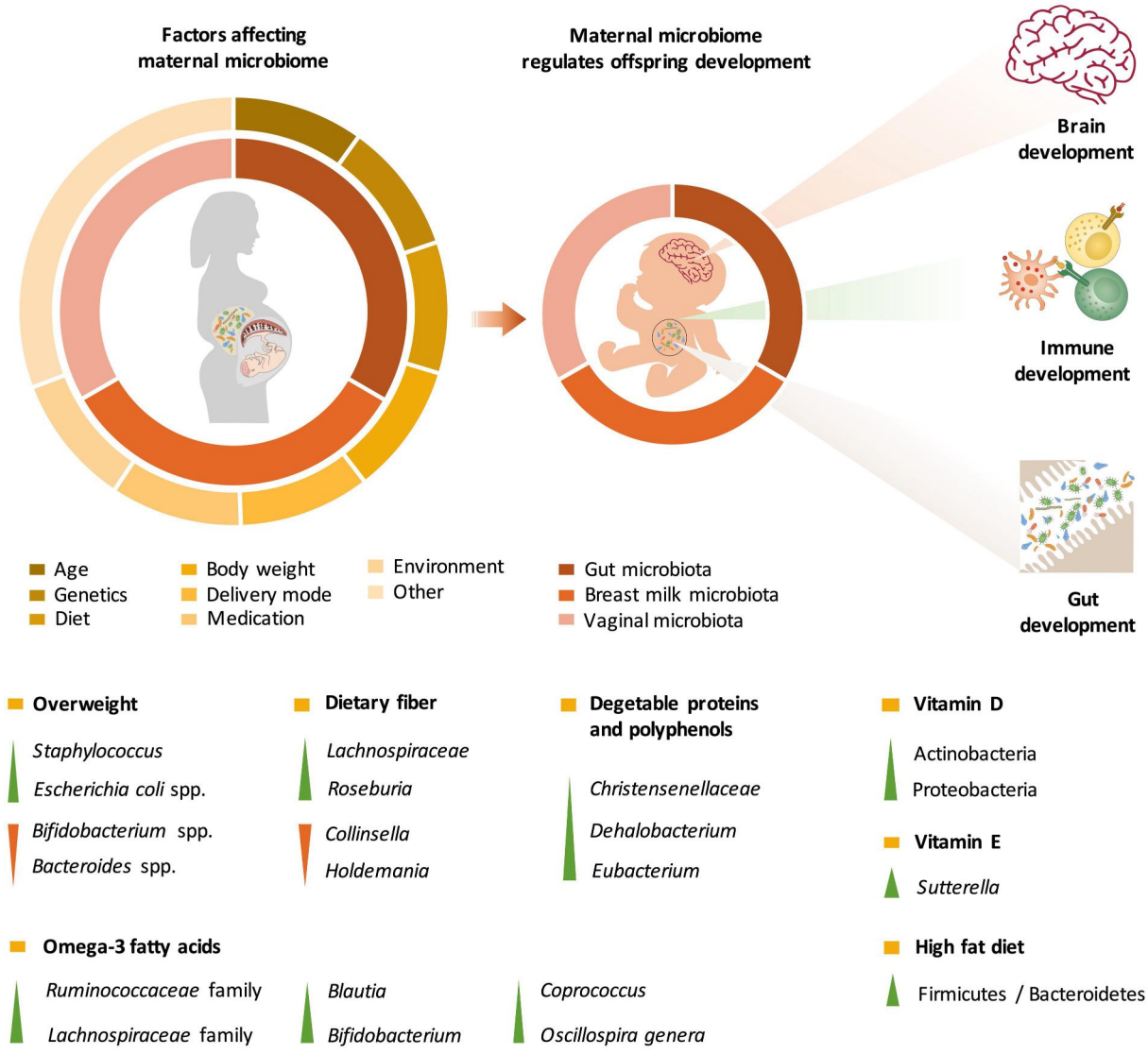

Figure 1. The maternal microbiota and its metabolites regulate fetal development before birth and during lactation. The maternal gut, breast milk and vaginal microbiota are major factors regulating offspring development. The maternal microbiome is influenced by a variety of factors, including age, genetics, diet, body weight, delivery mode, medication use, and the environment. The effects of various factors on the maternal microbiome may be passed on to the offspring through microbes, microbial products or metabolites, which regulate early gut colonization, immune maturation, and brain development in infants.

disrupt the maturation of the fetal immune system and cause neonatal sepsis and death.[52,53] Syphilis (caused by *Treponema pallidum* infection) leads to adverse pregnancy outcomes, and fetal transmission causes congenital diseases such as osteochondritis and meningitis in newborns.[54,55] Although the pathophysiology regarding the transplacental transmission of pathogenic bacteria remains unknown, the impact of bacterial infections on congenital malformations and serious diseases in the fetus has been clearly shown.

Recently, with the development of 16S rRNA sequencing, this general dogma has been challenged. Bacterial signals have been detected in amniotic fluid,[56,57] umbilical cord blood,[58] and fetal membranes[59] in babies who do not have infection or exhibit inflammation. These findings provide evidence for the transmission of the maternal microbiome to the fetus before birth. Bacteria from the maternal intestine can be harvested by dendritic cells and then migrate to the fetus through the blood (Figure 2). Although no consensus has been reached regarding the transfer of microbes before

Box 1. Is the womb sterile?

To verify this hypothesis, pregnant mice were inoculated with genetically labeled *Enterococcus faecium*.[157] Notably, genetically labeled *Enterococcus faecium* was detected in the meconium of fetuses delivered via sterile C-section, which provides primary evidence for maternal microbial transmission to the fetus.[157] However, recently, inconsistent results regarding in utero bacterial colonization among different studies indicate that the detection of a prenatal microbiome might just be due to contamination of reagents, sample handling, etc.[158] Furthermore, even though many studies have reported that bacterial DNA can be detected in first-pass meconium samples, most of the samples are collected hours to days after birth.[159] Meconium collected during cesarean deliveries indicates that healthy infants do not harbor a detectable gut microbiota.[159] Thus, the microbiotas previously identified in neonatal meconium might be acquired during and/or after birth. In addition, bacterial DNA does not indicate the existence of live microbes.

Recent evidence supports the 'sterile womb' hypothesis, as the existence of C-section-born germ-free animals strongly argues against the existence of microbes in the placenta. However, an intense debate about the existence of microbes in the fetus is ongoing. Recently, although strict contamination controls (environmental control, operator control, PBS buffer control, and reagent control) were carefully applied in an experiment,[160] weak but consistent microbial signals were still detected by 16S rRNA sequencing in fetal organs (including the gut, skin, placenta, and lungs) during the 2nd trimester of gestation. More importantly, these microbes were viable and could be cultured and propagated *in vitro*. These sparsely distributed microbes, such as *Staphylococcus* and *Lactobacillus*, induce the activation of memory T cells, which might contribute to the establishment of early immunity.[160] Currently, early fetal microbial colonization is thought to be affected by microbes from the maternal vagina or gut. The contradictions in the results regarding the sterile womb necessitate the collection and assessment of additional evidence in the future.

birth (discussed in Box 1), metabolites from the maternal microbiota are undoubtedly involved in the regulation of infant health and embryonic development and have long-term effects during postnatal life (Figure 1).[60,61] The physiological mechanisms by which the maternal microbiome affects fetal immunity and brain development are now comparatively well established and are discussed below.

2.3.2 Microbiota metabolites regulate fetal development during pregnancy

Metabolites from maternal bacteria can be absorbed into the bloodstream and then transported to the fetus through the placenta.[62] These metabolites regulate multiple biological functions in the fetus, such as metabolic homeostasis.[63] Recently, a growing amount of evidence has indicated that bacterially produced metabolites promote neural development and boost the immune system in the fetus (Figure 3).

2.3.2.1 Brain development. Extremely premature birth (gestational age<28 weeks) is associated with high morbidity and incomplete neurodevelopment.[64] The third trimester during pregnancy is the predominant period during which the maturation of the human brain and the establishment of cognitive capacities occur.[65] Maternal obesity not only affects maternal microbes but also might be associated with neurodevelopmental disorders in offspring, such as deficits in cognition and sociality.[66] A high-fiber diet alleviates maternal obesity-induced synaptic impairments in offspring by reshaping maternal gut microbes (increasing the abundances of *Bifidobacterium animalis*, *Prevotella*, and Clostridiales).[66] The modification of microbes by a high-fiber diet is associated with the enrichment of SCFAs, which further regulate microglial maturation, microglial cell-neuron interactions, and synaptic function in the hippocampus.[66] Recently, the biological functions of SCFAs in the fetus have been well characterized. SCFAs are more than an energy source; they also serve as signaling molecules to regulate cellular functions via the activation of GPR41 and GPR43 (G-protein coupled receptors that sense SCFAs). In addition to SCFA production, the process of axonogenesis is promoted by maternal microbes through the modulation of metabolites (such as trimethylamine-N-oxide (TMAO) and imidazole propionate (IP)) during pregnancy, which could increase axon numbers and promote thalamocortical axonogenesis in the fetal brain.[61] Furthermore, pathogen infection during pregnancy might negatively affect fetal brain development.[67] Bacterial infection during pregnancy can increase the secretion of proinflammatory cytokine interleukin (IL)-17a, which can be transferred through the circulation, affect fetal brain development and induce an abnormal cortical phenotype.[68] In rodent models, probiotic supplementation of the maternal diet during pregnancy promotes offspring brain development (Table 1). A deep understanding of the maternal microbiota and their metabolites that reach the

Table 1. Effect of maternal probiotic and prebiotic supplementation during pregnancy on brain development in offspring.

Probiotic and/or prebiotic	Time	Species	Impact on fetal brain development	Reference
Galacto-oligosaccharide (1.5 mg/ml)	During pregnancy and lactation	Mouse	Reduces expression of hippocampal glutamate receptor genes in offspring	155
Microbial metabolites	Gestational day 0.5 to day 14.5	Mouse	Supports fetal thalamocortical axonogenesis in the developing mouse offspring	61
Lactobacillus (10^9 CFU)	During gestation	Mouse	Rescues brain region-specific microglial dysfunction of the male offspring	161
A combination of 1.5675×10^7 CFU *Bifidobacteria* and 5.28×10^8 CFU *Lactobacillus helveticus*	Embryonic day 0.5 to postnatal day 21	Mouse	Prevents parvalbumin positive (PV+) neuron loss, and the decrease in the γ-aminobutyric acid levels in the prefrontal cortex of adult offspring	162
A combination of *Lactobacillus rhamnosus* R0011 and *Lactobacillus helveticus* R0052 (10^9 CFU/mL)	Postnatal day 2 to 14	Rat	Prevents precocious neural maturation in stressed infants	163
A combination of fructo- and xylooligosaccharides (3 g/kg/day)	During gestational days 0–19	Rat	Protects developing fetal brain against oxidative stress-mediated neurotoxicity	164
Oligofructose (10%)	During the 3rd week of pregnancy and throughout lactation	Rat	Prebiotics reverse maternal antibiotic use-induced decreased microglial response to LPS	165
A formulation containing *L. Fermentum 139*, *L. Fermentum 263* and *L. Fermentum 296* (ratio 1:1:1, 10^9 CFU ml/L)	During pregnancy and lactation	Rat	Improves the sympathetic tone and autonomic dysfunction in male and female offspring	166
Inulin (2 g/kg/BW)	During gestational days 0–19	Rat	Reduces oxidative stress-mediated developmental origins of neurodegenerative disorders	167
Inulin (2 g/kg/day)	During pregnancy	Rat	Protects fetal brain from oxidative dysfunctions; protects developing brain against oxidative stress-mediated neurotoxicity	168

fetus is crucial for facilitating clinical diagnoses and medical interventions. For instance, some metabolites (e.g., IL-17a) can be used as biomarkers for timely monitoring of fetal developmental brain abnormalities. Furthermore, other metabolites (e.g., SCFAs, TMAO, IP), which are beneficial to fetal brain development, could be used as maternal supplements to reduce neurodevelopmental disorders during pregnancy. Further research is needed to determine their clinical significance. In addition, it is crucial to assess the safety of these metabolites and to identify the optimal time for clinical intervention during pregnancy.

2.3.2.2 Immune developmentt. The intestinal commensal microbiota is crucial for the establishment of the host's innate and adaptive immunity. Recent evidence indicates that the maternal microbiome is involved in regulating the establishment of fetal immunity during pregnancy. Disruption of immunity might lead to allergic diseases, metabolic disorders, and infection in the fetus.[69] A reversible germ-free colonization system was established to decipher the potential role of maternal microbes during pregnancy.[70] With this system, transiently colonizing pregnant female mice with a live nonreplicating *E. coli* HA107 modulates the intestinal mucosal innate immune composition by increasing intestinal group 3 innate lymphoid cells and $F4/80^+CD11c^+$ mononuclear cells in their pups.[71] These effects are partially dependent on maternal IgG that potentially retains and transmits bacteria-derived AhR ligands to offspring during pregnancy.[71]

Recently, more metabolites produced by maternal intestinal flora that regulate fetal innate immunity have been identified. Microbiota-derived SCFAs, which play multiple physiological and pharmacological roles, have been reported to boost Treg cell numbers.[72] Specifically, bacteria-derived butyrate and propionate have been shown to increase the differentiation of extrathymic Treg cells in a CNS1-dependent manner in a mouse model.[72] Acetate could prevent the development of preeclampsia through the promotion of fetal thymic and regulatory T-cell development.[73] Furthermore, increased maternal dietary fiber intake during pregnancy has been reported to reduce asthma development in offspring through SCFAs, which might partially be due to the modulation of regulatory T lymphocytes in the fetal lung.[74] In addition, the maternal microbiome regulates fetal immunity through the secretion of inflammatory factors. Maternal infection by a pathogen (*Yersinia pseudotuberculosis*) has been shown to regulate fetal immunity through an increase in the levels of interleukin (IL)-6, which crosses the placental barrier, promotes intestinal Th17 cell responses and enhances

intestinal protective immunity in the fetus.[75] Taken together, these findings suggest that maternal microbial metabolites (AhR ligands and SCFAs) and inflammatory factors (IL-6) synergistically regulate the early establishment of innate immunity before birth. It is worth noting that these identified microbial metabolites cannot fully explain the complicated molecular mechanism involved in gestational microbial shaping. Targeted and untargeted metabolomic studies are required to further identify other crucial microbial metabolites that participate in the regulation of fetal innate immunity.

To date, whether gestational colonization of the microbiota regulates adaptive immunity is unclear, which might be due to the controversy surrounding the prenatal microbiome. Amplification and activation of B and T cells are triggered mainly by bacterial colonization.[76,77] Although colonization only during gestation does not affect adaptive immunity (populations of B or T cells) in infants,[78] pregnant mothers who undergo antibiotic treatment have been shown to have lower proportions of both $CD4^+$ and $CD8^+$ T cells.[79–81] Future research is needed to verify whether microbes are transferred from mother to fetus during the prenatal period, which could help clarify recent conflicting reports. Despite the inconsistent results regarding adaptive immunity regulation, it is worth noting that some preliminary evidence has indicated that probiotic supplementation of the maternal diet during pregnancy may reduce the risk of immune-mediated disease in infants (Table 2). Thus, modulation of the maternal diet, for instance, via direct supplementation with functional metabolites could be an efficient way to program the immunity of neonates.

3. Maternal microbiome and neonatal health and development after birth

After birth, neonates are exposed to commensal microbes from a variety of sources. Maternal oral, gut and vaginal microbes all contribute to the initial bacterial inoculum in the newborn.[82] Subsequently, antibiotic use, human milk, solid food, host genetics, and environmental exposure further affect the gut microbiome during early life.[83–85] The complicated sources and transmission routes of pioneering microbes in infants make it difficult to systematically understand pioneer colonizers in newborns. In this section, we discuss the current understanding of the influence of the maternal microbiome derived from the maternal gut, vagina, and milk on early microbial colonization in neonates and the role of these microbes in neonatal health and development.

3.1 Maternal-source microbiome

3.1.1 Maternal gut microbiome

The human maternal gut microbiota remains stable over the perinatal period (during late pregnancy and early lactation).[17] To the best of our knowledge, change that occurs in the intestinal microbial community of humans during lactation is unknown. As a good model for the human microbiota, swine gut microbes harbor more Firmicutes (Lachnospiraceae, Christensenellaceae, *Clostridium*, *Ruminococcus*, and *Lactobacillus*) and Bacteroidetes (*Prevotella* and Paraprevotellaceae) during lactation than during gestation, with a corresponding augmentation in SCFA production.[86] Similar to the situation that is observed during the gestational period, individual heterogeneity might also be a crucial factor that regulates the composition of the intestinal microbiome during lactation. The maternal gut microbiota regulates neonatal microbes via direct mother-neonate contact during vaginal delivery. In addition, *Bifidobacterium*, *Bacteroides*, *Parabacteroides* and members of Clostridia are shared among neonatal feces, maternal feces and breast milk,[87] which indicates that the maternal gut microbiota can be transferred to the neonate through breast milk during lactation (this concept is discussed in the section on the maternal milk microbiome in detail).

3.1.2 Vaginal microbiome

The vaginal microbiota during pregnancy is less diverse and comparatively stable throughout pregnancy.[88,89] Five bacterial community state types (CSTs) have been defined in the vagina.[90] In Asian and white women, CSTs are dominated by *Lactobacillus* species (*L. crispatus* (CST I), *L. gasseri* (CST II), *L. iners* (CST III) and *L. jensenii* (CST V)). CST IV (*Lactobacillus* spp.) is often observed in black and Hispanic women. This might partially

Table 2. Effect of maternal probiotic and prebiotic supplementation during pregnancy on immune development in offspring.

Maternal diet	Time	Species	Impact on fetal immune function	Reference
10^9 CFU of *Bifidobacterium lactis* and 10^9 CFU of *Lactobacillus rhamnosus*	14 days prior to the cesarian section	Human	Protects the infant against detrimental long-term immunologic disturbances	57
Galacto-oligosaccharides and inulin in a 9:1 ratio, 4%	During acclimation, mating, and gestation	Mouse	Increases the presence of B and T regulatory immune cell subsets in the lymph nodes of offspring	169
Galacto-oligosaccharides and inulin (at a 9:1 ratio, 4%)	During mating and pregnancy	Mouse	Increases the abundance of regulatory B and T cells in the fetus	170
Bifidobacterium breve M-16 V	Pregnancy days 14 to the end of the lactation	Mouse	Prevents and/or alleviates allergic lung inflammation	171
Inulin (10%)	During pregnancy	Mouse	Regulates the differentiation of regulatory T-cell in offspring	172
Settings Lactobacillus fermentum CECT5716 (10^{10} UFC/day)	Pregnancy day 0 to lactation day 14	Rat	Increases IgA and IgG2a concentrations in offspring plasma	173
Sodium formate, sodium propionate, or sodium butyrate (250 mM)	Prior to pregnancy until day 40 after KRV infection	Rat	Downregulates virus-induced proinflammatory responses in the intestine; alters the B- and T-cell compartments in Peyer's patches	174
Short-chain galacto- and long-chain fructo-oligosaccharides in a ratio of 9 : 1, 3%	From 2 weeks before mating until childbirth	Rat	Increases percentage of regulatory T cells and the concentrations of immunoglobulin (Ig)E and ovalbumin-specific IgG2a	175
Bifidobacterium animalis subsp lactis BB-12® (3×10^9 CFU/ml) and *Propionibacterium jensenii 702* (8.0×10^8 CFU/ml)	From 10 days before conception until postnatal day	Rat	Improves the plasma IgA and luminal IgA levels of stressed animals	176
Chitosan oligosaccharide (0.12/0.24 g/day)	During gestation and lactation	Pig	Increases the serum concentrations of IgM and sIgA in piglets	177
Mannan oligosaccharide (400 mg/kg)	From day 86 of gestation until weaning	Pig	Decreases concentrations of proinflammatory cytokines IL-2 and IL-4 in piglet serum	178
An insoluble/soluble fiber ratio of 3.89, 5.59, 9.12, and 12.81	During pregnancy	Pig	Improves antioxidative capacity and decrease the inflammatory response	179
Sugar beet pulp (20% SBP in gestation and 10% SBP in lactation)	Gestation and lactation	Pig	Ameliorates intestinal inflammation in piglets	180
Pregelatinized waxy maize starch plus guar gum (2.0%)	Pregnancy day 85 to end of lactation	Pig	Increases the plasma concentrations of IL-10 and TGF-β	181
Mannan oligosaccharide (400 mg/kg)	From d 86 of gestation until weaning	Pig	Increases the serum concentrations of IgA and IgG in piglets at weaning	182
Short-chain fructooligosaccharide (10 g sCFOS/d)	From last 4 weeks of gestation to the first 4 weeks of lactation	Pig	Improves the intestinal immune function of piglets	183
Seaweed extract (10 g/d)	Pregnancy day 107 to end of lactation	Pig	Increases piglet plasma IgG levels and improves intestinal inflammation	184

explain why vaginally born infants initially exhibit an enrichment in *Lactobacillus* spp. in the oral cavity, skin and gut.[91] During gestational progression, enriched *Lactobacillus* species might produce lactic acid and protect the vagina from bacterial vaginosis and pelvic inflammatory disease.[89,92] Disruption of the vaginal microbiome during pregnancy is associated with preterm birth. *Lactobacillus iners* is correlated with the risk of preterm birth,[93] while *Lactobacillus crispatus* dominance has a protective effect against preterm birth.[53] Furthermore, preterm birth has been found to be associated with proinflammatory cytokines in vaginal fluid, including eotaxin, IL-1β, IL-6 and macrophage inflammatory protein (MIP)-1β.[94]

The mode of delivery (C-section-born infants vs. vaginally born infants) considerably affects microbiota colonization in newborns. C-section is usually conducted under antibiotic treatment to prevent infection, which further affects the transmission of maternal microbes to offspring. Thus, neonates born by C-section are associated with an aberrant and distinct gut microbiome that differs from that in vaginally born neonates.[95–98] Intriguingly, microbes in vaginally born infants are affected mainly by maternal vaginal and fecal microbes, whereas microbes from C-section-born infants are more likely to be determined by maternal skin microbes.[91,99] Infants born by C-section have a decreased abundance of *Bacteroides* spp. and *Lactobacillus*. [100,101] The shift in the microbial community caused by C-section might subsequently increase the risk of childhood sickness, such as asthma, obesity, allergies, and autoimmunity.[83,117,118]

Both vaginal seeding and maternal fecal microbiota transplantation can partially restore the microbiota difference caused by C-section

delivery.[102] However, unlike maternal intestinal strains, maternal vaginal strains seem to contribute to only a small and transient fraction of the microbes compared to the neonatal intestinal microbiota after birth. Oral administration of a maternal vaginal microbiota has a weak effect on the early development of the neonatal gut microbiome after one month.[103] This might be because the vaginal environment is remarkably different from the gut environment, which makes the maternal vaginal strains difficult to seed. Currently, the understanding of neonatal microbiome seeding is limited, which prevents comprehensive assessment of the acute and lasting effects of the vaginal microbiome on newborns. Further research is needed to determine whether the maternal microbiota composition is optimal. Understanding the most effective combination of microbes and their metabolites may allow for the development of targeted probiotics that could be used for infants born by C-section.

3.1.3 Milk microbiome

As the optimal nutrient source for infants, breast milk is the most important postnatal link between the mother and infant. In addition to macronutrients (protein, fat, and lactose), micronutrients (minerals and vitamins), oligosaccharides and immune factors, maternal milk contains a microbiota. Breast milk-derived microbes help neonates build a beneficial microbiome.[104] The origins of bacteria in breast milk have not been fully elucidated, and these bacteria might be derived from maternal skin,[105] the infant oral cavity,[106] the peripheral environment,[107] and the maternal gut.[108] Currently, the neonatal oral cavity[109,110] and maternal gastrointestinal tract[108,111] are considered two major sources of milk microbes. Researchers have proposed "the enteromammary transfer hypothesis" and "the retrograde flow transfer hypothesis" to interpret the origin of the human milk microbiota (Figure 2).[112,113]

Both culture-based and sequence-based work suggest that streptococci and staphylococci are the most predominant taxa in human milk, but results regarding other bacterial taxa (such as lactobacilli) are inconsistent.[114] To date, it has been difficult to conclude that the variation in milk microbes is attributed to geographical location or contamination during sampling.[115–117] The milk microbiome is affected by lactation stage and influenced by maternal health status and BMI during pregnancy. In human milk, hindmilk (milk produced at the end of breastfeeding) has a higher bacterial load than foremilk (milk produced at the start of breastfeeding).[118] Obese mothers have lower numbers of *Bifidobacterium* and higher numbers of *Staphylococcus* in their breast milk than normal-weight mothers.[119] The effect of breast milk on infants lasts throughout the breastfeeding period. Approximately 33% and 23% of the bacterial taxa (especially *Streptococcus*, *Veillonella* and *Bifidobacterium* spp.) detected in infant feces are similar to those in maternal milk at 5 and 9 months, respectively.[118]

3.2 Maternal microbiome regulates neonatal health and development

3.2.1 Early microbial colonization regulates neonatal health

Microorganisms in the neonatal gut regulate infant development and immune system maturation. There is a close relationship between intestinal microbial disorders and the development of pediatric diseases, such as obesity and metabolic disease.[120,121] NEC and LOS are major causes of neonatal morbidity and mortality, and their occurrence is closely related to early microbial colonization. During early infancy, elevated levels of Enterobacteriaceae may contribute to the development of NEC.[122,123] The intestinal microbiota in LOS infants has limited diversity, with low levels of *Bacteroides* and *Bifidobacterium* and a predominance of enterobacteria.[124] In addition, early microbial colonization can affect asthma.[125] The relative abundance of *Lachnospira*, *Veillonella*, *Faecalibacterium* and *Rothia* in the first 100 days of life has been found to be significantly reduced in children at risk of asthma.[125] Furthermore, maternal group B S*treptococcus* colonization is related to increased aortic intima-media thickness in infants, which may have an impact on cardiovascular risk.[126] Currently, it remains unclear how maternal microbes modulate pediatric disease through early

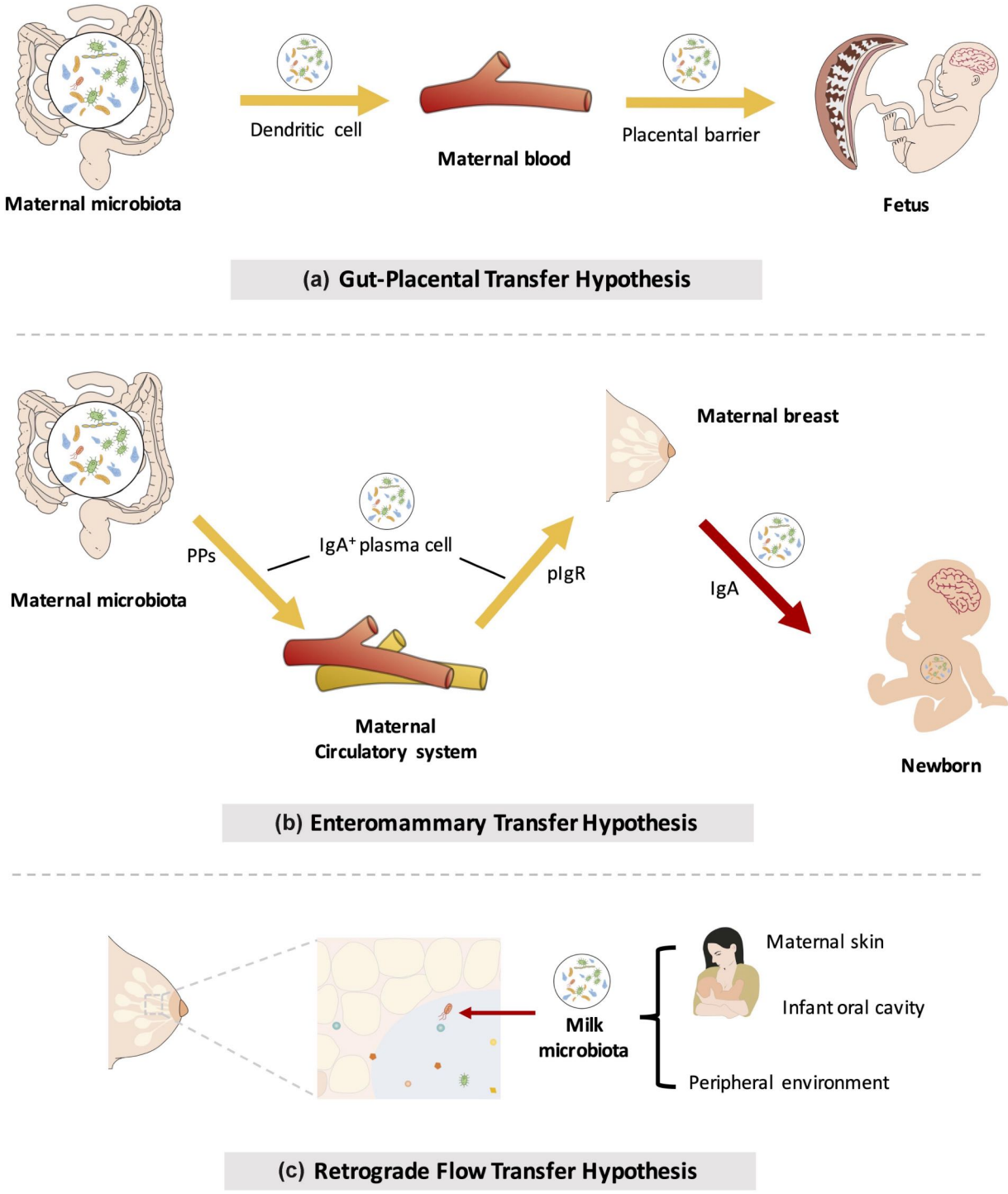

Figure 2. Mother-to-offspring bacterial transmission during pregnancy and lactation. (a) Gut-placental transmission hypothesis. This hypothesis states that transmission of the maternal microbiome to the fetus occurs before birth. Bacteria from the maternal intestine can be harvested by dendritic cells and then migrate to the fetus through the blood. (b) Enteromammary transfer hypothesis. Breast milk functions as the most important postnatal link between infants and mothers. In addition to macronutrients, micronutrients, oligosaccharides and immune factors, maternal milk contains a microbiota. This hypothesis states that the parent microbes combine with IgA in the gut to form a complex (IgA^+). IgA^+ plasma cells translocate to maternal mammary tissue via intestinal Peyer's patches (PPs) to release microorganisms and secrete IgA. Breastfeeding newborns acquire nutrients and microorganisms in milk. Microorganisms in milk are transferred to colonize the neonatal gut. (c) Retrograde flow transfer hypothesis. This hypothesis proposes that the microbiota from the maternal skin, fetal oral cavity and environment enter the maternal mammary gland during lactation, thereby composing the milk microbial community.

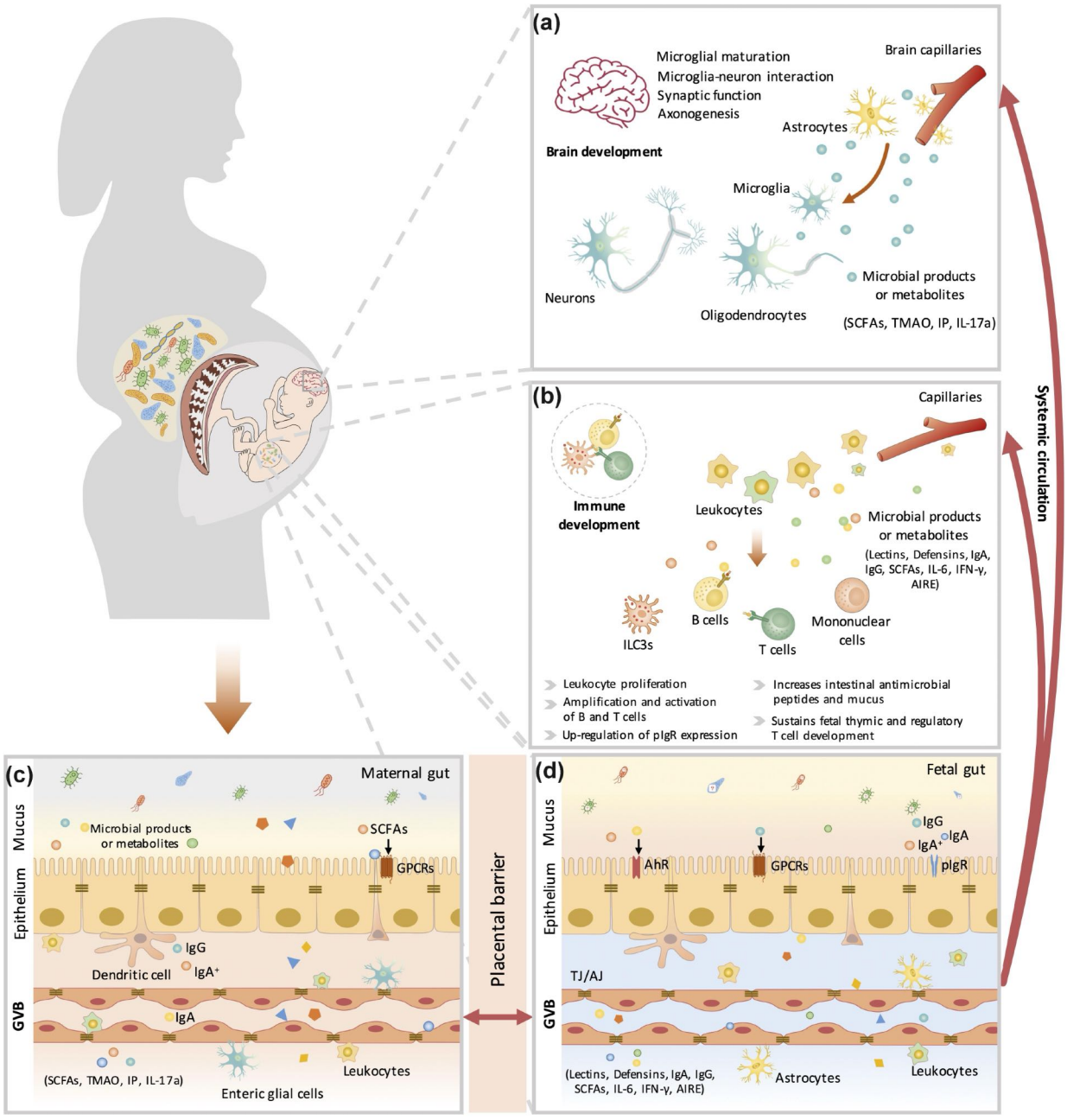

Figure 3. The maternal microbiota or microbiota metabolites regulate fetal immunity and brain development. Bacterial metabolites can be absorbed into the bloodstream and then transported to the fetus during placentation. These metabolites derived from maternal bacteria regulate multiple biological functions of the fetus. Short-chain fatty acids (SCFAs) produced by microbial fermentation of high-fiber diets may regulate microglial maturation, microglial cell-neuron interactions, and synaptic function in the hippocampus by activating fatty acid receptors (GPR41 and GPR43). During pregnancy, maternal microbes also increase axonal numbers and promote thalamocortical axon development in the fetal brain by modulating the levels of metabolites, such as trimethylamine-N-oxide (TMAO) and imidazole propionate (IP). In addition, pathogen infection during pregnancy increases proinflammatory cytokine IL-17a secretion, which is transmitted through the circulation, affects fetal brain development and induces abnormal cortical phenotypes. Maternal microbes are also involved in the regulation of fetal immune establishment during pregnancy. Maternal intestinal Escherichia coli HA107 colonization increases fetal intestinal leukocyte proliferation (that of type 3 innate lymphocytes (Ilc3s) and monocytes) as well as the production of intestinal antimicrobial peptides (C-type lectins and defensins of the REG family) and mucus. Some microbiota-derived compounds are microbial aryl hydrocarbon receptor (AhR) ligands, which promote the amplification of ILC3s. Maternal-derived SCFAs also regulate thymus and T-lymphocyte development. Maternal infection by a pathogen (Yersinia pseudotuberculosis) regulates fetal immunity through an increase in the levels of IL-6, which crosses the placental barrier and promotes intestinal Th17 cell responses and intestinal protective immunity in the fetus. Therefore, maternal microbial metabolites (AhR ligands and SCFAs) and the cytokine-mediated response (IL-6) synergistically regulate the early establishment of immunity before birth. AIRE, autoimmune regulator; AJ, adherens junction; GPCRs, G protein-coupled receptors; GVB, gut vascular barrier; IFN-γ, interferon γ; IgA, immunoglobulin A; IgA^+, IgA^+ plasma cells; IgG, immunoglobulin G; pIgr, polymeric immunoglobulin receptor; TJ, tight junction.

microbial colonization. However, there is growing evidence indicating that the maternal microbiome plays an important role in the regulation of neonatal immunity and neurodevelopment.

3.2.2 Maternal microbiome regulates neonatal development

3.2.2.1 Immune development. Beneficial microbes inherited from breast milk contribute to the establishment of the immune system in neonates, especially during the first 3 months of life (Figure 4).[127] *Lactobacillus reuteri* enrichment in milk promotes the production of IgA through the induction of T cells and type 3 innate lymphocytes (ILC3s) in neonates.[127] Milk-derived Bifidobacterium produces indolelactic acid via aromatic lactate dehydrogenase in infants.[128] Importantly, indolelactic acid can further induce the immune responses of $CD4^+$ T cells and monocytes through the activation of AhR and hydroxycarboxylic acid receptor 3.[128] Human breast milk contains a large amount of indigestible oligosaccharides (human milk oligosaccharides, HMOs), which can be used by commensal organisms as well as probiotics.[129–131] Bifidobacterium expressing HMO utilization genes can alleviate inflammatory responses by decreasing proinflammatory Th2 cells and promoting the expansion of anti-inflammatory regulatory T cells.[130] Thus, maternal milk-derived HMOs and beneficial microbes can coordinately boost the immunity of neonates.

The maternal microbiome also regulates neonatal immunity through the secretion of milk immunoglobulin (Ig). Maternal milk, especially colostrum, is enriched in IgA. In contrast to other organic components in milk, Ig is not synthesized by mammary epithelial cells. In the mammary gland, both pIgR expression and IgA plasma cell

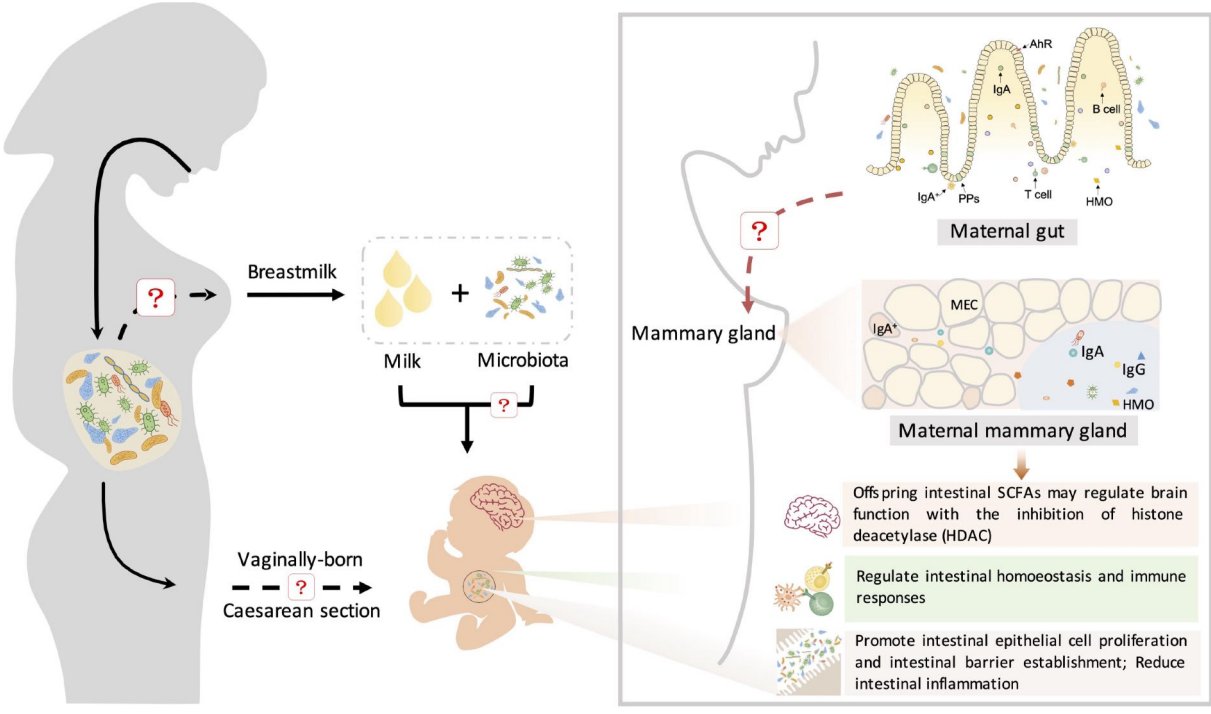

Figure 4. The maternal microbiota regulates neonatal immunity and brain development. Beneficial microbes inherited from breast milk contribute to the establishment of the immune system in neonates. Beneficial microbes from breast milk promote the production of immunoglobulins (IgA and IgG) and regulate intestinal homeostasis and immune responses (CD4+ T cells and monocytes) through the aryl hydrocarbon receptor (AhR). IgA^+ plasma cells are recruited from maternal intestinal Peyer's patches (PPs) and then translocate through the circulatory system to the mammary gland and secrete IgA. Furthermore, maternal intestinal Ig-coated bacteria can directly translocate to the mammary gland and then be enriched in maternal milk. Immunoglobulins in milk promote the differentiation of neonatal intestinal epithelial cells and the establishment of intestinal barrier function, reducing intestinal inflammation. During this process, immunoglobulins cooperate with gut microbes to regulate innate and adaptive immunity in neonates. In addition, maternal prebiotic intake increases the concentrations of offspring intestinal SCFAs, which may cross the blood – brain barrier and modulate brain function by inhibiting histone deacetylases (HDACs).

accumulation are positively correlated with the production of milk IgA.[132] Evidence suggests that milk IgA is either derived from serum or secreted from IgA^+ plasma cells that translocate to the mammary gland.[133] From pregnancy to the first few days of lactation, intestinal IgA^+ plasma cells are significantly enriched, accompanied by a similar increase in the number of IgA^+ plasma cells in the mammary gland.[134] It is now clear that mammary gland IgA^+ plasma cells are recruited mainly from intestinal Peyer's patches (PPs) but not supramammary lymph nodes (LNs).[135] Therefore, maternal gut microbes are involved in the regulation of milk IgA production.[135] Maternal milk IgA production in germ-free (GF) mice is markedly lower than that in wild-type mice.[135] *Bacteroides acidifaciens* and *Prevotella buccalis* cohabiting in the large intestine induce the synthesis of bacterium-specific IgA in milk.[135]

IgA binds to a variety of microbes, including four major phyla, namely, Firmicutes, Actinobacteria, Bacteroidetes, and Proteobacteria.[136] Recently, accumulating evidence has revealed the potential role of IgA in the neonatal gut, which contributes to the establishment of the intestinal barrier and regulates the innate and adaptive immunity of neonates by binding to intestinal bacteria. First, IgA coats harmful bacteria and prevents their colonization and propagation. The enrichment of Proteobacteria, especially Enterobacteriaceae, is a crucial factor that triggers the development of NEC, an inflammatory gastrointestinal disease, in preterm infants.[8] Milk IgA binds to Enterobacteriaceae and prevents the development of NEC in human infants.[137] Second, IgA facilitates the adherence of beneficial bacteria (such as *Bacteroides fragilis*) to intestinal epithelial cells and promotes their proliferation.[138] Third, IgA coating intestinal bacteria alleviates intestinal inflammation in neonates.[139] The IgA coating of the microbiota decreases the proportions of ROR-γt^+ Tregs and alleviates mucosal immune induction and inflammatory disease susceptibility.[140] Furthermore, maternal intestinal Ig-coated bacteria can directly translocate to the mammary gland and then be enriched in maternal milk.[136] IgA-coated Lachnospiraceae bacteria in milk can directly regulate the adaptive immune responses in the intestine.[141,142] Due to technical issues, such as those with anti-Ig antibodies (monoclonal or polyclonal) and sequencing methods, it is difficult to identify the dominant Ig-coated community in maternal milk.[143–145] In addition to IgA, IgG binds with bacteria and regulates gut immune function. Enteropathogenic *E. coli* (EPEC) is a foodborne pathogen that causes diarrhea in neonates.[146] EPEC induces Ig production through "attaching and effacing" (A/E) lesions located on the intestinal epithelium.[147] Interestingly, maternal pathogen-induced production of IgG but not IgA or IgM protects neonates against EPEC infection. Mechanistically, neutrophil phagocytosis is increased when pathogens are specifically coated with IgG.[148] Although the origins of milk Igs and their capacity to prevent bacterial pathogenicity are well established, their role in promoting beneficial microbes and regulating immunity needs to be clarified. In addition, future research is required to identify other potential bacteria that can bind with Igs and their role in the regulation of neonatal gut immune function.

Allergic diseases affect the respiratory system, digestive system, skin and other systems. There are potential associations between immune dysfunction (such as an imbalance toward a T-helper-2 response and exaggerated IgE responses) and allergic diseases in infants.[149] Atopic eczema is a strong predictor of allergic disease and occurs during childhood.[150] During early infancy, a reduction in the abundance of bacteria with the ability to produce butyrate and propionate was observed in infants with atopic eczema.[151] The World Allergy Organization (WAO) guideline panel suggests the use of probiotics in pregnant and breasting women with children at high risk for allergies.[152] This recommendation could efficiently prevent the development of atopic eczema during childhood. Additionally, initial microbial colonization is associated with other immune-mediated diseases, such as inflammatory bowel disease (IBD)[153] and asthma.[154] More research is needed to clarify whether and how maternal microbes modulate clinical disease.

3.2.2.2 Brain development. Recent evidence also indicates that the maternal microbiome regulates neonatal brain development (Figure 4). Maternal prebiotic intake reduces anxiety and promotes

brain development. The intestinal microbiome composition in the offspring of mice fed prebiotics differs from that in control offspring, with significant decreases in the abundances of *Bacteroides caecimuris, the Barnesiella* genus, and the *Bifidobacterium* genus.[155] Importantly, postnatal maternal prebiotic intake significantly increases the concentrations of intestinal SCFAs in offspring, which might cross the blood – brain barrier and regulate brain function via the inhibition of histone deacetylase (HDAC).[155] In contrast, maternal infection with *E. coli* O16:H48 has a negative effect on the growth of neonates in a mouse model.[156] *E. coli* O16:H48 can disrupt the production of precursors of neurotransmitters (such as serotonin), which negatively affects maternal behavior.[156] Poor maternal behavior blocks milk availability for neonates and subsequently affects the brain development of neonates.[156]

During pregnancy, the developing fetus is entirely dependent on maternally derived nutrients. However, during lactation, when maternal breast milk is insufficient, an alternative form of enteral nutrition for preterm or low birth weight (LBW) infants is artificial formula. Currently, artificial formula contains more nutrients than breast milk but might be deficient in some nonnutritive bioactive components. Fortification of artificial formulas with microbiota and functional metabolites such as milk-derived Bifidobacterium and *Lactobacillus reuteri* as well as indolelactic acid could be beneficial for preterm or LBW infants and might have great potential for alleviating clinical diseases such as IBD and asthma.

4. Conclusion and perspectives

A healthy maternal microbiota is required for the health and development of offspring. During the progression of gestation and lactation, the maternal gut microbe composition is comparatively stable. Individual heterogeneity is the main factor that shapes the maternal intestinal microbiome. These microbes do not act as silent passengers but have long-term effects that reduce the risk of immune-mediated illness and improve the cognitive outcomes of offspring. During pregnancy, maternal microbial metabolites can be transported from the placenta to the fetus to regulate immunity and recognition. Whether a small number of bacteria exist in the fetus before birth is uncertain, and further research is needed.

Microbial colonization of neonates begins at birth. The transfer of microbes from mother to newborn is influenced by many factors, such as birth route and nursing. Vaginal birth, breastfeeding, and skin-to-skin care are considered predominant factors in establishing the close relationship between the mother and infant microbiome. Maternal microbes that originate from the maternal gut but not the vagina contribute mainly to the early colonization of the microbiome. Maternal milk is rich in microbes, oligosaccharides and Igs, which boost immune and brain development. The disruption of maternal-to-child microbiome transfer might lead to short- and/or long-term adverse health issues.

Recently, great interest in modulating the maternal and neonatal microbiomes through therapeutic manipulation has emerged. Maternal probiotic and prebiotic supplementation during pregnancy have been found to influence the regulation of offspring brain and immune development (as shown in Tables 1 and 2). The analysis and modulation of specific bacterial metabolites, such as IL-17a, AhR ligands and SCFAs, may provide a noninvasive monitoring and intervention strategy to improve adverse prognoses and alleviate clinical symptoms. Further studies are needed to identify other crucial microbial metabolites that participate in regulating fetal immune and brain development using both targeted and untargeted metabolomics.

Outstanding questions

Numerous outstanding questions that deserve to be addressed in the future are listed below.

(1) Is the womb sterile or not?
(2) Are there key molecular, genetic or microbiome-based biomarkers available to test the "gut-placental transmission hypothesis", "entero-mammary transfer hypothesis" and "retrograde flow transfer hypothesis"?
(3) Which maternal-derived microbes participate in early colonization of the offspring's gut and what is the potential role of

maternal-derived microbes in later microbiota development?

(4) In the milk, are there any functional metabolites synthesized locally in the mammary gland?

(5) Could diet-derived maternal microbial metabolites in milk be modulated by dietary intervention or by direct supplementation with targeted metabolites?

Authors' contributions

MT and SHZ initiated the idea, scope, and outline of this review paper. MT, SHZ, QHL, THZ, SWY and FC studied and analyzed all of the publications cited in this paper and were involved in manuscript preparation. SHZ and WTG conducted the final editing and proofreading. All authors have read and approved the final manuscript.

Disclosure statement

No potential conflict of interest was reported by the authors.

Funding

This study was financially supported by the National Key R&D Program of China [No. 2021YFD1300700], Guangdong Basic and Applied Basic Research Foundation [No. 2021A1515010440], Science and Technology Program of Guangzhou [No. 202102020056], and National Natural Science Foundation of China [Nos. 31802067 and 31872364].

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Cite this: *Food Funct.*, 2023, **14**, 3290

Maternal supplementation with glycerol monolaurate improves the intestinal health of suckling piglets by inhibiting the NF-κB/MAPK pathways and improving oxidative stability†

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Glycerol monolaurate (GML) is a food safe emulsifier and a kind of MCFA monoglyceride that has been proven to confer positive benefits in improving animal health, production and feed digestibility as a feed additive. This study aims to evaluate whether supplementation of a sow diet with GML could affect the intestinal barrier function and antioxidant status of newborn piglets and to explore its regulatory mechanism. A total of 80 multiparous sows were divided into two groups, which were fed a basal diet or a basal diet supplemented with 0.1% GML. The results indicated that maternal supplementation with GML significantly increased fat, lactose and protein in sow colostrum, as well as fat and protein in sow 14-day milk ($P < 0.05$). The results showed that GML significantly reduced the concentrations of IL-12 in the duodenum, TNF-α, IL-1β and IL-12 in the jejunum, and IL-1β in the ileum of piglets ($P < 0.05$). Higher concentrations of T-AOC, T-SOD, GSH and GSH-Px and lower MDA in the intestine were observed in the GML group than in the control group. Correspondingly, the villi height, crypt depth and the ratio of villi height to crypt depth (V/C) in the jejunum and the V/C in the ileum in the GML group were significantly higher than those in the control group ($P < 0.05$). Moreover, the GML group displayed significantly increased protein abundance of zonula occludens (ZO)-1, occludin, and claudin-1 in the small intestine ($P < 0.05$), mRNA expression of mucins (MUCs) in the small intestine (*MUC-1*, *MUC-3* and *MUC-4*), and mRNA expression of porcine beta defensins (pBDs) in the duodenum (*pBD1* and *pBD2*), jejunum (*pBD1*, *pBD2* and *pBD129*) ($P < 0.05$), and ileum (*pBD2*, *pBD3* and *pBD114*) ($P < 0.05$). Further research showed that GML significantly reduced the phosphorylation of the NF-κB/MAPK pathways in the small intestine ($P < 0.05$). In addition, the results of 16S rDNA sequencing showed that maternal supplementation with GML altered the colonic microbiotic structure of piglets, and reduced the relative abundance of *Escherichia shigella*. In summary, a sow diet supplemented with GML enhanced the offspring's intestinal oxidative stability and barrier function and attenuated the offspring's intestinal inflammatory response, possibly by suppressing the activation of the NF-κB/MAPK pathways.

Received 5th January 2023,
Accepted 6th March 2023

DOI: 10.1039/d3fo00068k

rsc.li/food-function

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†Electronic supplementary information (ESI) available: The raw data of 16S sequencing analysis of microbiota involved in this study (**https://bigd.big.ac.cn/gsa/browse/CRA008645**). The following supporting information can be downloaded at: **https://www.rsc.org/**. Table S1: ingredients of glycerol monolaurate added in feed; Table S2: diet composition and nutrition level of lactating sows (As-fed basis); Table S3: analytical kit information used in this study; Table S4: primers used in real-time quantitative; Table S5: antibody information used in this experiment. See DOI: **https://doi.org/10.1039/d3fo00068k**

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1 Introduction

The development of the piglet gastrointestinal tract (GIT) before and after birth is a dynamic and evolving process.[1] The lactation phase and postweaning phase are the key stages of piglet GIT development.[2] Both pathogenic microorganisms in the environment and weaning stress are important triggers of gastrointestinal disease in piglets.[2,3] In the lactation phase, sufficient intake of colostrum and milk is a key factor in ensuring the intestinal health of piglets.[4–6] Previous studies have shown that the quality of a sow's milk is closely related to her diet during lactation.[2] Importantly, compelling evidence has indicated that immune agents and antioxidant protection can be transferred from mother to neonate *via* maternal colostrum

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and milk supply.[2,7] For example, recent evidence has suggested that supplementation with phytogenic actives and enzyme-hydrolyzed *Artemisia annua* (artemisinin) in sow diets significantly improved the antioxidant and anti-inflammatory capacity of offspring *via* maternal-to-fetal nutrition transmission.[8,9] Thus, it is feasible to enhance the intestinal oxidative stability of offspring and promote gastrointestinal health by formulating scientific nutrition strategies for sows.

Glycerol monolaurate (GML) is a monoglyceride composed of glycerol and lauric acid that naturally exists in milk, coconut oil and other natural sources and has a variety of biological activities.[10,11] GML is also a food safe emulsifier approved by the US Food and Drug Administration that will not be toxic even at a higher dose, and the content range is 10 to 2000 mg kg^{-1} in food and health care products (21 CFR GRAS 182.4505).[12] GML has been used in animal production because of its extensive immunomodulatory properties, animal friendliness and growth-promoting effects.[13,14] *In vitro*, GML inhibited T-cell receptor-induced signal transduction and T-cell effector function to reduce cytokine production.[15] A recent study showed that GML can be used to improve the intestinal health of piglets due to its established safety and antibacterial and immune regulation ability, and it was confirmed that it is an effective fungicide and antibiotic substitute.[16] Currently, no research has shown whether a sow diet supplemented with GML can improve the antioxidant status and barrier function of the GIT of offspring. Therefore, this study aims to evaluate whether the addition of GML to the diet of sows affects the intestinal barrier function and antioxidant status of offspring and to explore its regulatory mechanism to provide a theoretical basis to guide production.

2 Materials and methods

2.1 Animals, diets and management

All animal procedures conducted in this study were approved by the Animal Care and Use Committee of South China Agricultural University (no. SYXK2019-0136, Guangzhou, China). This trial was carried out on an experimental farm in Heyuan city, Guangdong Province, China. A total of 80 multiparous sows (Landrace × large white) were randomly allocated to 2 groups (40 sows in each group) by body condition, parity, and historical reproductive performance as follows: CON group (basal diet) and GML group. According to previous studies, an effective dose of GLM in pig trials is around 200–1600 mg kg^{-1};[13,16,17] thus 1000 mg kg^{-1} GML (purity of 94%, Table S1†) was chosen for this trial. The nutritional level of the basal diet meets the nutritional requirements of pregnant and lactating sows according to the National Research Council (NRC) (2012). No antibiotics were used during the entire trial. The feed composition and nutrient level of the basal diet are listed in Table S2.†

Animal feeding started from the 85th day of gestation and continued to the 21st day of lactation. The daily management and routine immunization procedures for all sows and piglets followed pig farm regulations. During late pregnancy (from day 85 to day 107 of pregnancy), each sow was housed in an individual crate (2.10 × 0.6 square meters) with free access to water and was fed twice a day (6:30 and 14:00). Sows were restricted from feeding, and the total diet of each sow was 3.0–3.5 kg per day. On the third day after parturition, sows were fed a 3 kg diet, which was then gradually increased by 0.5 kg per day to the maximum feed intake. Within 48 hours after delivery, piglets were only cross fed within the group, and the litter size was standardized to 10–12 piglets. During this study, no milk replacer or creep feed was provided to the piglets. Piglets were kept from sow feeders while the sows were eating by restricting the piglets to cages. Furthermore, we also cleaned the remaining feed from the sow feeder in a timely manner. At weaning, 12 piglets were slaughtered using humane euthanasia with sodium pentobarbital. In each treatment, 6 weaned piglets from different sows were selected and slaughtered for sampling (the weight was close to the average litter weight, half male and half female).

2.2 Sample collection

In each group, colostrum was collected from every teat within 24 h of postpartum (n = 6), 14 d milk was collected after injection of 30 IU oxytocin postpartum on day 14 of lactation. Approximately 20 mL were collected and stored at −80 °C until analysis. In each group, 6 weaned piglets from different sows were selected for slaughter sampling. These piglets represent the average level of the group, with half males and half females. Tissue samples of the duodenum, jejunum, ileum, and colon contents were collected and immediately stored at −80 °C. Parts of the duodenum, jejunum and ileum samples were fixed with 4% paraformaldehyde solution and then analyzed with hematoxylin and eosin (H&E) staining.

2.3 Nutritional composition in colostrum and milk

Colostrum and 14 d milk samples were analyzed by a fully automated milk composition analyser (ULTRA-MILER-UL40AC, China).

2.4 Chemical analyses

The intestinal tissue (50 mg) was homogenized in 0.45 mL of 1× PBS using an automatic low-temperature homogenizer (Jingxin, Shanghai, China). After ultrasonic crushing, the homogenate was centrifuged at 3000 rpm for 10 minutes at 4 °C to obtain the supernatant. According to the instructions, the total antioxidant capacity (T-AOC), total superoxide dismutase (T-SOD), glutathione (GSH), glutathione peroxidase (GSH-Px) and malondialdehyde (MDA) in the supernatant were detected with kits purchased from Nanjing Jiancheng Biological Research Institute. After the same homogenate procedure, the homogenate used to detect inflammatory factors was centrifuged at 5000g for 5 minutes at 4 °C. The concentrations of interleukin (IL)-1, IL-6, IL-12 and tumor necrosis factor (TNF)-α in the small intestine were analyzed with a Porcine ELISA Kit purchased from ML Bio Company (Shanghai, China). All kits used in this study are listed in Table S3.†

2.5 Intestinal morphology

The fixed intestinal tissue was removed from the 4% paraformaldehyde solution. With reference to standard paraffin embedding technology, the intestinal samples were embedded in paraffin and sliced with a Leica slicer (Leica RM2235, Germany). Then, the slices (5 μm) were stained with hematoxylin and eosin staining solution and evaluated histopathologically. The slices were photographed with a Nikon imaging system (100×, Nikon YS100; Nikon, Tokyo, Japan). Nine well-oriented crypt–villi units were randomly selected from 3 slices to measure the villi height and crypt depth, and the ratio of villi height to crypt depth was calculated.

2.6 Total RNA extraction and real-time quantitative PCR

100 mg of intestinal tissue was added into 1 mL of lysis buffer (EZB-RN001, EZBioscience, USA) and homogenized using an automatic low-temperature homogenizer. Then, the supernatant was separated from the homogenate by centrifugation at 12 000 rpm at 4 °C for 5 min. Total RNA was isolated from the supernatant using an RNA Purification Kit (EZB-RN001, EZBioscience, USA), and RNA quality was determined by electrophoresis on 1.0% agarose gel. The concentration and purity of total RNA were determined with a NanoDrop ND-1000 spectrophotometer. Subsequently, 1 μg of RNA was used to synthesize first-strand cDNA according to the manufacturer's protocol of a Colour Reverse Transcription Kit (with gDNA Remover) (A0010CGQ, EZBioscience, USA). Then, these cDNAs were quantitatively analyzed with a Colour SYBR Green qPCR Master Mix kit (A0012-R2, EZBioscience, USA) using an ABI Prism 7500 sequence detection system (Applied Biosystems, Carlsbad, CA, USA). The 20 μL PCR volume was composed of 2× Colour SYBR Green qPCR Master Mix (10 μL), PCR primers (10 μM, 0.4 μL of each), cDNA (2 μL), and dd water (7.2 μL), with a supporting reaction procedure of denaturation at 95 °C for 5 min, followed by 40 cycles of 95 °C for 10 s, 60 °C for 30 s, and 72 °C for 30 s. The relative mRNA expression was calculated using the relative quantification method ($2^{-\Delta\Delta Ct}$). The primers used in this study are listed in Table S4.†

2.7 Western blotting analysis

The total protein of the small intestine (100 mg of tissue to 0.5 mL of lysis buffer) was extracted with a mixture of RIPA lysis buffer (Beyotime, Shanghai, China) containing 1% phosphatase inhibitors and 1% PMSF protease inhibitor. The total protein concentration was determined with a BCA protein assay kit (Beyotime, Shanghai, China). A total of 25 μg of total protein from each sample was separated on 10% SDS-PAGE gels, electrically transferred to polyvinylidene difluoride membranes (Millipore, Bedford, MA), and blocked with 5% BLOT-Quick Blocker (Sangon, Shanghai, China) for 40 min. After washing with TBST buffer, these membranes were immersed in primary antibody diluent at 4 °C for 12 h. Details of the antibodies used in this study are listed in Table S5.† Subsequently, these membranes were rinsed and incubated in the corresponding secondary antibody diluent at room temperature for 1.5 h. After incubation with the antibody, these membranes were rinsed again 5 times. Then, band signals of these membranes were captured using an ECL Plus chemiluminescence detection kit (Applygen Technologies Inc., Beijing, China) and a Chemiluminescence Image Analysis System (Tanon, Shanghai, China). Finally, the gray values of these bands were analyzed with Image Processing Software (Image-Pro Plus 6.0) (Rockville, MD), and the relative protein abundance of the target protein was calculated.

2.8 Bacterial data analysis

High-throughput 16S rDNA sequencing technology was selected to analyze the colonic microbiomes of the piglets. First, genomic DNA was isolated from the colon contents. Subsequently, the barcode specific primers (341 Forward: CCTACGGNGGCWGCAG and 806 Reverse: GGACTACHVGGG-TATCTAAT) were used to amplify the V3–V4 region of 16S rDNA. Then, the purified amplification products were connected to a sequencing adapter to construct a sequencing library, which was sequenced on a NovaSeq 6000 instrument (PE250, Illumina Inc., CA, USA). This analysis was conducted by the Gene Denovo company (Guangzhou, China). Finally, the Omicsmart (Gene Denovo, Guangzhou, China) online tool was used to analyze 16S rDNA sequencing data. The raw data of the 16S sequencing analysis of microbiota involved in this study can be accessed at the National Genome Data Center (Beijing, China), under the registration number CRA 008645.

2.9 Statistical analysis

All the data were analyzed using the independent sample T test of IBM SPSS statistical software (IBM Version 22.0, Armonk, New York, USA). $P \leq 0.05$ indicated statistical significance, while $0.05 < P < 0.10$ was considered a significant trend. All data are shown as the mean ± standard error (SEM).

3 Results

3.1 Nutritional composition in colostrum and milk

The nutritional composition changes in sow colostrum and 14-day milk are shown in Fig. 1A–D. The result indicated that maternal supplementation with GML significantly increased fat, lactose and protein in sow colostrum, and fat and protein in sow 14-day milk ($P < 0.05$).

3.2 Proinflammatory factor levels in the small intestine

In the duodenum (Fig. 2A), the concentration of IL-12 in the GML group was significantly lower than that in the CON group ($P < 0.05$). In the jejunum (Fig. 2B), compared with those in the CON group, the concentrations of TNF-α, IL-1β and IL-12 in the GML group were significantly decreased ($P < 0.05$). In the ileum (Fig. 2C), the IL-1β concentration in the GML group was lower than that in the CON group ($P < 0.05$).

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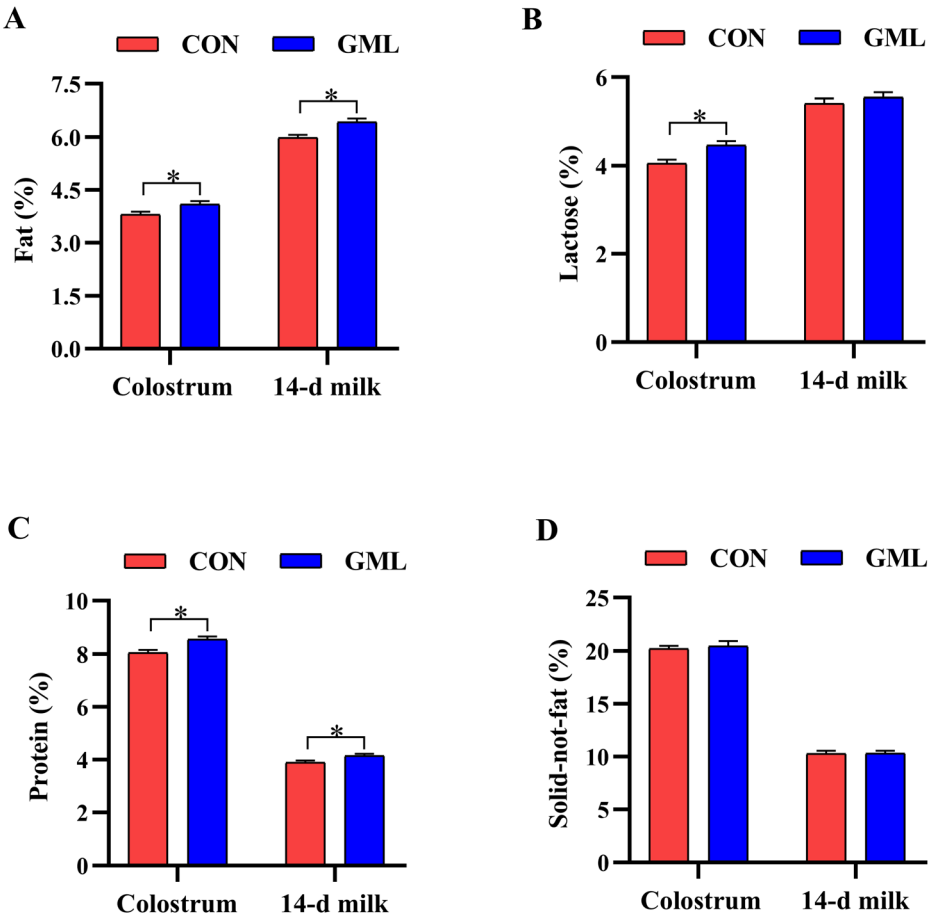

Fig. 1 Effects of maternal glycerol monolaurate (GML) supplementation on the nutritional composition changes in sow colostrum and 14-day milk (A–D) (n = 6). * P < 0.05.

3.3 Antioxidant status of the small intestine

The antioxidant status of the small intestine is shown in Table 1. In the duodenum, the concentrations of T-SOD and GSH in the GML group were significantly higher than those in the CON group (P < 0.05). The GSH-Px concentration in the duodenum of the GML group was slightly higher than that of the CON group (P = 0.072). Moreover, the MDA concentration in the duodenum of the GML group was lower than that of the CON group (P = 0.087). In the jejunum and ileum, the concentrations of T-AOC and T-SOD in the GML group were significantly higher than those in the CON group (P < 0.05). The concentrations of GSH and GSH-Px were slightly higher than those in the CON group (P = 0.066, P = 0.065, respectively). Furthermore, the MDA concentrations in the jejunum and ileum of the GML group were lower than those of the CON group (P = 0.092, P = 0.065, respectively).

3.4 Intestinal morphology

The intestinal villi height and crypt depth of piglets were measured to investigate the effect of GML on intestinal integrity (Fig. 3A–D). In the jejunum, the villi height and the ratio of villi height to crypt depth in the GML group were greater than those in the CON group (P < 0.05). However, the crypt depth was significantly lower than that of the CON group (P < 0.05). In the ileum, the ratio of villi height to crypt depth in the GML group was higher than that in the CON group (P = 0.094).

3.5 Expression of the tight junctions in the piglet intestine

Intestinal integrity is an important indicator of animal intestinal health. Fig. 4 shows the results of glycerol monolaurate regulating the expression of intestinal tight junction proteins. In the duodenum (Fig. 4A), jejunum (Fig. 4B) and ileum (Fig. 4C) of the piglets, higher protein abundance of zonula occludens (ZO)-1, occludin and claudin-1 was observed in the GML group compared with that in the CON group (P < 0.05).

3.6 mRNA expression of beta defensins and mucins in the piglet intestine

The relative mRNA expression of beta defensins is shown in Fig. 5A–C. In the duodenum (Fig. 5A), the mRNA expression of *pBD1* and *pBD2* in the GML group was higher than that in the CON group (P < 0.05). In the jejunum (Fig. 5B), the mRNA

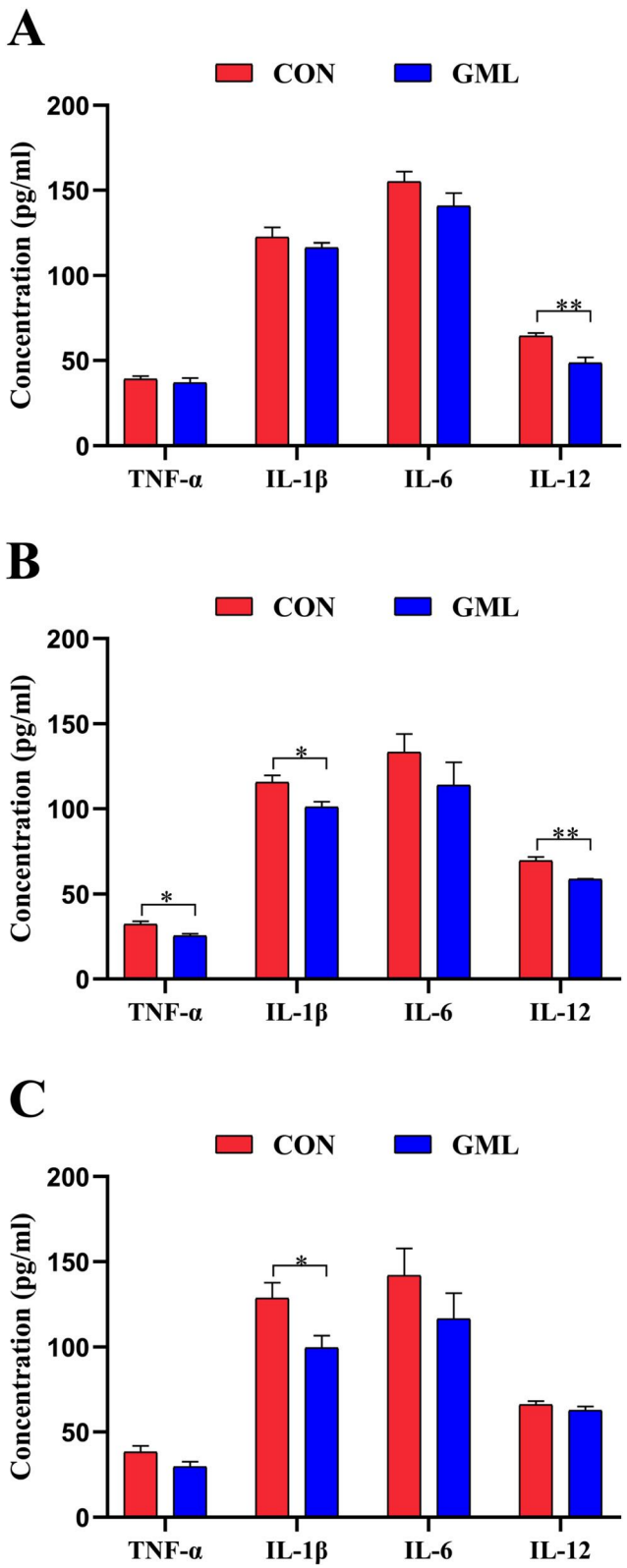

Fig. 2 Effects of maternal glycerol monolaurate (GML) supplementation on proinflammatory factor levels in the duodenum (A), jejunum (B) and ileum (C) of piglets (n = 6). * $P < 0.05$, ** $P < 0.01$.

Table 1 Effects of maternal GML supplementation on small intestinal antioxidant status of weaned piglets (n = 6)

Item	CON	GML	SEM	*P*-Value
Duodenum				
T-AOC, U mg^{-1}	8.85	9.17	0.14	0.205
T-SOD, U mg^{-1}	51.57	57.08	1.04	0.030
GSH-Px, U mg^{-1}	545.62	568.71	6.07	0.072
GSH, μmol g^{-1}	14.71	16.58	0.48	0.054
MDA, nmol mg^{-1}	4.43	4.22	0.07	0.087
Jejunum				
T-AOC, U mg^{-1}	9.01	10.33	0.19	< 0.01
T-SOD, U mg^{-1}	56.56	66.38	1.57	0.021
GSH-Px, U mg^{-1}	580.31	605.27	6.16	0.059
GSH, μmol g^{-1}	15.64	17.41	0.49	0.066
MDA, nmol mg^{-1}	4.58	4.12	0.13	0.092
Ileum				
T-AOC, U mg^{-1}	8.96	9.93	0.15	0.013
T-SOD, U mg^{-1}	57.34	63.27	1.05	0.015
GSH-Px, U mg^{-1}	578.35	595.77	8.44	0.066
GSH, μmol g^{-1}	16.38	17.16	0.19	0.065
MDA, nmol mg^{-1}	4.50	4.22	0.08	0.065

The data presented are obtained from 6 animals in each group (n = 6). Differences were statistically significant when $P < 0.05$. T-AOC, total antioxidant capacity; T-SOD, total superoxide dismutase; GSH-Px, glutathione peroxidase; GSH, glutathione; MDA, malondialdehyde.

expression of *pBD1*, *pBD2* and *pBD129* in the GML group was higher than that in the CON group ($P < 0.05$). Compared with the CON group, the mRNA expression of *pBD2* and *pBD114* in the GML group was significantly increased in the ileum (Fig. 5C) ($P < 0.05$). Moreover, the mRNA expression of mucin (*MUC*)-*1*, *MUC-3* and *MUC-4* was determined in the intestine (Fig. 5D–F). In the duodenum (Fig. 5D), jejunum (Fig. 5E) and ileum (Fig. 5F), the mRNA expression of *MUC-1*, *MUC-3* and *MUC-4* was significantly increased in the GML group compared with that in the CON group ($P < 0.05$).

3.7 Microorganisms in the colonic contents of the piglets

Across all samples, a total of 1 133 030 effective sequences were successfully generated, and 10 340 OTUs were obtained for subsequent analysis. For beta diversity, PCoA analysis based on Bray distance showed that the microbiota in the GML group obviously tended to separate from the CON groups (Fig. 6A). The four major bacterial phyla in the colonic contents were Firmicutes (31.47% *vs.* 46.95%, $P < 0.05$), Proteobacteria (34.10% *vs.* 28.09%), Bacteroidetes (25.80% *vs.* 17.95%, $P < 0.05$), and Euryarchaeota (5.43% *vs.* 1.75%), accounting for more than 94% of the total bacterial community (Fig. 6B and D). The results of microbial data analysis indicated that the major colonic bacterial communities in different treatments showed obvious differences. The relative abundance of Firmicutes in the GML group increased by 15.48% compared with that in the CON group. Compared with the CON group, the relative abundance values of Proteobacteria, Bacteroidetes and Euryarchaeota decreased by 6.00%, 7.85% and 3.68%, respectively. Within Firmicutes, the increase in the *Eubacterium_coprostanoligenes_group* was the

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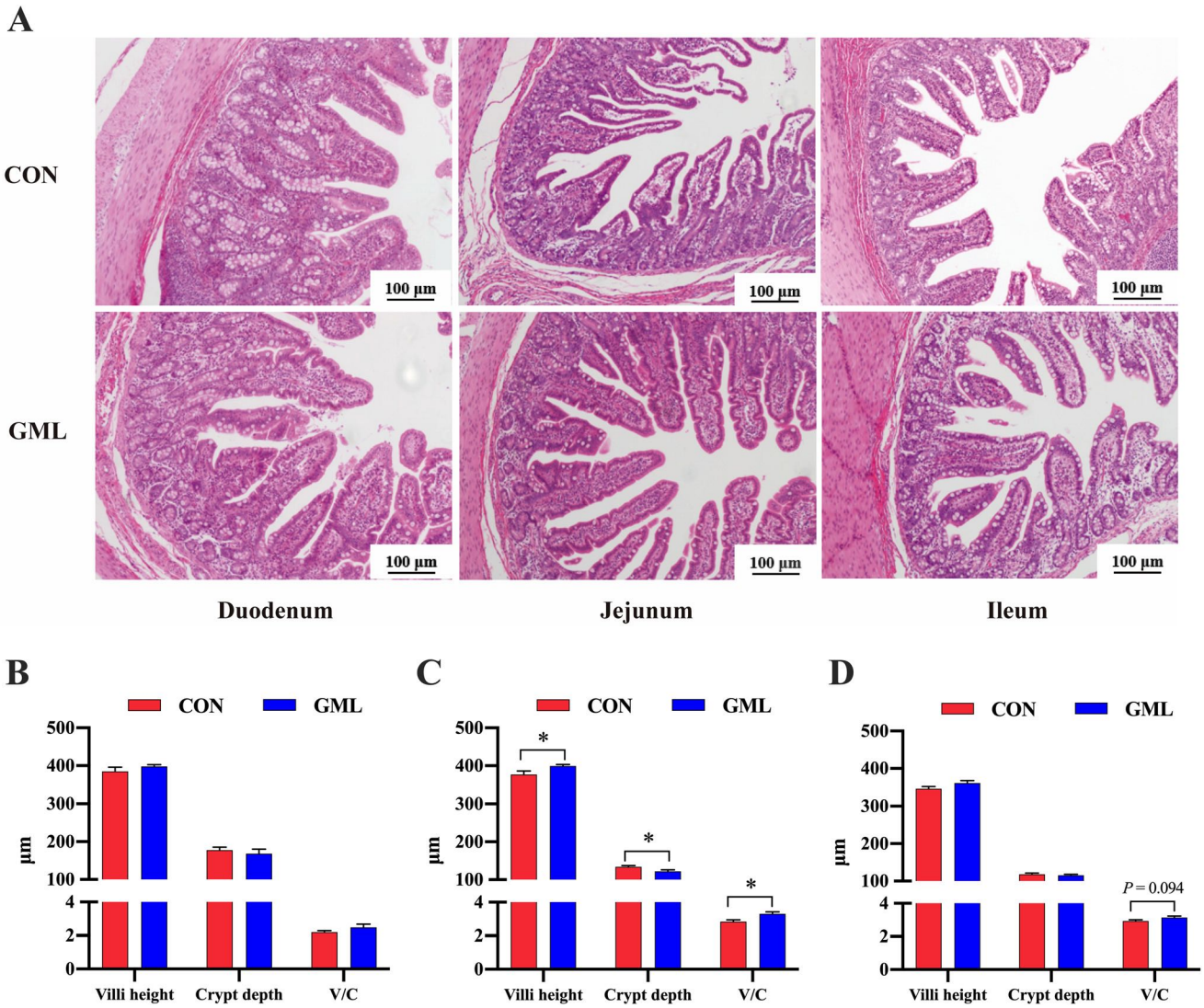

Fig. 3 Effects of maternal GML supplementation on the villi height and crypt depth of the small intestine in piglets (n = 6). (A) H&E staining of piglet small intestine. (B–D) Analysis of villi height and crypt depth in the small intestine of piglets. * $P < 0.05$.

main factor leading to the change in the relative abundance of bacteria (Fig. 6C and E). In addition, *Escherichia shigella* was detected with high relative abundance in the colonic contents (Fig. 6C). However, compared with the control group, *Escherichia shigella* in the GML group decreased from 31.28% to 25.09%.

3.8 NF-κB/MAPK pathway

To investigate the potential mechanism of GML in regulating intestinal inflammation and improving intestinal barrier function, the nuclear factor kappa B (NF-κB) and mitogen-activated protein kinase (MAPK) pathways in the piglet small intestine were determined. In the small intestine (Fig. 7A–C), lower phosphorylation of NF-κB, JNK, ERK and P38 in the GML group was determined and compared with that in the CON group ($P < 0.05$).

4 Discussion

Piglets are directly faced with tremendous changes in the growth environment, such as diet, temperature, and pathogenic bacteria, after birth.[18] Providing suitable temperatures, clean houses, clean drinking water, adequate nutrition (milk) and formulating a scientific diet transition plan for neonates are very important to improve the growth performance and survival rate of piglets.[19] Studies and practices have indicated that the health status of piglets is the leading factor affecting their growth and survival, not external conditions.[18] However, the immune system development of piglets is rudimentary after birth.[20] Therefore, it is necessary for piglets to obtain nutrition and immunity from their mothers in time to resist challenges from the surrounding environment.[8,21] Milk, especially colostrum, contains energy-rich nutrients, maternal

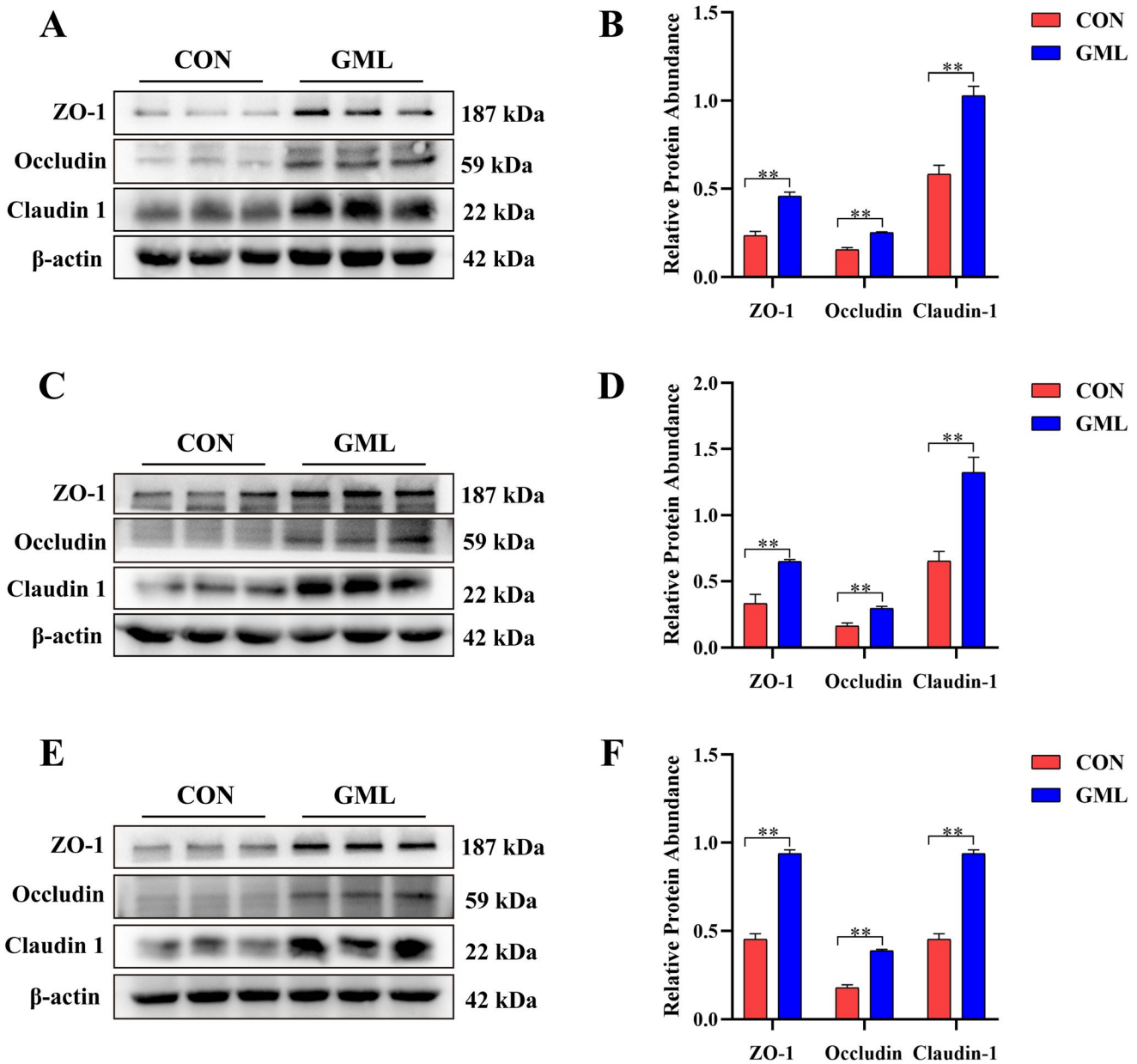

Fig. 4 Effects of maternal GML supplementation on the protein abundance of tight junctions in the duodenum (A and B), jejunum (C and D), and ileum (E and F) of piglets (n = 6). * $P < 0.05$, ** $P < 0.01$.

antibodies and growth factors, which are very important for the growth and development of newborns in the lactation phase and their survival in the transition period.[22] Thus, the quality of sow milk is particularly critical. Many previous studies have confirmed that the nutritional composition of the diets of lactating sows is closely related to the quality of their milk.[23–26] Importantly, immune agents and antioxidant protection can be transferred from mother to offspring through maternal colostrum and milk,[7,8,27,28] which makes it possible to regulate the health of suckling piglets through nutritional strategies. In this study, the results indicated that maternal supplementation with GML significantly increased fat, lactose and protein in sow colostrum, and fat and protein in sow 14-day milk. Based on these results, we think the potential mechanism of action of GML is to promote the intestinal health of piglets by improving the nutritional composition in sow colostrum and milk.

The GIT is an organ for digesting and absorbing nutrients and is also an important immune organ.[29] The destruction of intestinal integrity will directly reduce the ability of piglets to digest and absorb nutrients, ultimately leading to a decrease in their growth rate, limiting their lifelong growth potential.[30,31] Antioxidant protection and immune agents from the mother can directly protect the intestinal tract of piglets from damage.[32] Previous studies have proven that GML and medium chain fatty acids (MCFAs) manifest good effects

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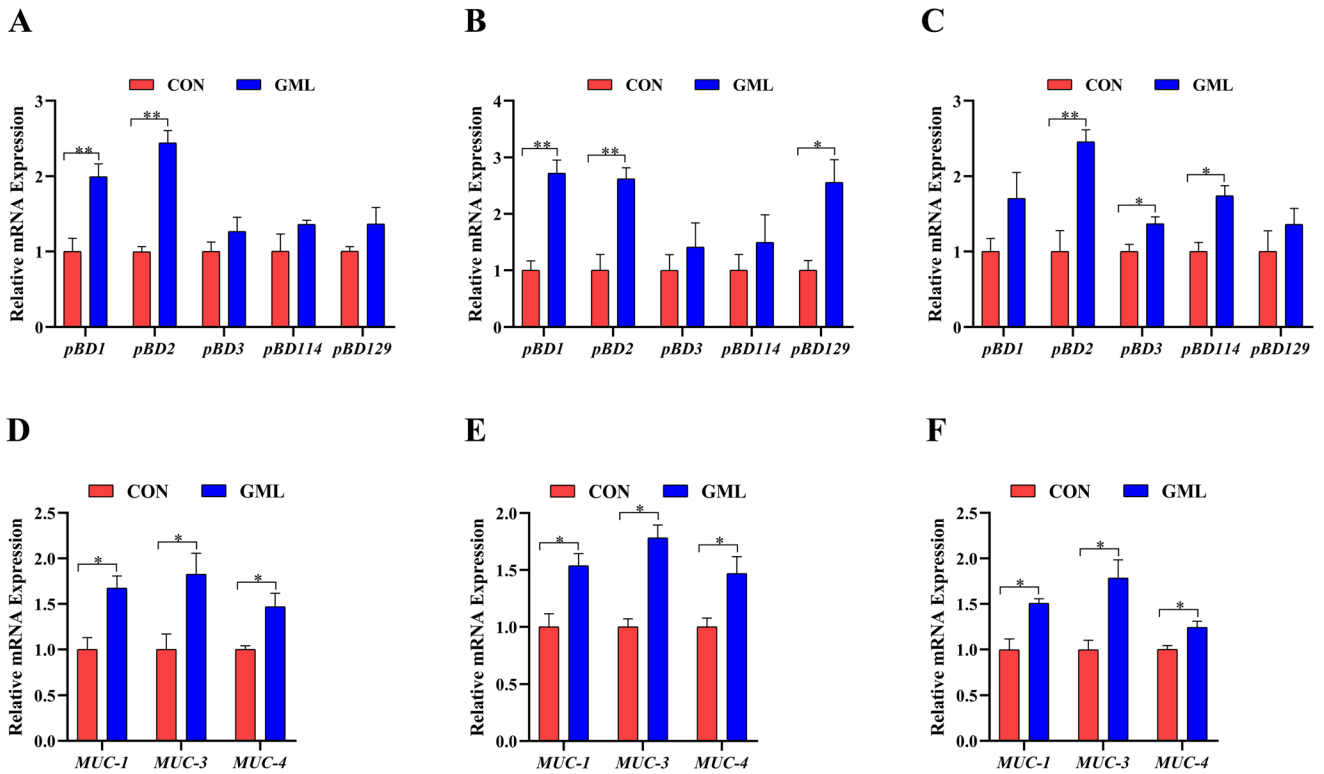

Fig. 5 Effects of maternal GML supplementation on the mRNA expression of beta defensins and mucin proteins in the small intestine of piglets (n = 6). (A and D) Duodenum. (B and E) Jejunum. (C and F) Ileum. * $P < 0.05$, ** $P < 0.01$.

in relieving inflammation.[33] *In vitro*, GML can inhibit the inflammatory processes of T-cells by changing lipid dynamics and reducing the production of proinflammatory factors, thus playing an immunoregulatory role.[15] Notably, in a recent study on weaned piglets, α-GML reduced the TNF-α concentration and increased the IL-10 concentration in serum.[13] By detecting the concentrations of proinflammatory factors, the present study found that a sow diet supplemented with GML reduced the levels of proinflammatory factors in the small intestine of the offspring, which proved that maternal anti-inflammatory protection could be transmitted to offspring and protect their intestinal health. In addition, oxidative stress is often accompanied by inflammation, which is one of the main factors that destroys the intestinal barrier function of piglets. In the present study, the decrease in MDA in the small intestine of the piglets and the increases in T-AOC, T-SOD, GSH and GSH-Px activities indicated that GML improved the antioxidant capacity of the small intestine of the piglets. Recent studies have also proven that GML increases the activity of serum GSH-Px and reduces the content of MDA in weaned piglets.[13] Thus, a sow diet supplemented with GML can alleviate intestinal inflammation and enhance oxidative stability in offspring.

Inflammation and oxidative stress will directly lead to changes in intestinal morphology, which are defined as important indicators to evaluate intestinal function and health.[34] In the present study, a sow diet supplemented with 0.1% GML significantly improved the ratio of villi height to crypt depth in the jejunum and ileum of the offspring, indicating that GML has positive significance in improving the intestinal development of offspring.[13] GML is a kind of MCFA monoglyceride that has been proven to convey positive benefits in improving animal health, production and feed digestibility as a feed additive. Świątkiewicz M *et al.* evaluated the effects of a sow diet supplemented with coconut oil and adding medium chain fatty acid triglycerides (MCTs) to the diets of offspring (after weaning) on the growth performance of the offspring.[35] A sow diet supplemented with coconut oil significantly increased the content of C12 MCFAs in milk.[35] In a subsequent study, dietary supplementation with MCTs increased the survival rate of weaned piglets and improved their growth performance and intestinal villi height.[35] Another study also showed that using 2.5–5% MCT-containing oil or 5% cuphea seeds (a natural source of MCT) as feed additives to piglet diets can improve intestinal health and growth performance.[36,37] Recent credible evidence showed that GML significantly increases the apparent total tract digestibility of dry matter, gross energy, crude protein and ether extract in a weaned piglet experiment.[13] Moreover, GML significantly increased duodenal villi height/crypt depth and jejunum and ileum villi height in piglets.[13]

The intestinal barrier is a key factor for protecting piglets. Inflammation and oxidative stress can result in intestinal barrier dysfunction.[31] Therefore, intestinal integrity is an important indicator to evaluate the intestinal health of piglets.

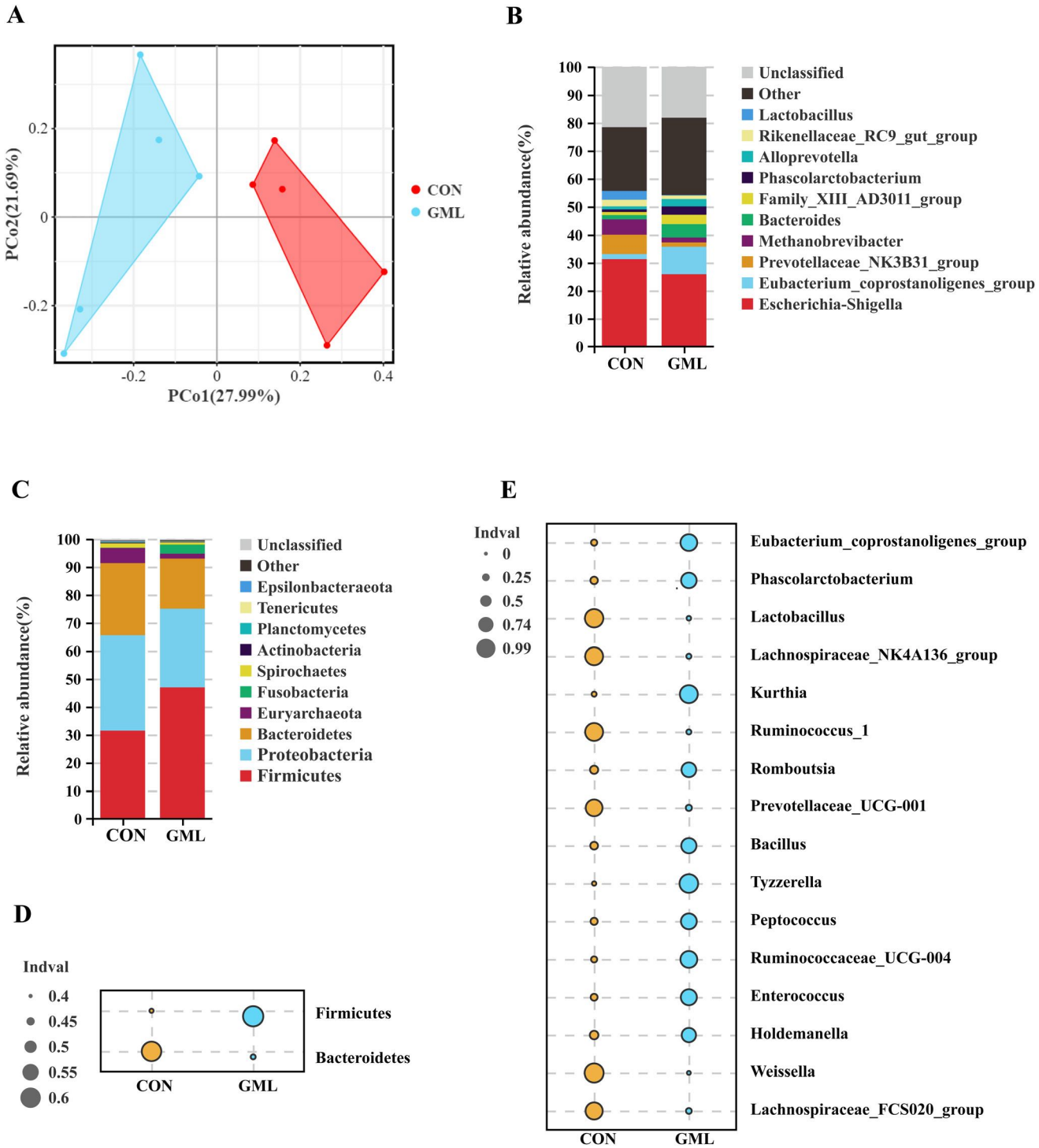

Fig. 6 Effects of maternal GML supplementation on the colonic microbial composition and beta diversity of piglets. (A) Beta diversity. (B) Relative abundance of microbiota at the phylum level. (C) Relative abundance of microbiota at the genus level. (D) Indicator species analysis at the phylum level. (E) Indicator species analysis at the genus level.

The intestinal barrier of piglets mainly includes mechanical barriers, immune barriers and biological barriers.[38] Intestinal tight junctions are the first pathway by which piglets resist the invasion of external pathogenic factors, which allows the passage of soluble small molecules and prevents the passage of macromolecules and microorganisms.[31] In this study, maternal GML supplementation significantly improved the relative protein abundance of intestinal tight junctions in the offspring, indicating that maternal GML supplementation was beneficial for strengthening the mechanical barrier function

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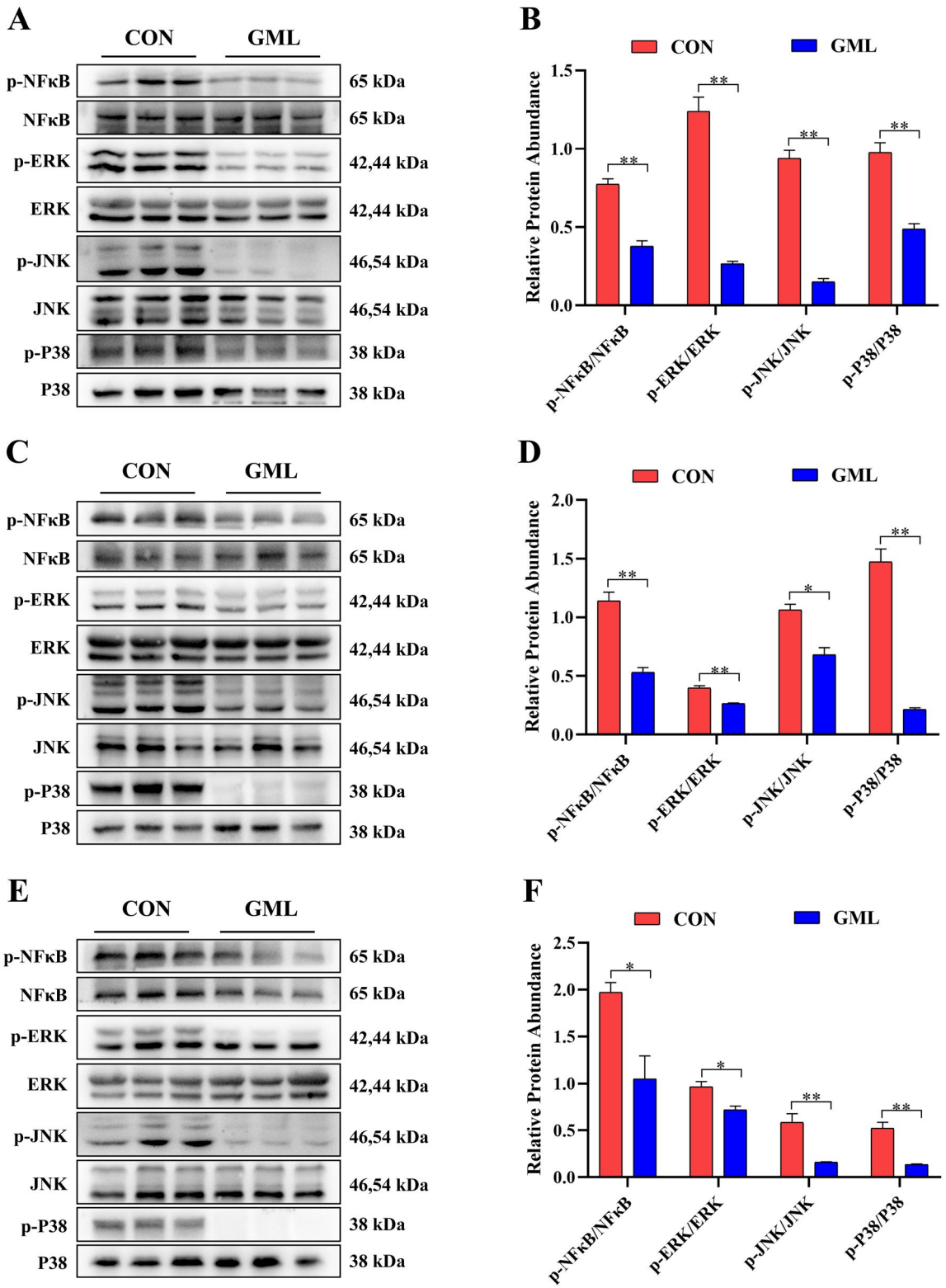

Fig. 7 Effects of maternal GML supplementation on the phosphorylation of the NF-κB/MAPK pathways in the duodenum (A and B), jejunum (C and D) and ileum (E and F) (n = 6). * $P < 0.05$, ** $P < 0.01$.

of the offspring. Similar to this result, Chen *et al.* showed that the addition of 7.75 g kg^{-1} MCFA to the sow diet significantly increased the content of MCFA (*e.g.*, lauric acid) in the sow's milk and promoted the expression of claudin-1 and ZO-1 in the jejunum of the offspring.[39] In addition, in a study of a low-protein diet supplemented with MCTs and GML, MCT treat-

ment significantly increased the expression of claudin-1 and ZO-1 in piglets' intestines, while GML treatment significantly increased the levels of claudin-1, occludin and ZO-1.[40] These results showed that the intestinal barrier function of weaned piglets could be improved by adding MCTs to the diets of sows and weaned piglets. Furthermore, the present study also observed the positive role of dietary supplementation with GML in improving the intestinal immune barrier of offspring. A sow diet supplemented with GML significantly increased the expression of β-defensins and MUCs in the offspring's intestine. Similar results have been observed in previous *in vitro* studies. MCFAs (including lauric acid) can significantly improve the immune response of porcine intestinal cell lines.[41] Wang *et al.* also showed that octanoic acid, an MCFA, can improve the expression levels of endogenous host defense peptides to enhance intestinal barrier function in an *in vitro* cellular model.[42]

Gut microbes are a crucial intestinal barrier in piglets.[43] The early colonization of beneficial microorganisms in the intestinal tract of piglets is crucial to their intestinal health and growth.[44] To explore the effect of GML on intestinal bacterial colonization in piglets, this study analyzed the microorganisms in colon contents by high-throughput 16S rDNA sequencing. For beta diversity, PCoA analysis based on Bray distance showed that the microbiota in the GML group obviously tended to separate from the CON groups. The results of species composition analysis and indicator species analysis showed that GML mainly reduced the relative abundance of *Escherichia shigella* (approximately 5.4%) and the *Prevotellaceae_NK3B31_group*, and increased the relative abundance of the *Eubacterium_coprostanoligenes_group* (approximately 8.0%) and *Phascolarctobacterium*. GML inhibited the colonization of pathogenic bacteria (such as *Escherichia shigella*) in the intestine. Previous studies have shown that MCFAs and monoglycerides exhibit strong antibacterial and antiviral abilities and exhibit potent inhibitory activity against a wide range of pathogens, including Gram-negative and Gram-positive bacteria and envelope viruses.[33] GML was reported to promote metabolic disorder and gut microbiota dysbiosis in low-dose supplementation upon low-fat-diet feeding.[12] However, it is worth noting that GML ameliorated high-fat diet (HFD)-induced gut microbiota dysbiosis, with an increase in *Achmania*, *Bacteroides uniformis*, *Bifidobacterium* and *Lactobacillus* and decreases in *Escherichia coli*, *Flexispira*

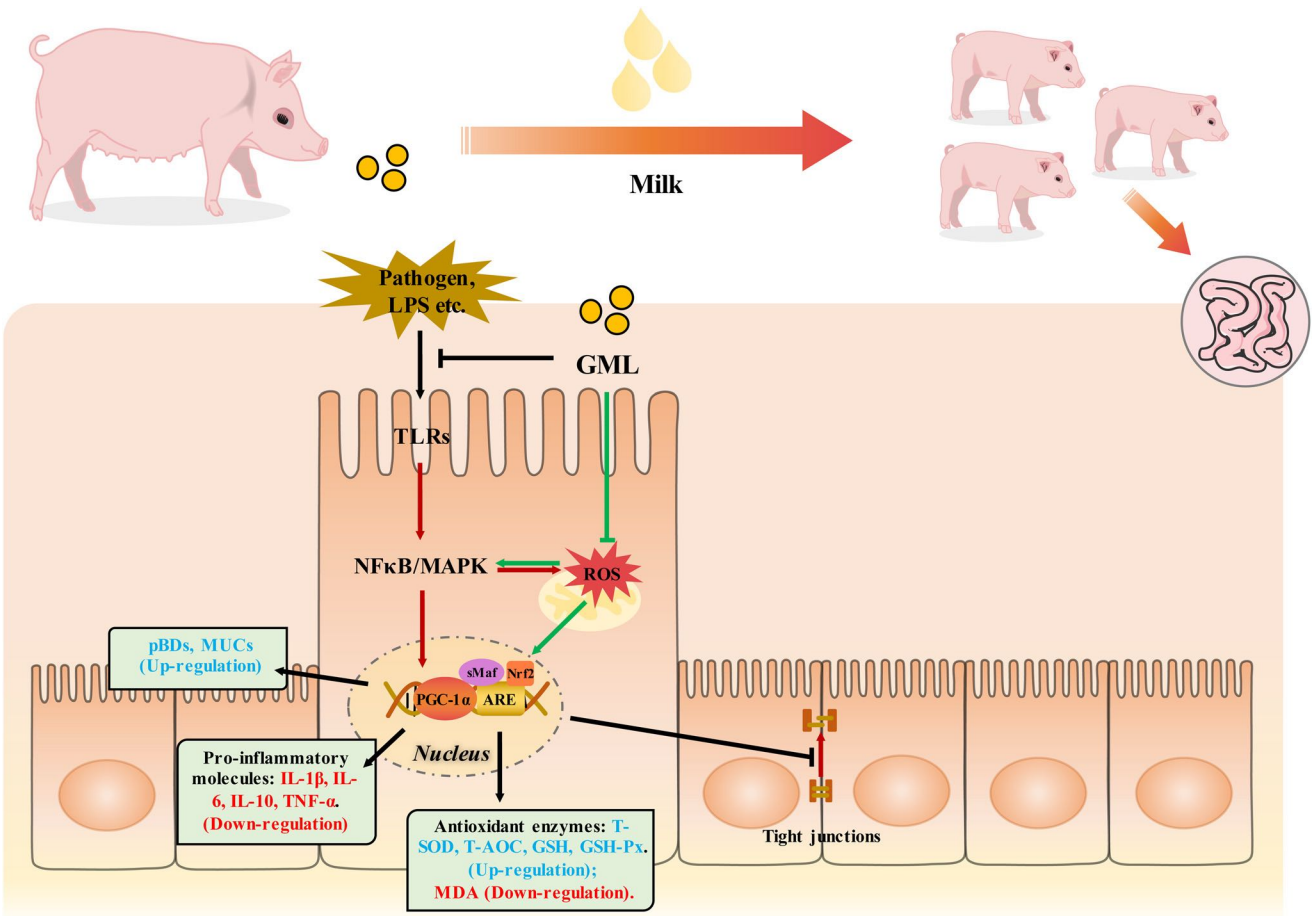

Fig. 8 Maternal diet supplemented with 0.1% GML enhanced offspring intestinal oxidative homeostasis and barrier function, and attenuated offspring intestinal inflammatory response, possibly through suppressing the activation of the NFκB/MAPK pathways.

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and *Lactococcus*.[45] Similarly, Mo *et al.* found that high doses of GML upregulated favorable microbial taxa without inducing systemic inflammation or metabolic disorders of glucose and lipids.[17] In weaned piglets, GML significantly increased the relative abundance of Firmicutes in cecal contents, but decreased the relative abundance of Bacteroidota and Proteobacteria. Among them, the relative abundance of *Lactobacillus* and *Blautia species* increased significantly, while those of *Campylobacter*, *Eubacterium_rectale_ATCC_33656* and *uncultured_bacterium_Alloprevotella species* decreased significantly.[13] In summary, in different studies, the effects of GML on intestinal microorganisms are quite different, which may be related to its dosage and experimental design. Therefore, more data are needed to evaluate the effectiveness of GML in regulating intestinal microorganisms.

In addition, the NF-κB/MAPK signaling pathway has also been proven to play an important role in the regulation of intestinal inflammation and oxidative stability by GML. In the present study, a sow diet supplemented with GML significantly inhibited the phosphorylation of NF-κB, ERK, JNK and P38 in the small intestine of the offspring. In a study conducted by Ren *et al.*,[16] a mixture of formic acid and monolaurin attenuated intestinal inflammation in piglets by inhibiting the NF-κB/MAPK pathways.

According to our research results, maternal supplementation with GML from the 85th day of gestation to the 21st day of lactation has positive significance for the intestinal health and growth of the offspring. It is understood that the current market price of GML is about 5500 dollars per ton. At present, the large-scale use of GML in pig production will obviously increase the production cost; however it can be used in some key periods, such as lactation. Predictably, with an improvement in the GML production process and a reduction in production cost, GML will have broad application prospects in animal production.

5 Conclusions

A maternal diet supplemented with 0.1% GML enhanced offspring intestinal oxidative homeostasis and barrier function and attenuated the offspring's intestinal inflammatory response, possibly by improving the nutritional composition in sow colostrum and milk (as shown in Fig. 8). Furthermore, maternal 0.1% GML supplementation may inhibit the colonization of pathogenic bacteria in the intestine. Taken together, the results of this study proved that it is feasible to regulate the intestinal health of offspring through maternal nutrition strategies, which is of great significance to the improvement of diet recommendations during pregnancy and lactation.

Author contributions

Conceptualization, HZ, MT, SZ and WG. Data curation, formal analysis and methodology, HZ, TL, SZ, XY, XL and LX. Funding acquisition, WG. Supervision, FC, SZ and WG. Visualization, MT and HZ. Writing – original draft, MT and HZ. Writing – review & editing, SZ and WG.

Institutional review board statement

All animal procedures in this study were performed according to the rules of the Administration of Experimental Animals (National Science and Technology Commission of the People's Republic of China), and Guiding Opinions on the Treatment of Experimental Animals (GKFCZ [2006] No. 398). All of the procedures performed in animal using and caring during this study were approved by the South China Agricultural University Animal Care and Use Committee (Guangzhou, China).

Data availability statement

Please contact the author if further information is required.

Conflicts of interest

All authors declare no conflict of interest.

Acknowledgements

This study was supported by the Project of Swine Innovation Team in Guangdong Modern Agricultural Research System (2020KJ126), National Key Research and Development Program (No. 2021YFD1300401-2), Guangdong Basic and Applied Basic Research Foundation (No. 2021A1515010440), Science and Technology Program of Guangzhou (No. 202102020056).

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Nutrition Research Reviews (2020), 33, 287–297
doi:10.1017/S0954422420000074
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Amino acid transportation, sensing and signal transduction in the mammary gland: key molecular signalling pathways in the regulation of milk synthesis

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Abstract

The mammary gland, a unique exocrine organ, is responsible for milk synthesis in mammals. Neonatal growth and health are predominantly determined by quality and quantity of milk production. Amino acids are crucial maternal nutrients that are the building blocks for milk protein and are potential energy sources for neonates. Recent advances made regarding the mammary gland further demonstrate that some functional amino acids also regulate milk protein and fat synthesis through distinct intracellular and extracellular pathways. In the present study, we discuss recent advances in the role of amino acids (especially branched-chain amino acids, methionine, arginine and lysine) in the regulation of milk synthesis. The present review also addresses the crucial questions of how amino acids are transported, sensed and transduced in the mammary gland.

Key words: **Mammary gland: Amino acids: Milk protein: Milk fat: Signalling pathways**

(Received 17 October 2019; revised 19 February 2020; accepted 3 March 2020; accepted manuscript published online 10 March 2020)

Introduction

Milk is primarily composed of milk protein, fat, lactose, vitamins and minerals, which are important nutrient sources for neonates. Maternal nutrients are considered as building blocks for milk synthesis. In humans, breast-feeding decreases the risk of neonatal acute illnesses, diarrhoea and overweight/obesity (breast-feeding for more than 6 months)[1]. However, in pigs, sufficient and quality colostrum (first 24 h) supplies are important for neonates to gain higher weaning weight and have better growth performance later in life[2]. Calves fed adequate colostrum during the first week of their life exhibit significantly enhanced metabolic and immunological status[3]. Thus, understanding nutritional strategies to regulate milk synthesis and its underlying mechanism is important for human beings and other mammals.

Amino acids are not only basic components of proteins, but also act as functional regulators in a variety of biological processes. The functions of amino acids have been extensively studied in the gut, liver, muscle and adipose tissue, especially in the field of protein[4] and fat metabolism[5]. In the mammary gland, amino acid uptake from blood is almost equal to milk output based on a nitrogen basis in dairy cows[6–8], goats[9], sows[10] and ewes[11]. However, the destinies of different amino acids in mammary cells are different[6–8,10–12]. The mammary amino acid uptake:output ratios could be larger (for example, valine, isoleucine, leucine and arginine), equal (for example, methionine and histidine) or less than 1 (for example, asparagine and proline) (mammary amino acid uptake:output ratios in cows, goats, sows and ewes are shown in Table 1)[6–8,10–12]. Those amino acids with uptake:output ratios greater than 1 can be metabolised to CO_2, urea, polyamine or simply other non-essential amino acids[13]. In addition, various amino acids (especially branched-amino acids, methionine and arginine) are involved in the regulation of milk synthesis. A variety of signalling molecules have been proposed to cooperate with amino acids to regulate biological functions in the mammary gland. Signalling pathways regulating mammary

Abbreviations: Akt, protein kinase B; BCAA, branched-chain amino acid; CAT-1, cationic amino acid transporter-1; ERK, extracellular signal-regulated kinase; FABP5, fatty acid-binding protein 5; GATOR, GTPase-activating protein activity toward Rags; GNG12, guanine nucleotide-binding protein subunit γ-12; GPRC, G-protein-coupled receptor; HEK 293, human embryonic kidney 293 cells; LAT1, L-type amino acid transporter 1; LAT2, L-type amino acid transporter 2; LeuRS, leucyl-tRNA synthetase; mTORC1, mammalian target of rapamycin complex 1; PI3K, inositol 1,4,5-trisphosphate 3-kinase; SAM, *S*-adenosylmethionine; SNAT, Na-coupled neutral amino acid transporter; SREBP-1, sterol regulatory element-binding protein 1.

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Table 1. Mammary amino acid uptake:output ratios in different mammals

Species	Uptake: output ratios	Amino acids	References
Cows	= 1	Methionine, histidine, tryptophan, phenylalanine and tyrosine	(6–8)
	> 1	Valine, isoleucine, leucine, lysine and arginine	
	< 1	Non-essential amino acids	
Goats	= 1	Methionine, histidine, tryptophan, phenylalanine and tyrosine	(12)
	> 1	Valine, isoleucine, leucine, lysine and arginine	
	< 1	Serine, glutamine, proline and asparagine	
Sows	= 1	Methionine, histidine and lysine	(10)
	> 1	Arginine, leucine, isoleucine, valine, tryptophan, phenylalanine, glutamate, glutamine, alanine and glycine	
	< 1	Proline, aspartate and asparagine	
Ewes	= 1	Methionine, phenylalanine, tyrosine, threonine and histidine	(11)
	> 1	Valine, isoleucine, leucine and arginine	
	< 1	Non-essential amino acids (except for serine)	

epithelial cell proliferation and differentiation have been well characterised previously[14]. However, the underlying mechanisms and signalling pathways by which amino acids regulate milk and fat synthesis were largely unknown until recently. The aim of the present review is to describe how amino acids are transported, sensed and transduced in the mammary gland, as well as their functions in the regulation of milk synthesis.

Branched-chain amino acids

Transportation and metabolism of branched-chain amino acids in the mammary gland

The plasma membrane transport system L is the most critical amino acid transporter system for branched-chain amino acids (BCAA) in mammary cells[15]. Transporters from the L system have been well characterised to regulate cell growth and proliferation by directly transporting branched or aromatic amino acids into the cytoplasm in a range of cell lines[16–18]. These transporters are heterodimeric proteins, which comprise of a catalytic subunit (L-type amino acid transporter 1 (LAT1), encoded by *SLC7A5*, or L-type amino acid transporter 2 (LAT2), encoded by *SLC7A6*) and a glycoprotein 4F2 heavy chain (4F2hc). Both LAT1 and LAT2 are highly expressed in the mammary tissues[15], but which subtype is predominately expressed in the mammary gland seems to be different among species[19–21]. In rats, the gene expression of LAT1 in the mammary gland is greater than that of LAT2 during the lactation period[19]. The gene expression of LAT2, but not LAT1, is significantly increased with the progression of lactation in sows[21]. In bovine mammary glands, more research has been focused on the effects of LAT1[20,22]. Depletion of LAT1 in the bovine mammary gland dephosphorylates and inhibits the activity of mTORC1 (mammalian target of rapamycin complex 1), thereby blunting cell viability and β-casein synthesis[20]. Additionally, the inhibition of mTORC1 can be rescued by re-expressing LAT1[20]. However, whether LAT2 also plays an important role in the bovine mammary gland has not been determined and remains to be studied.

BCAA catabolism in mammary tissue is similar to that in other tissues (such as muscle, liver and intestine)[23]. Leucine, isoleucine and valine share a number of BCAA catabolic enzymes, such as branched-chain aminotransferase (BCAT) and branched-chain α-keto acid dehydrogenase (BCKD)[24]. In the porcine mammary gland, the protein levels of BCAT are higher than those in the small intestine, skeletal muscle and liver[23], which indicates that BCAA are actively metabolised in the mammary gland. Mammary BCAA catabolism primarily produces glutamine and aspartate[23]. Notably, as BCAA share the same catabolic enzymes, excessive supplementation of either BCAA might affect the metabolism of other BCAA.

Potential signalling pathway of branched-chain amino acids in the mammary gland

Leucine. In the mammary gland, leucine regulates various biological processes, such as cell proliferation and milk synthesis (α_s-casein, β-casein and κ-casein) (as shown in Table 2). Numerous studies have demonstrated that mTOR functions as a critical regulator of these processes. Until recently, it was not clear of how leucine regulates mTOR until recently (Fig. 1). Before scientists started studying the effect of leucine on mTOR signalling in mammary cells, most pioneering studies were conducted in human embryonic kidney 293 (HEK 293) cells, which is a classical cell line for research investigating the cellular signalling pathway. In HEK 293 cells, it has been demonstrated that leucine regulates the mTOR signalling pathway primarily through mTOR complex 1 (mTORC1) which consists of regulatory-associated protein of mTOR (Raptor), mammalian lethal with SEC13 protein 8 (mLST8), 40 kDa proline-rich protein kinase B (Akt) substrate (PRAS40) and DEP domain-containing mTOR-interacting protein (DEPTOR). After being absorbed into the cytosol, leucine beings to regulate mTORC1 activation by first dephosphorylating Sestrin2[25,26]. Dephosphorylated Sestrin2 induces the dissociation of GTPase-activating protein activity toward Rags 2 (GATOR2), which further inhibits the function of GTPase-activating protein activity toward Rags 1 (GATOR1, a negative regulator of RagA/B)[27]. Finally, activated RagA/B promotes the translocation of mTORC1 to lysosomes for further activation[27].

Recent advances also strongly suggest that the leucine-regulated mTOR signalling pathway is also conserved in the mammary gland. In bovine mammary glands, overexpression of Sestrin2 depresses mTORC1 activity and synthesis of casein, indicating that Sestrin2 plays a major physical role in mammary gland cells[28]. *Danio rerio* SH3-domain binding protein 4 (SH3BP4) has been previously reported to abrogate mTORC1 activation by hydrolysing GTP to GDP of RagB in HEK 293 cells[29]. In the mammary gland, SH3BP4 is also proposed to play a vital role between Sestrin 2 and Rag GTPase[28]. Interestingly, not only leucine but also other essential amino acids and

https://doi.org/10.1017/S0954422420000074 Published online by Cambridge University Press

Table 2. Effects of branched-chain amino acids (BCAA) on mammary gland function and its potential signalling pathways

Amino acids	Cell lines/animal species	Functions	Potential signalling pathways	References
BCAA (–)	Cow mammary gland (*in vivo*, 105 ± 12 d of lactation for 5 d)	Milk yield ↓	Inhibit mTORC1/eIF2Bε/eIF2α signalling pathway	(94)
Leucine (+)	Bovine mammary epithelial cells/bovine mammary tissue slices	Milk protein synthesis ↑	Activate mTOR/S6K1 signalling pathway	(38)
Leucine (+)	Mouse mammary gland (*in vivo*, from parturition to day 17 of lactation)	–	Activate Akt/mTOR signalling pathway	(39)
Leucine (+)	Mouse mammary epithelial cells	Proliferation ↑	LAT1 and leucyl-tRNA synthetase	(34)
Leucine (+)	Mouse mammary gland (*in vivo*, from parturition to day 17 of lactation)	β-Casein synthesis ↑	Activate mTOR signalling pathway	(39)
Leucine (+)	Bovine mammary epithelial cells	α_s-, β-, κ-casein synthesis ↑	Activate mTOR signalling pathway	(98)
Leucine (+)	Bovine mammary epithelial cells	Expression of casein genes (CSN1S1, 2, 3) ↓	Activate JAK2/STAT5 and mTOR signalling pathway Inhibit AMPK phosphorylation	(99)
Leucine (-)	Mid-lactation Holstein cows (*in vivo*, 108 ± 11 d of lactation for 5 d)	Milk protein yield ↓ Synthesis of α_{s1}, β, κ-casein ↓	–	(100)
Leucine (-)	Bovine mammary epithelial cells/bovine mammary tissue slices	Milk protein synthesis ↓ Protein synthesis rates ↓	Inhibit mTOR/S6K1 signalling pathway	(38)
Isoleucine (+)	Bovine mammary epithelial cells/bovine mammary tissue slices	Milk protein synthesis ↑	Increase mTOR/S6K1 signalling pathway	(38)
Isoleucine (+)	Mouse mammary gland (*in vivo*, from parturition to day 17 of lactation)	–	Increase Akt/mTOR signalling pathway	(39)
Isoleucine (–)	Bovine mammary epithelial cells/bovine mammary tissue slices	Milk protein synthesis ↓ Protein synthesis rates ↓	Inhibit mTOR/S6K1 signalling pathway	(38)
Valine (+)	Porcine mammary epithelial cells	Milk fat synthesis ↑	Akt/mTOR/SREBP-1 pathway	(40)
Valine (+)	Porcine mammary epithelial cells	α-Lactalbumin and β-casein synthesis ↑	mTOR and Ras/ERK signalling pathways	(101)

mTORC1, mammalian target of rapamycin complex 1; eIF, eukaryotic initiation factor; mTOR, mammalian target of rapamycin; S6K1, S6 kinase 1; Akt, protein kinase B; LAT1, L-type amino acid transporter 1; CSN1S1, 2, 3, casein αs1, 2, 3; JAK2, Janus kinase 2; STAT5, signal transducers and activators of transcription 5; AMPK, AMP-activated protein kinase; SREBP-1, sterol regulatory element-binding protein 1; ERK, extracellular signal-regulated kinase.

non-essential amino acids can also regulate mTORC1 through Sestrin2 in the mammary gland[30], which is inconsistent with observations in HEK 293 cell lines and warrants further investigation.

In the mammary gland, the other potential leucine-mediated mTORC1 signalling pathway is through guanine nucleotide-binding protein subunit γ-12 (GNG12) and leucyl-tRNA synthetase (LeuRS). GNG12 regulates mTORC1 via interaction with Regulator[31], which affects the translocation of mTORC1 to lysosomal membranes[32]. LeuRS acts as a vital intracellular leucine sensor that can directly bind to Rag GTPase and activate mTORC1[33]. In mouse mammary cells, LeuRS activates the mTOR signalling pathway and increases cell proliferation[34]. In bovine mammary cells, GNG12 enhances cell growth and milk protein synthesis by activating the mTORC1 signalling pathway[31].

Valine and isoleucine. In addition to L-leucine, L-isoleucine and L-valine are also proposed to regulate milk synthesis. Dietary supplementation of L-isoleucine and L-valine during the whole lactation period enhances milk synthesis in sows and supports increased weaning weight of their litters[35,36]. Similarly, L-isoleucine and L-valine deficiency during the mid-lactation period inhibits milk synthesis in dairy cows[37]. Furthermore, recent advances indicate that both L-isoleucine and L-valine can activate the mTOR signalling pathway and have the potential to enhance milk protein synthesis[38–40]. Additionally, L-valine enhances fatty acid synthesis through activation of the mTOR/sterol regulatory element-binding protein 1 (SREBP-1) pathway[40]. SREBP-1 is a transcription factor and is proposed to be an important regulator of mammary gland fat synthesis in sheep[41], cows[42] and mice[43]. Lipin 1, a phosphatidic acid phosphatase, is a critical link between mTOR and SREBP-1[44]. When mTORC1 is activated, it phosphorylates lipin 1, which releases SREBP-1 and activates SREBP-1-regulated lipogenetic gene expression[44]. As L-valine regulates lipogenesis through activation of mTORC1, this finding strongly suggests that L-leucine and L-isoleucine might also participate in lipogenesis progression. The isotope tracing experiment showed that the carbon from L-leucine can incorporate into milk fat on goat mammary explants, which provides direct evidence that L-leucine is involved in milk fat production[45].

Recently, mTORC1 stimulation was observed before amino acid absorption into the cytostome[46–48]. T1R1/T1R3, a G-protein-coupled receptor (GPCR) in the cell membrane, is an important player in this process[46]. It has been demonstrated that amino acids regulate milk protein synthesis through T1R1/T1R3 in the mouse mammary gland[47,48]. Activation of the G-protein-coupled receptors (GPRC) T1R1/T1R3 increases phospholipase Cβ (PLCβ) activity and further enhances an influx of intracellular Ca^{2+}[49,50]. Extracellular signal-regulated kinase (ERK) 1 and 2 (ERK1/2), which are increased with Ca^{2+} concentration, regulate the activation of mTORC1 directly via the phosphorylation of Raptor (regulatory-associated protein of mTOR)[51]. The other possible pathway by which ERK1/2 activates mTORC1 signalling is through the inactivation of tuberous sclerosis complex 2 (TSC2),

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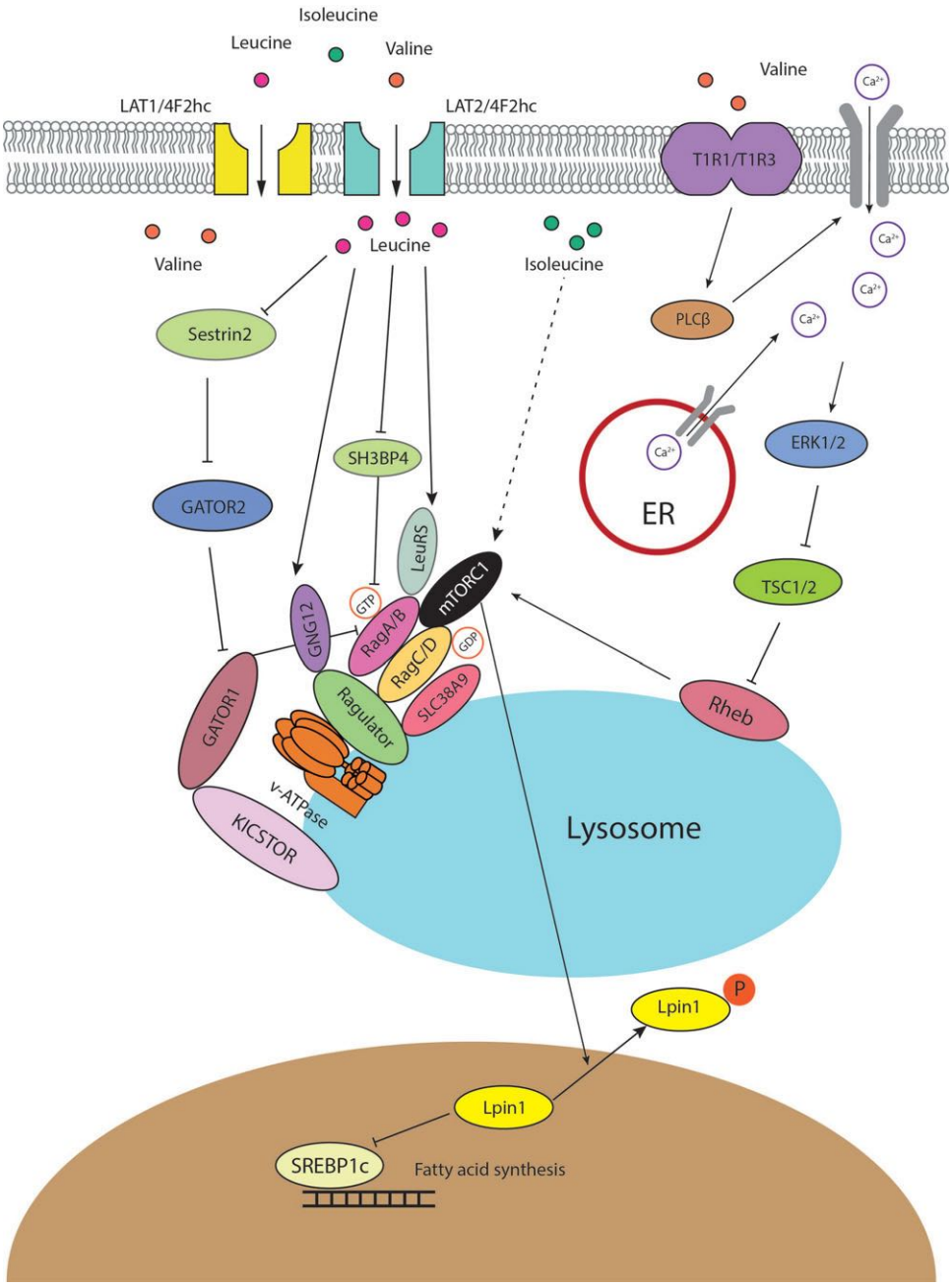

Fig. 1. Branched-chain amino acid (BCAA) and mammalian target of rapamycin complex 1 (mTORC1) signalling networks in the mammary gland. Note: L-type amino acid transporter 1/4F2 heavy chain (LAT1/4F2hc) and L-type amino acid transporter 2 (LAT2)/4F2hc derived from transporter system L are highly expressed and play a dominant role in BCAA transportation in the mammary gland. All three BCAA activate mTORC1 pathways in mammary glands. Leucine induces dephosphorylation of Sestrin2 and further promotes mTORC1 activation through GTPase-activating protein activity toward Rags (GATOR) 2, GATOR1 and RagA/B. In addition, GCG12, SH3-domain binding protein 4 (SH3BP4) and leucyl-tRNA synthetase (LeuRS) are crucial regulators in leucine-related mTORC1 activation. Extracellular valine activates G-protein-coupled receptors (GPRC) T1R1/T1R3, increases phospholipase Cβ (PLCβ) activity and further enhances an influx of intracellular Ca^{2+}. Increased Ca^{2+} regulates the mTORC1 signalling pathway through extracellular signal-regulated kinase 1/2–tuberous sclerosis complex 1/2–Rheb (ERK1/2–TSC1/2–Rheb) signalling. Intracellular isoleucine activates mTORC1 through an unknown mechanism. In the mammary gland, activated mTORC1 not only increases milk protein synthesis but also milk fat synthesis through lipin 1 (Lpn1)–sterol regulatory element-binding protein 1c (SREBP-1c) pathways. ER, endoplasmic reticulum. Please refer to the main text for details.

which is an inhibitor of mTORC1[52]. As T1R1/T1R3 can be widely activated by L-amino acids[46], it is supposed that all of the BCAA (leucine, valine and isoleucine) could activate mTORC1 through this signalling pathway in the mammary gland. However, in bovine mammary glands, valine, not isoleucine and leucine, regulates the mTOR signalling pathway through the membrane GPRC receptor T1R1/T1R3[53], which might be due to insufficient supplementation with isoleucine and leucine. Future experiments are warranted to verify these results in the mammary cells of other species.

https://doi.org/10.1017/S0954422420000074 Published online by Cambridge University Press

Table 3. Effects of methionine on mammary gland function and its potential signalling pathways

Items	Cell lines/animal species	Functions	Potential signalling pathways	References
Methionine (+)	Cows (*in vivo*, periparturient period)	Antioxidation ↑	Activate NFE2L2 signalling pathway	(102)
Methionine (+)	Cow mammary gland epithelial cells	Functional phosphoproteins ↑	Post-translational modification of proteins	(103)
Methionine (+)	Porcine mammary epithelial cells/mammary tissue slices	Mammary gland protein synthesis ↑	Activate mTOR/S6K1/4E-BP1 signalling pathway	(104)
Methionine (+)	Bovine mammary epithelial cells	Milk fat synthesis ↑	Activate FABP5/SREBP-1c signalling pathway	(65)
Methionine (+)	Bovine mammary epithelial cells	Milk fat synthesis ↑ Protein synthesis ↑ Cell proliferation ↑	Activate SNAT2/PI3K signalling pathway	(57)
Methionine (+)	Bovine mammary epithelial cells	–	Activate T1R1/T1R3/Ca^{2+}/mTOR signalling pathway	(53)
Methionine (+)	Bovine mammary epithelial cells	Amino acid transporter LAT1/4F2hc expression ↑	Activate mTORC1 signalling pathway	(22)
Methionine (+)	Holstein cows (*in vivo*, periparturient period)	Glucose and amino acid transporter ↑	Activate Akt signalling pathway	(64)
Methionine (+)	Bovine mammary epithelial alveolar cells	Regulate histone methylation	–	(105)
Methionine (+)	Mouse mammary gland (*in vivo*, from parturition to day 17 of lactation)	–	Increase Akt and mTOR signalling pathway	(39)

NFE2L2, nuclear factor erythroid 2-like 2; mTOR, mammalian target of rapamycin; S6K1, S6 kinase 1; 4E-BP1, eukaryotic initiation factor 4E-binding protein 1; FABP5, fatty acid-binding protein 5; SREBP-1c, sterol regulatory element-binding protein 1c; SNAT2, Na-coupled neutral amino acid transporter 2; PI3K, inositol 1,4,5-trisphosphate 3-kinase; mTORC1, mammalian target of rapamycin complex 1; Akt, protein kinase B.

Methionine

Transportation system of methionine in the mammary gland

In the mammary gland, three amino acid transporter systems are involved in methionine transportation, namely systems A, ASC and L[54]. System A consists of Na-coupled neutral amino acid transporter 1 (SNAT1; detected in pigs) and Na-coupled neutral amino acid transporter 2 (SNAT2; detected in rats and cows)[55]. System ASC primarily contains Na-dependent alanine cotransport 1 (ASCT1) (detected in humans, mice, cows and pigs) and Na-dependent alanine cotransport 2 (ASCT2) (detected in rats and cows)[55]. System L is composed of two heteromeric Na^+-independent transporters LAT1/4F2hc (detected in humans, rats, mice, cows) and LAT2/4F2hc (detected in rats and cows)[56]. In bovine mammary glands, SNAT2 inhibition strongly prevents the activation of mTORC1 caused by decreased methionine transportation[57]. The functional evaluation of the importance of specific methionine transporters is still insufficient and warrants further research to demonstrate which transporter system may play a dominant role in the mammary gland.

Potential signalling pathway of methionine in the mammary gland

The effects of methionine on milk synthesis in the mammary gland have been demonstrated for many years (Table 3). It has been shown through meta-analyses that methionine is one of the first two limiting amino acids in dairy cows[58,59] and is an important limiting amino acid in lactating sows[60,61]. Intriguingly, the effect of methionine on milk fat synthesis is not linked to the use of methionine carbon in fatty acid synthesis since its ratio of mammary uptake to milk output is always at 1 in dairy cows[62]. Advanced research in HEK 293 cells has demonstrated that when methionine is deficient, the cellular methyl donor *S*-adenosylmethionine (SAM) level will be reduced, which further increases the association of SAMTOR (SAM sensor) with GATOR2 and inhibits mTORC1 signalling[63]. However, to the best of our knowledge, whether SAMTOR also acts as a conserved SAM sensor in the mammary gland has not been determined and merits further research.

Two other potential methionine-regulated mTORC1 signalling pathways have been verified in the mammary gland (Fig. 2). One possible approach is the inositol 1,4,5-trisphosphate 3-kinase (PI3K)/Akt signalling pathway. Activated PI3K/Akt/mTORC1 significantly stimulates milk protein synthesis[57,64]. Furthermore, methionine also plays a crucial role in milk fat synthesis. Fatty acid-binding protein 5 (FABP5) is a crucial regulator that activates SREBP-1c for milk fatty synthesis[65], which can be partly activated by PI3K[66,67]. All of this information indicates that methionine may regulate milk lipid synthesis through the PI3K/Akt/FABP5/SREBP-1c signalling pathway. The other novel and crucial signalling pathway is the T1R1/T1R3 signalling pathway. Similar to isoleucine and valine, methionine also increases the influx of intracellular Ca^{2+} and regulates the effects of mTORC1 through T1R1/T1R3 in the mammary gland[53].

PI3K/Akt was previously demonstrated to be regulated by hormones, but not amino acids, in cell models. One possible cause of this discrepancy is that an unknown link between methionine and PI3K exists in the mammary gland. As IGF-1/PI3K/Akt/mTOR is the canonical pathway in cells[68,69], the other possible cause is that dietary methionine deficiency might indirectly inhibit PI3K activation via decreased IGF-1 secretion[70–72].

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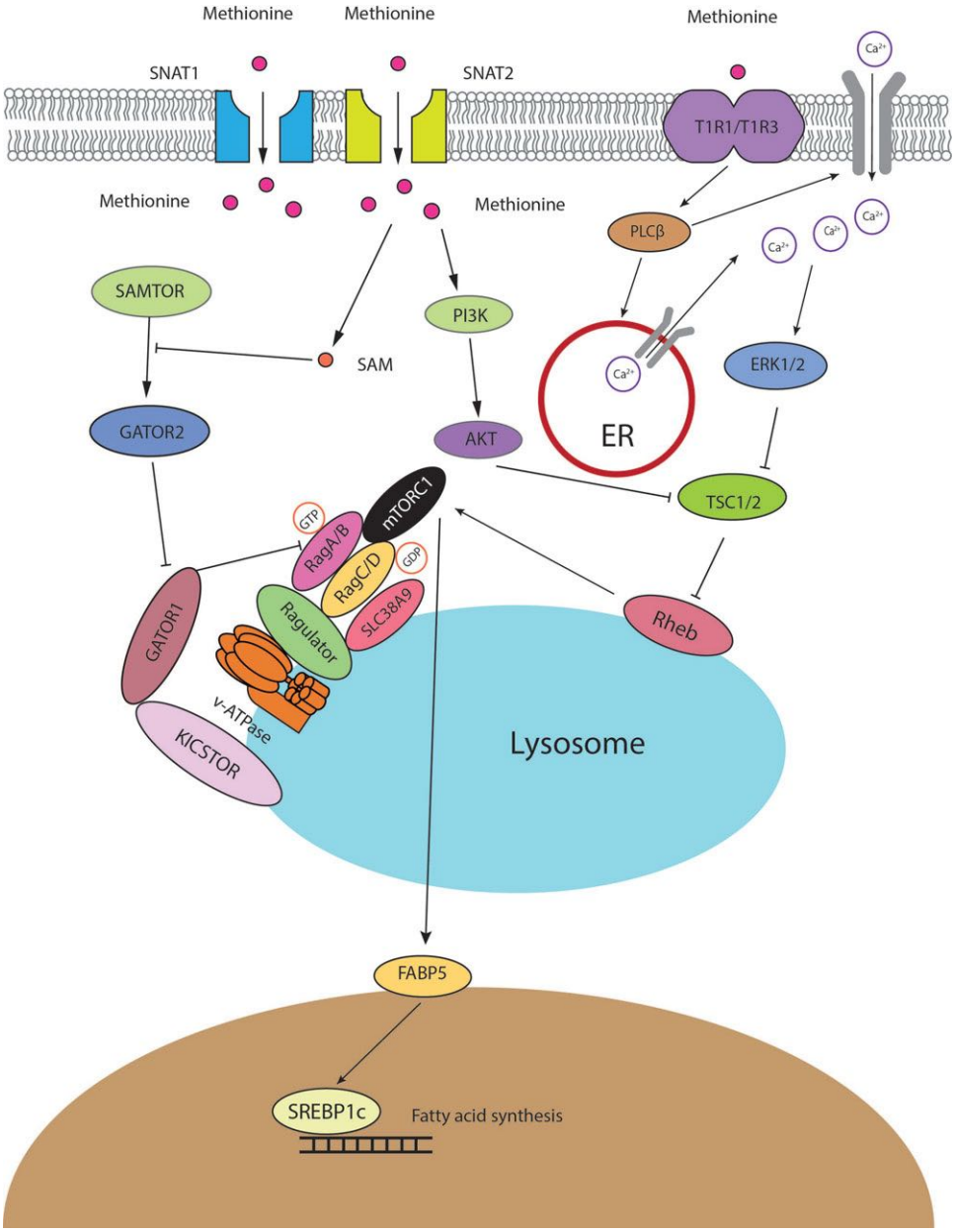

Fig. 2. Methionine and mammalian target of rapamycin complex 1 (mTORC1) signalling networks in the mammary gland. Note: sodium-coupled neutral amino acid transporter 1 (SNAT1) and SNAT2 originate from transporter system A and are crucial methionine transporters in the mammary gland. Intracellular methionine increases cellular *S*-adenosylmethionine (SAM) levels, which decreases the association of SAMTOR (SAM sensor) with GTPase-activating protein activity toward Rags 2 (GATOR2) and inhibits the mTORC1 signalling pathway. In addition, intracellular methionine regulates mTORC1 through the inositol 1,4,5-trisphosphate 3-kinase/protein kinase B/Rheb (PI3K/Akt/Rheb) signalling pathway. Extracellular methionine activates the G-protein-coupled receptors (GPCR) T1R1/T1R3, increases phospholipase Cβ (PLCβ) activity and further enhances the influx of intracellular Ca^{2+}. Increased Ca^{2+} regulates the mTORC1 signalling pathway through extracellular signal-regulated kinase 1/2–tuberous sclerosis complex 1/2–Rheb (ERK1/2–TSC1/2–Rheb) signalling. Activated mTORC1 increases milk protein synthesis and regulates milk fat synthesis through sterol regulatory element-binding protein 1 (SREBP-1) and fatty acid-binding protein 5 (FABP5). ER, endoplasmic reticulum. Please refer to the main text for details.

Arginine and lysine

Transportation system of arginine and lysine in the mammary gland

Arginine and lysine are both cationic amino acids and have the same amino acid transporter systems in the mammary gland[73]. Four cationic amino acid transporter (CAT) systems have been identified in the mammary gland as follows: (1) y^+ system: CAT-1 (detected in humans, cows, pigs and rats) and CAT-2 (detected in pigs); (2) y^+L system: y^+LAT1 (detected in pigs and cows) and y^+LAT2 (detected in pigs); (3) $b^{0,+}$ system: $b^{0,+}AT$ (detected in pigs); and (4) $B^{0,+}$ system: $ATB^{0,+}$ (detected in pigs, humans, rats)[74–77]. Among all transporters, CAT-1 seems to play a central role in arginine uptake in the mammary gland. In mammary MCF-7 cells, when 50 % cellular CAT-1 was knocked down, arginine uptake was inhibited by 35–40 %[78,79]. Furthermore, blocking $ATB^{0,+}$ also inhibits arginine uptake in the MCF-7 cell line[80]. Recently, $ATB^{0,+}$ has also been considered

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Table 4. Effects of arginine on mammary gland function and its potential signalling pathways

Items	Treatments	Functions	Potential signalling pathways	References
Arginine (+)	Porcine mammary epithelial cells	Cell proliferation ↑ Protein synthesis ↑	Activate mTOR signalling pathway	(86)
Arginine (+)	Bovine mammary epithelial cells	Milk synthesis ↑ Cell proliferation ↑ Amino acid transporter ↑	–	(106)
Arginine (+)	Lactating dairy cows (*in vivo*, 20 ± 2 d of lactation for 7 d)	Milk protein synthesis ↑ Amino acid transporters ↑	–	(107)
Arginine (+)	Lactating sows (*in vivo*, from parturition to day 21 of lactation)	Mammary tissue angiogenesis ↑	–	(91)
Arginine (+)	Lactating cows (mid-lactation for 14 d)	Increase casein ↑	–	(108)
Arginine (+)	Bovine mammary epithelial cells	Inflammatory response ↓ Casein secretion ↑	–	(109)
Arginine (+)	Primary bovine mammary epithelial cells	Milk protein ↑ Fat synthesis ↑	Inhibit GCN2/eIF2α pathway	(110)
Arginine (–)	Primary bovine mammary epithelial cells	Mammary gland autophagy ↑	Activate GCN2 signalling pathway	(111)
Arginine (–)	Mid-lactation Holstein cows (*in vivo*, 108 ± 11 d of lactation for 5 d)	Milk protein yield ↓ Synthesis of α_{s1}, β, κ-casein ↓	–	(100)
Arginine (+)	Bovine mammary epithelial cells	Casein synthesis ↑	mTOR signalling pathway	(87)
Arginine (+)	Bovine mammary epithelial cells	Casein synthesis ↑	–	(112)

mTOR, mammalian target of rapamycin; GCN2, general control non-derepressible 2; eIF, eukaryotic initiation factor.

as the most crucial lysine transporter in the mammary gland. In bovine mammary gland epithelial cells, lysine activates the mTOR signalling pathway, which is inhibited by blockade of the lysine transporter $ATB^{0,+}$[81]. In the sow mammary gland, the transportation of lysine is partly inhibited by excessive arginine supplementation[82]. This evidence indicates that arginine and lysine have the same critical transporter system (CAT-1 and $ATB^{0,+}$) in the mammary gland.

Potential signalling pathway of arginine in the mammary gland

Positive effects of arginine on placental growth and fetal survival and growth have been demonstrated in pigs, rats, mice and sheep[83], whereas its functions in the mammary gland were not determined until recently (Table. 4). In the mammary gland, arginine catabolism produces proline, ornithine, urea, glutamate, glutamine, CO_2 and polyamines (putrescine, spermidine and spermine)[84]. In sows, supplementation with 0·5 or 1·0 % L-Arg-HCl activates the milk synthesis and increases the litter weight of sucking piglets[85]. Similar to other amino acids, arginine also regulates milk protein synthesis through mTORC1[86,87]. The central regulator linking arginine to the mTORC1 signalling pathway is cellular arginine sensor for mTORC1 (CASTOR1)[88,89]. Sufficient arginine dissociates GATOR2 from CASTOR1 and further activates the mTOR signalling pathway[88,89] (Fig. 3). The other crucial function of arginine in the mammary gland is primarily achieved through its metabolite NO[90]. Briefly, NO increases the mammary blood vessel density and diameter, which might enhance the transportation of nutrients to the mammary gland and support milk synthesis[91].

Potential signalling pathway of lysine in the mammary gland

Similar to methionine, lysine is one of the first two limiting amino acids both in dairy cows[59,92] and sows[60,61]. In the bovine mammary gland, the use of lysine is dose dependent as it can be the first limiting amino acid. In case of low supply, lysine is mainly utilised in milk protein synthesis with the ratio of lysine uptake to lysine output close to 1[8]. However, when sufficient amount of lysine is provided through the diet, it can also be used to either synthesise non-essential amino acids[62,93] or be oxidised into CO_2 as BCAA[81]. When lysine is deficient, milk protein synthesis is inhibited[94], with a decrease in mTORC1 activity in dairy cows[95]. However, the function of lysine in the mammary gland has largely not been determined. Recent advances have found that lysine increases milk fat synthesis through the GPRC6A/PI3K/FABP5 signalling pathway[67]. GPRC6A is a G protein-coupled receptor that is specific for cationic amino acid sensing[96]. As a Gαi/Gαq receptor, GPRC6A has the potential to regulate cellular cAMP levels and activate the MAPK signalling pathway[97]. Thus, both GPRC6A/PI3K/Akt/mTOR and GPRC6A/ERK/mTOR can be the potential signalling pathways for lysine to regulate milk protein and fat synthesis in the mammary gland.

Conclusion

Amino acids play crucial roles in the synthesis of milk protein and fat in the mammary gland. The dominant amino acid transposers (BCAA, methionine, lysine and arginine) of the mammary gland are summarised in the present review. In addition, our review has focused on a number of canonical and novel signalling molecules involved in amino acid signalling pathway in the mammary gland. Remarkably, mTORC1 acts as the central node of the amino acid-regulated signalling pathway and can be activated intracellularly and extracellularly (through a G-protein-coupled receptor (GPCR)). Currently, the amino acid signalling pathway in the mammary gland still warrants further investigation. Achieving a better understanding of the amino acid signalling pathway might help us to optimise the amino acid profiles in maternal diets for human beings and other mammals in the future.

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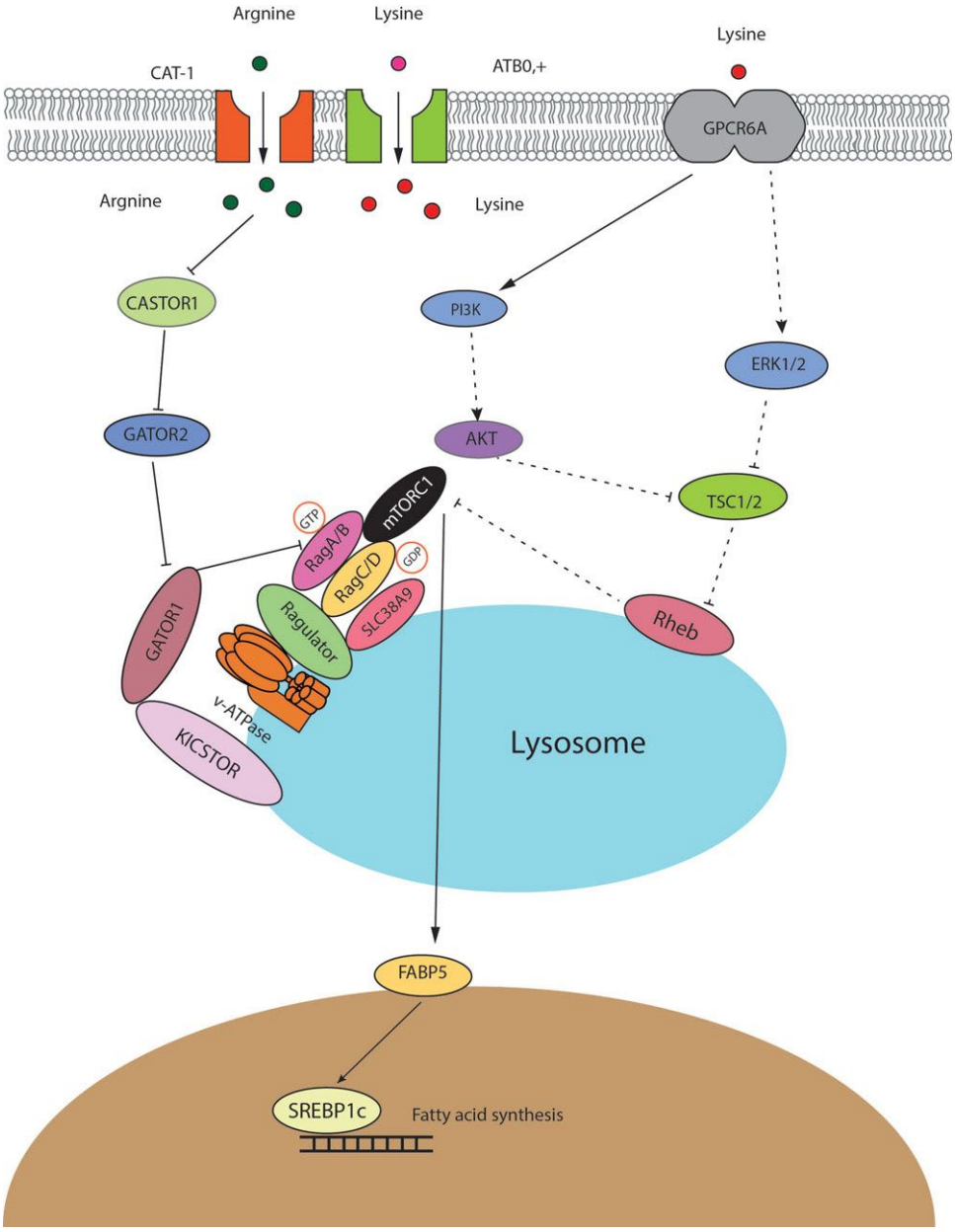

Fig. 3. Lysine and arginine regulate the mammalian target of rapamycin complex 1 (mTORC1) signalling network in the mammary gland. Note: cationic amino acid transporter-1 (CAT-1) and $ATB^{0,+}$ are critical cationic amino acid transporters for arginine and lysine transportation in the mammary gland. The intracellular arginine regulator mTORC1 acts through the cellular arginine sensor for mTORC1–GTPase-activating protein activity toward Rags 2–GTPase-activating protein activity toward Rags 1–RagA/B (CASTOR1–GATOR2–GATOR1–RagA/B) signalling pathway, whereas extracellular lysine regulates mTORC1 through the G-protein-coupled receptor (GPCR) GPCR6A. As a Gαi/Gαq receptor, GPCR6A can activate milk protein synthesis through the GPRC6A–inositol 1,4,5-trisphosphate 3-kinase–protein kinase B–tuberous sclerosis complex 1/2–Rheb (GPRC6A–PI3K–Akt–TSC1/2–Rheb) and GPRC6A–extracellular signal-regulated kinase 1/2 (ERK1/2)–TSC1/2–Rheb pathways. Dashed lines represent potential signalling pathways that have not been verified in the mammary gland. FABP5, fatty acid-binding protein 5; SREBP-1, sterol regulatory element-binding protein 1. Please refer to the main text for details.

Acknowledgements

The present review was supported by the National Natural Science Foundation of the People's Republic of China (no. 31802067 and 31872364) and the Natural Science Foundation of Guangdong Province (no. 2018A030310201).

S. Z. initiated the idea, the scope, and the outline of this review paper. Z. W., J. H., M. T., H. S. and F. C. studied and analysed all of the publications cited in this paper and were involved in manuscript preparation. W. G. conducted the final editing and proofreading. All authors read and approved the final manuscript.

The authors declare that they have no competing interests.

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https://doi.org/10.1017/S0954422420000074 Published online by Cambridge University Press

Animal Nutrition 6 (2020) 397–403

Contents lists available at ScienceDirect

Animal Nutrition

journal homepage: http://www.keaipublishing.com/en/journals/aninu/

Review Article

Dietary fiber and microbiota interaction regulates sow metabolism and reproductive performance

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ARTICLE INFO

Article history:
Received 12 August 2020
Received in revised form
13 September 2020
Accepted 2 October 2020
Available online 8 October 2020

Keywords:
Dietary fiber
Gut microbe
Reproductive performance
Sow

ABSTRACT

Dietary fiber is a critical nutrient in sow diet and has attracted interest of animal nutritionists for many years. In addition to increase sows' satiety, dietary fiber has been found to involve in the regulation of multiple biological functions in the sow production. The interaction of dietary fiber and gut microbes can produce bioactive metabolites, which are of great significance to sows' metabolism and reproductive performance. This article reviewed the interaction between dietary fiber and gut microbes in regulating sows' gut microbial diversity, intestinal immune system, lactation, and production performance, with the aim to provide a new strategy for the use of dietary fiber in sow diets.

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1. Introduction

During gestation, sows are restrictively fed to maintain normal reproductive performance. However, excessive feeding restrictions makes it difficult for sows to produce satiety and sows show abnormal behaviors such as vacuum chewing, frequent rising and lying during pregnancy, which leads to a negative impact on their reproductive performance (Sekiguchi and Koketsu, 2004). In addition, the feed intake of sows during pregnancy is closely related to their voluntary feed intake during lactation. Excessive feeding restriction during pregnancy will result in a decrease in sows' voluntary feed intake during lactation (Matte et al., 1994; Sun et al., 2014, 2015). Dietary fiber supplementation is an efficient way to maintain proper satiety for sows. Dietary fiber is not easily digested by digestive enzymes in the gastrointestinal tract, thus avoiding the sow from taking in excessive energy (Huang et al., 2020).

Interestingly, dietary fiber not only increases the satiety, but also improves reproductive performance and lactation performance of sows. Previous studies have found that adding dietary fiber to sow diets during pregnancy increased the total litter birth, weaning litter weights, and milk fat content in colostrum (Che et al., 2011; Loisel et al., 2013; Matte et al., 1994; Veum et al., 2009). Moreover, sows fed with dietary fiber during multiple reproductive cycles have better reproductive and lactation performances (Che et al., 2011; Matte et al., 1994; Veum et al., 2009). The physiological metabolism of sows undergoes complex changes during pregnancy. As a result, the stability of the intestinal flora structure is fragile and easily broken (Newbern and Freemark, 2011). The destruction of intestinal flora stability results in intestinal epithelium damage and intestinal inflammation. The gut microbiota is sensitive to the physical and chemical properties of specific dietary fibers. Many recent studies have reported that the addition of dietary fibers to diets during pregnancy effectively alters sow gut microbiota composition and diversity, and their interaction affects the sow's

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Peer review under responsibility of Chinese Association of Animal Science and Veterinary Medicine.

Production and Hosting by Elsevier on behalf of KeAi

https://doi.org/10.1016/j.aninu.2020.10.001

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nutritional, physiological, and immune processes in a variety of ways (Cheng et al., 2018a; Jarrett and Ashworth, 2018; Wu et al., 2020; Xu et al., 2020a; Zhuo et al., 2020). The present review summarized the effects of dietary fiber on the composition and diversity of sows' gut microbes, as well as their interactions on the regulation of sows' gut health, and reproductive and lactation performances.

2. Changes of gut microbiota composition during different stages of pregnancy

During the reproductive cycle, the physiology and metabolism of the sow undergo complex changes. Gut microbes play a vital role in the function and health of their host (such as, nutrient absorption, metabolism, immune system, and resistance to pathogens) and are also affected by changes in the physiological state of their host. Therefore, the relationship between pregnancy and gut microbes is of particular concern. With the increase of gestational age, the alpha diversity of gut microbiota is also increased. Kong et al. (2016) and Ji et al. (2017) analyzed the ileal and colonic luminal microbiota composition of Huanjiang mini-pigs during pregnancy. From the first to third trimester of pregnancy, the dominant bacteria in the colon of Huanjiang mini-pigs are Firmicutes and *Bacteroides*, and the dominant genera include *Lactobacillus*, *Treponema*, *Ruminococcus*, *Clostridium*, and *Prevotella* (Kong et al., 2016). And, Firmicutes (account for 69.99% to 85.44% of the total reads) and Proteobacteria (5.82% to 15.17%) are dominant in the ileum (Ji et al., 2017). From the 45th day to the 75th day of pregnancy, the number of Firmicutes and *Lactobacillus* bacteria in the ileum is decreased, which is contrast to the number of Proteobacteria, Enterobacteriaceae, and *Bacteroides*. The significant increase of Proteobacteria (mainly pathogenic bacteria) is an indicator of ileal inflammation (Flint et al., 2008; Koren et al., 2012; Wu et al., 2011).

These changes of intestinal microbes could further regulate the metabolism of the host. Recently, Huang et al. (2019) analyzed the gut microbe characteristics of sows from late pregnancy (5 d before delivery) to postpartum (within 6 h after delivery). The results showed that the ratio of *Bacteroidetes* to Firmicutes and the relative abundance of *Prevotella* were significantly reduced, and the dominant genus *Lactobacillus* was significantly increased. Consistent with these changes, the predicted functional capacities of the gut microbe associated with glycan biosynthesis, amino acid metabolism, and the metabolism of cofactors and vitamins were significantly decreased. However, the abundance of the functional capacities related to carbohydrate and lipid metabolism were increased. Intriguingly, it has also been reported that the progression of pregnancy is related to several carbohydrate-degrading bacteria (*Prevotella*, *Succinivibrio*, *Bacteroides*, and *Parabacteroides*) (Ji et al., 2019). In addition, from pregnancy to parturition, changes in the composition of gut microbes also modified the intestinal levels of pro-inflammatory IL-6 and anti-inflammatory IL-10 (Cheng et al., 2018b). Thus, the interaction of gut microbes at different stages of pregnancy affects the metabolism of the host, which may increase nutrient metabolism and regulate sows' lactation performance.

3. Dietary fiber regulates the abundance and diversity of sow gut microbes

Dietary fiber has an intimate relationship with the intestinal microbes (Makki et al., 2018). Generally, fiber is divided into soluble fiber and insoluble fiber. The amount of soluble and insoluble fiber varies in different feed ingredients (Jarrett and Ashworth, 2018). Soluble fiber (such as inulin) is hardly digested in the mouth, stomach, and small intestine. This fiber is transported to the hindgut as intact molecules and broken down by the coliform, which produce a small amount of energy and various bioactive substances (Makki et al., 2018). Insoluble fiber (such as cellulose) is difficult to be fermented by intestinal microbes, which could enhance intestinal health by promoting intestinal peristalsis (Capuano, 2017). Firmicutes and Actinobacteria are dominant responders in the gut to break down dietary fiber (Deehan et al., 2017). The digestibility for fiber could be different in the different breeds of sows. For example, Rongchang sows are more capable of digesting high-fiber feed compared with Landrace sows, which is related to the abundant Ruminococcaceae in the gut of Rongchang sows (Liu et al., 2019).

Difference in dietary fiber sources and gut microbe sensitivity partly determine the complex microbial system of the gut. In sows, Bacteroidetes and Firmicutes are the most dominant phyla in the intestine, accounting for about 73% of the total sequence of microorganisms (Niu et al., 2019). Moreover, *Lactobacillus, Oscillibacter*, and *Treponema* accounted for more than 49% of the total sequence (Niu et al., 2019). There is a close relationship between the gut microbiota composition and the apparent digestibility of crude fiber. For example, the abundance of some microorganisms in sow fecal samples was positively correlated with the apparent digestibility of crude fiber (such as *Anaerofustis* and *Robinsoniella*), neutral detergent fiber (such as *Collinsella* and *Sutterella*), and acid detergent fiber (such as *Clostridium*, *Collinsella*, *Robinsoniella*, and *Turicibacter*). Dietary fiber is the main source of energy for gut microbiota, which means that adding an appropriate amount of dietary fiber can increase the abundance of specific microorganisms (Sappok et al., 2015). In addition, dietary fiber also increases the proportion of beneficial bacteria and reduces the proportion of pathogenic bacteria in the gut (Guan et al., 2019; Jiang et al., 2019; Wu et al., 2020; Xu et al., 2020a).

Therefore, dietary fiber can improve the utilization of protein and fat by regulating the abundance of specific gut microbes. The strategy for dietary fiber to regulate protein fermentation in the intestine has been summarized before (Jha and Berrocoso, 2016). Briefly, dietary fiber effectively relieves the adverse effects of certain amino acid fermentation products (for example, ammonia, histamine) and decreases the harmful strains (such as, enterotoxigenic strains of *Escherichia coli*) on the intestinal tract. Furthermore, dietary fiber reduced the sow weight gain and fat accumulation that are caused by adding fat in diets (Zhou et al., 2017), which might be caused by the modification of gut microbial structure. These results indicate that dietary fiber can regulate the utilization of protein and fat by intestinal microorganisms, which further promotes sows' intestinal health.

In summary, the addition of different levels and types of dietary fiber are related to the gut microbiota abundance and diversity. However, for the level of dietary fiber added to the diet, there is currently insufficient data to form a reference standard. Moreover, the research on the effect of dietary fiber types on the gut microbes of sows is still in the preliminary exploratory stage and a large amount of data is still needed to enrich this aspect.

4. Biological effects of dietary fiber fermentation by-products in the gut

Dietary fiber is a critical energy source for cecal and colonic epithelial cells (Fig. 1). Under specific intestinal environmental conditions, dietary fiber is fermented and utilized by anaerobic bacteria and produces a variety of metabolites, such as short-chain fatty acids (SCFA), especially acetate, propionate and butyrate (Jarrett and Ashworth, 2018; Patil et al., 2020). They are an important substrate of gluconeogenesis and participate in the regulation of central metabolism. Short-chain fatty acids trigger

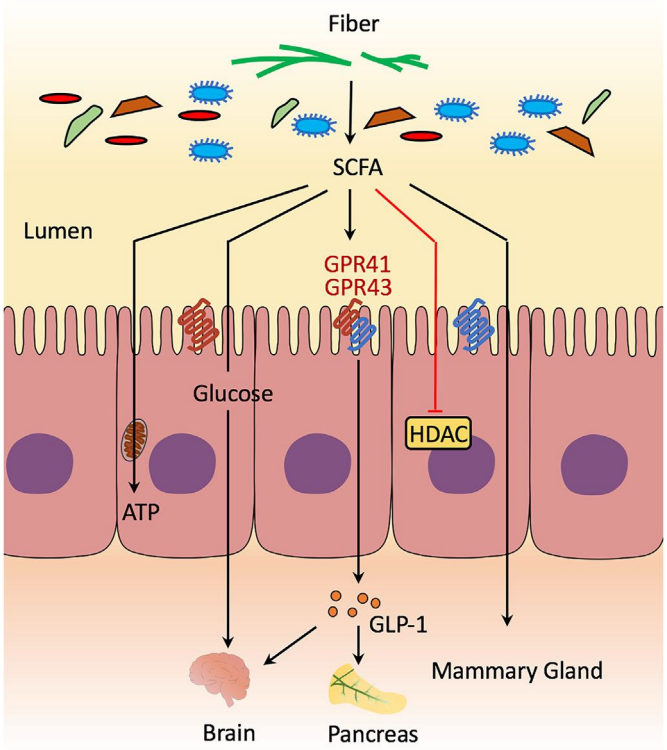

Fig. 1. Mechanisms of signaling from the gut microbiota to the sow. SCFA = short-chain fatty acids; GPR = G protein-coupled receptors; HDAC = histone deacetylase; ATP = adenosine triphosphate; GLP-1 = glucagon-like peptide 1.

host signals by inhibiting histone deacetylase (HDAC) or activating G protein-coupled receptors (such as GPR41 and GPR43), which are activated to regulate the release of glucagon-like peptide 1 (GLP-1) and neuropeptide Y which lead to satiety.

Short-chain fatty acids involved in the regulation of sow metabolism, immunity, and cell proliferation (Li et al., 2019; Wu et al., 2020; Xu et al., 2020a). Dietary supplementation of 3% purified fiber-mixture significantly increase the concentrations of butyrate and propionate in the feces of sows, which was related to the increase in the abundance of *Eubacterium-hallii-group* and *Bacteroides* in the gut (Wu et al., 2020). Different types of dietary fiber contribute to the variation of SCFA (Tokach et al., 2019). Short-chain fatty acids are transported to the peripheral circulation through the portal vein, thereby acting on the liver and peripheral tissues. They have been proposed to act as signal molecules to regulate different physiological activities of the host (Koh et al., 2016), such as regulating immunity and the expression of antioxidant enzymes, inflammatory and pro-inflammatory factors (Li et al., 2019; Shang et al., 2019; Guo et al., 2020; Xu et al., 2020a).

5. Embryo development and survival

To date, it has been widely accepted that dietary fiber supplementation improves the survival rate of sow embryos during pregnancy. Oocytes are very sensitive to maternal nutrient levels. Dietary nutrients intake changes the physiological levels of circulating hormones and metabolites, which ultimately affects the function of the ovaries (Ashworth et al., 2009). Administration of fiber promotes the maturation of follicles and oocytes in the sow ovary (Ferguson et al., 2007; Renteria-Flores et al., 2008; Weaver et al., 2013). The proportions of oocytes reaching Metaphase II were higher in the diet fiber group than in the control group (Weaver et al., 2013).

The modification of ovary function may be due to the dietary fiber fermentation products in the gut. Short-chain fatty acids could bind with G protein-coupled receptors GPR41 and GPR43 and activate their downstream target proteins in the gut. Adenosine 5′-monophosphate-activated protein kinase (AMPK)/mammalian target of rapamycin complex 1 (mTORC1) is a classic signaling pathway which largely regulates cellular biology and metabolism. This pathway also has an important significance for the activation and survival of primordial follicles (Zhuo et al., 2019). Short-chain fatty acids mediates multiple beneficial effects on the host metabolism through the activation of AMPK (Hu et al., 2010). AMPK is a negative regulator of mTORC1. Consistently, the phosphorylation level of mTORC1 and its downstream target S6 in the ovary decrease linearly with the increase of dietary fiber (Cao et al., 2019). In addition, the extracellular signal-regulated protein kinases 1/2 (ERK1/2) signal is also suppressed as the level of dietary fiber increases (Cao et al., 2019). Inhibition of ERK1/2 decrease the mTORC1 signaling, which participates in the activation of primordial follicles. These results indicate that increasing the level of dietary fiber intake can preserve a greater reserve of primordial follicles. Interestingly, these results show that the mTOR signaling pathway is inhibited. Whether this result is really beneficial to the reserve of primordial follicles still needs more evidence.

The other potential pathway for dietary fiber to regulate follicular maturation is through neuropeptide Y. This may be the result of the fermentation of dietary fiber by microorganisms in the intestine. However, to date, there is no direct evidence that microorganisms play a key role in this process. Neuropeptide Y has been shown to be involved not only in regulating food intake, but also related to the secretion of gonadotropin releasing hormone. A high-fiber diet changes the circulating concentrations of estradiol and luteinizing hormone, which are important for oocyte maturation (Ferguson et al., 2007). These results also explain why long-term feeding of dietary fiber increases the total number of births of sows, whereas short-term feeding does not. Because sows fed dietary fiber for a long time not only retain a larger reserve of primordial follicles, but also create a better physiological environment for follicle maturation than short-term feeding sows and gilts.

6. Milk composition

Milk is a unique nutrition source for newborns. During pregnancy and lactation, the mammary gland is one of the most active tissues in sows. In order to provide enough nutrients for newborns, a large amount of nutrients absorbed by the gut are transported to the mammary glands through the blood circulation to synthesize milk. Dietary nutrient level is an important factor affecting the synthesis and composition of sow milk. Although dietary fiber cannot be used directly by sows, its fermentation product is an important source of nutrients for sow milk synthesis. As we mentioned before, dietary fiber could be fermented by gut microorganisms to produce SCFA (Fig. 2). Mammary glands use SCFA as precursors for *de novo* synthesis of milk fat. Compared with the low-fiber group, the high-fiber group had increased milk fat content in the colostrum (Loisel et al., 2013; Feyera et al., 2019).

The effects of dietary fiber on milk immunoglobin (Ig) secretion is inconsistent. Loisel et al. (2013) and Feyera et al. (2019) found the concentrations of IgA and IgG in colostrum of sows in the high-fiber group was reduced. Whereas, Shang et al. (2019) reported the concentration of IgA in sow colostrum is significantly increased

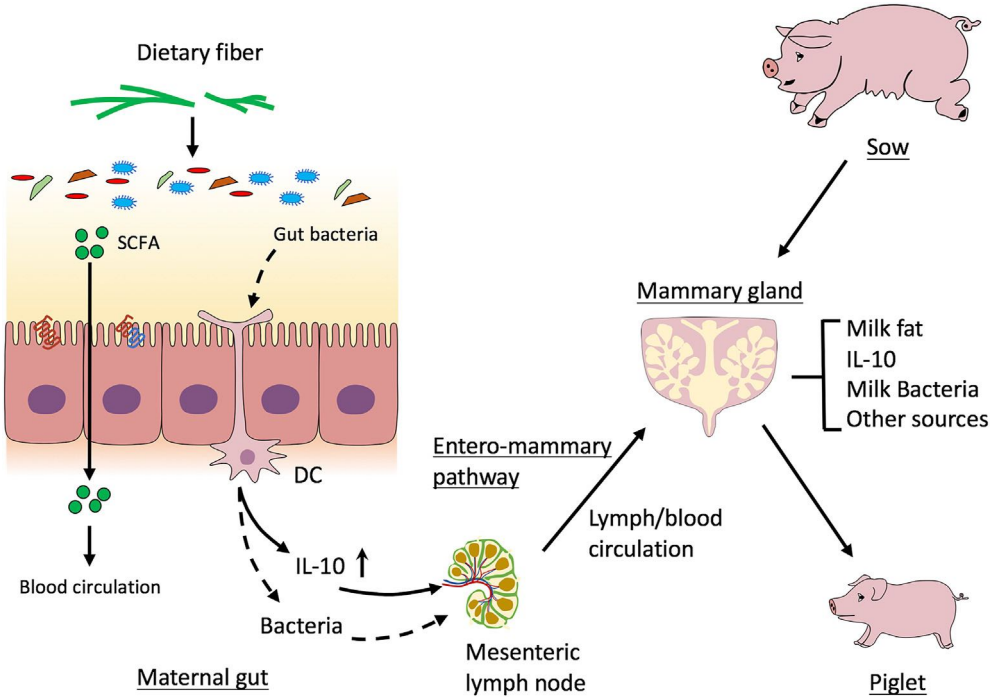

Fig. 2. Dietary fiber and gut microbe interaction changes the composition of milk. Dietary fiber and gut microbe interaction produces a variety of fermentation products, including short-chain fatty acids (SCFA). Parts of the SCFA are transferred to the mammary gland through the blood circulation, then used as substrates for *de novo* synthesis of milk fat. Also, some immune factors (e.g. IL-10) from gut are transported to the mammary gland through the intestinal-lymphatic circulation system. Interestingly, there is a possibility that gut microbe can enter the lymph nodes through dendritic cells (DC) and then reach the mammary gland through the intestinal-lymphatic circulation system. These factors result in the changes of sow milk composition. Ultimately, the beneficial effects of the interaction between dietary fiber and gut microbes are passed from mother to offspring through lactation.

when sows were fed the high-fiber diet. These inconsistent results may be due to the different types of dietary fibers (Loisel et al., 2013; Shang et al., 2019). Recent studies also reported that dietary fiber increases the IL-10 concentration of sow colostrum and normal milk, which is critical to promote piglet intestinal health (Lv et al., 2018; Shang et al., 2019). There is no doubt that the utilization of dietary fiber by sows requires intestinal microbial fermentation, which leads to the changes of related physiological indicators. Unfortunately, these studies did not explore the role of gut microbes in this process.

In addition to nutrients, milk also contains a lot of microorganisms. It is proposed that a portion of the milk microorganisms may derive from the gut, but there is yet a definite conclusion (Moossavi and Azad, 2019; Rodríguez, 2014; Zhang et al., 2018). During the third trimester of pregnancy and lactation, there is an important outflow of intestinal immune cells to the mammary glands (Macpherson and Uhr, 2004; Newburg, 2005; Rescigno et al., 2001; Vazquez-Torres et al., 1999). Gut microbes might transfer into the lymph nodes with the help of dendritic cells and then reach the mammary gland through the intestinal-lymphatic circulation system (Fernández et al., 2013; Rodríguez, 2014). Certain bacteria in the gut have been found to co-exist in maternal peripheral blood and lymphocytes isolated from milk (Avershina et al., 2018; Perez et al., 2007). The main dominant bacteria in sow milk are Ruminococcaceae, *Streptococcus*, *Lactobacillus*, and Clostridiales, which largely exist in the gut of animals (Chen et al., 2018). Intriguingly, *Ruminococcaceae* and *Lactobacillus* are important bacterial genera for the dietary fiber fermentation in the gut (Cheng et al., 2018a). These studies provide some primary evidence that dietary fiber composition may change the microbial composition of milk.

7. Weaning-to-estrus interval

The weaning-to-estrus interval (WEI) is a crucial indicator to evaluate the reproductive performance of sows. During the reproductive cycle, a relatively fixed WEI (approximately 5 to 7 d) is essential for sows to ovulate efficiently and maintain the embryo survival rate during pregnancy. Estrus is a complex physiological activity and regulated by a variety of reproductive hormones, such as follicle stimulating hormone, luteinizing hormone, and estradiol. The bidirectional interaction between gut microbes and the secretion and metabolism of host sex hormones has been demonstrated (Markle et al., 2013; Melvin, 2016). In rats, transplanting *Lactobacillus* or faecal microbes from healthy rats into polycystic ovary syndrome rats increases the abundance of *Lactobacillus* in the gut and significantly increases the plasma concentrations of estradiol and estrone (Guo et al., 2016). In sows, the plasma reproductive hormone levels (oestradiol, follicle-stimulating hormone and luteinizing hormone) in sows with a short WEI (not more than 7 d) are significantly higher than those with a long WEI (>14 d) sows (Xu et al., 2020b). Importantly, in the gut of sows with short WEI, *Prevotella* and *Bacteroides* are lower at the genus level, whereas Firmicutes and Lentisphaerae are higher at the phylum level (Xu et al., 2020b). *Prevotella* and *Bacteroides* are important genera that degrade carbohydrates in the gut, which indicates dietary fibers might regulate WEI of sows. To our surprise, most studies have shown that increasing dietary fiber has no significant

effect on the WEI of sows. Thus, the minor effects of microorganisms on the secretion of reproductive hormones might not efficiently modify the WEI of sows.

8. Effects on offspring piglets

The beneficial effects of the interaction between dietary fiber and gut microbes can be passed from the mother to the offspring. Dietary fiber is reported to enhance the reproduction performance of sows. Wu et al. (2020) fed gestational sows with a diet supplemented with 3% purified fiber-mixture, which significantly increased the number of live-born piglets. Veum et al. (2009) reported feeding sows a diet supplemented with wheat straw for a long period significantly increased the number of weaned piglets per litter, the weight of total litter births, and weaning litter. Furthermore, a short-term addition of dietary fiber in the late pregnancy also reduced the proportion of stillborn piglets and the total mortality of piglets during lactation (Feyera et al., 2017). The effects of dietary fiber on reproduction performance of sows might be partially dependent on the physical condition of sows and dietary fiber level. Che et al. (2011) fed sows with 3 fiber levels (10.8%, 15.8% and 20.8% neutral detergent fiber) for 2 reproductive cycles. In the first parity, the total litter births, litter births alive, and litter weight in the low-fiber group were significantly higher than those in other groups; likewise, the low-fiber group and medium-fiber group had greater litter weights at parturition and weaning. However, in the second parity, the total number of births, the total litter births and litter births alive, and litter weight in the medium-fiber group were significantly higher than those in other groups. Interestingly, the high-fiber group had the highest survival rate and heavier weight of piglets at weaning (after 22 d of lactation) compared with other groups. These results indicate that sows fed dietary fiber-added diets for a long time is more conducive to improving its production performance, and the addition level of fiber in the diet can be appropriately increased with the extension of feeding time.

Some recent studies reported that the changes of sow production performance caused by dietary fiber is related to the modification of gut microbes. Intestinal microbes may lead to the changes of intestinal antioxidant capacity (Zhou et al., 2017). Adding inulin to high-fat diets enhanced the activities of superoxide dismutase (T-SOD) and glutathione peroxidase (GSH-Px) and reduced the malondialdehyde (MDA) concentrations in the serum of sows (Wang et al., 2016). The production performance and antioxidant capacity of sows are positively correlated with *Bacteroides* of Bacteroidaceae, but negatively correlated with *Phascolarctobacterium* and *Streptococcus* (Wang et al., 2019a,b; Wang et al., 2018). It worth noting that T-SOD, GSH-Px, and MDA could be transferred from sows to piglets through milk (Chen et al., 2019a; Chen et al., 2019b; Chen et al., 2020; Wang et al., 2019a,b). Thus, there is a close relationship between dietary fiber, gut microbes, antioxidant abilities, and sow production performance.

9. Conclusion

The application value of dietary fiber in improving the satiety of sows has long been recognized. However, some recent reports suggested that dietary fiber has many other benefits in production of sow. With the reduction in the use of antibacterial drugs on a global scale, the application value of dietary fiber is particularly valued. Dietary supplementation of fiber can change the abundance and diversity of gut microbiota and thereby promote gut health and reduce the need for antibiotics. In addition, the application of dietary fiber to sow diets provides opportunities to improve sow production and reproduction in several aspects. Dietary fiber and gut microbe interaction affects the diversity and abundance of gut microbes, immune system, milk composition, embryonic development, and the production performance of sows. However, there are still many problems that need to be resolved: 1) The source of dietary fiber is rich and the composition is complex, which makes it difficult to determine a reference standard in production; 2) People's understanding of gut microbes mostly stays at the level of phylum and genus, which makes it difficult to isolate the key bacteria related to dietary fiber fermentation; 3) A large number of obligate anaerobic bacteria are present in the milk and passed to the piglets. Do these bacteria come from the gut of the sow? Therefore, although dietary fiber and gut microbes play an important role in improving sow performance, a lot of data are still needed to explain the regulation mechanism in the future.

Author contributions

Min Tian and Shihai Zhang initiated the idea, scope, and outline of this review paper. Min Tian, Shihai Zhang, Jiaming Chen, Jiaxin Liu, and Fang Chen studied and analyzed all of the publications cited in this paper and prepared the initial manuscript. Shihai Zhang and Wutai Guan made the final revision. All authors read and approved the final manuscript.

Conflict of interest

We declare that we have no financial and personal relationships with other people or organizations that can inappropriately influence our work, there is no professional or other personal interest of any nature or kind in any product, service and/or company that could be construed as influencing the content of this paper.

Acknowledgements

This work was supported by National Natural Science Foundation of China (No. 31802067 and 31872364) and the Natural Science Foundation of Guangdong Province (No. 2018A030310201).

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JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

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A Combination of Formic Acid and Monolaurin Attenuates Enterotoxigenic *Escherichia coli* Induced Intestinal Inflammation in Piglets by Inhibiting the NF-κB/MAPK Pathways with Modulation of Gut Microbiota

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Cite This: *J. Agric. Food Chem.* 2020, 68, 4155−4165

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ABSTRACT: This study determined the potential of formic acid plus monolaurin (FA + ML) as an alternative to antibiotics in diet when piglets are challenged with ETEC. Piglets fed the FA + ML diet had lower fecal score and rectal temperature after the ETEC challenge. In addition, FA + ML supplementation induced lower plasma TNF-α, IL-6, and IL-1β concentration postchallenge, downregulated the mRNA expression of TNF-α, IL-1β, IL-6, and TLR4 in the ileum and TLR4 and CFTR in the jejunum. Phosphorylation levels of NF-κB p65 and MAPK p38 were reduced in the ileum of piglets fed FA + ML diet. Supplementation of FA + ML increased the relative abundance of genera *Lactobacillus* especially *Lactobacillus amylovorus* species and decreased the genus abundances of *Actinobacillus*, *unidentified Enterobacteriaceae*, *Moraxella*. Collectively, the combination of formic acid and monolaurin in diets have the potential to be an antibiotic alternative to mitigate inflammatory response in piglets challenged with ETEC.

KEYWORDS: *monolaurin, formic acid, Escherichia coli, piglets*

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INTRODUCTION

Enterotoxigenic *Escherichia coli* (ETEC) is the most common cause of postweaning diarrhea, resulting in high mortality and huge economic loss in swine production.[1] Antibiotics play a significant role in livestock industries as prophylaxis and growth promoters.[2] However, approximately 52% of total antibiotics are consumed by animals.[3] The abuse of antibiotics in the livestock production threatens human health due to the problem of antibiotic resistant bacteria.[4] Therefore, alternatives to antibiotics with less side effects to the environment are desperately needed.

Organic acids have effective antimicrobial properties against Gram-positive and Gram-negative bacteria.[5] Various studies have shown that dietary supplementation of organic acids can increase body weight, improve the feed conversion efficiency, and maintain gut health in piglets.[6−8] Similarly, our recent research also demonstrated that the organic acid supplementation alleviates the ETEC infection in piglets.[9] Monolaurin also functions as an antibacterial component, which naturally is present in coconut oil and palm kernel.[10] Previously, monolaurin has been reported against pathogens like *Clostridium perfringens*,[11] *Listeria monocytogenes*,[12] but not *Escherichia coli* (*E. coli*)[11,13] and *Salmonella sp.*[11] Supplementation of monolaurin increases growth performance and improves meat quality in broilers.[14,15]

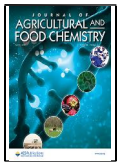
The cumulative antibacterial effect of monolaurin was observed when it is used with organic acids. For *Staphylococcus aureus* challenged mice, higher survival rate was observed when they were treated with the combination of origanum oil and monolaurin (62.5%), compared with individually being treated with either origanum oil (37.5%) or monolaurin (50%).[16] Intriguingly, the combination of monolaurin and organic acids might also have synergistic antibacterial function. It has been reported that 1.0 mM lauric acid or organic acids has negligible antimicrobial effect against *E. coli*, whereas 1.0 mM lauric acid combined with organic acids (acetic acid, lactic acid, malic acid, and citric acid) show a significantly synergistic antibacterial function against *E. coli* (2.52, 5.21, 5.50, and 6.27 log CFU/mL reduction, respectively).[17] All the evidence implies the potential role of monolaurin as prophylaxis with the addition of organic acids against bacteria-induced infection in the animal diet.

In addition, formic acid has been shown to be more effective in antibacterial activity against *E. coli* compared with other organic acids *in vitro*.[18] Thus, the aim of this study was to investigate the effect of the combination of monolaurin and formic acid in diets on protecting weaning pigs challenged with ETEC and its underlying mechanisms.

MATERIALS AND METHODS

Ethics Approval. The animal use and care protocols were approved by the South China Agricultural University Animal Care and Use Committee (no. 20110107-1, Guangzhou, China).

Received: March 1, 2020
Revised: March 21, 2020
Accepted: March 23, 2020
Published: March 23, 2020



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Animals and Experimental Design. Thirty individually housed male piglets (age 25 ± 1 day, Duroc × Landrace × Yorkshire) weighing 7.5 ± 0.5 kg were randomly assigned to one of the five experimental diets: (1) basal diet (CONT); (2) basal diet with antibiotics (50 mg/kg quinocetone, 75 mg/kg chlortetracycline, 50 mg/kg kitasamycin; ATB); (3) basal diet with 0.6% formic acid (85% formic acid and 15% water, BASF, Germany; FA); (4) basal diet with 0.2% monolaurin (purity ≥98%; ML); (5) basal diet with 0.6% formic acid and 0.2% monolaurin (FA + ML). The basal diet was formulated to meet the nutritional requirements for a 6−10 kg pig (NRC, 2012) and its ingredient composition and nutrient levels are presented in Table 1.

Table 1. Compositions of Basal Diet and Nutrient Profile, As-Fed Basal

ingredient composition	content (%)	calculated nutrient profile	content
corn	47.25	digestible energy (MJ/kg)	15.05
wheat powder	7.50	cp (%)	19.20
whey	7.50	crude fat (%)	8.03
sucrose	2.50	crude fiber (%)	1.76
glucose	1.25	ash (%)	3.01
soybean meal	8.50	calcium (%)	0.77
extruded soybean	10.00	phosphorus (%)	0.53
fish meal	7.00	available phosphorus (%)	0.34
soybean oil	2.00	lysine (%)	1.35
yeast extract	2.50	methionine + cysteine (%)	0.74
choline chloride (50%)	0.10	threonine (%)	0.79
calcium hydrophosphate	0.38	tryptophan (%)	0.22
limestone	0.8		
salt	0.30		
L-lysine HCl	0.50		
DL-methionine	0.22		
L-threonine	0.25		
L-tryptophan	0.02		
L-valine	0.1		
vitamin premix[a]	0.035		
mineral premix[b]	0.23		
unite bran	1.065		
total	100.00		

[a]Supplied per kilogram of diet: 11375 IU of vitamin A, 3500 IU of vitamin D_3, 26.3 IU of vitamin E, 3.5 mg of vitamin K_3, 3.5 mg of vitamin B_1, 8.8 mg of riboflavin, 5.4 mg of vitamin B_6, 0.03 mg of vitamin B_{12}, 17.5 mg of pantothenic acid, 35.0 mg of niacin; 1.75 mg of folacin, 0.14 mg of biotin. [b]Supplied per kilogram of diet: 64.4 mg of Cu (cupric glycinate), 165.4 mg of Fe (iron glycine), 47.8 mg of Mn (manganese glycinate), 47.8 mg of Zn (zinc glycinate), 0.54 mg of Se (yeast selenium), 0.68 mg of I (calcium iodate), 0.1 mg of Co (cobaltous sulfate).

Each pen was equipped with a feeder and a nipple drinker to allow pigs to have *ad libitum* access to the feed and water, and diets were offered to pigs as mash. Room temperature was maintained at 30 ± 1 °C.

Bacteria Preparation and Oral Challenge. Enterotoxigenic *Escherichia coli* (CVCC225, serotype O149:K91:K88ac; toxins LT and ST;) was purchased from Chinese Veterinary Medicine Collection Center. The ETEC was cultured overnight in nutrient broth (NB, Huankai, Guangzhou) and diluted to 5×10^9 CFU/mL for challenging piglets. The experimental procedure is listed in SI Figure S1. In brief, the pigs were allowed to adapt to the surroundings for 3 days and then were switched to experimental diets for 7 days. After 7 days feeding with experimental diets, all pigs were challenged with 10 mL nutrient broth containing 5×10^9 CFU/mL ETEC. All pigs were slaughtered by euthanasia with sodium pentobarbital 1 d after ETEC challenge. Body weight and feed intake were recorded in the experiment (SI Table S1).

Rectal Temperature and Fecal Score. The rectal temperature of all pigs was recorded by rectal thermometer (Omron MC-347) at 0, 9, and 24 h postchallenge. Fecal scores were evaluated at 0, 9, and 24 h postchallenge according to the fecal scoring system reported by Marquardt et al. (0-normal, hard form; 1-soft feces, soft form; 2-mild diarrhea, no form, loose, puddles; 3-severe diarrhea, watery) and were measured by someone who had no prior knowledge of the dietary treatment allocation.[19]

Cytokines and Urea Nitrogen in Plasma. At 0, 9, and 24 h postchallenge, blood was sampled via the vena cava puncture using EDTA anticoagulative tube. The plasma samples were obtained by centrifuging the blood at 1000 × *g* for 10 min. Plasma concentrations of IL-1β, IL-6, IL-10, IFN-γ, and TNF-α were measured using porcine-specific multiplex assay (Millipore Multiplex; Billerica, MA, #PCYT-MAG-23K-05) according to Vieira-Potter et al.[20] In addition, the concentrations of insulin-like growth factor-1 (IGF-1) and plasma urea nitrogen (PUN) were measured using commercially available porcine Elisa kits (Huamei, Wuhan) following the manufacturer's instructions.

Tissue Sample and Intestinal Morphololgy. Two centimeter sections of ileum (10 cm proximal to the ileocecal junction) and jejunum (40 cm proximal to the ileocecal junction) were collected according to Xu et al.[21] The slides of jejunal and ileal tissue were made according to the procedure descried by Fan et al.[22] The 2 cm jejunal and ileal tissue samples were flushed with ice-cold PBS to remove intestinal contents and then fixed in 4% buffered formalin solution. After 2 d of fixation, the small intestinal tissues were embedded in paraffin and then were cut into circular slices by Leica slicer with 5−10 μm thick (Leica RM2235, Germany). The slices were stained with hematoxylin and eosin for intestinal morphology. The villous height and crypt depth from five well-oriented crypt-villi units per sample were measured and ratio of villous height/crypt depth was calculated.

Gene Expression of Jejunal and Ileal Mucosas by q-PCR. Jejunal and ileal mucosal samples were collected with glass slides, snap-frozen in liquid nitrogen, and stored at −80 °C for further analysis of mRNA expression. Total RNA was extracted using Trizol reagent (Takala) and RNA quality and purity was confirmed by electrophoresis on 1.0% agarose gel. The concentration of total RNA was determined by Nano-Drop ND-1000 spectrophotometer. Subsequently, 1 μg RNA

Table 2. Primers Used for q-PCR Analysis

gene	forward nucleotide sequence primers (5′-3′)	reverse nucleotide sequence primers (5′-3′)	product size (bp)
TNF-α	CCACCAACGTTTTCCTCACT	TAGTCGGGCAGGTTGATCTC	211
IL-1β	CCAAAGAGGGACATGGAGAA	TTATATCTTGGCGGCCTTTG	160
IL-6	TGGCTACTGCCTTCCCTACC	CAGAGATTTTGCCGAGGATG	132
IL-12	CAACCCTGTGCCTTAGCAGT	AGAGCCTGCATCAGCTCAGT	151
IL-10	GCTGAAGACCCTCAGGCTGA	TTGCTCTTGTTTTCACAGGGC	66
IFN-γ	AATGGGACAAAAGTGGCTTG	CCAGCTGTGTGTTTGCAAGT	201
TLR-4	GCCATCGCTGCTAACATCATC	CTCATACTCAAAGATACACCATCGG	108
CFTR	TTCCTCGTAGTCCTCGCC	GGTCAGTTTCAGTTCCGTTTG	204
ZO-1	AAGATCTGCTTCCGTGGAGA	TTCACTTGGGGTTTTTGAGG	183
Claudin-1	GATTTACTCCTACGCTGGTGAC	CACAAAGATGGCTATTAGTCCC	199
Occludin	GTAGTCGGGTTCGTTTCC	GACCTGATTGCCTAGAGTGT	167

https://dx.doi.org/10.1021/acs.jafc.0c01414
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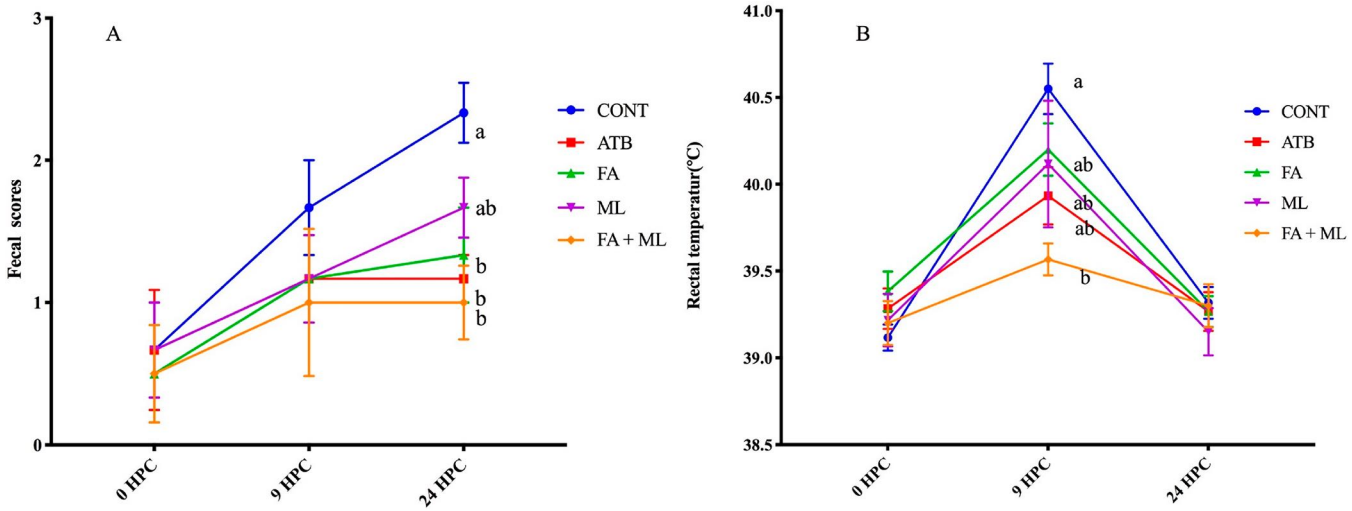

Figure 1. Effect of formic acid and monolaurin on the fecal scores and rectal temperature of piglets challenged with enterotoxigenic *Escherichia coli* K88. The data in each group is expressed as the mean ± SE ($n = 6$). Different letters mean statistically significant difference among the groups ($P < 0.05$).

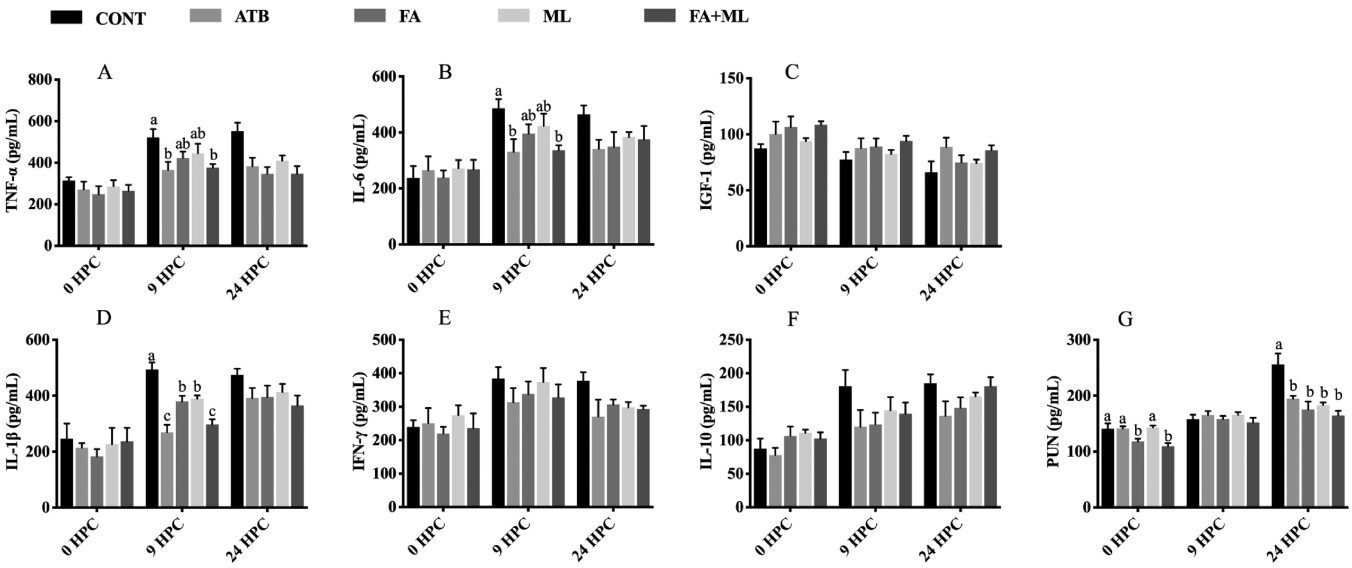

Figure 2. Effect of formic acid and monolaurin on the cytokines in plasma of piglets challenged with enterotoxigenic *Escherichia coli* K88. The data in each group is expressed as the mean ± SE ($n = 6$). Different letters mean statistically significant difference among the groups ($P < 0.05$).

sample was used to synthesize complementary DNA using PrimeScript RT reagent Kit (Takara) according to manufacturer's protocol. The q-PCR analysis was performed using the Power SYBR Green PCR Master Mix and the 7500 Fast Real-Time PCR System (Applied Biosystems and Life Technology, Inc.). Primers were designed using Primer 3.0 (http://www.ncbi.nlm.nih.gov/tools/primer-blast) and are listed in Table 2. The reference gene β-actin was used as the internal control. The q-PCR procedures were 30 s initial temperature at 95 °C, 40 cycles of denaturing at 95 °C (15 s), and annealing at 60 °C. Data was analyzed using $2^{-\Delta\Delta Ct}$ method to determine the relative expression of target genes.

Protein Abundance in the Ileal Mucosa Analyzed by Western Blot. The ileal samples were homogenized and lysed in cold RIPA buffer (Beyotime, China). Total protein was extracted with Protein Extraction Kit (Beyotime, China) and the protein concentration was determined by a bicinchoninic acid assay on BioRad protein microassay procedure (BioRad, Hercules, CA). Equal amounts of protein extracts (30 μg) were diluted and heated for 5 min and then electrophoresed on 10% SDS-PAGE. The protein was transferred to polyvinylidene difluoride membranes and blocked with 5% skim milk solution at room temperature for 2 h and then incubated with rabbit p65 (Proteintech, American), phospho-p65 (CST, American), phospho-p38 (CST, American) polyclonal antibody, Mouse p38 (Proteintech, American), and β-actin (Proteintech, American). After incubation with horseradish peroxide-conjugated secondary antibody and ECL, proteins were visualized by exposing the blot to an X-ray film. The net intensities of the individual bands were performed using Quantity One (version 4.6.2) and protein abundance was normalized to β-actin.

Extraction of Genomic DNA in Ileal Chyme, PCR Amplification, Purification, and Sequencing. Total genomic DNA of five ileal contents from each group was extracted using CTAB/SDS method, and DNA was diluted with sterile water according to the concentration. The distinct V4 regions in 16S rRNA genes were amplified used specific primers 515F (5′-GTGCCAGCMGCCGCGGTAA-3′) and 806R (5′-GGACTACHVGGGTWTCTAAT-3′). Sequencing libraries were generated using Ion Plus fragment library kit (Thermo Fisher Scientific, U.S.A.) according to the manufacturer's protocol. At last, the library was sequenced on an Ion S5 XL platform and 400bp/600bp single-end reads were generated (Novogene, Beijing, China).

Bacterial Data Analysis. Data quality filtering on the raw reads were performed to obtain the high-quality clean reads according to the

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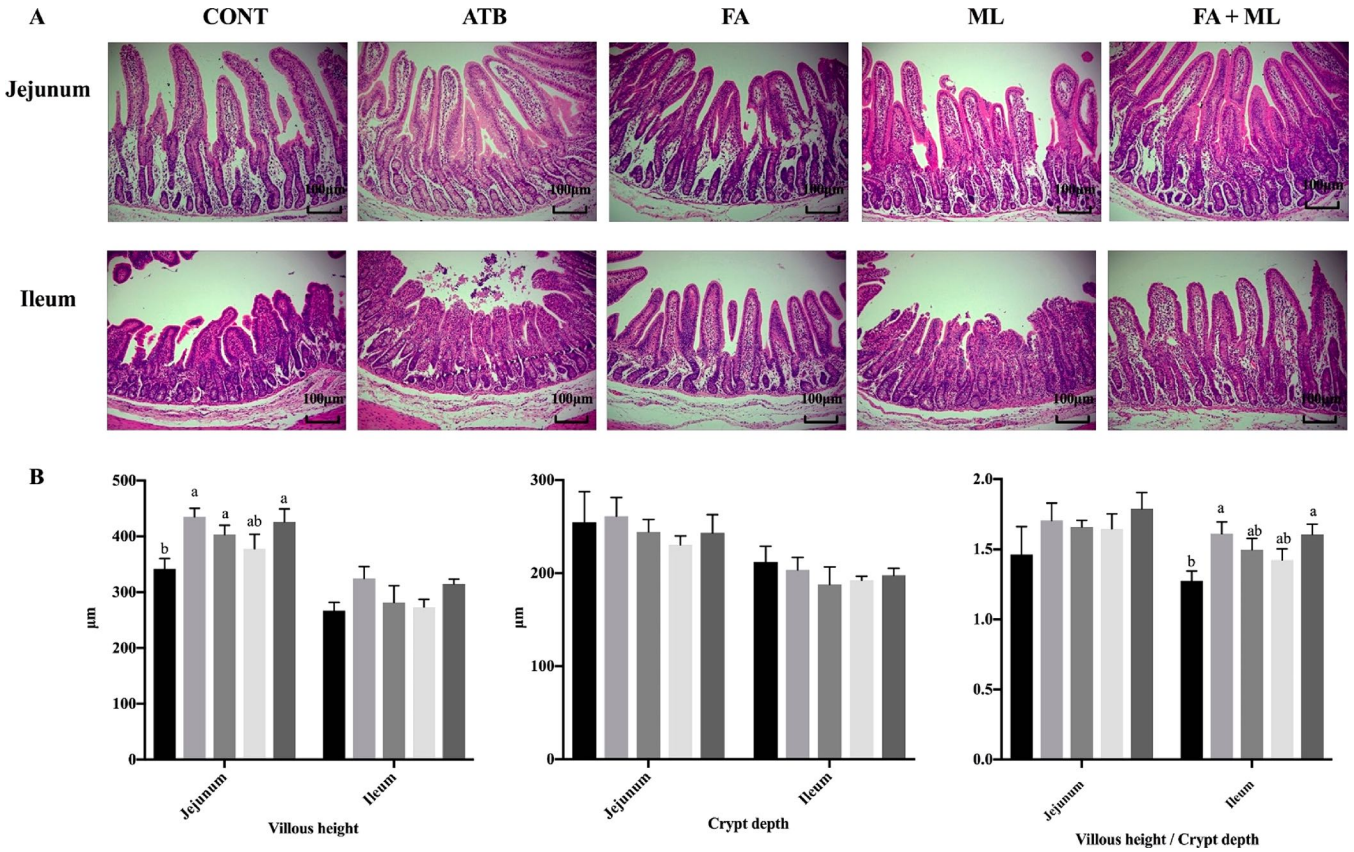

Figure 3. Effect of formic acid and monolaurin on morphology of piglets challenged with enterotoxigenic *Escherichia coli* K88. (A) HE staining. (B) Villous height, crypt depth, and ratio of villous height to crypt depth. The data in each group is expressed as the mean ± SE ($n = 6$). Different letters mean statistically significant difference among the groups ($P < 0.05$).

Cutadapt (Martin., 2011) (version 1.9.1) quality-controlled process. The clean sequences were assigned to the same OTUs with ≥97% similarity by Uparse software (Uparse v7.0.1001). For each representative sequence, the Silva Database (https://www.arb-silva.de/) was used based on Mothur algorithm to annotate taxonomic information. Alpha diversity, including ACE, observed species, and chao1, were calculated to reflect the bacterial diversity with the Mothur program (version 1.31.2). Beta diversity on both weighted and unweighted unifrac was calculated by QIIME software (Version 1.7.0). Principal coordinate analysis (PCoA) based on weighted Unifrac and unweighted Unifrac was performed by R software (Version 2.15.3) to get principal coordinates and visualize complex data. Tax4Fun functional prediction was achieved by the nearest neighbor method based on the minimum 16S rRNA sequence similarity by extracting the KEGG database prokaryotic whole genome 16S rRNA gene sequence and aligning it to the Silva SSU ref NR database using BLASTN algorithm (BLAST Bitscore >1500). The sequenced samples were clustered out of the OTU using the Silva Database sequence as a reference sequence to obtain functional annotation information.

Statistical Analysis. Data were analyzed as completely randomized design using GLM procedures with SPSS (SPSS So ware Release 22, IBM Inc., USA). Alpha and beta diversities were analyzed using QIIME v.1.7.0 (http://qiime.org/1.7.0/) and displayed using R v.2.15.3 software. Group data were analyzed using one-way ANOVA and comparisons between dietary treatments were made when a significance was found using LSD procedure. The individual pig served as the experimental unit. All results are presented in the figures as least-squares mean ± standard error of the means. Statistical significance was considered when $P < 0.05$.

RESULTS

Body Weight, Feed Intake, Rectal Temperature, and Fecal Scores. Body weight and feed intake of piglets were not affected by experimental diets (SI Table S1). There were no significant differences among the five treatments in the fecal scores at 0 and 9 h postchallenge ($P > 0.05$) (Figure 1A). Piglets from ATB, FA, and FA + ML treatments had lower fecal scores at 24 h postchallenge compared with those from the CONT treatment ($P < 0.05$). Compared with the CONT treatment, rectal temperature was significantly lower in the piglets treated with FA + ML at 9 h postchallenge ($P = 0.002$) (Figure 1B).

Inflammatory Cytokines, IGF-1, and PUN in Plasma. In comparison with the CONT treatment, plasma levels of pro-inflammatory cytokines (TNF-α, IL-6, and IL-1β) were significantly lower in ATB treatment at 9 h postchallenge ($P < 0.05$) (Figure 2A,B,D). Similarly, plasma levels of pro-inflammatory cytokines (TNF-α, IL-6, and IL-1β) in the FA + ML treatment were also lower than those in the CONT treatment at 9 h postchallenge ($P < 0.05$). There was a lower PUN concentration in piglets fed FA or FA + ML diets compared with the piglets fed CONT or ATB diets at 0 h postchallenge ($P < 0.05$), whereas a lower PUN concentration was observed in pigs fed FA + ML, FA, ML or ATB diets than the CONT tretament at 24 h postchallenge ($P < 0.05$) (Figure 2G).

Gut Morphology and Tight Junction Protein Genes Expression. In the jejunum, FA and FA + ML treated piglets showed higher villus height compared to the CONT piglets ($P < 0.05$), which did not differ from the ATB treated piglets ($P > 0.05$) (Figure 3B). In the ileum, the ratio of villous height to

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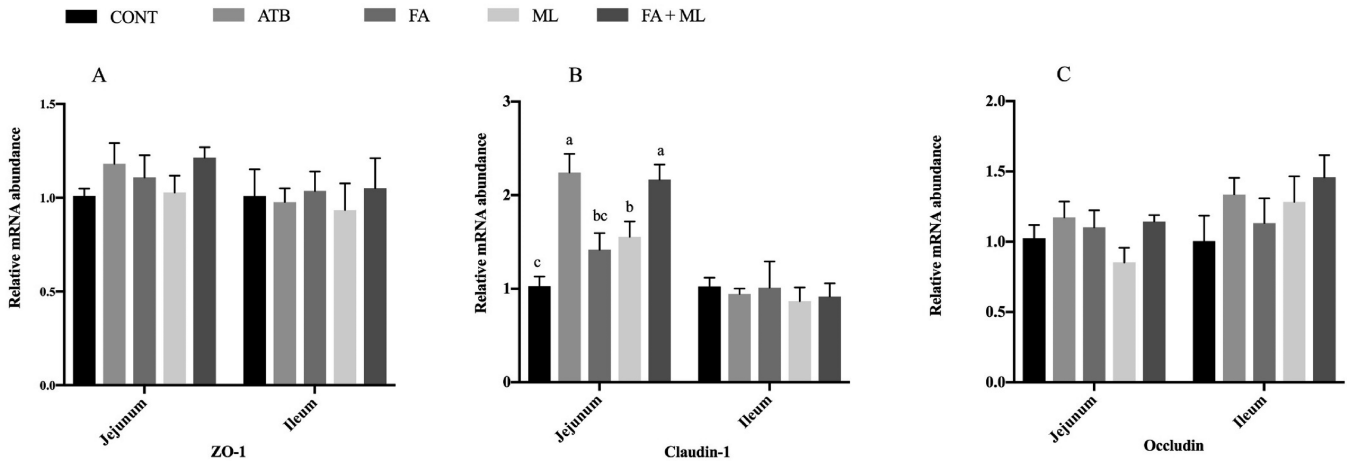

Figure 4. Effect of formic acid and monolaurin on genes expression of intestinal tight junction protein of piglets challenged with enterotoxigenic *Escherichia coli* K88. The data in each group is expressed as the mean ± SE ($n = 6$). Different letters mean statistically significant difference among the groups ($P < 0.05$).

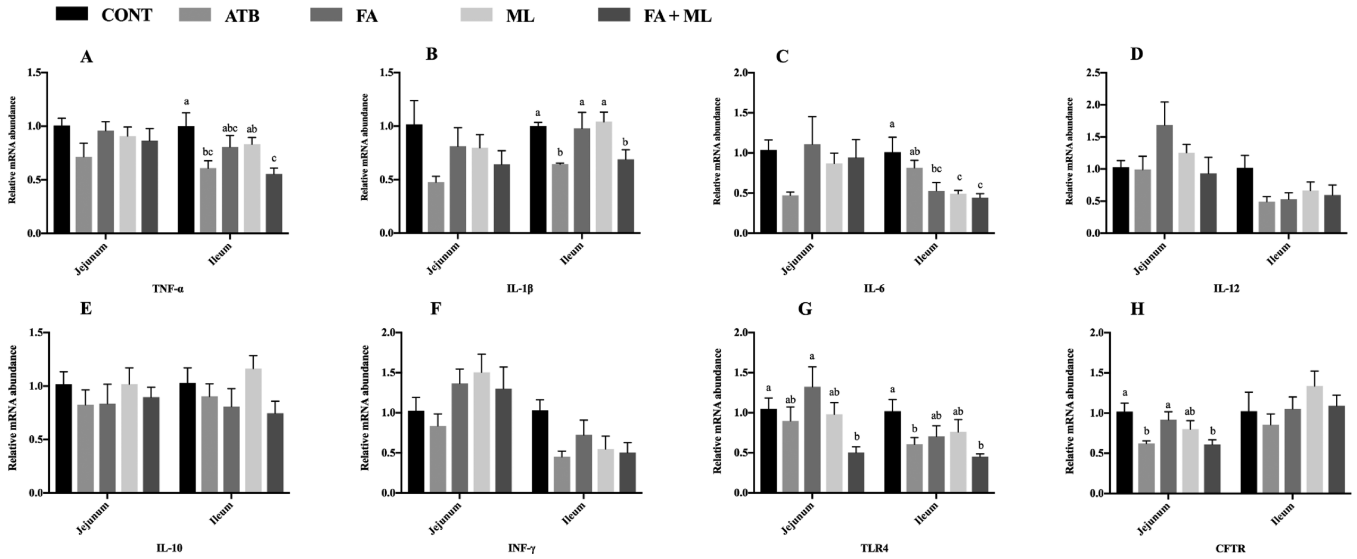

Figure 5. Effect of formic acid and monolaurin on relative genes expression of piglets challenged with enterotoxigenic *Escherichia coli* K88. The data in each group is expressed as the mean ± SE ($n = 6$). Different letters mean statistically significant difference among the groups ($P < 0.05$).

crypt depth in pigs from ATB and FA + ML treatments was greater than that of piglets from the CONT treatment (Figure 3B).

Relative to the CONT treatment, the addition of ML or FA + ML to the diet upregulated claudin-1 gene expression ($P < 0.05$) in the jejunum, while there was no signicant difference in claudin-1 gene expression in the jejunum between FA + ML and ATB treatments ($P = 0.075$) (Figure 4B). There were no significant differences in the expression of ZO-1 and occludin among five groups ($P > 0.05$) (Figure 4A,C).

Expression of Genes Related to Immune Status and Diarrhea. Compared with CONT piglets, the expression of TNF-α, IL-1β, and TLR4 in the ileum and the expression of TLR4 and CFTR in the jejunum was decreased in the piglets fed FA + ML diet compared with that of the piglets fed CONT diet ($P < 0.05$), which was not different from ATB treatment ($P > 0.05$) (Figure 5A,B,G,H). In the ileum, piglets fed diets with FA + ML as well as FA or ML alone downregulated the mRNA expression of IL-6 when compared with CONT group ($P < 0.05$) (Figure 5C).

Abundance of Protein Related to NF-κB/MAPK Signal Pathway. Compared with CONT group, pigs fed FA + ML had a lower ratio of phosphorylated nuclear factor kappa B (NF-κB) p65 to total p65 and ratio of phosphorylated mitogen-activated protein kinase (MAPK) p38 to total p38 in the ileum ($P < 0.05$) (Figure 6). Moreover, phosphorylated levels of p65 and p38 in the FA + ML treatment was lower than those in FA or ML treatment ($P < 0.05$), which did not differ from the ATB treatment ($P > 0.05$).

Microbiota. Data from sequencing can be accessed online on SAR database with accession number PRJNA607169. For all ileal samples, 3321 OTUs were detected at the distance level of 0.03 (97% similarity), and 241, 797, 183, 43, and 104 elements were unique to the CONT, ATB, FA, ML and FA + ML treatments, respectively (Figure 7A). The Alpha diversity of ileal microbiota indicated by ACE, observed species, chao1, is shown in Figure 7B−D. FA + ML treatment tended to decrease ACE compared with FA group ($P = 0.0954$). There were no significant differences in observed species and chao1 among treatments ($P > 0.05$). To measure the degree of microbial

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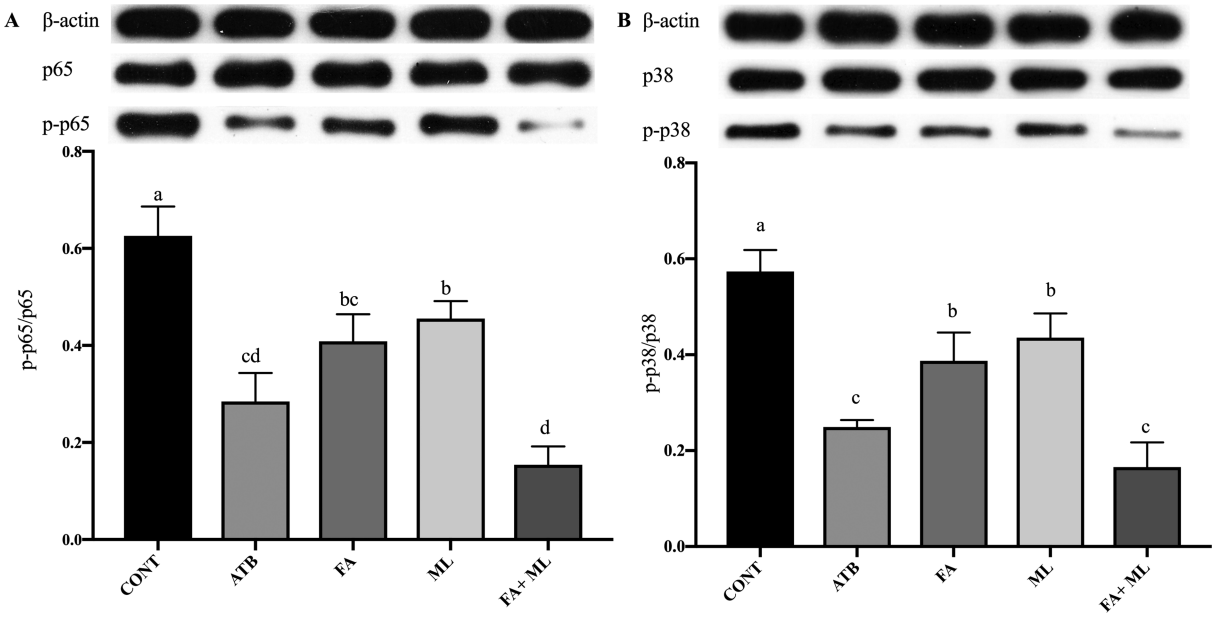

Figure 6. Effect of formic acid and monolaurin on NF-κB/MAPK signaling pathways of piglets challenged with enterotoxigenic *Escherichia coli* K88. (A) Phosphorylation levels of nuclear factor kappa B (NF-κB) p65. (B) Phosphorylation levels of mitogen-activated protein kinase (MAPK) p38. The data in each group is expressed as the mean ± SE ($n = 6$). Different letters mean a statistically significant difference among the groups ($P < 0.05$).

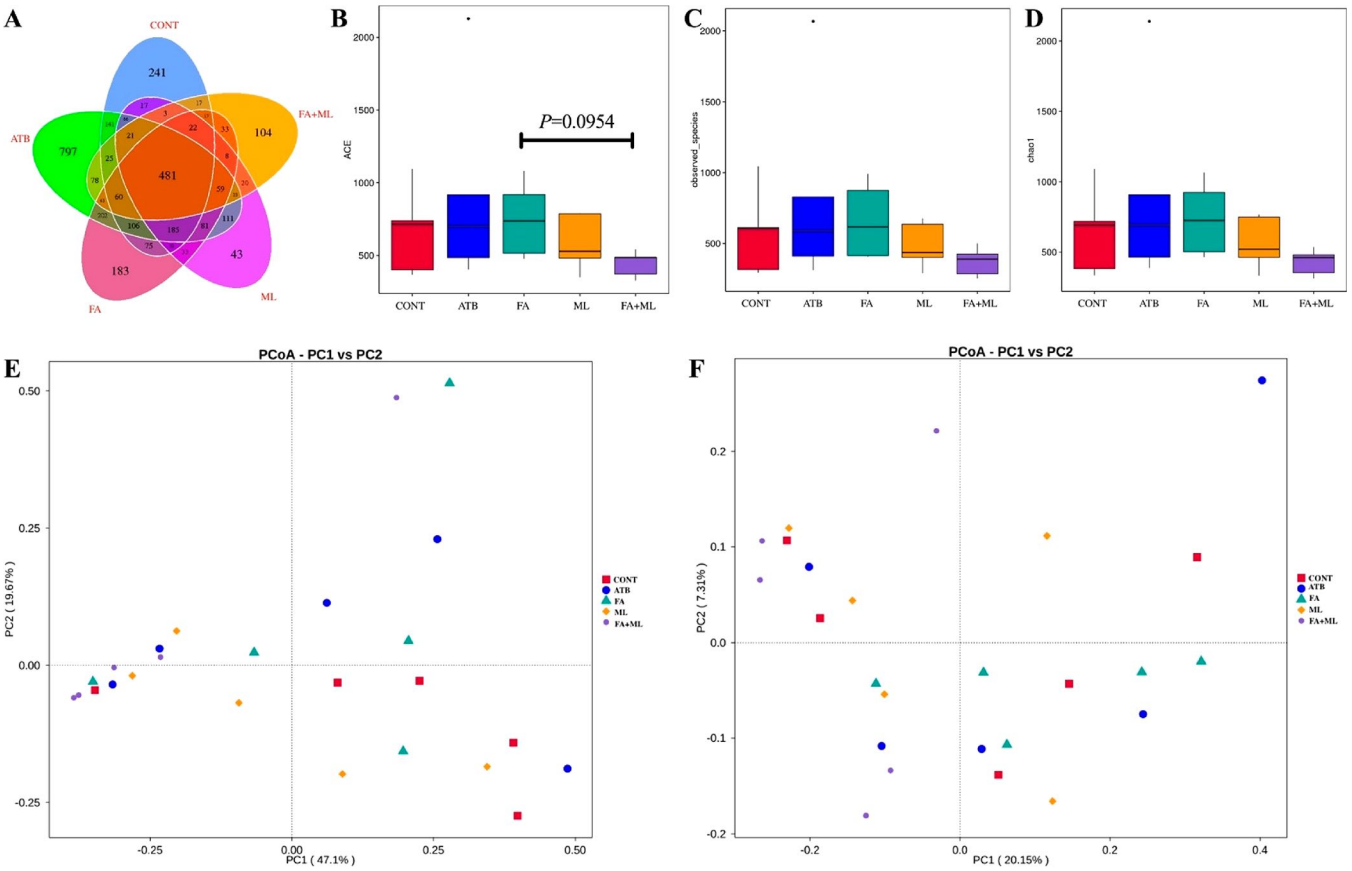

Figure 7. Effect of formic acid and monolaurin on alpha diversity and beta diversity of ileal microbiota of piglets challenged with enterotoxigenic *Escherichia coli* K88 ($n = 5$). (A) Venn graph. (B−D) Alpha Diversity index: ACE, chao1, observed_species. (E) Principal Coordinates Analysis based weighted Unifrac. (F) Principal Coordinates Analysis based unweighted Unifrac.

community similarity among treatment groups, beta diversity metrics were calculated. Results from PCoA based on unweighted UniFrac distance metrics and weighted UniFrac distance are showen in Figure 7E,F, respectively. No significant difference in beta diversity of ileal microbiota among treatment groups were observed ($P > 0.05$).

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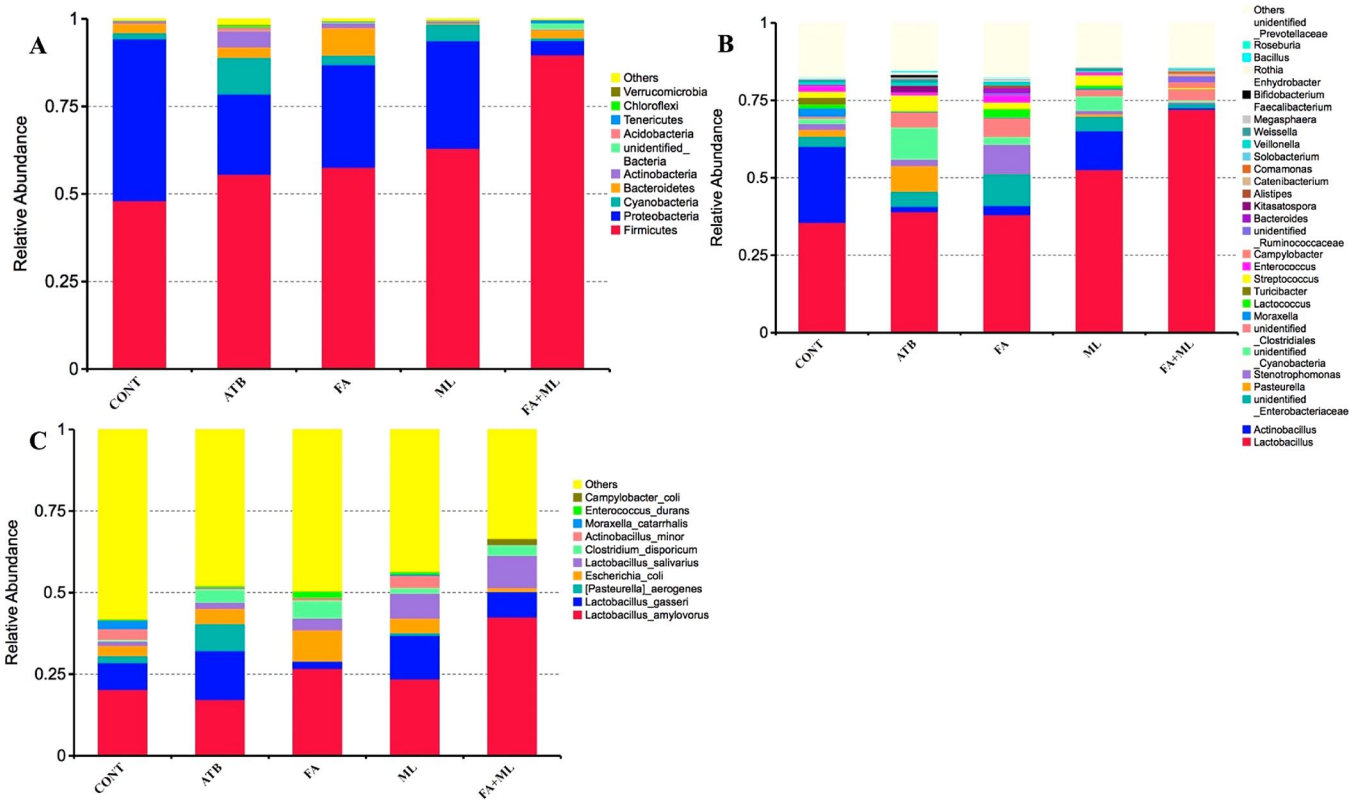

Figure 8. Effect of formic acid and monolaurin on microbial composition of ileal microbiota of piglets challenged with enterotoxigenic *Escherichia coli* K88 ($n = 5$). (A) Relative abundance in genus. (B) Relative abundance in species.

Five major bacteria phyla in the ileal contents of piglets were Firmicutes, Proteobacteria, Cyanobacteria, Bacteroidetes, and Actinobacteria, which accounted for more than 95% of the total ileal bacterial community (Figure 8A). However, their respective proportion varied among different treatments. Compared with the CONT group, the relative abundance of Firmicutes increased from 48.09% to 57.60% and 63.03% with the addition of FA alone and ML alone, respectively, and the abundance of Firmicutes was increased to 89.75% with the addition of FA + ML. Within the changes of Firmicutes, at the genus level the relative abundance of genera *Lactobacillus* was 72.10% and 52.6% in the FA + ML and ML group, respectively, while the CONT group was 35.58% (Figure 8B). A major source of change in the genus level was the species of *Lactobacillus amylovorus, Lactobacillus salivarius*, and *Lactobacillus reuteri* (Figure 8C). The results of genus level showed that FA + ML supplementation decreased genus abundance of *Actinobacillus* (species *Actinobacillus minor*), *unidentified Enterobacteriaceae* (species *Escherichia coli*), *Moraxella* (species *Moraxella catarrhalis*), and *Pasteurella* (species *Pasteurella aerogenes*) (Figure 8B,C).

Changes in the Functional Predictions of Ileal Microbiota. In the present study, the functional predictions of ileal microbiota were analyzed by Tax4Fun. The top five relative microbial abundant pathways at KEGG pathways level 3 in all treatments included transporters, DNA repair and recombination proteins, two component system, tRNA biogenesis, and purine metabolism (Figure 9A). The distribution of the top 35 functional predictions of ileal microbiota is shown in Figure 9B. Among the top 10 abundant pathways, the relative abundance of 2 component system, DNA repair and recombination proteins, purine metabolism, pyrimidine metabolismm and amino acid related enzymes was increased, and the relative abundance of transporters was decreased in the FA + ML treatment compared to CONT ($P < 0.05$).

DISCUSSION

Enterotoxigenic *Escherichia coli* can secret enterotoxins, which induces diarrhea in piglets.[23] The present study showed that the incidence of diarrhea in piglets was increased after challenge in the control group, which showed mild diarrhea. The pigets fed a diet with a combination of formic acid and monolaurin appeared to have a lower fecal score, which was similar to ATB treatment. Heat labile enterotoxin (LT) increased production of intracellular cyclic adenosine monophosphate and heat stable enterotoxin (ST) induced uncontrolled production of cyclic guanosine monophosphate, resulting in activation of the cystic fibrosis transmembrane conductance regulator (CFTR) and ultimately causing chloride and water secretion.[24] In this study, there was decreased mRNA expression of CFTR in the jejunum of piglets with a combination of formic acid and monolaurin, the effect of which did not occur in piglets with formic acid or monolaurin alone. The results suggest that there is a synginestic effect of formic acid and monolaurin on CFTR mRNA expression of ETEC challenge piglets. The reduction of fecal scores may be due in part to the bactericidal effect of formic acid and monolaurin. In addition, the inhibition of CFTR may attribute to the reduced virulence activity of ETEC by monolaurin and organic acid; as stated in previous studies, subminimal inhibitory concentration of short chain fatty acids suppressed the gene expression of virulence factors in pathogens.[25−27]

The lipopolysaccharide of ETEC can trigger the inflammatory response by recognition of Toll-like receptor 4 (TLR4) and

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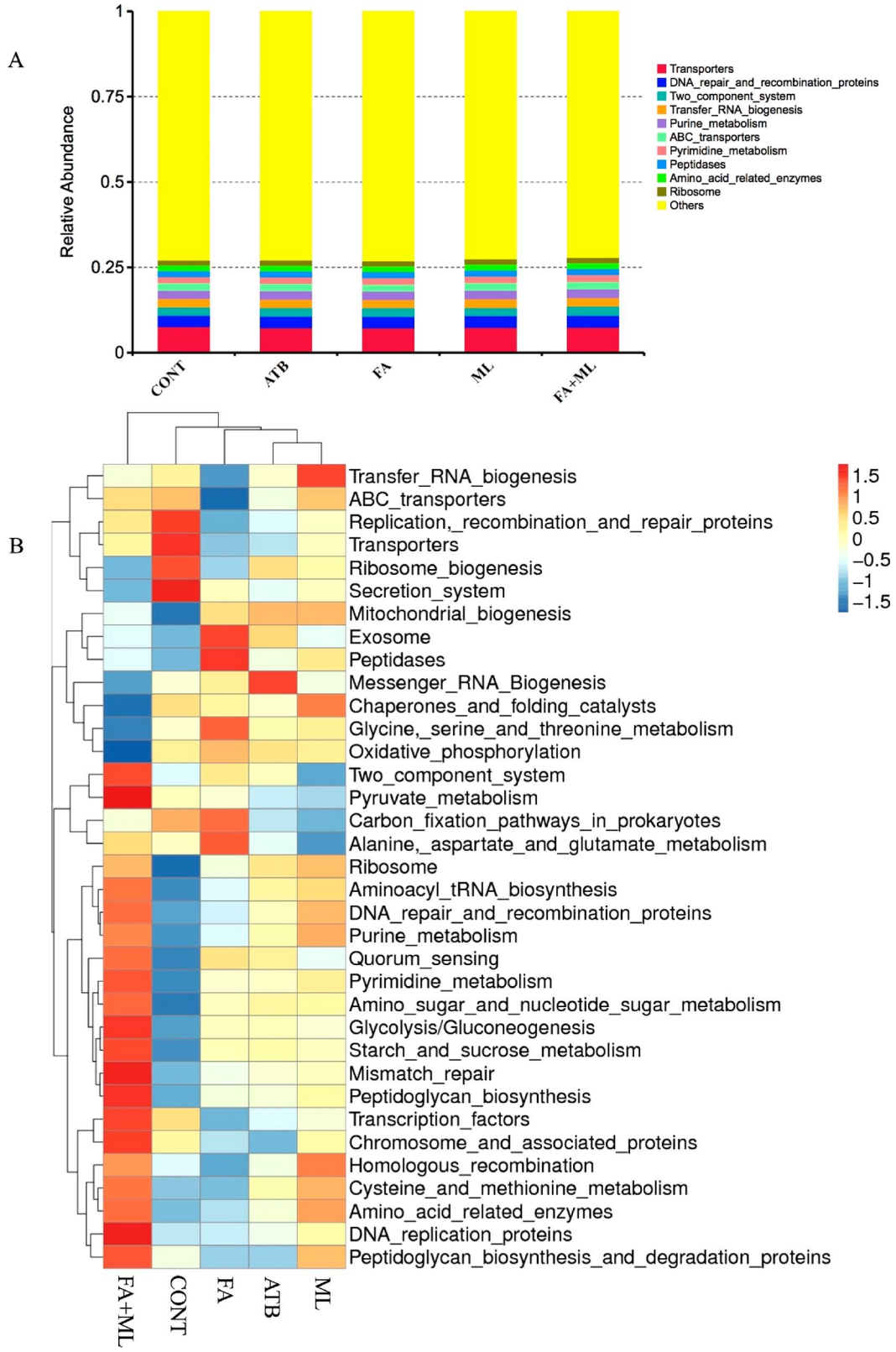

Figure 9. Effect of formic acid and monolaurin on changes in the metabolic functions of ileal microbiota of piglets challenged with enterotoxigenic *Escherichia coli* K88 ($n = 5$). (A) The top ten relative microbial abundant pathways at KEGG pathways level 3. (B) The distribution of the top 35 relative microbial abundant pathways at KEGG pathways level 3.

elicitation of TLR4 leading to excessive proinflammatory cytokines secretion via activation of nuclear factor-kappa B (NF-κB) signaling pathway.[28] Under stimulated conditions, NF-κB proteins regulate the expression of genes involved in innate immunity, meanwhile, the immunes cytokines can produce heat which lead to increase rectal temperature.[29] After the ETEC challenge, rectal temperature increased rapidly in response to intestinal infection.[30] In the present study, pigs challenged with

https://dx.doi.org/10.1021/acs.jafc.0c01414
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ETEC had increased rectal temperature in the control group and lower rectal temperature was observed in the piglets fed a diet with a combination of formic acid and monolaurin, but not by formic acid or monolaurin alone, indicating the additive effect of formic acid and monolaurin was used to decrease the rectal temperature of piglets. Furthermore, the results from plasma proinflammatory cytokines also substantiated that formic acid and monolaurin showed anti-inflammatory activity in the intestinal bowel of ETEC challenged piglets. There was clear evidence in the literature that formic acid reduced the pH value of the gastrointestinal tract and compensated for the deficiency of gastric acid secretion in weaning pigs to activate trypsin and ultimately promote protein deposition.[31] Consistently, in the present study the nitrogen retention was enhanced with decreased plasma urea nitrogen (PUN) in formic acid treated piglets after 7 days of dietary treatment. However, at 24 h postchallenge, PUN concentration in control piglets was remarkably increased, which was probably related to synthesizing the regulators involved in innate immunes.[32] Although formic acid and monolaurin supplementation improved the nitrogen retention, the concentration of growth hormone IGF-1 was not affected with the addition of formic acid and monolaurin.

In order to investigate the anti-inflammatory mechanism of formic acid and monolaurin in the intestinal bowel of ETEC challenged piglets, TLR4 (as critical sensors to LPS) as well as NF-κB and MAPK signaling pathways were examined. The LPS of ETEC activates TLR4 through a series of interactions with binding protein CD14 and MD-2.[33] Here, we observed a dietary combination of formic acid and monolaurin significantly down-regulated the expression of TLR4. Upon activation by LPS, TLR recruits its downstream adaptors through Toll-interleukin-1 receptor domains and then initiates the MyD88 (myeloid differentiation factor 88) dependent pathway and the MyD88 independent pathway to activate NF-κB and MAPK.[34] Consistently, a combination of formic acid and monolaurin decreased the phosphorylation levels of both NF-κB and MAPK in the intestinal tract, indicating the preventive effect of a combination of formic acid and monolaurin on ETEC-stimulated intestinal inflammation was through down-regulation of NF-κB and MAPK signal pathways. The translocation of the transcription factor NF-κB and activation of the downstream MAPK pathways control the expression of proinflammatory cytokines.[35] Therefore, the expression of pro-inflammatory cytokines in intestinal mucosa were measured. The results demonstrated that the levels of pro-inflammatory cytokines were decreased in piglets treated with a combination of formic acid and monolaurin. Collectively, the results in the present study indicated that supplementation of a combination of formic acid and monolaurin mediated intestinal inflammation genes expression through inhibiting the activation of NF-κB and MAPK signaling pathways by decreasing the activation of TLR4. Many cytokines are involved in the intestinal structure and function defects.[36] Hence, we hypothesized that dietary combination of formic acid and monolaurin could alleviate the intestinal damage induced by ETEC via reduction of pro-inflammatory cytokines. As expected, the combination of formic acid and monolaurin improved the intestinal morphology and increased claudin-1 gene expression in this study.

As strong interactions between a combination of organic acid and monolaurin with bacteria *in vitro*[12] and the correlation of microbiome change with intestinal inflammation,[37] the microbiota in the ileal content was determined in this study. As reflected by the alpha diversity and beta diversity, the combination of formic acid and monolaurin did not notably alter the structure of ileal microflora. Dietary combination of formic acid and monolaurin remarkably enriched the relative abundance of Firmicutes. Furthermore, the change of Firmicutes' relative abundance mainly came from *Lactobacillus* at the genus level, especially for the species of *Lactobacillus amylovorus* and *Lactobacillus salivarius*, which showed an increase in relative abundance with addition of formic acid and monolaurin. *Lactobacillus amylovorus* has been reported to inhibit the TLR4 inflammatory signaling triggered by ETEC[38] and *Lactobacillus salivarius* is beneficial for the maturation of the intestinal mucosal immune system and elicites local immunomodulatory activities.[39] Moreover, a combination of formic acid and monolaurin decreased the relative abundance of Proteobacteria mainly via reduction of relative abundance of *Actinobacillus* at the genus level and species of *Escherichia coli*. These bacteria have been identified as pathogens that can induce inflammatory response in piglets.[40−42] The abundance of pathways of the two component system, DNA repair and recombination proteins, and purine metabolism were upregulated and the pathway of transporters was downregulated with addition of formic acid and monolaurin. The results indicate that the combination of formic acid and monolaurin regulates metabolism and nutrient uptake of bacteria in the gut. Collectively, supplementation of the combination of formic acid and monolaurin enriched the abundance of beneficial intestinal bacteria and decreased the abundance of pathogenic bacteria, which enhanced the intestinal mucosal immune system and reduced the incidence of inflammation.

In summary, dietary supplementation of formic acid and monolaurin altered the microbiota in the ileum and alleviated the inflammatory response via the inhibition of NF-κB/MAPK signaling pathways in the ETEC challenged piglets. Meanwhile, ETEC-induced diarrhea and febrile were alleviated with the addition of the combination of formic acid and monolaurin, which exerted similar effects as antibiotics. This evidence suggests that the combination of formic acid and monolaurin is a potential alternative to antibiotics in pig production.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.jafc.0c01414.

Table S1. The body weight and intake of piglets during experiment (kg). Figure S1. The experimental procedure (PDF)

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Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

This research was funded by the National Key R&D Program of China (No. 2018YFD0500600).

■ ABBREVIATIONS USED

ATB, antibiotic; CFTR, cystic fibrosis transmembrane regulator; *E. coli*, *Escherichia coli*; ETEC, enterotoxigenic *Escherichia coli*; FA, formic acid; IFN-γ, interferon-γ; IGF-1, insulin-like growth factor-1; IL-10, interleukin-10; IL-1β, interleukin-1β; IL-6, interleukin-6; LPS, lipopolysaccharide; LT, labile enterotoxin; MAPK, mitogen-activated protein kinase; ML, monolaurin; MyD88, myeloid differentiation factor 88; NF-κB, nuclear factor kappa B; Principal coordinate analysis, (PCoA); PUN, plasma urea nitrogen; ST, stable enterotoxin; TLR4, toll like receptor 4; TNF-α, tumor necrosis factor-α.

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https://dx.doi.org/10.1021/acs.jafc.0c01414
J. Agric. Food Chem. 2020, 68, 4155−4165

frontiers in Immunology

REVIEW
published: 21 January 2022
doi: 10.3389/fimmu.2021.758525

Maternal Nutrition During Late Gestation and Lactation: Association With Immunity and the Inflammatory Response in the Offspring

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Reviewed by:
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Specialty section:
This article was submitted to Nutritional Immunology, a section of the journal Frontiers in Immunology

Received: 14 August 2021
Accepted: 20 December 2021
Published: 21 January 2022

Citation:
Li Q, Yang S, Zhang X, Liu X, Wu Z, Qi Y, Guan W, Ren M and Zhang S (2022) Maternal Nutrition During Late Gestation and Lactation: Association With Immunity and the Inflammatory Response in the Offspring. Front. Immunol. 12:758525. doi: 10.3389/fimmu.2021.758525

The immature immune system at birth and environmental stress increase the risk of infection in nursing pigs. Severe infection subsequently induces intestinal and respiratory diseases and even cause death of pigs. The nutritional and physiological conditions of sows directly affect the growth, development and disease resistance of the fetus and newborn. Many studies have shown that providing sows with nutrients such as functional oligosaccharides, oils, antioxidants, and trace elements could regulate immunity and the inflammatory response of piglets. Here, we reviewed the positive effects of certain nutrients on milk quality, immunoglobulin inflammatory response, oxidative stress, and intestinal microflora of sows, and further discuss the effects of these nutrients on immunity and the inflammatory response in the offspring.

Keywords: maternal nutrition, neonate, growth, disease resistance, inflammatory, immunoglobulin

INTRODUCTION

During gestation and lactation, maternal nutrition is a predominant factor to regulate the growth and immunity of piglets (1, 2). Since neonates are born without brown fat reserves, timely intake of colostrum is the guarantee of energy supply for piglets. In addition, colostrum also provides bioactive molecules such as immunoglobulins and inflammatory factors to piglets (3). Even though maternal immunoglobulins cannot cross the placental barrier (4), these immunoglobulins could transfer to piglets through colostrum and milk (5). Maternal diets regulate the composition of colostrum and milk, which further affect the maturation of immune system in neonates (6). Furthermore, maternal milk-derived cytokines also regulate the immunity of neonates (6). It is worth noting that maternal intestinal microflora play a crucial role in regulation of immune development and response during the neonatal period (7). Transferring the intestinal flora of sows during pregnancy into sterile mice improved the intestinal innate immunity and reduced the inflammatory response in their offspring (8). Interestingly, newborn intestinal bacteria is derived from maternal microbiota during delivery and lactation (9). Thus, the regulation of maternal

intestinal microflora by nutrients indirectly affect the offspring immunity and inflammatory response.

Maternal infection or inflammatory exposure during pregnancy impairs the innate response of newborns and increases their susceptibility to infection (10). During pregnancy, sows undergo dramatic changes of physiological metabolism and immunity (11), with markedly increased oxidative stress and inflammatory response (12). Imbalanced inflammatory response are closely related to reproductive disorders, including constipation, abortion and intrauterine growth retardation (9). In addition, inflammatory factors could transfer from maternal to fetus and regulate immunity and inflammatory response. Thus, modification of dietary components of sows during pregnancy might affect neonate intestinal development, immunity, and inflammation. In this review, we summarized the recently published data regarding prebiotic and nutrient supplementation to sow diets during late gestation (mainly during G85-G114) and lactation on maternal milk quality, inflammatory response, oxidative stress and then discuss their effect on the inflammatory response and immunity in the offspring.

SOLUBLE DIETARY FIBER

As indigestible carbohydrate, dietary fiber (DF) is partially or completely fermented by microorganisms in the large intestine, which could be categorized into insoluble and soluble fiber (13). Insoluble fiber speeds up the intestinal circulation, reduces constipation and increases the intestinal volume (14). While soluble fiber is fermented to produce numerous functional metabolites, such as short-chain fatty acids (SCFAs), which could be transmitted from maternal to offspring. Among them, acetate regulates intestinal permeability and anti-inflammatory effect (15). Butyrate improves intestinal morphology, promotes beneficial bacterial growth, and enhances immune defense (16, 17). It has also been shown that maternal DF supplementation could promote T cell differentiation and reduce the inflammatory response in the offspring by regulating the intestinal microbial composition (18). In this review, we focused on the effects of several representative DF supplementation in sow diets (**Table 1** and **Figure 1**).

Isomaltooligosaccharide (IMO) has been reported to activate the immune system (27) and promotes the proliferative potential of beneficial bacteria (particularly *Bifidobacterium*) of sows (28). A recent study reported that feeding sow IMO during late pregnancy (G85-G110) could promote milk GH, IgA and IgG concentrations, increase litter average daily gain (ADG) of piglets, and reduce backfat loss in sow during lactation (19). Similarly, another study showed that IMO given to sows during late pregnancy increased the concentration of IgA, IgG and IgM in colostrum and reduced the diarrhea rate of piglets (29).

Chitosan oligosaccharide (COS) has good water solubility and performs antioxidant (30), anti-inflammatory (31), and immunity-enhancing functions (32). During gestation and lactation (G85-L21), sows given to COS (100 mg/kg) have higher milk production as well as IgM and lactose concentration in colostrum. In addition, COS (100 mg/kg) increased total number of piglets born and weaning weight per litter (20). Importantly, feeding sows with 30 mg/kg or 100 mg/kg COS both increase the serum IgG concentration of piglets, which indicates the enhancement of immune function in neonates (26, 33).

Sugar peat pulp (SBP) contains large amounts of soluble fibers such as pectin and dextran (34). Feeding SBP could increase the feed intake of sows during lactation by improving insulin sensitivity, which is beneficial to the serum GH and IGF-1 levels and growth of piglets (35). SBP supplementation (20% during gestation and 10% during lactation) reduced pro-inflammatory cytokines (IL-6 and TNF-α) in serum of sow. Consistently, pro-inflammatory cytokines (IL-6 and TNF-α) in colostrum, milk and piglet serum are also decreased. Moreover, SBP supplementation in sow diet increase intestinal SCFA and colostral IgA levels, which might be beneficial for reducing inflammatory response in piglets (21).

Seaweed extracts (SWEs) mainly consists of seaweed polysaccharide (SDP), laminarin, and fucoidan (36). Supplementation with SWEs from late gestation to weaning increased colostrum IgG and IgA concentrations. Correspondingly, higher serum IgG concentrations were observed in piglets, which indicates the increased immune function (23). Sudden weaning of piglets is often accompanied by adverse morphological changes in the structure of the small intestine, including villous atrophy and crypt hyperplasia (37). Recent studies have shown the addition of seaweed-derived polysaccharides (10 g/d) to sow feed significantly increased the VH and ratio of villi/crypt (VH:CD) of weaned piglets. In addition, maternal SWE supplementation increases anti-inflammatory (TGF-β1) and inhibits pro-inflammatory factors (IL-6 and IL-8) in the ileum and colon of piglets. Accordingly, the diarrhea score of the piglets during lactation was decreased (22). Furthermore, SWEs diet reduced the number of *Enterobacteriaceae* in sow feces at delivery and the number of *Escherichia coli* in piglet feces at weaning (38). These benefits might be attributed to laminarin could agglutinate certain pathogens and inhibit their adhesion to mucosal epithelial surfaces (39).

Guar gum is a kind of galactomannan extracted from guar endosperm. It has high viscosity and water solubility, which is widely used as a stabilizer and thickener in foods (40). Feeding 2.0% guar gum diet to sow during the gestation and lactation period (G85-L21) could improve the intestinal barrier function, accelerate the growth and reduce the diarrhea rate of piglets. In addition, guar gum increases the abundance of *Lactobacilli* and decrease the abundance of *Bilophila spp* in intestine. Importantly, IL-10 and TGF-β levels were increased in piglets, which avoids over-activated immune system in piglets (24).

Mannan oligosaccharide (MOS), derived from the cell wall of Saccharomyces cerevisiae, has been used as a prebiotic for a long time (41). Recent supplementation of MOS in sow diets has been reported to regulate immunity and the inflammatory response in the offspring. Compared with the control treatment, MOS treatment (400 mg/kg) shortened the weaning estrous of the sows and increased the weaning weight of the piglets. Besides, sows fed MOS increased IgA, IgG, IgM in colostrum, and serum IgA and IgG levels in suckling piglets (25). Additionally, another study shows that the addition of MOS (400 mg/kg) to sow diet could significantly

TABLE 1 | Maternal microbial and soluble dietary fiber intake in the regulation of neonatal infection, immunity and production performance.

Breed, feeding time and products	Reproductive and lactation performance	Immune and oxidative stability of sows and piglets	Intestinal health and inflammation	Others	References
Breed: Large White × Landrace **period:** G85-G110 **Product:** isomaltooligosaccharide 5.0 g/ kg IMO 0.2 g /kg B. subtilis 0.2 g/ kg B. licheniformis	**Reproductive performance** ↑weaning BW (45.63-55.18 kg) Average litter gain (28.43-35.87 kg) **Milk** ↑Total milk yield (113.73-143.46 kg) IgM (1 794.18-1 894.73 g/ mL) on L0 IgA (607.50-922.07 g /mL) on L17	**N/A**	**N/A**	**Sow plasma (L17)** ↓ALT (37.23-35.49 U/ L) ALP (40.23-31.82 U /L)	(19)
Breed: Yorkshire **period:** G85-L21 **Product:**chitosan oligosaccharide (100 mg/kg COS)	**Reproductive performance** ↑daily BW gain: (1.90-2.21 kg) piglet weaning weight: (53.63-60.04 kg) **Colostrum (L1)** ↑Solids-not-fat: (128.07 -153.33 g/kg) IgM: (3.27-4.76 g/L) **Milk (L21)** ↑Lactose: (44.12 -56.10 g/kg) Solids-not-fat: (85.44-101.82 g/kg)	**Sow serum (L1)** ↑CAT: (14.25-20.49 U/mL) T-AOC: (5.93 -8.79 U/mL) IL-10: (50.57-67.73 pg/mL) IgA: (71.31-91.48 μg/mL) IgM: (92.53-117.86 μg/mL) ↓MDA: (16.97-11.90 nm/mL)	**N/A**	**N/A**	(20)
Breed: Yorkshire × Landrace **Period:** G85-L21 (weaning) **Product:** Sugar beet pulp (SBP) 20% SBP in gestation and 10% SBP in lactation	**Piglet at weaning (L21)** ↑Litter weight: (56.94-64.39 kg) Weaning weight: ((5.74-6.26 kg) ADG: (196-221 g/d) **Colostrum (L1)** ↑IgA: (7.94-9.17 g/L)	**Piglet serum (L21)** ↓DAO: (5.68-3.60 U/L) Endotoxin: (0.60-0.47 EU/ml) IL-6: (178.49-154.30 pg/mL) TNF-α: (102.45-80.28 pg/mL) ↑IL-10: (4.55-5.13 pg/mL) **Piglet ileum (L21)** ↓TNF-α: (1-0.6) IL-6: (1-0.6) ↑IL-10: (1-1.3) SIgA: (0.8-1.5 μg/mg)	**Piglets Ileal Tight Junction (L21) mRNA expression** ↑Occluding: (1-1.3) ZO-1: (1-1.4) **Piglet Jejunum** ↑Villus height: (387-447 μm) **Intestinal microbiota (Piglet)** the relative abundance of Christensenellaceae was increased significantly	↑Sow ADFI: (4.80-5.48 kg/d) **Piglet serum (L21)** ↑GH: (3.37-4.23) IGF-1(156.09-187.86)	(21)
Breed: Yorkshire × Landrace **Period:** G83-L28 (weaning) **Product:** seaweed-derived polysaccharides (10·0 g SDP/d)	↑gestation period: (113.5-114.5 d)	**Piglet ileum (weaning) gene expression** ↓PEPT1: (1.71-0.43) GLUT1: (0.98-0.65) GLUT2: (1.76-0.41) ↑IL-1: (0.99-2.85) IL-12A (p35): (0.97-1.93) TNF-α: (0.93-1.66) ↓IL-10: (0.82-0.29) IL-6: (1.12-0.64) IL-8: (1.15-0.77)	**Log GCN/g of sow faece** ↓Enterobacteriaceae: (8.55-7.76) parturition **Piglet weaning** ↑villus height: (347-466 μm) ileum villus height: (317-454 μm) jejunum ↓crypt depth: (144-108) ileum	Piglets had a lower diarrhoea score during the lactation period	(22)
Breed: Large White x Landrace **Period:** G107-L26 **Product:** seaweed extract (10 g/d)	**Colostrum** ↑IgA: (8.02-11.61 mg/mL)	**Piglet serum (L14)** ↑IgG: (8.59-11.36 mg/ml)	**piglet intestinal microbiology (weaning)** ↓colonie E.col: (6.45-5.11 Log cfu/g) ↑lactobacilli: E.coli: (1.21-1.45 Log cfu/g)	**log cfu/g of sow feces (farrowing)** ↓En-terobacteriacea: (8.60-7.26)	(23)
Breed: Landrace sows **Period:** G85-L21 (weaning) **Product:** 2.0% pregelatinized waxy maize starch plus guar gum (SF)	**Piglet at weaning (L21)** ↑Final BW: (6.49-7.09 kg) ADG: (233.66-261.20 g/day)	**Piglet serum (L14)** ↓IL-6: (310-290 pg/ml) ↑TGF-β: (650-750 pg/ml) IL-10: (110-140 pg/ml)	**Piglet plasma (L14)** ↓Zonulin: (700-550 ng/ml) Endotoxin: (0.7-0.5 Eu/ml) Diamine oxidase: (10-9 U/L) **↓lipocalin-2 (80-58μg/g feces)** **Intestinal microbiota (Piglet)** strong increase in relative abundance of the Lactobacillus genus	**Piglet Diarrhea rate:** (13.69-10.35%) **plasma hormone of piglets (L14)** ↑GH: (587.65-657.49 pg/ ml) IGF-1: (309.04-374.63 ng/ml)	(24)

(Continued)

TABLE 1 | Continued

Breed, feeding time and products	Reproductive and lactation performance	Immune and oxidative stability of sows and piglets	Intestinal health and inflammation	Others	References
Breed: Large White × Yorkshire **Period:** Sow: G86-L20 Piglet: D7-D35 **Product** Mannan oligosaccharide Sow: 400 mg/kg Piglet: 800 mg/kg	**N/A**	**Piglet serum (D35)** ↓IL-2: (146.58-107.83 ng/L) IL-4: (18.21-12.09 ng/L) IFN-γ: (535.58-448.88 ng/L) ↑IL-10: (65.82-76.04 ng/L)	**Intestinal microbiota (Piglet on D35)** log10 counts of Lactobacillus, E. coli ↓E. coli: (6.83-6.43 Jejunum) ↑Lactobacillus:(7.63-8.44 in Jejunum) (7.82-8.76 in Cecum) **immunoglobulin A in piglet jejunum** ↑sIgA: (4.48-6.77 mg/g pro)	**N/A**	(25)
Breed: Landrace×Yorkshire **Period:** G86-L21 **Product:** chitosan oligosacchari (30 mg/kg)	**Colostrum (L1)** IgM: (0.95-1.3 g/L) **Umbilical cord blood** IgM: (38.36-43.26 g/L)	**Piglet serum (D21)** ↑IL-10: (57.04-65.29 ng/L) IgG: (163.81-192.29 mg/L) C3: (211.35-254.35 mg/L)	**N/A**	**N/A**	(26)

↑, increase; ↓, decrease; N/A, No Value; BW, body weight; IgA, Immunoglobulin A; IgG, Immunoglobulin G; IgM, Immunoglobulin M; T- AOC, Total antioxidant capacity; CAT, Catalase; MDA, Malondialdehyde; IL-10, interleukin 10; IL-6, interleukin 6; IL-8, interleukin 8; IL-4, interleukin 4; IL-2, interleukin 2; TNF-α, tumor necrosis factor-α; GH, growth hormone; IGF-1, insulin like growth factor 1; ZO-1, zonula occludens-1; ALT, cereal third transaminase; ALP, alkaline phosphatase; ADG, average daily gain; PEPT1, peptide-transporters 1; GLUT1, glucose transporter-1; GLUT2, glucose transporter-2; TGF-β, transforming growth factor β; IFN-γ, interferon-γ; C3, complement 3; sIgA, secretedimmunoglobulin A.

increase the sIgA content in jejunum mucosa and reduce the intestinal inflammatory response of piglets by inhibiting the TLR2/TLR4/NF-κB p65 pathway. Furthermore, MOS supplementation in sow diet increased the number of *Lactobacilli* and decreased the number of *Escherichia* coli in the jejunum of piglets, which is beneficial for reducing diarrhea (42).

Besides soluble fiber, insoluble dietary fiber also plays a crucial physiology role in sow. Insoluble dietary fiber accelerates gastrointestinal motility, reduces constipation and increases satiety of sows (43). Wheat bran (WB) is a insoluble fiber rich in arabinoxylan and cellulose, and widely used in the sow diet (44). A recent study showed that feeding WB to sows during late pregnancy and lactation (from G110 and L21) reduced inflammatory responses with the downregulation of serum IL-6 concentrations (21). In addition, the addition of wheat bran (25% during gestation and 14% during lactation) to sow diets increase the duodenal villi and higher colonic and ileal VH:CD ratios of the weaning piglets (45).

However, excessive level of dietary fiber could negatively affect total tract nutrient digestibility in pigs (46). As soluble fiber might increase digesta viscosity and slow down the diffusion of digestive enzymes in the small intestine (47). While insoluble fiber could promote the passage rate of chyme and reduce the mixing time of digestive enzymes and dietary ingredients (47). Therefore, overmuch high-fiber diet may cause reduced nutrient absorption by sows, which is detrimental to piglets. And the optimal dosage of fiber supplement in the diet of gestational sows needs further study.

OILS

During late pregnancy and lactation period, sows require more nutrients and energy for fetal growth and milk synthesis. Oil supplementation in sow diets could prevent excessive mobilization of body reserves (48), shorten the estrous interval, improve milk quantity (49), and increase the survival rate and daily weight gain of weaned piglets (50). In addition, some specific types of fatty acids also participate in metabolic regulation and perform antibacterial and anti-inflammatory effects (51). In this section, we discussed the role of three wildly used oils (soybean oil, fish oil and olive oil) in sow diet.

Soybean oil is rich in linoleic acid. The addition of 2% soybean oil during pregnancy increased the content of protein and lipid-free solids in colostrum (**Table 2**). Furthermore, supplementation of soybean oil in the lactating diets of sows also resulted in higher concentrations of protein in maternal milk (54), which may be due to fatty acids stimulate the development of mammary duct and alveolar structure (55). In addition, maternal soybean oil supplementation also improved the intestinal morphology, digestive enzyme activities, serum growth factor concentrations and even intestinal immune function of piglets with the upregulation of immune-related genes (*TLR-4*, *TLR-9* and *MyD88*) in the ileum (52, 56).

Fish oil (FO) is rich in long-chain n-3 polyunsaturated fatty acids, such as eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), which have anti-inflammatory effects both *in vivo* and *in vitro* (57) (**Figure 2**). Maternal supplementation of FO accelerated immune system maturation and enhanced anti-inflammatory response of piglet (58). The addition of 3-5% fish oil to sow feed during lactation promoted the growth of piglets during lactation (59–61), which might partly due to the increased secretion of milk fat and immunoglobulins (IgM and IgG) (62, 63). Furthermore, fish oil also reduced the transmission of pro-inflammatory cytokines (IL-1β) from the sow to the piglets, and up-regulated the expression of IL-10 in the liver and pro-inflammatory cytokines (IL-6, TNF-α) in the skeletal muscle of piglets to alleviate the inflammatory response of the

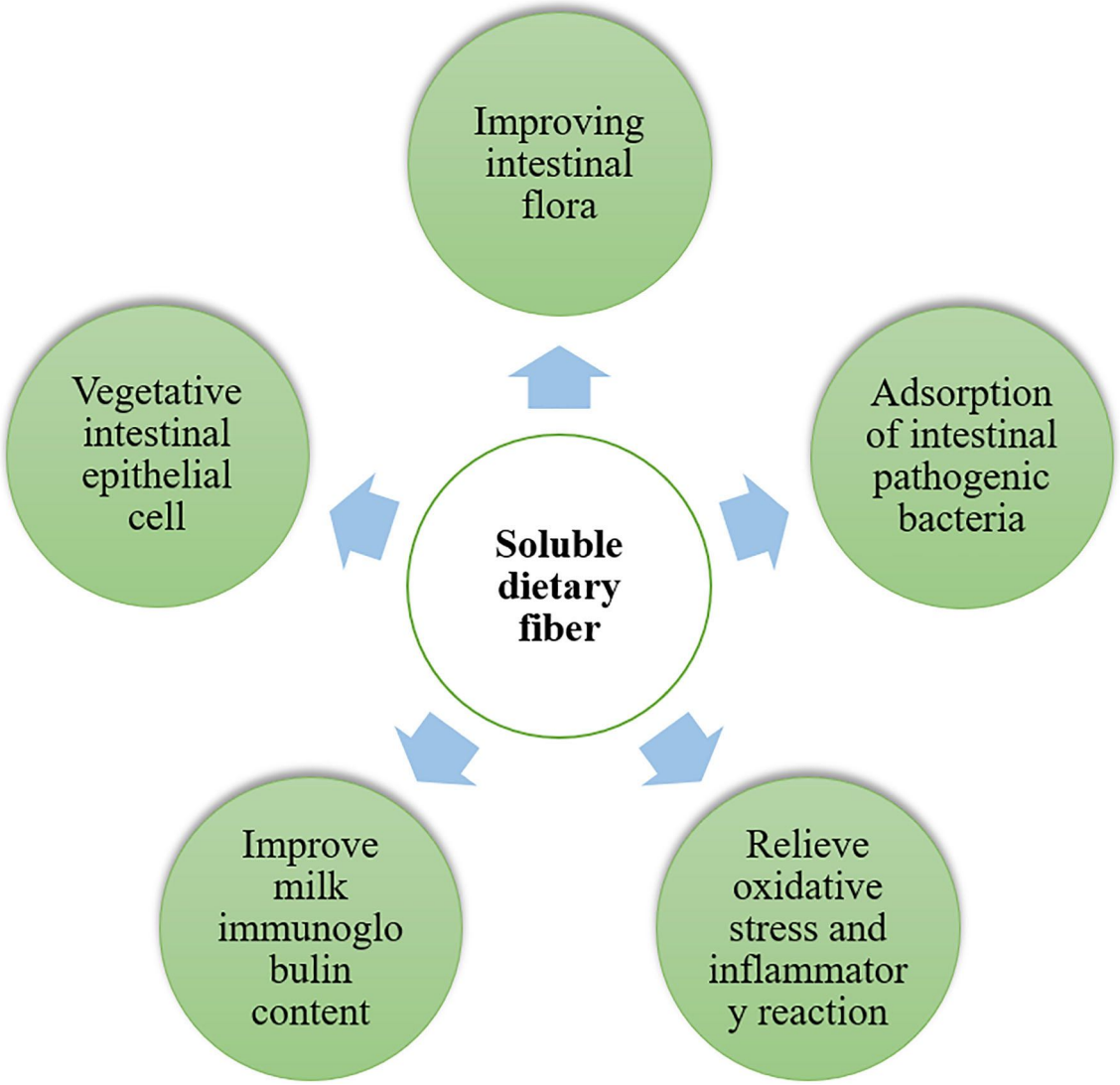

FIGURE 1 | The soluble dietary fibers beneficial to intestinal health of sow, improves colostrum quality, enhance antioxidant capacity of sows and reduces inflammatory reaction of piglets.

piglets (64, 65). However, addition of fish oil to sow diets could increase the sensitivity to oxidative stress in sows and piglets (66, 67). MDA is an indicator of lipid peroxidation, which is higher in the plasma of pregnant sows after feeding FO (53). This might due to unsaturated bonds in EPA and DHA were easily attacked by free radicals (68). Similar to fish oil supplementation, addition of n-3 PUFA during late pregnancy and lactation (G82-L22) reduced the weaning-estrous interval of sows, increased the concentrations of fat, protein and immunoglobulins (IgA, IgG and IgM) in milk (69). Furthermore, n-3 PUFA supplementation improved the intestinal barrier, reduced the diarrhea rate, and minimized the mortality of suckling piglets (69). Besides, changing the ratio of n-6/n-3 PUFA in the diet of lactating sows also affect the immune system and antioxidant status of piglets (70, 71).

Olive oil (OO) is rich in monounsaturated fatty acids (72), as well as antioxidant and anti-inflammatory components such as tocopherols, triterpenoid alcohols, phytosterols and phenolic compounds (73). Sows fed with olive oil (2% OO) diet during late pregnancy and lactation resulted in greater milk fat content, and higher birth weight and survival rate of piglets (53). This might be due to sows distributed a larger proportion of nutrients for fetus and neonate growth instead of using them for fat deposition. In addition, OO significantly reduced the contents of IL-1β, IL-6, MDA and TNF- α in milk, and improved the plasma levels of IL-1β and TNF-α in piglets (53). However, lower feed intake in sows was caused by OO feeding, which might be due to olive oil derived oleic acid upregulated plasma oleoyl ceramide (OEA) levels and caused anorexia in sows (74).

TABLE 2 | Maternal fats intake in the regulation of neonatal infection, immunity and production performance.

Breed, feeding time and products	Reproductive and lactation performance	immune and oxidative Stability of sows and piglets	Intestinal health	others	References
Breed: Landrace × Yorkshire **Period:** G0-L20 **Product:** 2% soybean oil	**Colostrum** ↑No-fat solids: (15.53-22.90%) Protein: (5.85-8.79%)	**Piglet ileum (After farrowing) Gene Expression** ↑TLR-4: (1.00-1.48) TLR-9: (1.00-1.40) MyD88: (1.00-1.22)	**Piglet Jejunum (After farrowing)** ↑Villous height: (717-923 μm) Crypt depth: (76-88 μm) **Piglet Colon (After farrowing)** ↑Crypt depth: (32-41 μm) ↓VCR: (6.53-4.40) (villous height to crypt depth ratio)	**Sow plasma (After farrowing)** Prolactin: (262.00-432.70 ng/mL)	(52)
Breed: Large White × Landrace **Period:** G109-weaning (L26) **Product:** fish oil and seaweed extract (100 g of FO/d, 10.0 g of SWE/d)	**Colostrum (SWE)** ↑IgG: (63.27-69.84 mg/ml) **Milk (L12) (SWE)** ↑CP: (5.17-5.39%) **Milk (L12) (FO)** ↑Total n-34: (1.73-4.62%) Ratio n-6:n-3: (9.75-3.80%)	**Piglet serum (L5)** ↑IgG (SWE): (19.31-22.9 mg/ml) IgA (SWE): (2.51-3.13 mg/ml) ↓IgA (FO): (3.12-2.52 mg/ml) **Piglet serum (L12)** ↑IgG (SWE): (9.98-12.04 mg/ml)	**N/A**	**Piglet serum (L26)** ↑Total n-6: (0.99-0.16%) Total n-3: (1.43-0.030%) Ratio n-6:n-3: (0.61-0.232%)	(23)
Breed: large white × landrace **Period:** G84-L21 **Product:** Fish Oil (2%) Or Olive Oil (2%)	**Litter Performance** ↑Piglet BW: (1.33-1.58 kg) OO ↑Piglet mortality: (7.2-12.3%) FO ↓Piglet mortality: (7.2-2.2%) OO **Colostrum** ↑Fat: (4.84-5.69%) OO MDA: (3.9-5.8 nmol/ml) FO IL-1β: (14-20 ng/L) FO **Milk (OO)** ↑Fat: (6.77-8.08%) L10 Fat: (5.86-7.99%) L21 ↓IL-1β: (20-10 ng/L) L10 IL-1β: (18-6 ng/L) L21 **Milk (FO)** ↑MDA: (3.9-8 nmol/ml) L10 MDA: (3.8-8 nmol/ml) L21	**Sow plasma (FO)** ↑MDA: (2-2.25 nmol/ml) L0 MDA: (2-3.5 nmol/ml) L10 MDA: (1.5-2 nmol/ml) L21 **Piglet serum (FO)** ↑MDA: (2.75-4 nmol/ml) L0 MDA: (3-4 nmol/ml) L21 GSH-Px: (275-300 U/ml) L0 **Piglet serum (OO)** ↓IL-1β: (12-10 ng/L) L21 TNF-α:(90-80 ng/L) L21	**N/A**	**N/A**	(53)

↑, increase; ↓, decrease. N/A, No Value; TLR-4, toll-like receptor 4; TLR-9, toll-like receptor 9; MyD88, myeloiddifferentiationfactor88 IgG, Immunoglobulin G; IgA, Immunoglobulin A; IL-10, interleukin 10; TNF-α, tumor necrosis factor-α; MDA, malondialdehyde; IL-1 β, interleukin-1 β; T- AOC, total antioxidant capacity; GSH-Px, glutathione peroxidase IL-6, interleukin 6.

It is worth noting that high fat-induced obese sows have lower number of live-born piglets (75), piglet birth weight and weaning weight (76). Moreover, these piglets showed reduced responses to infection (77). One of the possible reasons is that obesity lead to lipotoxic placental environment (78, 79), which results in placenta proinflammatory response and oxidative stress (80, 81). The other reason is obese sow has higher plasma pro-inflammatory cytokines TNF-α, IL-1β, and IL-6 (75, 82). Maternal inflammation and oxidative stress further increase the expression of intestinal pro-inflammatory cytokines (83) and disrupts the homeostasis of immune cells (such as the number of T cells and macrophages) in the offspring (84), which makes them more vulnerable to inflammatory bowel disease. These data indicate that the excessive high-energy feed have catastrophic consequences for health of sows and piglets. Therefore, oil additive dosage should be considered in actual production.

ANTIOXIDANTS

During late pregnancy,rapid fetal development increases the metabolic burden and induces systemic oxidative stress of pregnant sows (85).Severe oxidative stress leads to postpartum hemorrhage, decreases neonate's birth weight and even causes fetal death (86). Furthermore, oxidative stress usually causes inflammation and reduces immune system function in sows, which leads to growth-retarded fetuses (87, 88). The detrimental effect of maternal infection or inflammation on fetus development might be due to maternal inflammatory cytokines that transmitted from maternal to fetus (89, 90). Therefore, nutritional strategies to relieve oxidative stress in sows is crucial to improve fetus and neonate development (**Table 3**).

Vitamin E, one of the most effective antioxidants, could directly react with free radicals and stimulate the expression of antioxidant enzyme genes, like GSH-Px and CAT (94). In addition, vitamin E enhances cellular and humoral immune responses in a variety of animals, including pigs (98, 99). During last week of gestation and lactation, vitamin E (250 IU/kg) supplementation in sow diet increased the levels of IgG, IgA, and fat in sow milk and enhanced antioxidant and immune capacity in piglets with the upregulation of plasma IgG, IgA, T-AOC and CAT levels (94). Similarly, injection of 1000 IU vitamin E during gestation also increases serum IgG in sows (100).

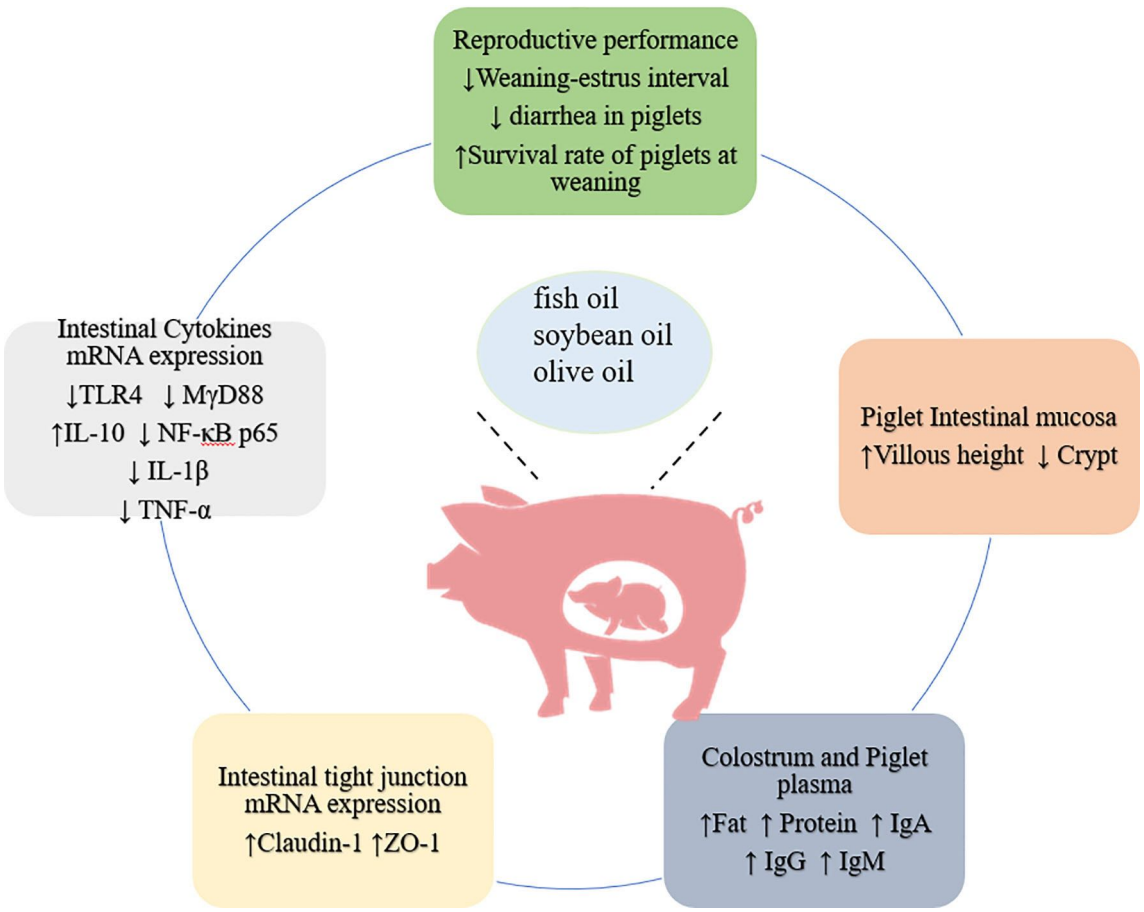

FIGURE 2 | Beneficial effects of adding fat in feed of pregnant sow on piglets.

Polyphenol is a bioactive substance with antioxidant, anticancer, anti-inflammatory and antibacterial properties (101). Supplementation of grape seed polyphenols (GSP) (300mg/kg) during late pregnancy and lactation reduced the number of dead fetuses, improved farrowing and pre-weaning survival (91). This might due to GSP increased antioxidant ability, progesterone and estradiol levels as well as the content of colostral IgM and IgG in sow (91). Intriguingly, effects of GSP on colostral immunoglobin production is better than vitamin E (91). Supplementation herbal extracts during pregnancy and lactation also enhance the immune function and antioxidant capacity of next generation through maternal-offspring transmission. *Forsythia suspensa* extract (FSE) is a medicinal herb extract that mainly consists of forsythiaside A, forythialan A, phillyrin and phillygenin. FSE has been shown to perform antioxidant (102), intestinal microflora-regulating, and anti-inflammatory effects (103). Dietary supplementation with FSE (100mg/kg) in sows from the G85 to farrowing could upregulate the milk fat, milk protein and IgM level in colostrum, and increase the immune ability of the piglets (104). Mechanistically, FSE limits the inflammatory response with the inhibition of NF-κ B signaling and the activation of Nrf2/HO-1 pathway (105). In addition, GE has an anti-inflammatory effect by inhibiting the expression of chemokines (106). The sow feed GE could improve the content of antioxidant and phenolic compounds in piglets' plasma, and enhance the immune function by improve the concentration of IgG in colostrum and the plasma of the piglets (107). Resveratrol is a plant polyphenol with anti-inflammatory and antioxidant properties (108). Resveratrol (300 mg/kg) supplementation in sow diet improved the intestinal morphology and reduced intestinal inflammation as well as diarrhea in the offspring (109).

As an essential trace element for sows, selenium (Se) is incorporated into selenopsroteins and subsequently prevent intestinal inflammation by alleviating oxidative stress (110). In addition, selenoproteins such as glutathione peroxidase (GPX) and thioredoxin reductase (TXNRD) play an important role in the regulation of immune function (111). Organic Se compounds are more bioavailable than inorganic Se forms (112, 113). Supplementing sow gestation diets with HMSeBA (0.3 mg Se/kg) increases the expression of antioxidant-related selenoprotein genes in the placenta (*GPx2*, *GPx3*) and liver of neonates (*GPx1*, *GPx2*, *GPx3* and *TXNRD2*). Furthermore, administration of HMSeBA decreased the gene expression of *IL-1β*, *IL-6* and *IL-*

TABLE 3 | Maternal antioxidant and other substrates intake in the regulation of neonatal Infection, immunity and production performance.

Breed, feeding time and products	Reproductive and lactation performance	immune and oxidative Stability of sows and piglets	Intestinal health	others	References
Breed: Large White × Landrace **period:** G80-L21 **Product:** grape seed polyphenols (300 mg/kg GSP)	**Reproductive performance** ↓dead fetuses: (1.19-0.63) ↑Farrowing survival: (81.47-89.32%) Preweaning survivability: (91.85-95.23%) **Colostrum** ↑IgM: (2.5-6 g/L) IgG: (38-80 g/L)	**Sow plasma (G110)** ↑SOD: (37.51-66.21 IU/mL) GSH-Px: (417.83-620.33 IU/mL)	**N/A**	**Sow plasma (G110)** ↑P4: (35-45 ng/ml) E2: (40-50 pg/ml)	(91)
Breed: Landrace × Yorkshire **period:** G85-L21 **Product:** fully oxidised β-carotene (8 mg/kg)	**Milk (14)** ↑Lactose: (5.67-6.09%) IgM: (0.024-0.057 g/L) **Colostrum** ↑IgM: (2.55-4.52 g/L) IgG: (29.91-33.22 g/L) IgA: (2.39-5.11 g/L) ↓TNF-α: (0.34-0.08 ng/mL) IL-8: (1079.06-605.46 pg/ml)	**N/A**	**N/A**	**N/A**	(92)
Breed: Landrace × Yorkshire **period:** G90-L21 **Product:** Rare Earth Elements (200 mg REE mixture/kg)	**Reproductive performance** ↓Within-litter birth weight CV: (0.21-0.18%) ↑Weight at 21st day: (5.71-6.21 kg) Daily weight gain: (223.06-241.75 g/day)	**Sow plasma (farrowing)** ↑GSH-Px: (650-700 U/ml) CAT: (4.8-6.5 U/mL) ↓TNF-α: (200-130 pg/ml) **Piglet plasma (weaning)** ↑SOD: (120-130 U/mL) ↓TNF-α: (120-80 pg/ml)	**Fecal Microbiota (lactating sows)** ↑Firmicutes: (78.2-81.0%) Bacteroidetes: (13-19.1%) **Piglet Fecal Microbiota (weaning)** ↓Proteobacteria phylum: (14.8-6.7%)	**Piglet plasma (weaning)** ↑IGF-1: (180-210 ng/ml)	(93)
Breed: Large White × Landrace **period:** G107-L21 **Product:** vitamin E (250 IU/kg)	**Reproductive performance** ↑BW of weaned piglets:(4·89-5·67 kg) Piglet Day 0-21 ADG: (160-194 g/d) **Colostrum** ↑Fat: (44·35-53·80 g/kg) IgG: (52·78-63·45 g/l) IgA: (8·02-9·01 g/l) α-tocopherol: (18·51-26·97 μg/l) **Milk** ↑Fat: (67·01-79·13 g/kg) IgG: (0·89-0·96 g/l) IgA: (3·81-4·11 g/l) α-tocopherol: (4.16-7.97 μg/l)	**Piglet plasma (L21)** ↑IgG (0·44-0·49 g/l) IgA (0·33-0·36 g/l) T-AOC (6·82-7·65 IU/ml) CAT (7·38-8·78 U/ml)	**N/A**	**N/A**	(94)
Breed: Yorkshire × Landrace **period:** G75-L21 **Product:** Taurine (1%)	**Reproductive performance** ↑Average daily gain: (194.62-230.11 g) Weaning weight: (5.35-6.29 kg) **Milk** ↑T-AOC: (106.21-165.16 U/ml) on L1 T-AOC: (34.45-105.93 U/ml) on L10 GP-x: (103.75-174.03 U/ml) on L1 CAT: (0.69-0.74 U/ml) on L10 T-SOD: (23.71-29.48 U/ml) on L10	**Piglet plasma (L1)** ↑T-SOD: (35.53-104.92 U/ml) T-AOC: (23.45-41.22 U/ml) CAT: (0.34-0.38 U/ml)	**Piglet Villous height** ↑Duodenum: (249.10-503.08 μm) on L1 Ileum: (318.61-467.21 μm) on L21 Jejunum : (358.39-524.045 μm) on L7 **villus height-to-crypt depth ratio** ↑Duodenum: (1.47-2.81) on L1 Jejunum: (1.38-1.99) on L7	**N/A**	(95)
Breed: Yorkshire × Landrace **period:** G85-L21 **Product:** lysozyme (300 g/t)	↓Stillborn: (0.89-0.15) Diarrhea rate: (2.24-1.41%) **Colostrum** ↑IgA: (3.21-3.51 mg/mL) **Milk (L7)** ↑IgA: (1.84-2.11 mg/mL)	**Sow plasma (L1)** ↑IgM: (0.81-0.98 mg/mL) **Piglet plasma (L21)** ↑IL-10: (209.60-239.21 ng/L) IgA: (2.16-2.56 mg/mL) IgG: (2.25-2.65 mg/mL) IgM: (23.98-28.87 mg/mL)	**N/A**	**N/A**	(96)
Period: G43-weaning **Product:** wheat bran (25% of WB in	**N/A**	**Piglet Ileal mRNA expression** ↑PPARγ: (1-1.37) IL6: (0.61-1)	**Piglet Small Intestine** ↑villi height: (380-450 μm) duodenum villi/crypt: (1.4-2) duodenum	**N/A**	(45)

(Continued)

TABLE 3 | Continued

Breed, feeding time and products	Reproductive and lactation performance	immune and oxidative Stability of sows and piglets	Intestinal health	others	References
gestation and 14% of WB in lactation.)			villi/crypt: (1.4-1.6) jejunum ↓crypts depth: (250-200 μm) jejunum		
Breed: Large White × Landrace **Period:** G85-L20 **Product:** Yeast-based nucleotide (4 g YN/kg diet)	**Piglet at Weaning (D20)** ↑litter size: (9-10) ADG: (190-200 g) Sow total milk yield: (130-150 kg)	**Gene expression of Intestinal cytokine (neonatal piglets)** **Ileal** ↑(IL)-17: (1-1.8) IL-8: (1-1.5) TNF-α: (1-1.8) **Jejunal** ↑(IL)-17: (1-1.8) IL-6: (1-2.5) IL-8: (1-1.7) IFN-γ: (1-1.6) TNF-α: (1-1.8) **Duodenal** ↓IL-6: (1-0.5) ↑IL-1β: (1-1.6)	**Ileum (neonatal piglets)** ↑average villus height: (550-600 μm) villus height-to-crypt depth (V:C): (5-6) sIgA: (5-6.5 μg/g) **Intestinal tight junction (neonatal piglets) mRNA expression** **Ileal** ↓ZO-1: (1-0.6) **Jejunal** ↓ZO-1: (1-0.7) claudin-1: (1-0.5) **Duodenal** ↓claudin-1: (1-0.5)	↓ Diarrhoea rate of piglets: (4.5-3%)	(97)

↑, increase; ↓, decrease. N/A, No Value; SOD, superoxide dismutase; GSH-Px, glutathione peroxidase; P4, progesterone; E2, estradiol; IgM, Immunoglobulin M; IgG, Immunoglobulin G; IgA, Immunoglobulin A; TNF-α, tumor necrosis factor-α; IL-8, interleukin 8; IL-6, interleukin 6; IL-10, interleukin 10; IL-17, interleukin 17; IFN-γ, interferon-γ; CAT, catalase; IGF-1, insulin like growth factor 1; T- AOC, total antioxidant capacity; T-SOD, total Superoxide dismutase; ZO-1, zonula occludens-1; IL-1 β, interleukin-1 β; ADG, average daily gain; sIgA, secretedimmunoglobulin A; PPARγ, peroxisome proliferator-activated receptor γ.

8 in placentas and IL-6 serum concentration in neonatal piglets. Therefore, HMSeBA supplementation in sows during late pregnancy increased the antioxidant capacity of piglets and reduced maternal and fetal inflammation (114). Similarly, another study reported that HMSeBA (0.3 mg Se/kg) supplementation to sows during pregnancy could up-regulate GPX1, GPX4 and selenoprotein expressions in the thymus and spleen of the offspring. Besides, the levels of inflammation, autophagy and endoplasmic reticulum stress were reduced, suggesting favorable outcomes in the immune function of offspring (115). Moreover, provision of maternal hydroxy-selenomethionine (OH-SeMet) (0.3 mg Se/kg) during G84 to L21 showed a significantly increase of IgG level in piglets at weaning (2).

Taurine (Tau), a metabolite of methionine and cysteine, have anti-inflammatory and antioxidant properties (116, 117). Tau effectively promotes mammalian growth and intestinal development (118). Supplementation with Tau (1%) in sow diets from G75 to weaning could significantly increase the activity of antioxidant enzymes (T-SOD, T-AOC, and CAT) in piglet serum and weaning body weight of the piglets. Besides, the height of jejunal villi, the ratio of villi height to crypt depth (VCR) and the expression of tight junction were also increased (95).

Oxidized β-carotene (OxBC) is a complex mixture produced by complete and spontaneous oxidation of β-carotene. The addition of OxBC (8 mg/kg) to the perinatal diet (G85-L21) improved the litter weight and individual body weight of the weaned piglets. This might be due to OxBC increased the immune status of sows, which further affect the growth of piglets. This is evidenced by decreased levels of cytokines (TNF-α and IL-18) and increased levels of immunoglobulin (IgM, IgA, and IgG) in colostrum (92).

OTHER NUTRITIONAL STRATEGIES

In this section, we describe some other nutrients which are advantageous to regulate the immunity and inflammation of piglets when supplemented in sow diets such as rare earth elements, lysozyme, and yeast nucleotides etc (**Table 3**).

Rare earth elements (REEs) includes 15 elements such as lanthanum (La) and cerium (Ce) (119). In addition to promote growth and feed conversion rate, rare earth elements also have anti-inflammatory and antioxidant properties (120, 121). A recent study showed that maternal supplementation with REEs (200 mg/kg) during late gestation could improve the antioxidant capacity and immune system through the up-regulation of serum CAT and GSH-Px level and downregulation of the serum TNF-α level of sow. In addition, piglets from REEs fed sow, have higher uniformity of birth weight and weaning weight, which might be related to the higher serum IGF-1 level (93). Furthermore, increased abundance of beneficial bacteria (*Christensenellaceae* and *Ruminococcaceae*) and decreased abundance of opportunistic pathogenic bacteria (*Proteus* and *Campylobacter*) were also found in the intestinal tract of piglets (93).

Lysozyme (LZM) is a natural antibacterial enzyme found in the tears, saliva and milk of mammals (122). Previous studies have shown that lysozyme has multiple beneficial effects on piglets, including improving intestinal morphology (123), regulating the intestinal microflora (124), and improving immunity (125). Sows fed diets containing lysozyme (300 g/t) from late gestation to weaning exhibited shorter weaning-estrous intervals and less stillbirths. In addition, serum IgM, IgA, IgG and IL-1 in sow were increased during lactation. Correspondingly, serum IgA, IgG, IgM, and IL-10 concentrations were also increased in piglet (96). Besides, piglets showed reduced rates of

diarrhea, which may be due to a decreased number of *campylobacter* in the feces (126).

Nucleosides could promote the growth and development of intestinal epithelial cells (127). The addition of nucleotides to infant formula has a protective effect in preventing diarrhea and improving immunity (128). As a byproduct of yeast degradation, yeast-based nucleotides (YN) are rich in nucleotides. Supplementation of yeast cultures during pregnancy and lactation decrease of diarrhea and improve the growth performance of piglets (129). In detail,administration of yeast-based nucleotide (4 g YN/kg) during late pregnancy and lactation (G85-L20) of sow improved the development of intestinal morphology, and increased innate immunity with upregulation of intestinal IL-17, IL -8, IL -1β, IL -10 and TNF-α expressions in neonatal piglets (97).

Spray-dried plasma (SDP) is a protein-rich feed additive that contains immunoglobulins, peptides, glycoproteins and other active ingredients (130). Previous studies have shown that supplementation of SDP improved the immune response of pigs (131). From late pregnancy to weaning (G85-L27), maternal supplemented with 1% SDP reduced the serum concentrations of TNF-α, TGF-β1 and cortisol in sows and serum concentrations of TNF-α, TGF-β1 and cortisol in piglets. Additionally, the average daily gain of piglets at weaning was greater, and serum concentrations of cortisol, TGF-β1, TNF-α and C- reactive protein were lower (132).

CONCLUSION AND OUTLOOK

Dietary fiber regulates inflammatory and immune response in the offspring by modulating the maternal intestinal microflora and milk immunoglobulin content. The antioxidant substances could directly react with the free radicals and enhance the maternal antioxidant capacity, thereby indirectly reducing infection in the offspring. The oil and fat products not only provide adequate energy to sows, but also supply functional fatty acids to alleviate infection and enhance the immune function in the offspring by exerting the anti-inflammatory and anti-oxidant effects. In summary, maternal nutrition intervention is an effective way to regulate the inflammatory response and immunity in the offspring.

In this review, we mainly focus on the positive effects of nutrients in the regulation of immunity and inflammatory response of sows and piglets during pregnancy and lactation. It worth noting that these effects would be affected by timing and/or dosage of nutrient supplementation. Moreover, it is well known that excessive addition of fat usually has a negative effect on pigs. The toxic effects of excessive addition of other products, such as vitamin E and selenium (133) are also worthy of attention. Therefore, we have given the current dosage of these products. However, the adverse effects of excessive maternal supplementation of such products on the immune system of piglets still need further research. In addition, applying nutrients to piglets and sows at the same time during lactation could produce better results (93). Even though nutrient mixture might produce synergistic and addictive effects, but economic cost should be considered in pig production. Future study needs to identify the best time and dosage for nutrient supplementation in sow diet. In addition, current studies only observe the change of phenotypic indicators, *in vitro* cell experiments are required to clarify the potential mechanism. Lastly, whether the metabolites of these nutrients were involved in the regulation of immunity and inflammation in the offspring is still unclear and require more research.

AUTHOR CONTRIBUTIONS

QL, SZ, and MR initiated the idea, the scope, and the outline of this review paper. QL, SY, XZ, XL, ZW, YQ, WG, MR, and SZ studied and analyzed all of the publications cited in this paper and were involved in the manuscript preparation. SZ and MR conducted the final editing and proofreading. All authors contributed to the article and approved the submitted version.

FUNDING

This study was financially supported by the National Natural Science Foundation of the P.R. of China (No. 31872364 and No. 31802067), Guangdong Basic and Applied Basic Research Foundation (No. 2021A1515010440), Science and Technology Program of Guangzhou (No. 202102020056), Anhui Provincial Science and Technology Major Special Project (201903a06020002).

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frontiers in Veterinary Science

ORIGINAL RESEARCH
published: 25 March 2022
doi: 10.3389/fvets.2022.843673

Dietary Supplementation of Enzymatically Treated *Artemisia annua* L. Improves Lactation Performance, Alleviates Inflammatory Response of Sows Reared Under Heat Stress, and Promotes Gut Development in Preweaning Offspring

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OPEN ACCESS

Edited by:
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Guangdong Academy of Agricultural Sciences, China

Reviewed by:
Shenfei Long,
China Agricultural University, China
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Specialty section:
This article was submitted to Animal Nutrition and Metabolism, a section of the journal Frontiers in Veterinary Science

Received: 26 December 2021
Accepted: 16 February 2022
Published: 25 March 2022

Citation:
Xiong L, Zhang W, Zhao H, Tian Z, Ren M, Chen F, Guan W and Zhang S (2022) Dietary Supplementation of Enzymatically Treated Artemisia annua L. Improves Lactation Performance, Alleviates Inflammatory Response of Sows Reared Under Heat Stress, and Promotes Gut Development in Preweaning Offspring. Front. Vet. Sci. 9:843673. doi: 10.3389/fvets.2022.843673

Artemisia annua L., which is known for its antimalarial compound artemisinin, has commonly been used for its anti-inflammatory and antibacterial functions. Enzymatically treating *Artemisia annua* L. can improve its bioavailability. The purpose of this study was to investigate the effects of dietary enzymatically treated *Artemisia annua* L. (EA) supplementation in late gestation and lactation diets on sow performance, serum hormone, inflammatory cytokines, and immunoglobulin level of heat-stressed sows. A total of 135 multiparous sows (Large White × Landrace) on day 85 of gestation were selected and randomly distributed into 3 groups with 45 replicates per group. The control group was reared under standard conditions (temperature: 27.12 ± 0.18°C, THI (temperature-humidity index): 70.90 ± 0.80) and fed with basal diet. The heat stress (HS) and HS + EA groups were raised in heat-stressed conditions (temperature: 30.11 ± 0.16°C, THI: 72.70 ± 0.60) and fed with basal diets supplemented with 0 or 1.0 g/kg EA, respectively. This trial lasted for 50 consecutive days until day 21 of lactation. Compared with the control group, HS increased the concentrations of serum endotoxin and heat shock protein 70 (HSP-70), and inflammatory cytokines in serum, colostrum, and 14 day milk of sows. Meanwhile, the EA supplementation decreased levels of serum endotoxin, HSP-70, and inflammatory cytokines in both sows and offspring and increased serum triiodothyronine (T_3) level and average daily feed intake (ADFI) of sows. In addition, EA significantly improved average daily gain (ADG) and altered intestinal morphology with an increased villus height in the duodenum and ileum of piglets. Collectively, EA

supplementation at 1.0 g/kg in late gestation and lactation diets alleviated the adverse effects of HS, which were reflected by enhancing ADFI and decreasing endotoxin as well as inflammatory cytokine levels in the serum and colostrum of heat-stressed sows, while promoting ADG and gut development of their offspring.

Keywords: ***Artemisia annua*** **L., heat stress, hormone level, inflammatory factor, intestinal morphology**

INTRODUCTION

Heat stress (HS) has been reported to seriously affect the production performance (1), immune status (2), plasma hormone profiles (3), intestinal integrity and function (4), oxidative stress (5), and inflammation and heat shock response (6) of livestock, and is estimated to lose over US $450 million annually (7). In addition, global warming worsens the already serious problem of HS for the pig industry in tropical and subtropical regions around the world (8). Due to a lack of functional sweat glands and the existence of a thicker layer of subcutaneous adipose tissue that impedes radiant heat loss, sows are more susceptible to HS (9). HS normally reduces appetite and feed intake in sows which results in a negative energy balance and nutrient supply deficit for sows and impairs lactation performance further (9). Many detrimental effects of HS can be attributed to HS-induced intestinal barrier dysfunction (10). HS exposure directly impairs gut integrity and increases endotoxin concentration, which causes a severe immune inflammatory response and cytokine-induced febrile response in sows (11). The long-term effects of maternal HS exposure might induce intestinal dysfunction of offspring, especially gut permeability and barrier function (12).

Currently, supplementing animal diets with natural phytogenic additives, which can alleviate HS, have received extensive attention in consideration of the safety of animal-origin food (13–15). These extracts can be used as anti-inflammatory agents due to the ability of scavenging free radicals or alleviating inflammatory response (16, 17). *Artemisia annua* L. (*A. annua*) is a natural herb belonging to the genus artemisia of Compositae, famous for its antimalarial compound artemisinin (Qinghaosu) (18). Approximately 600 secondary metabolites have been identified and separated in *A. annua*, including sesquiterpenes, monoterpenes, flavonoids, and phenolic compounds. Among these compounds, artemisinin characterizes the biological action of *A. annua* (19). In addition, many previous studies have shown that *A. annua* powder or extract could enhance growth performance (20), anti-inflammation (21–23), redox status, and innate immunity in broilers and rats (24, 25). Nevertheless, the plant cytoderm restricts the release of phytochemicals inside the cytoplasm, which leads to inefficient nutritional ingredient utilization in natural herbs. Previous studies have shown that enzymatic hydrolysis technology has positive effects, such as: (i) promotion in the release and purification of biologically active substances in plant cells; (ii) shorter extraction time, less usage of solvents, and higher yield and quality of product extract than physical and chemical extraction; and (iii) improvement in absorption efficiency to the animal (26).

Our lab recently found that the dietary supplementation of enzymatically treated *Artemisia annua* L. (EA) at 1.0 g/kg greatly alleviated the oxidative stress in sows, and improved the antioxidant capacity (15). Until now, little information is available regarding the effect of EA on ameliorating the adverse effects caused by HS in sows and offspring. Therefore, we speculated that the EA supplementation in late gestation and lactation diets could ameliorate the adverse effects of HS on the lactation performance, serum hormone secretion, inflammation, and immunoglobulin transfer of multiparous sows and promote intestinal development of their preweaning offspring.

MATERIALS AND METHODS

Preparation of EA

Artemisia annua was produced in China and harvested during the prosperous period. The dried whole plant (stems and leaves) of *A. annua* was pulverized and mixed thoroughly with the 0.6% compound enzymatic hydrolysate (50000 U/g cellulase: 30000 U/g pectinase = 4:1) at 55°C and a pH of 4.5 for 4 h. Following enzymatic hydrolysis, filtrate and residue were collected, dried (filtrate of enzymatic hydrolysis of *A. annua* for 3 h at 60°C; residue of enzymatic hydrolysis of *A. annua* for 5 h at 90°C), and pulverized separately, then mixed to obtain final products. The EA contained 45.02, 972.96, and 335.17 g/kg of CP (AOAC, 2005, method 991.20), DM (AOAC, 2005, method 926.12), and ether extract (AOAC, 2005, method 920.39), respectively. Total phenolic content and total flavonoid content were measured as previously described (27). In addition, the main active ingredients of EA include 1.68 ± 0.13 mg of rutin equivalents (RE)/g flavonoids, 4.77 ± 0.22 mg of gallic acid equivalents (GAE)/g phenols, 156.2 ± 0.20 mg/kg of arteannuinic acid, 62.0 ± 0.08 mg/kg of deoxyartemisinin, and 26.0 ± 0.06 mg/kg of artemisinin.

Animals and Experimental Design

Overall, 135 sows (Large White × Landrace) of similar parity and weight on day 85 of gestation (G85) were selected and randomly distributed into 3 treatment groups with an initial 45 sows per treatment. The three treatment groups were as follows: control (Con) group, in which sows were raised in normal conditions (temperature: 27.12 ± 0.18°C, THI: 70.90 ± 0.80) and fed the basal diet. The heat-stressed (HS) and HS + EA groups, in which sows were raised in heat-stressed conditions (temperature: 30.11 ± 0.16°C, THI: 72.70 ± 0.60), and fed basal diets supplemented with 0 or 1.0 g/kg EA, respectively. The trial lasted for 50 consecutive days until day 21 of lactation (L21). The ultimate numbers of sows for analysis were 40, 41, and 42 in the Con,

HS, and HS + EA groups in the present trial, respectively. The numbers of sows which were abortive, diseased (refer to metritis, lameness, poor health, etc.), deceased, and returned to estrus were 1, 1, 0, and 3 in the Con group, 0, 0, 1, and 3 in the HS group, and 1, 0, 0, and 2 in the HS + EA group, respectively.

This trial was conducted in a subtropical city in Guangdong Province in South China (subtropical climate) from July to October 2018. Comfortable control rooms contained a pad curtain with cycling water on one end of a completely closed room and large fans on the wall of the opposite end, while HS rooms were conventional rearing rooms, which are in a semi-opened building. It is worth noting that the normal adaptation temperature of sows generally should be no more than 25°C (28). In this experiment, the temperature of the Con group was higher than the normal range, although it was 3°C lower than the HS group. However, during our experiment, we did not observe any abnormal behavior of sows under 27°C, which might be due to the possibility that sows in Guangdong have partially adapted to the surrounding environment.

Feeding and Management

The experimental basic diet is a corn-soybean diet that can meet the nutritional needs of pregnant sows and lactating sows (NRC 2012). The composition and nutritional levels of the basal diet are shown in **Table 1**. During the experiment, the temperature (T) and relative humidity (RH) were recorded hourly using an auto temperature and humidity recorder (W-series, Henan, China). The temperature-humidity index (THI) was calculated according to the formula of Wegner et al. (29).

From the day 85 to day 107 of gestation of the sows, every sow was housed in individual crates (2.10 × 0.6 m^2) in the gestation facility, and the sows were allowed to drink freely and were fed twice a day (6:30 and 14:00). On day 108 of gestation, all sows were transferred into respective farrowing rooms, where all experimental settings were held constant during the gestational period. They had *ad libitum* access to their respective experimental diets, which were fed to them until weaning of their offspring on L21. One week after the piglets were born, they were assisted with creep feeding, and they were free to eat until L21. Sows were housed in individual farrowing crates (2.3 × 2.4 m^2). Piglets were cross-fostered only within 48 h postpartum in each treatment group, and the number of litters was standardized to 10 ± 1.

TABLE 1 | Ingredient composition and nutritional levels of the basic diet (g/kg, as-fed) basis).

Items	Content
Ingredients	
Corn	522.0
Soybean meal, 420 g CP/kg	240.0
Wheat bran, 157 g CP/kg	60.0
Fish meal, 640 g CP/kg	25.0
Wheat standard powder	75.0
Soybean oil	40.0
Dicalcium phosphate	12.0
Limestone	9.0
Salt	3.0
Vitamin and mineral premix[a]	3.0
Choline chloride, 500 g choline/kg	2.0
Sodium bicarbonate	2.0
Mold removal agent[c]	0.5
Mold inhibitor[d]	2.0
Carrier	4.5
Total	1,000.0
Analyzed nutritional levels	
Dry matter, g/kg	871.0
Crude protein (CP), g/kg	181.3
Ash, g/kg	57.3
Ether extract, g/kg	67.9
Neutral detergent fiber, g/kg	110.1
Acid detergent fiber, g/kg	47.8
Ca, g/kg	7.6
Total P, g/kg	6.6
Calculated nutritional levels[b]	
Digestible energy, MJ/kg	14.1
Digestible Lys, g/kg	8.6
Digestible Met + Cys, g/kg	5.1
Digestible Thr, g/kg	6.0
Digestible Trp, g/kg	1.8

[a] *Vitamin and mineral premix supplied per kilogram of complete diet: 50.0 mg Zn ($ZnSO_4 \cdot H_2O$), 80.0 mg Fe ($FeSO_4 \cdot H_2O$), 20.0 mg Mn ($MnSO_4 \cdot H_2O$), 5.00 mg Cu ($CuSO_4 \cdot 5H_2O$), 0.14 mg I (CaI_2O_6), 0.30 mg Se (Na_2SeO_3), 13,500 IU vitamin A, 4000 IU vitamin D_3, 90 IU vitamin E, 4 mg vitamin K_3, 4 mg vitamin B_1, 10 mg vitamin B_2, 4.8 mg vitamin B_6, 40 mg niacin, 0.034 mg vitamin B_{12}, 20 mg D-pantothenate, 0.16 mg D-biotin, 2.0 mg folic acid.*

[b] *Calculated values according to the Chinese Feeding Standard of Swine (2004).*

[c] *The main components of the mold removal agent are saccharomyces cerevisiae and sodium alginate.*

[d] *The main components of the mold inhibitor are propionic acid and ammonium propionate.*

Data and Sample Collection

Sow and Litter Performance

Feed intakes of sows were recorded during lactation. The estrus rate of sows was recorded 7 days post-weaning of offspring. Sow backfat thickness was measured at the P_2 position (at the last rib and 66 mm away from spine) using a digital backfat indicator (Renco Lean-Meater®, Renco Corporation, Minneapolis, USA) on G85, G114, and L21, which were used to calculate the backfat loss of lactation. Within the first 24 h after farrowing, the litter size at birth (total, live, healthy, weak, and stillborn) was recorded in this study. Moreover, individual piglet body weight (BW) as well as litter size at weaning and individual piglet BW were recorded on L21 and shown in our published research (15). These data were used to calculate the average daily gain (ADG) and survival rate of piglets during lactation in this study.

Serum Sample of Sows and Piglets

Blood samples (∼8 mL per sow) were collected by ear venipuncture from a random subset of sows (n = 10 per treatment) on G85, G114, L14, and L21. Sows were fed at 06:30, the blood samples were taken 3.5 h after morning feeding (around 10:00). The sows that had blood collected at each time

point were the same. On L1 and L21, six piglets were randomly selected from each treatment. At the postprandial time, blood samples were collected *via* jugular venipuncture. Blood samples were collected into sterile vacuum tubes, followed by centrifuged at 3000 × g at 4°C for 10 min. The serum was separated, transferred into micro-tubes, and then frozen immediately at −80°C for subsequent biochemical analysis.

Colostrum and Milk Samples of Sows

Colostrum was collected from all functional teats within 12 h postpartum; 14 day milk was collected after intramuscular injection of 30 IU oxytocin on L14. Approximately 25 mL was collected at each time period, and samples were frozen immediately at −80°C until further analysis.

Small Intestine Samples of Piglets

Whole gastrointestinal tracts were rapidly removed after piglets were sacrificed by exsanguination. The small intestine was peeled from the mesentery and placed on a cold stainless-steel tray. Then, segments of ∼2 cm in length of the middle of the duodenum, jejunum, and ileum were immediately isolated, respectively. The intestinal tissues were gently flushed with cold phosphate-buffered saline and then placed in 10% formalin solution for morphological examination.

Laboratory Analysis

Serum Hormone Profile, Endotoxin, and HSP-70 Concentrations

Serum hormone concentrations (corticosterone, insulin, glucagon, T_3 (triiodothyronine), T_4 (thyroxine), leptin, and prolactin), endotoxin, and heat stress protein 70 (HSP-70) were assayed as described in previous studies (30–32) using commercial radio-immunoassay kits (CUSABIO Biotech Company, Wuhan, China), then read on a spectrophotometer.

The Levels of Cytokines in Serum and Immunoglobulins in Colostrum and Milk

The levels of inflammatory cytokines [IL-1β (interleukin-1β), TNF-α (tumor necrosis factor-α), sCD14 (soluble cluster of differentiation 14), IL-6 (interleukin-6), IL-8 (interleukin-8), and IL-18 (interleukin-18)] in serum, and immunoglobulin G (IgG), IgA, and IgM levels in colostrum and 14 day milk of sows were analyzed as described by a previous method (33) using commercially available porcine ELISA kits (CUSABIO Biotech Company, Wuhan, China). These indices were measured with the corresponding assay kits according to the manufacturer's instructions.

Before analyzing inflammatory cytokines, lipids in colostrum and 14 day milk were removed following centrifugation at 4°C, 3000 × g for 25 min as described by Hu et al. (34). Somatic cell counts (SCC) were determined by a Fossomatic 5000 (Foss Electric, Hillerød, Denmark).

Intestinal Morphology

The collected duodenum, jejunum, and ileum segments were dehydrated, embedded in paraffin, then stained with hematoxylin as well as eosin according to the procedures described by Song et al. (35). Villus height and crypt depth were determined using an image processing and analysis system (Leica Imaging Systems Ltd, Cambridge, UK).

Statistical Analysis

Statistical analyses were conducted using one-way ANOVA in SPSS 17.0 software (SPSS, INC., Chicago, IL, USA), differences among groups were evaluated by Duncan's test. The individual sow and its litter were used as the experimental unit. Hormone and inflammatory cytokines in sow serum and milk were analyzed as repeated measures. The results were expressed as mean and its pooled standard errors. The significance of all data analysis was defined as $P < 0.05$.

RESULTS

Lactation Performance of Sows

As shown in **Table 2**, higher ADG during lactation was observed in the HS + EA group compared to the HS group and Con group ($P < 0.05$). Compared with the Con group, HS decreased the average daily feed intake (ADFI); conversely, a higher ADFI was observed when the HS group was fed an EA-supplemented diet during lactation ($P < 0.05$). Sow backfat loss and the estrus rate over 7 days post-weaning and piglet survival rate did not differ among treatments ($P > 0.05$).

Hormone, Endotoxin, and HSP-70 Levels in Serum of Sows

As shown in **Tables 3**, **4**, compared with the Con group, sows in the HS group had lower levels of T_3 (L14, L21) and higher levels of endotoxin (G114, L14) and HSP-70 (G114) ($P < 0.05$). In contrast, dietary EA supplementation resulted in higher levels of T_3 (L14, L21) and lower concentrations of endotoxin (G114, L14) and HSP-70 (G114) compared with the Con and HS groups ($P < 0.05$).

Inflammatory Cytokines Levels in Serum, Colostrum, and 14 Day Milk

Compared with the Con group, the HS group had higher levels of TNF-α (G114) and IL-6 (G114, L14) in the serum of sows, as well as TNF-α (L1) and IL-6 (L1) levels in the serum of piglets ($P < 0.05$). Dietary EA supplementation resulted in lower levels of TNF-α and IL-6 levels compared with the Con and HS groups in the serum of sows and their offspring ($P < 0.05$; **Tables 5**, **6**). Compared with the Con group, the HS group had higher levels of TNF-α, IL-6, and SCC in the colostrum of sows ($P < 0.05$). Dietary EA supplementation resulted in lower levels of TNF-α, IL-6, and SCC in the colostrum compared with the Con and HS groups ($P < 0.05$; **Table 7**).

Immunoglobulin Levels in Colostrum, 14 Day Milk of Sows, and Serum of Nursing Piglets

As shown in **Tables 8**, **9**. There were no treatment effects on IgA, IgG, and IgM levels in the colostrum, 14 day milk of sows, and serum of nursing piglets ($P > 0.05$).

TABLE 2 | Effects of dietary supplementation with EA on lactation performance of sows.

Items	Con	HS	HS + EA	SEM	*P*-value
Observations of sows, *n*	40	41	42		
Sow performance					
Sow BW, kg					
G85	195.60	194.95	196.50	5.950	0.980
G114	236.50	232.95	235.20	8.650	0.780
L21	217.60	211.90	215.80	5.650	0.670
Backfat thickness, mm					
G85	18.56	19.03	18.72	0.131	0.896
G114	20.00	20.53	20.13	0.162	0.525
L21	17.69	18.19	17.78	0.170	0.614
Backfat increase during late gestation	1.44	1.50	1.41	0.193	0.326
Backfat loss during lactation	2.35	2.38	2.34	0.070	0.572
ADFI, kg	4.41^{b}	4.16^{c}	4.64^{a}	0.051	0.001
Estrus rate over 7 days post-weaning, %	91.12	94.44	91.11	1.901	0.572
Litter performance					
Litter size at birth, total	11.36	10.86	11.03	0.161	0.963
Litter size at birth, live	9.64	10.13	10.38	0.150	0.368
Litter size at birth, healthy	9.44	9.95	10.27	0.161	0.266
Litter size at birth, weak	0.18	0.18	0.11	0.030	0.276
Litter size at birth, stillborn	0.66	0.62	0.64	0.090	0.491
Piglet ADG, g/day	198.09^{b}	187.90^{c}	210.15^{a}	2.450	0.037
Piglet survival rate, %	90.90	85.76	87.96	1.030	0.882

Different superscript letters (a, b, c) indicate that there are significant differences between the groups ($P < 0.05$).
EA, enzymatically treated Artemisia annua L.; Con, control group; HS, heat stress group.

Intestinal Morphology of Piglets

As shown in **Table 10**. Compared with the Con group, the villus height decreased in the duodenum at birth (L1) and in the ileum at weaning (L21) ($P < 0.05$) in the HS group. Compared with the Con and HS groups, EA supplementation increased the villus height in the duodenum at birth and in the ileum at weaning ($P < 0.05$).

DISCUSSION

HS exposure in later pregnancy induces oxidative stress (36), reduces digestive enzyme activities and immunity capability (37), causes inflammation and intestinal damage (38), and leads to an insufficient nutrient supply for sows, which have a series of detrimental effects on the reproductive and lactation performance as well as health condition of sows (39). Therefore, it is particularly important to find a suitable method to alleviate the adverse effects of HS on sows and their offspring.

When the ambient temperature exceeds the sow's critical temperature for evaporation, the sow is under HS (40). HS reduced the appetite and feed intake which impairs the compensatory capacity of sows (41) and sows usually consume less feed in order to reduce metabolic heat production (42). Disrupted physiological balance and reduced milk production and feed conversion efficiency of sows under HS have also been observed (43). To date, many previous studies have shown that diets supplemented with *A. annua* extract or *A. annua* leaf powder increased ADG and ADFI (14, 23, 44) and alleviated HS-induced body weight reduction and intestinal morphology impairment in broilers (19). Beyond this, emerging evidence also demonstrated that a diet directly supplemented with EA increased ADG and nutrient digestibility in weaned pigs (45, 46). Similar to this trial, the higher ADG in weaned piglets and ADFI in sows fed EA-supplemented diets was observed during lactation compared with the Con and HS groups. The crude protein, essential amino acids, minerals, vitamins, and flavonoids in EA delivered through the colostrum are important for growth and development of piglets, which is probably the reason for the enhancement of ADG (14, 45). The higher ADFI in the sows fed with supplemental EA might be due to the fact that artemisinin and phenolic compounds, including curcumin, resveratrol, and gallate could arouse appetite and reduce the heat from immuno-inflammatory responses.

A balanced physiological state is key for animal health. HS causes endocrine hormone increases in sows during pregnancy and lactation (3). T_3 is mainly derived by deiodination of T_4 which is known to stimulate lactation. HS could induce decreases in deiodinase activity to decrease T_3 expression (47). In addition, published experimental research indicated that HS negatively affected thyroid status, including depression in pituitary-thyroid axis activity and peripheral metabolism of T_3 and T_4 through LPS-induced pro-inflammatory stimulus (48). In addition, HS injures the barrier of the intestine and induces the increment of endotoxins (i.e., LPS) from bacteria flow across

TABLE 3 | Effects of dietary EA supplementation on hormone levels in the serum of sows.

Items	Con	HS	HS + EA	SEM	*P*-value
Corticosterone, ng/mL					
G85	195.83	189.71	189.70	3.960	0.778
G114	189.71	208.06	146.89	15.331	0.539
L14	342.63	367.11	330.40	19.170	0.378
L21	330.40	329.23	330.40	32.041	0.887
Insulin, mIU/L					
G85	117.50	78.67	85.64	16.120	0.771
G114	34.42	42.21	39.37	2.270	0.714
L14	34.54	54.92	33.42	26.480	0.895
L21	57.54	42.08	57.36	39.754	0.684
Glucagon, ng/L					
G85	1406.47	1437.95	1399.73	70.855	0.755
G114	1507.23	1364.85	1355.20	59.940	0.443
L14	1390.72	1362.67	1412.22	57.300	0.514
L21	1306.90	1391.69	1305.42	70.552	0.372
T_3, ng/mL					
G85	3.70	3.55	3.60	0.230	0.693
G114	7.23	6.98	7.56	0.111	0.205
L14	2.54^b	2.34^c	2.99^a	0.136	0.004
L21	3.50^b	3.00^c	5.65^a	0.440	0.001
T_4, ng/mL					
G85	41.25	37.13	40.80	3.070	0.838
G114	41.03	42.78	37.80	2.950	0.615
L14	46.43	47.55	41.99	3.610	0.254
L21	42.75	50.63	48.79	7.560	0.881
Leptin, ng/mL					
G85	12.85	13.74	11.07	0.630	0.314
G114	11.28	12.70	11.42	0.470	0.403
L14	12.36	11.95	12.38	0.360	0.863
L21	13.09	11.08	10.46	0.600	0.518
Prolactin, ng/mL					
G85	770.38	571.43	811.23	129.412	0.881
G114	1166.07	1008.45	1079.18	35.440	0.170
L14	703.31	795.50	898.69	61.220	0.324
L21	1149.08	1057.07	1141.47	17.121	0.228

The superscript letters (a, b, c) indicate that there are significant differences between the groups ($P < 0.05$). T_3, triiodothyronine; T_4, tetraiodothyronine; G85, day 85 of gestation; G114, day 114 of gestation; L14, day 14 of lactation; L21, day 21 of lactation.

TABLE 4 | Effects of dietary supplementation with EA on endotoxin and HSP-70 levels in the serum of sows.

Items	Con	HS	HS + EA	SEM	*P*-value
Endotoxin, EU/L					
G85	455.94	470.84	368.40	39.850	0.426
G114	515.78^b	576.25^a	508.84^c	26.130	0.026
L14	508.28^b	516.33^a	326.16^c	42.371	0.045
L21	369.07	392.41	282.96	45.121	0.131
HSP-70, ng/mL					
G85	2.32	2.52	2.30	0.110	0.440
G114	4.40^b	4.60^a	2.16^c	0.530	0.038
L14	2.87	3.06	2.54	0.641	0.266
L21	1.09	1.23	1.12	0.243	0.523

The superscript letters (a, b, c) indicate that there are significant differences between the groups ($P < 0.05$). HSP-70, heat shock protein 70.

the mesenteric-drained viscera. The transfer of endotoxins to the interior circulation contributes to systemic inflammation (49). A high content of HSP-70 is widely considered as a pointer of various environmental stress responses such as HS (50). Many previous studies have shown that HS reduced T_3 concentration (51) and activity and increased endotoxin (33) and HSP-70 levels (4) in the blood of broilers and sows. In this study, HS reduced the T_3 level in the serum of sows. Meanwhile, HS significantly increased the expressions of endotoxin and HSP-70 in the serum, which indicated that the HS model was established in sows in the current study. Our results were consistent with the findings reported in previous studies (4, 51). Whereas, EA supplementation effectively increased the T_3 level and reduced the endotoxin level, which might be due to the fact that artemisinin and arteannuinic acid have antioxidant activity (24), bactericidal and antibacterial functions (52), and improve deiodinase activity. Thus, EA directly or indirectly alleviated the inflammatory response triggered by HS. The downregulation of HSP-70 level in heat-stressed sows following supplemental EA might be associated with the modulation of reduced endotoxin-induced pro-inflammatory cytokine expression in the present study.

The gastrointestinal tract is predominantly responsive to HS which destroys the intestinal mucosal structure (53). Subsequently, pathogens and toxins enter the damaged mucosal barrier, which stimulates the immune inflammatory response and leads to the flow of inflammatory cytokines into the colostrum and milk *via* blood circulation (54). The levels of inflammatory cytokines (such as IL-1β, IL-6, and TNF-α) in blood are considered to be an indicator to assess inflammation (23). HS upregulates IL-1β, IL-6, and TNF-α levels in the blood of pigs (55), which manifests as a serious proinflammatory stimulus. Song et al. (4, 56) reported that EA reduced proinflammatory cytokines and restored intestinal mucosal integrity caused by HS in broilers. Niu et al. (45) also showed that EA decreased the concentrations of IL-1β, IL-6, and TNF-α in the small intestine of weaned pigs. Accumulating evidence suggested that artemisinin and phenolic compounds (such as gallate, curcumin, resveratrol, and chlorogenic acids) in EA could exhibit a greater ability to prohibit the inflammatory response induced by lipopolysaccharide (LPS) (21, 57). Our recent studies have shown that EA extractive artemisinin inhibited LPS-induced activation of the mitogen-activated protein kinase (MAPK) and nuclear factor-κB (NF-κB) signaling pathways in porcine mammary epithelial cells (58). Our findings were consistent with the findings of previous studies (21, 56–58). The mechanism has been observed that sesquiterpenes (artemisinin and arteannuinic acid) as well as flavonoids (casticin

TABLE 5 | Effects of dietary supplementation with EA on the inflammatory cytokine levels in the serum of sows.

Items	Con	HS	HS + EA	SEM	*P*-value
TNF-α, ng/mL					
G85	1.29	1.25	1.24	0.110	0.835
G114	1.71[b]	2.28[a]	1.23[c]	0.260	0.045
L14	0.82	0.87	0.76	0.100	0.728
L21	0.61	0.68	0.60	0.080	0.895
IL-1β, pg/mL					
G85	185.77	181.33	172.25	6.080	0.342
G114	129.55	143.10	126.40	1.080	0.907
L14	116.21	159.15	120.29	1.740	0.316
L21	95.34	83.86	110.87	6.614	0.622
sCD14, ng/mL					
G85	7.68	7.25	7.76	0.155	0.943
G114	3.23	3.49	4.02	0.520	0.686
L14	2.47	2.49	2.08	0.480	0.665
L21	1.65	1.75	1.63	0.113	0.885
IL-6, pg/mL					
G85	63.59	62.65	62.12	0.361	0.954
G114	31.01[b]	43.37[a]	26.66[c]	0.422	0.025
L14	10.76[b]	15.25[a]	8.31[c]	0.162	0.049
L21	9.46	9.16	8.91	0.132	0.754
IL-8, pg/mL					
G85	1010.85	1093.28	1094.37	98.320	0.987
G114	1145.96	1098.16	931.07	65.411	0.371
L14	823.51	872.25	892.46	75.762	0.985
L21	724.02	739.50	720.56	91.960	0.979
IL-18, pg/mL					
G85	1580.38	1453.33	1448.69	81.903	0.666
G114	601.26	611.50	679.36	64.150	0.665
L14	413.04	441.62	339.27	50.160	0.506
L21	404.08	393.35	391.14	57.980	0.898

The superscript letters (a, b, c) indicate that there are significant differences between the groups (P < 0.05). TNF-α, tumor necrosis factor-α; IL-1β, interleukin-1β; sCD14, soluble cluster of differentiation 14; IL-6, interleukin-6; IL-8, interleukin-8; IL-18, interleukin-18.

TABLE 6 | Effects of maternal supplementation with EA on the inflammatory cytokine levels in the serum of piglets.

Items	Con	HS	HS + EA	SEM	*P*-value
L1					
TNF-α, ng/mL	0.04[b]	0.08[a]	0.03[c]	0.001	0.049
IL-1β, pg/mL	7.25	8.52	6.68	0.560	0.420
sCD14, ng/mL	0.062	0.074	0.073	0.001	0.390
IL-6, pg/mL	3.53[b]	4.57[a]	2.15[c]	0.061	0.037
IL-8, pg/mL	229.75	269.81	223.29	13.920	0.350
IL-18, pg/mL	115.28	129.49	102.46	7.980	0.379
L21					
TNF-α, ng/mL	0.03	0.04	0.04	0.001	0.598
IL-1β, pg/mL	6.75	6.65	6.83	0.040	0.318
sCD14, ng/mL	0.079	0.086	0.082	0.002	0.260
IL-6, pg/mL	1.64	1.61	1.65	0.150	0.996
IL-8, pg/mL	276.43	300.99	264.83	26.981	0.855
IL-18, pg/mL	137.92	154.23	138.65	19.363	0.932

The superscript letters (a, b, c) indicate that there are significant differences between the groups (P < 0.05). TNF-α, tumor necrosis factor-α; IL-1β, interleukin-1β; sCD14, soluble cluster of differentiation 14; IL-6, interleukin-6; IL-8, interleukin-8; IL-18, interleukin-18; L1, day 1 of lactation.

TABLE 7 | Effects of dietary supplementation with EA on the inflammatory cytokine and SCC levels in the colostrum and 14 day milk of sows.

Items	Con	HS	HS + EA	SEM	*P*-value
Colostrum					
TNF-α, ng/mL	0.25[b]	0.43[a]	0.20[c]	0.021	0.043
IL-1β, pg/mL	31.03	32.51	32.55	2.610	0.809
sCD14, ng/mL	0.44	0.61	0.50	0.041	0.753
IL-6, pg/mL	6.64[b]	9.72[a]	5.70[c]	0.950	0.045
IL-8, pg/mL	893.96	937.49	893.10	45.920	0.917
IL-18, pg/mL	426.57	412.03	422.00	27.001	0.765
SCC, 10^6/mL	4.98[b]	5.34[a]	4.86[c]	0.030	0.046
14 day milk					
TNF-α, ng/mL	0.08	0.07	0.10	0.004	0.505
IL-1β, pg/mL	30.67	34.03	36.81	4.840	0.695
sCD14, ng/mL	0.02	0.04	0.04	0.006	0.206
IL-6, pg/mL	3.42	2.32	3.94	0.280	0.933
IL-8, pg/mL	1061.65	913.31	1018.68	176.810	0.929
IL-18, pg/mL	204.64	261.01	335.39	30.180	0.425
SCC, 10^6/mL	3.78	3.81	3.63	0.040	0.109

The superscript letters (a, b, c) indicate that there are significant differences between the groups (P < 0.05). TNF-α, tumor necrosis factor-α; IL-1β, interleukin-1β; sCD14, soluble cluster of differentiation 14; IL-6, interleukin-6; IL-8, interleukin-8; IL-18, interleukin-18; SCC, somatic cell count.

and chrysosplenol D) from EA can attenuate the NF-κB/nod-like receptor family pyrin domain containing 3 (NLRP3) pathways as well as toll-like receptor 4 (TLR4)/NF-κB and MAPK signaling pathways to further inhibit the expression of various inflammatory cytokines genes, including TNF-α and IL-1β (59, 60). In the present study, the addition of EA in sow diets effectively reduced the increase in inflammatory cytokine levels in the serum, colostrum, and milk of sows and piglets and further relieved the inflammation caused by HS. Hence, we speculated that EA attenuated the LPS-induced inflammatory pathways in the gut and mammary glands to alleviate inflammation in heat-stressed sows. Furthermore, the reduction resulting from EA in the colostrum might indicate the lower incidence of LPS-induced mastitis in sows during lactation (58).

The small intestine is not only the main place for the body to digest and absorb nutrients, but also the largest immune organ of the body (54). Oxidative and inflammatory stress brought by HS damages the intestinal villus structure, reduces villus height (33), increases intestinal permeability, and subsequently results in diarrhea and growth retardation (61). Small intestine mucosal villus height and crypt depth are important indicators for evaluating intestinal development (46). Intestinal villus height is positively correlated with the surface area in contact with the chyme, which is an indicator of the intestinal absorption capacity

TABLE 8 | Effects of dietary supplementation with EA on immunoglobulin levels in the colostrum and 14 day milk of sows.

Items	Con	HS	HS + EA	SEM	*P*-value
Colostrum, mg/mL					
IgA	8.33	8.69	8.88	1.051	0.970
IgG	46.90	45.70	47.55	2.750	0.988
IgM	0.23	0.16	0.18	0.011	0.795
14 day milk, mg/mL					
IgA	1.65	1.49	1.44	0.050	0.519
IgG	0.68	0.99	0.87	0.070	0.405
IgM	0.021	0.021	0.019	0.001	0.486

IgA, immunoglobulin A.

TABLE 9 | Effects of maternal dietary supplementation with EA on immunoglobulin levels in the serum of nursing piglets.

Items	Con	HS	HS + EA	SEM	*P*-value
L1, μg/mL					
IgA	10.20	10.61	10.22	0.581	0.954
IgG	58.24	57.11	56.22	0.602	0.412
IgM	3.37	3.56	3.49	0.071	0.627
L21, μg/mL					
IgA	12.71	12.53	12.81	0.160	0.796
IgG	58.45	59.47	58.22	0.261	0.112
IgM	3.70	3.79	3.83	0.060	0.629

IgA, immunoglobulin A; L1, day 1 of lactation.

TABLE 10 | Effects of maternal dietary supplementation with EA on small intestinal morphology of nursing piglets.

Items	Control	HS	HS + EA	SEM	*P*-value
Duodenum					
L1					
Villus height, μm	490.15[b]	462.33[c]	584.33[a]	27.350	0.045
Crypt depth, μm	65.55	59.04	76.52	5.980	0.321
Villus/crypt	7.47	7.86	7.64	0.760	0.821
L21					
Villus height, μm	288.38	315.91	293.89	12.700	0.461
Crypt depth, μm	142.79	132.08	148.42	6.780	0.412
Villus/crypt	2.07	2.45	1.99	0.151	0.274
Jejunum					
L1					
Villus height, μm	462.90	496.53	497.85	47.150	0.806
Crypt depth, μm	55.80	60.01	62.21	3.791	0.371
Villus/crypt	8.30	8.26	8.01	0.510	0.592
L21					
Villus height, μm	300.33	283.43	306.26	10.250	0.466
Crypt depth, μm	132.20	138.76	123.11	8.020	0.526
Villus/crypt	2.27	2.05	2.49	0.200	0.138
Ileum					
L1					
Villus height, μm	463.37	498.41	532.63	40.730	0.826
Crypt depth, μm	48.44	46.87	50.10	1.090	0.890
Villus/crypt	9.56	10.62	10.63	1.070	0.891
L21					
Villus height, μm	322.93[b]	308.17[c]	378.63[a]	13.710	0.048
Crypt depth, μm	91.21	82.71	101.60	5.310	0.600
Villus/crypt	3.54	3.72	3.73	0.150	0.249

The superscript letters (a, b, c) indicate that there are significant differences between the groups ($P < 0.05$). L1, day 1 of lactation; L21, day 21 of lactation.

(62). Studies have shown that HS affected the structural integrity and barrier function of piglet intestines through maternal effect directly and colostrum intake indirectly (10, 61). In the current study, we investigated the vertically transmitted effect of maternal effect on offspring's small intestinal health. Maternal supplementation with 1.0 g/kg of EA effectively alleviated the damage of HS to the small intestine of offspring by increasing intestinal villus height. However, Niu et al. (46) reported that diet supplemented with 2.0 g/kg of EA (but not 1.0 g/kg of EA) decreased crypt depth and increased villus height and villus height to crypt depth ratio in both the jejunum and ileum of piglets. The different results between these two studies indicates the optimal dose of EA for weaning piglets and sows is controversial. Different from the direct effect of EA on the gut health of sows, EA could indirectly protect mucosal health in piglets by means of reducing oxidative and inflammatory stimulus caused by milk from sows (15). The above results suggested that the decreased oxidative and inflammatory stress in the colostrum and milk in this trial might be the reason why EA protected gut health in piglets.

CONCLUSION

Our present study demonstrated for the first time that dietary supplementation with enzymatically treated *Artemisia annua* L. at 1.0 g/kg exhibited beneficial effects on the lactation performance, serum hormone level, and inflammatory response of sows under HS and the gut health of their offspring (**Figure 1**). The results of this study provided a new perspective for alleviating the inflammatory response of sows, and at the same time supplied a new nutritional management strategy for alleviating the HS response in animal production.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding authors.

ETHICS STATEMENT

The animal study was reviewed and approved by South China Agricultural University Animal Care and Use Committee (Guangzhou, China) (SCAU-AEC-2010-0416).

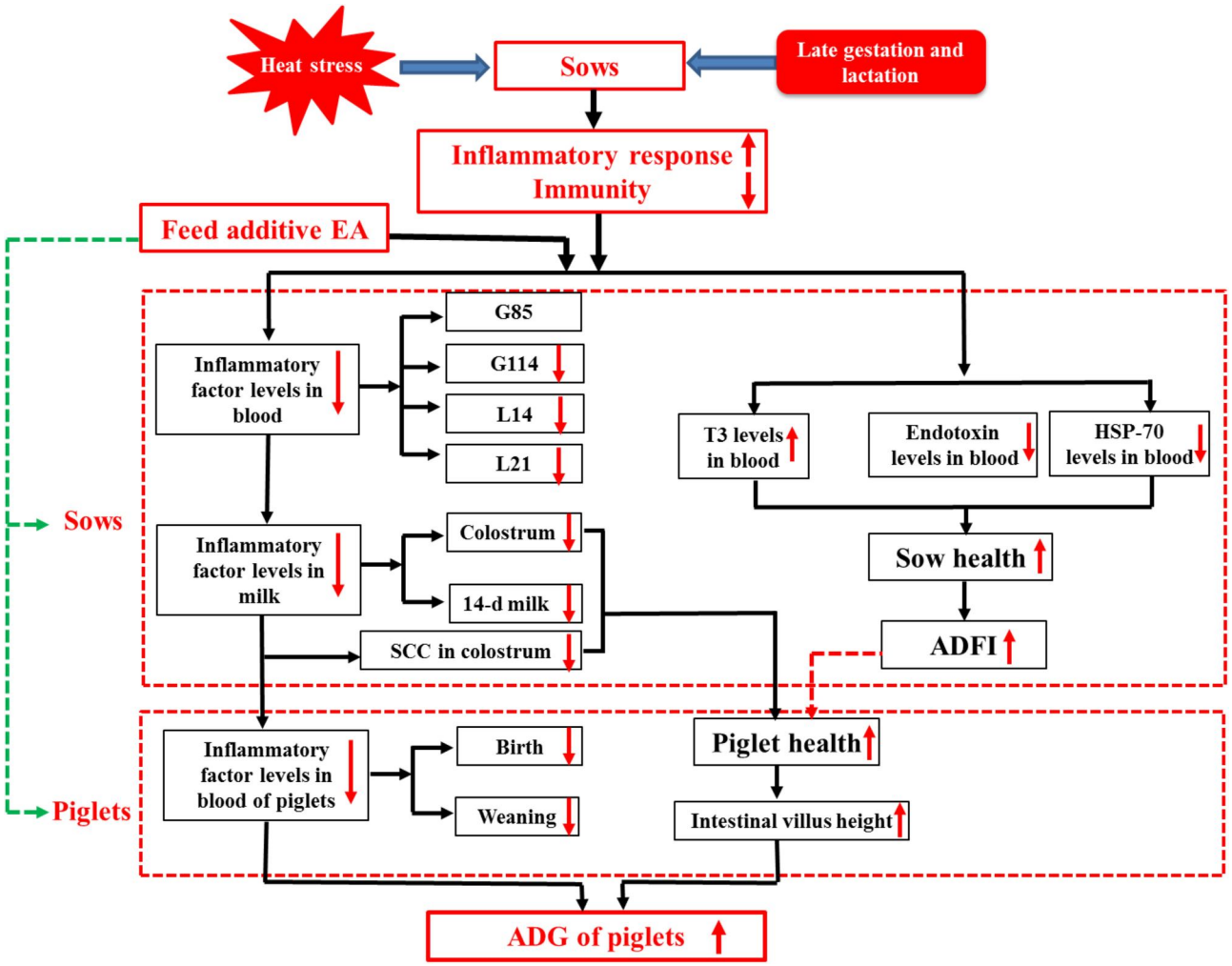

FIGURE 1 | Dietary enzymatically treated *Artemisia annua* L. (EA) supplementation at 1.0 g/kg during late gestation and lactation increased ADFI, decreased endotoxin and inflammatory factor levels in the serum and colostrum of heat-stressed sows during lactation, and increased ADG and improved the gut development of their offspring.

AUTHOR CONTRIBUTIONS

LX, WZ, FC, SZ, and WG designed the experiments. WZ, LX, HZ, and ZT performed the experiments. WZ analyzed the data and wrote the manuscript. FC, MR, WG, and SZ conducted the final proofreading. All authors have read and agreed to the published version of the manuscript.

FUNDING

This study was financially supported by the National Key R&D Program of China (No. 2021YFD1300700), the National Natural Science Foundation of the P. R. of China (Nos. 31872364 and 31802067), Guangdong Basic and Applied Basic Research Foundation (No. 2021A1515010440), Science and Technology Program of Guangzhou (No. 202102020056), and Major Science and Technology Projects in Anhui Province (201903a06020002).

ACKNOWLEDGMENTS

Special thanks for the Guangzhou Cohoo bio-tech Research & Development Center (Guangzhou, China) for their strong support.

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Check for updates

Received: 18 March 2022 | Accepted: 15 July 2022

DOI: 10.1002/jcp.30841

REVIEW ARTICLE

Journal of Cellular Physiology WILEY

A critical role of AMP-activated protein kinase in regulating intestinal nutrient absorption, barrier function, and intestinal diseases

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Funding information
National Natural Science Foundation of the P.R. of China; Guangdong Basic and Applied Basic Research Foundation; Science and Technology Program of Guangzhou

Abstract

As one of the most important organs in animals, the intestine is responsible for nutrient absorption and acts as a barrier between the body and the environment. Intestinal physiology and function require the participation of energy. 5′-adenosine monophosphate-activated protein kinase (AMPK), a classical and highly expressed energy regulator in intestinal cells, regulates the process of nutrient absorption and barrier function and is also involved in the therapy of intestinal diseases. Studies have yielded findings that AMPK regulates the absorption of glucose, amino acids, and fatty acids in the intestine primarily by regulating transportation systems, as we detailed here. Moreover, AMPK is involved in the regulation of the intestinal mechanical barrier and immune barrier through manipulating the expression of tight junctions, antimicrobial peptides, and secretory immunoglobulins. In addition, AMPK also participates in the regulation of intestinal diseases, which indicates that AMPK is a promising therapeutic target for intestinal diseases and cancer. In this review, we summarized the current understanding regarding how AMPK regulates intestinal nutrient absorption, barrier function, and intestinal diseases.

KEYWORDS

AMPK, intestinal barrier, intestinal diseases, intestine, nutrition absorption

1 | INTRODUCTION

The intestine is mainly responsible for absorption function and barrier function and is critical for animals to obtain adequate nutrition and resist diseases. As a key nutrient sensor, the intestine can sense (Everard et al., 2014) and regulate amino acid, fatty acid, and glucose (Dyer et al., 2003) absorption, which are considered as the most crucial macronutrients for animals. In addition to regulating nutrient absorption, the intestine also acts as a mechanical barrier and/or an immune barrier to maintain the integrity of the intestinal tract and prevent pathogen invasion. The mechanical barrier mainly functions through the expression of tight junction proteins, while the immune

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barrier mainly depends on the secretion of antimicrobial peptides and secretory immunoglobulins.

Recent advance indicates multiple signaling pathways involved in the regulation of intestinal nutrient absorption and barrier function, and 5'-adenosine monophosphate-activated protein kinase (AMPK) is one of them. AMPK is a heterotrimer, which comprised of catalytic subunits (α1 and α2) and regulatory subunits (β1, β2 and γ1, γ2, γ3; B. Xiao et al., 2011).

The dominant intestinal AMPK subunits are varied among species, which are responsible for maintaining intestinal homeostasis and preventing intestinal diseases. In mice, AMPKα1 is an important factor in maintaining the integrity of gastrointestinal barrier and protecting the intestine from injury (Deng et al., 2018; Hayes et al., 2021). In weaned piglets, both AMPKα1 and AMPKα2 are expressed in the small intestine which involved in maintaining water and ion homeostasis (He et al., 2017). In rodents, AMPKα1 and γ1 are closely associated with the biological function of intestinal epithelium and are considered as therapeutic targets to prevent cellular senescence and radiation ulcers (Z. Wang et al., 2019). In addition, in humans, the subunit composition of AMPK (α2β2γ1) is highly correlated with the survival of colorectal cancer cells (Fisher et al., 2015). Taken together, different subunits have various effects on intestinal function, and some subunits (such as AMPK beta) have not been understood well and warrant more research.

Mechanistically, AMPK is activated (phosphorylation at Thr172) in response to cellular AMP level, upstream kinase stimulation, and drug stimulation (5-aminoimidazole-4-carboxamide1-β-D-ribofuranoside (AICAR) or metformin; McFadden & Corl, 2009) and stressful conditions such as hunger. Since AMPK is an important energy sensor in eukaryotic cells, its activation indicates an energy-deficient state in cells, which might trigger the transportation of energy-supplying substances (Carling, 2017). Given amino acids (Suzuki et al., 2021), fatty acids (Turner et al., 2014), and glucose (Sylow et al., 2017) are important energy sources, AMPK signaling is proposed to regulate intestinal nutrient absorption. Furthermore, AMPK has been shown to regulate intestinal barrier function (Mihaylova & Shaw, 2011; Sun et al., 2017). In addition to the functions mentioned above, AMPK also has great potential to be a therapeutic target of intestinal diseases, which cause life-threatening damage to humans around the world. In this review, we will focus on the current understanding of intestinal AMPK in regulating nutrient absorption and barrier function, as well as its potential role in the treatment of intestinal diseases.

2 | AMPK REGULATES INTESTINAL NUTRIENT ABSORPTION

2.1 | Glucose

Glucose, as a critical energy source, has a predominant effect on the regulation of cellular energy balance (Deng & Yan, 2016). It is well known that glucose is absorbed directly into the bloodstream through mesenteric cells located in gut (Tricò et al., 2019). AMPK, as an energy sensor and regulator in intestinal cells, has been demonstrated to regulate glucose absorption. For instance, metformin, a common AMPK activator, has been reported to increase glucose uptake in the small intestine and colon of rats (Koffert et al., 2017). It also promoted intestinal glucose utilization through the activation of anaerobic glucose metabolism (Bailey et al., 1994). In addition, the other AMPK activator AICAR also enhanced net glucose uptake in mouse jejunum, which might be related to phosphorylation of p38 mitogen-activated protein kinase (Walker et al., 2005). Besides AMPK agonists, some biologically active substances also regulate the glucose absorption through AMPK activation. For example, consuming long-chain n-3 polyunsaturated fatty acids during pregnancy increased intestinal glucose absorption through acute activation of AMPK (Gabler et al., 2009). However, resveratrol (RSV) was observed to increase glucose transport in the small intestine of pigs, which may be associated with the increased phosphorylation of AMPK (Klinger & Breves, 2018). In general, AMPK activation induced by agonists or biologically active substances accelerates intestinal glucose uptake.

Glucose absorption is dependent on glucose transporters, which are mainly divided into three categories: glucose transporters (GLUTs), sodium-dependent glucose transporters (SGLTs), and the recently identified Sugars Will Eventually be Exported Transporters (SWEETs; Chen et al., 2015). GLUTs are a group of transmembrane transporters, which mainly mediate glucose uptake through a diffusion process along a concentration gradient (Wright et al., 2011). Currently, 14 GLUT family members (GLUT1-14) have been identified in humans (Thorens & Mueckler, 2010). SGLTs are mainly expressed in the intestine and renal cortices (Yan, 2015). As high-affinity transporters, SGLTs are responsible for the glucose uptake through the apical membrane of intestinal cells, using the energy released by Na+ flow (Wood & Trayhurn, 2003). Because SWEETs are mainly existed in plants, but have not been identified in mammals so far, it will not be discussed in this review.

In the intestine, GLUT2 and SGLT1 are considered as the dominant glucose transporters in their families (Abbasi et al., 2016). SGLT1 is located in the brush-like boundary membrane of intestinal cells (Hediger et al., 1987). While the location of GLUT2 mainly depends on the concentration of glucose in the intestinal lumen (Ferraris et al., 2018; Koepsell, 2020; Stelmańska, 2009). When glucose concentration is low, GLUT2 is the major basolateral glucose transporter subtype (Mueckler & Thorens, 2013), which is responsible for transporting intracellular glucose to blood vessels. Whereas when glucose concentration is high, GLUT2 is partially translocated to the brush-like boundary membrane of the cell and collaborates with SGLT1 for the maximum glucose uptake (Mueckler & Thorens, 2013).

Upon activation, AMPK increases the localization and expression of GLUT2 in the apical membrane of intestinal epithelial cells (IECs; Sakar et al., 2010; Walker et al., 2005). Additionally, in mice, AMPK activation increased jejunum GLUT2-mediated glucose uptake, which does not require ATP to facilitate transport (Walker et al., 2005; Ward et al., 2014). Intriguingly, AMPK activation reduces the activity and

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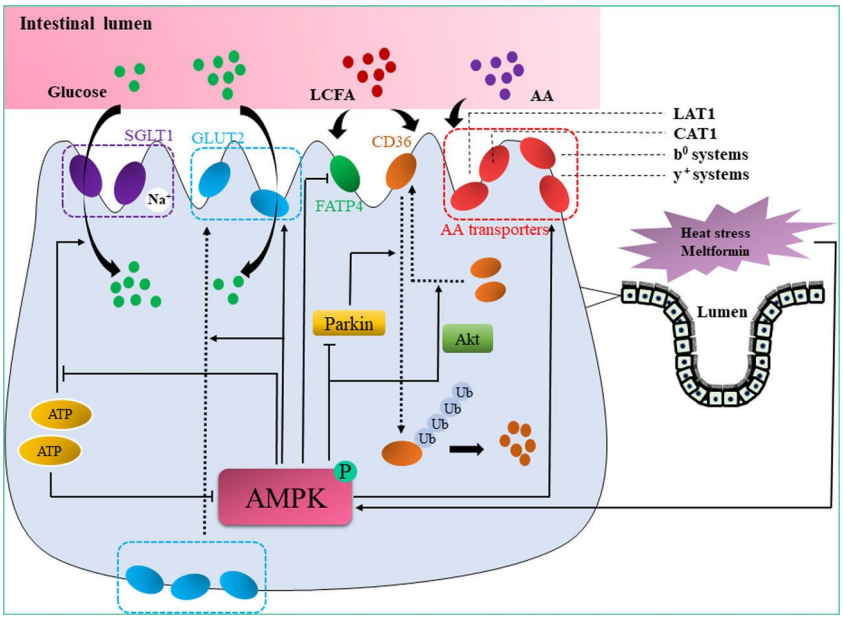

FIGURE 1 AMPK regulates nutrient (glucose, amino acids, and fatty acids) absorption in intestinal epithelial cells. AMPK activation upregulated GLUT2 expression, but decreased SGLT1 expression to reduce the energy supply. In addition, AMPK activation promotes the expression of amino acid transporters. For fatty acids, AMPK promotes the colonization of CD36 and inhibits its ubiquitination, thereby promoting the uptake of LCFAs, whereas AMPK activation inhibits FATP4 expression. LCFA, long-chain fatty acid.

expression of the ATP-dependent transporter SGLT1 (Bravard et al., 2021; Sakar et al., 2010; Ward et al., 2014). Taken together, AMPK activation upregulates the expression of GLUT2 but downregulates the expression of SGLT1 in the intestine. However, the underlying mechanism is still unclear. A possible model is when AMPK is activated, the animal body receives a signal of "energy deficiency," thereby increasing activities of glucose transporter (GLUT2) that does not require ATP consumption and reducing the activities of energy-consuming one (SGLT1). In addition, there is a compensatory mechanism by which GLUT2 can partially compensate for the lack of glucose absorption mediated by SGLT1 in animals (Chaudhry et al., 2012).

2.2 | Amino acids

As building blocks for protein synthesis (Battezzati & Riso, 2002), amino acids are mainly absorbed in the small intestine (Pappenheimer, 1993). Amino acid absorption efficiency highly depends on the activity of the amino acid transporters, and recent advance indicates that AMPK activation stimulates intestinal amino acid absorption with the upregulation of amino acid transporters (Chopra et al., 2011). AMPK activation enhances the mRNA level of L-type amino acid transporter 1 (LAT1), which mainly transports neutral amino acids, including branched amino acids (BCAAs; Hodson et al., 2017). While AMPK inhibition decreases the mRNA level of LAT1 and sodium-dependent neutral amino acid transporter 2 (SNAT2; Yamamoto et al., 2017). In vivo, when broiler chickens suffer from chronic heat stress (with subsequent activation of AMPK), the amino acid transporters (y + LAT1 and cationic amino acid transporter 1(CAT1)) in their jejunum are significantly increased (Al-Zghoul et al., 2019). Moreover, dietary lysine concentration is positively related to AMPK activation (Yin et al., 2018). Dietary lysine supplementation in pig diet increases CAT-1 expression in the small intestine (X. Q. Wang et al., 2012), indicating that lysine may trigger AMPK to promote intestinal CAT-1 expression. A similar situation also occurred in chickens, where increased dietary lysine upregulated the expression of the b0 and y + systems (Bröer, 2008; Torras-Llort et al., 1998). Collectively, AMPK activation could upregulate the expression of amino acid transporters in the intestine.

2.3 | Fatty acids

Digestion and absorption of dietary lipids is a complex multistep process that begins in the stomach and ends in the small intestine. Research has shown that long-chain fatty acid (LCFA) translocases were detected in the duodenum, jejunum, and ileum, with the highest expression in the jejunum (Poirier et al., 1996). There is increasing evidence that demonstrated that AMPK enhances intestinal lipid absorption (Chopra et al., 2011). Here, we discussed the mechanism by which AMPK regulates intestinal lipid absorption. In existing studies, the most studied LCFA transporters in rodents and human intestines are CD36 and FATP4 (Cifarelli & Abumrad, 2018). As a transmembrane protein widely distributed in various tissues (Yang et al., 2017), CD36 is mainly involved in lipid uptake, especially for LCFAs (Silverstein & Febbraio, 2009). Mechanistically, CD36 exodomains bind closely to LCFAs to form the CD36-LCFA complex, which facilitates LCFA transfer to the plasma membrane through internal channels (Hsieh et al., 2016). Additionally, intestinal CD36 regulates lipid-mediated satiety (Naville et al., 2012) and releases intestinal peptides (Kuda et al., 2013), which may promote intestinal uptake of LCFAs. AMPK activation in IECs promoted LCFA uptake by upregulating CD36 membrane transport through Akt signaling and inhibiting CD36 ubiquitination, revealing the role of AMPK in regulating intestinal LCFA uptake (W. Wu, Wang, et al., 2020). The details of the above mechanism are shown in Figure 1.

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FATPs, a family of membrane proteins originally identified from mouse adipose cells, also contribute to the uptake of LCFAs (Schaffer & Lodish, 1994). There are six members of FATPs in humans (FATP1–6; Hirsch et al., 1998), among which only FATP4 is significantly expressed at the apex of mature intestinal cells (Stahl et al., 1999). As an obesity marker in humans (Gertow et al., 2006), FATP4 promotes the uptake of LCFAs and very-long-chain fatty acids (VLCFAs) by intestinal cells (Schreck & Melzig, 2018). In isolated enterocytes, a 48% deletion of FATP4 resulted in a 40% reduction in LCFA uptake (Gimeno et al., 2003), indicating that FATP4 is a dominant intestinal LCFA transporter. AMPK activation has been shown to upregulate FATP4 expression in the placenta (Ma et al., 2011; O'Tierney-Ginn et al., 2015), blastocyst (Schindler et al., 2017), adipose tissue (Fritzen et al., 2015), and muscle (Bonen et al., 2007). In the intestine, however, the situation is different. An experiment showed that metformin can restore the high expression of FATP4 in the jejunum and ileum induced by high-fat and high-sucrose diets to basal levels (Bravard et al., 2021). Moreover, in energy-deficient piglets, intestinal AMPK was significantly activated and FATP4 expression was significantly decreased compared to piglets with adequate energy intake (Tian et al., 2020). In summary, even although AMPK positively regulates LCFA uptake in IECs, whereas its effect on FA transporter seems to depend on the specific type of FA transporter. More studies are needed to uncover the detailed mechanism.

3 | AMPK REGULATES INTESTINAL BARRIER FUNCTION

3.1 | Intestine mechanical barrier

The intestinal mechanical barrier is also known as a physical barrier, which is based on the mucosal epithelium, lamina propria, and muscularis mucosa. In the intestinal epithelium, tight junctions (TJs) tightly bind epithelial cells to protect the intestinal tract from the outside world (Rowart et al., 2018), which is of great importance for maintaining the intestinal mechanical barrier. The TJ family consists of the zonula occludins (ZO) proteins (Stevenson et al., 1986) and transmembrane proteins (including claudins (Furuse et al., 1998), occludins (Furuse et al., 1993) and junctional adhesion molecules (JAMs; Martìn-Padura et al., 1998)). It is now clear that claudins contain 24 members (Morita et al., 1999), JAMs have three subtypes (JAM-A [Martìn-Padura et al., 1998], JAM-B [Aurrand-Lions et al., 2001], and JAM-C [Aurrand-Lions et al., 2000]), while occludins have two subtypes (Muresan et al., 2000). These three types of transmembrane proteins are embedded in the cell membrane of IECs, and transmembrane proteins of the same or adjacent cells are connected to each other through the ZO-1 protein, resulting in a tight connection between epithelial cells that effectively blocks the entry of bacteria, viruses, and endotoxins (Rowart et al., 2018).

AMPK plays a critical role in regulating epithelial barrier function (Zhu et al., 2018), specifically by regulating tight junction assembly. For example, AMPK activation enhances intestinal barrier function by promoting the assembly of tight junctions, (Nozu et al., 2019; Peng et al., 2009; Zhu et al., 2018). In addition, AMPK activation also regulates epithelial barrier function through upregulating tight junction protein expression, such as ZO-1, occluding, and claudin1 (Rowart et al., 2018; W. Wu, Wang, Liu, Shan, et al., 2018). For instance, metformin supplementation improves barrier function in interleukin (IL-10)-deficient mice because AMPK activation increases the expression of barrier-promoting Claudin-3 in the ileum (Xue et al., 2016). Furthermore, AMPK activated by L-glutamine upregulates the levels of occludin, Claudin-3, claudin4, ZO-1, ZO-2, ZO-3, and JAM-A (B. Wang et al., 2016).

It is worth noting that intestinal AMPK activation is closely related to the intestine microbiome. As an important part of intestinal microecology, probiotics also have a significant effect on intestinal barrier function. In a recent study, lesions scores of duodenum and jejunum were significantly reduced in broilers fed probiotics compared with the control group. And the expression level of Claudin-3 was higher, and the expression level of Occluden-2 tended to increase. Importantly, the mRNA expression of AMPK-α1 in jejunum of broilers fed probiotic was significantly higher than that of the control group (Emami et al., 2019). This study showed that probiotic supplementation reduced lesions and increased TJs expression with the activation of AMPK signaling. However, it is still unclear whether intestinal microbes directly activate intestinal AMPK signaling or indirectly through its metabolites. Intriguingly, AMPK activator might directly regulate gut microbes. A well-organized study reported that metformin (a highly effective AMPK activator) has a metabolic binding point with intestinal flora at the intestinal mucosal interface (Jones & Molloy, 2021). These preliminary evidence indicate there might be an intimate relationship between AMPK signaling and intestine microbiome.

Collectively, all of the evidence indicate that AMPK activation positively regulates the intestinal mechanical barrier by promoting tight junction assembly and tight junction protein expression, as shown in Figure 2.

3.2 | Intestine immune barrier

The intestinal immune barrier is also called the intestinal mucosal immune barrier, as its function mainly depends on the intestinal mucosa. The intestinal mucosa has selective permeability, which not only absorbs nutrients, but also creates a barrier between the external and the internal environment (Turner, 2009). The crucial components of the intestinal immune barrier include antimicrobial peptides (AMPs) and secretory immunoglobulins (SIgs).

AMPs are cationic molecules that are involved in the regulation of innate immunity (Pasupuleti et al., 2012; H. Yi et al., 2016). In addition, AMPs play a crucial role in defending against intestinal

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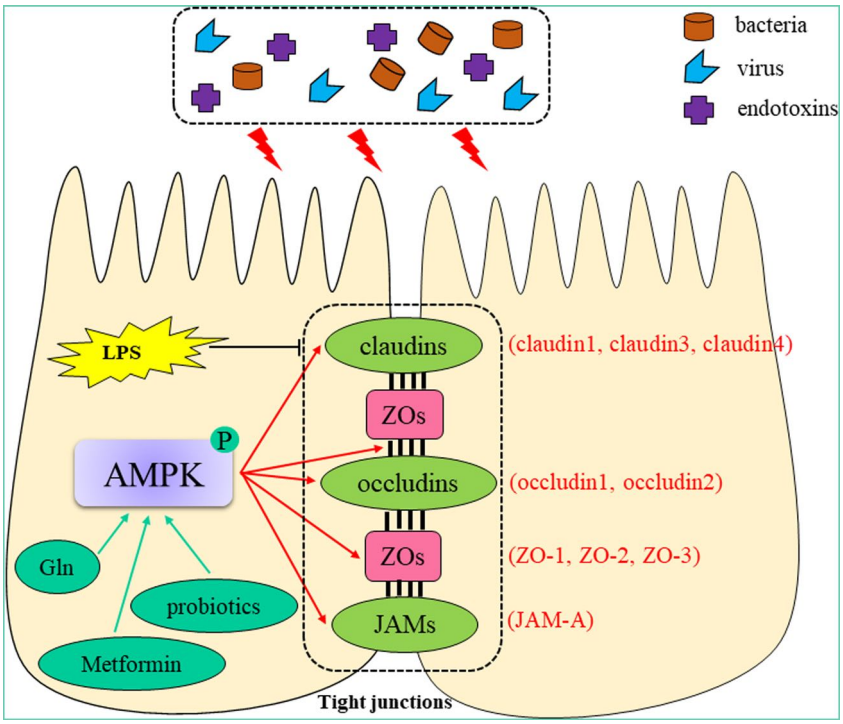

FIGURE 2 AMPK activation positively regulates the intestinal mechanical barrier by promoting tight junction assembly and tight junction protein expression. AMPK activated by drugs, amino acids, or probiotics promotes the assembly of tight junctions and promotes the expression of tight junction proteins (ZOs, claudins, occludins, and JAMs) to resist the invasion of external pathogens.

pathogens (Salzman et al., 2010). The available evidence has revealed that AMPK activation is involved in the regulation of AMP expression (Rekha et al., 2015). The level of AMPs goes down with a decrease in AMPK phosphorylation, suggesting that AMPK activation appears to upregulate AMP expression (Gonzalez et al., 2019). In addition, in mouse models, AMPK deletion decreased the ability of AMPs to alleviate intestinal damage (W. Wu, Wang, Liu, Wang, et al., 2018). Furthermore, AMPs mitigated the attack of LPS on the host depending on the activation of the LKB1-AMPK pathway (W. Wu, Wang, Liu, Wang, et al., 2018). In summary, AMPK activation positively regulates the expression of AMPs.

Defensins, which belong to AMPs, are widely expressed in neutrophils and epithelial cells and act as a natural immune defense system in animals (Zhao & Lu, 2014). Based on the disulfide topology, mammalian defensins are divided into three subfamilies, α, β, and θ-defensins (Zasloff, 2002). Notably, human β-defensin-1 (hBD1) is significantly expressed and constitutively secreted in the intestinal epithelium (Zheng et al., 2015). In IEC lines, the constitutive expression of hBD1 is dependent on basic HIF-1α signaling (Kelly et al., 2013). Interestingly, HIF-1α interacts with other oxygen response signaling pathways, such as AMPK (Yan et al., 2012), indicating that AMPK might indirectly regulate hBD1 expression and secretion through AMPK-HIF-1α signaling. Low levels of α-defensin and β-defensin due to reduced numbers of Paneth cells can be upregulated by metformin in a mouse model (Xue et al., 2016). BCAAs are reported to increase the β-defensin content (Bifari et al., 2017). Intriguingly, AMPK activation also promotes β-defensin expression with inhibition of the catabolic activity of BCAAs (Lian et al., 2015). According to the existing literature, we preliminarily concluded that AMPK activation positively regulates defensin expression and secretion.

Secretory immunoglobulin A (SIgA), a dimer antibody, is the most abundant immunoglobulin in mucosal secretions (Macpherson et al., 2012). SIgA binds to the polymeric Ig receptor (pIgR) on the basolateral surface of IECs, which is then released into the lumen and interacts with various intestinal antigens, thereby limiting the ability of symbiotic bacteria and soluble antigens to attack the intestinal epithelium (Kabat et al., 2016). Importantly, SIgA plays an irreplaceable role in maintaining intestinal homeostasis by regulating the immune response through a variety of mechanisms (Corthésy, 2013a, 2013b). AMPK has been reported as a crucial regulator of SIgA secretion. For instance, GABA-activated AMPK might promote the secretion of SIgA to attenuate ETEC-induced intestinal damage (Xia et al., 2019; Zhao et al., 2020). Furthermore, it has been reported that the ileum mucosa SIgA content of pigs fed pectin (PEC) was significantly increased (W. Wu, Zhang, et al., 2020). It is because the fermentation of PEC by intestinal microflora will produce a large number of short-chain fatty acids, which could further activate AMPK (Elamin et al., 2013; Pongkorpsakol et al., 2017; Shtriker et al., 2018). Glutamine (Gln) supplementation increased the abundance of SIgA in the intestinal tract of a variety of hosts, including rats (Shu et al., 2014; Takechi et al., 2014), mice (Fan et al., 2009; Santos et al., 2014), and pigs (Zou et al., 2010). This might be due to Gln activates CaMKK2-AMPK signaling pathways (B. Wang et al., 2016). Mechanistically, Gln improves the $Ca2^{+}$ uptake, which finally activates AMPK through the $Ca2^{+}$/calcium-sensing receptor (CaSR)/CaMKK2 pathway (Jouret et al., 2013). Conversely, when $Ca2^{+}$ is consumed or CaMKK2 is inhibited, the effect of Gln on AMPK activity is apparently

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weakened (B. Wang et al., 2016). Taken together, AMPK activation promoted the expression of SIgA in the intestine.

4 | AMPK IS A POTENTIAL TARGET FOR THE THERAPY OF INTESTINAL DISEASES

The intestine is a significant immune organ, and impaired barrier function can lead to inflammation and intestinal diseases (Camilleri et al., 2012). Common intestinal diseases include inflammatory bowel disease (IBD) and cancer, which take a heavy toll on people's economic burden and health. More than 3.5 million people in the United States and Europe suffer from IBD, and the number continues to increase in Asia and South America, according to a 2015 survey (Kaplan, 2015). Even more threatening than IBD is gastric cancer (GC), which is one of the most common cancers in both men and women worldwide, with a mortality rate of 70%, far higher than prostate cancer and breast cancer (Machlowska et al., 2020). Similar to GC, colorectal cancer is a common cancer with an increased incidence with age, and up to 20% of patients have metastatic disease (such as metastasis to the liver) at the time of diagnosis (Haraldsdottir et al., 2014). Considering the dangers of intestinal diseases, research on new treatments and drugs has become an urgent task worldwide. A large number of studies have shown that AMPK agonists (such as metformin, phenformin, AICAR, and A769662) can inhibit or delay tumorigenesis in animal models, revealing that AMPK may be a promising target for cancer therapy (Luo et al., 2010). Thus, the AMPK signaling pathway not only plays an important role in the energy balance of intestinal cells, but also involved in the prevention and treatment of intestinal diseases (Meng et al., 2018).

4.1 | Inflammatory bowel disease

IBD is classified as ulcerative colitis (UC) and Crohn's disease (CD), which is characterized by chronic and recurrent episodes (Nishida et al., 2018). It has been found that specific loss of AMPK villin worsens colitis (Sun et al., 2017), indicating that the presence of AMPK is critical to alleviate colitis. Correspondingly, the AMPK activator AICAR is effective in the treatment of acute and recurrent experimental colitis because AMPK activation may downregulate the immune response of colitis in mice (Bai et al., 2010). Similarly, metformin-activated AMPK significantly controlled the progression of colitis in mice by maintaining the integrity of tight junctions in the colonic epithelium (Chen et al., 2018). Interestingly, in addition to the typical AMPK agonists, other compounds or plant extracts can also activate AMPK to alleviate IBD, as described in Table 1 (Chang & Kuo, 2015; Gurung et al., 2020; Park et al., 2019; Takahara et al., 2019; H. T. Xiao et al., 2019; Zhao et al., 2015). It is worth mentioning that graphene oxide induces apoptosis of IECs by activating the ROS/AMPK/p53 pathway (Liu et al., 2021). It indicates the risk of graphene oxide exposure for patients with IBD from the perspective of prevention. Collectively, these findings suggest that AMPK is a promising target for the prevention and treatment of IBD.

4.2 | Gastric cancer

GC is a widely spread and an intractable disease worldwide. Therefore, how to effectively prevent gastrointestinal cancer has attracted the great attention of medical experts for a long time. A large number of studies have reported that GC and AMPK are closely related. For example, lncRNA MACC1-AS1 is a valuable biomarker for the diagnosis and prognosis of GC, and its metabolic process is thought to be coordinated by the AMPK/Lin28 pathway (Zhao et al., 2018). Moreover, hepatocyte nuclear factor-4 α (HNF-4α), a key signaling pathway in the development of GC, is downregulated by metformin-activated AMPK (Chang et al., 2016). Importantly, AMPK agonists have a positive effect on GC therapy. AICAR has been shown to be a chemotherapeutic sensitizer for GC due to its ability to induce apoptosis of GC cells (Y. Wu et al., 2016). Metformin also induced apoptosis of p53-deficient HCT116 colon cancer cells and inhibited the growth of colon cancer cell transplants (Buzzai et al., 2007).

In addition to AMPK agonists, other biologically active substances also effectively promote GC apoptosis and inhibit GC by activating AMPK. For instance, tetramethylpyrazine (TMPZ)

TABLE 1 Additives that alleviate IBD via AMPK

Additives	Diseases	Involved pathways	References
Indigo naturalis	Colonic oxidative stress, colitis	AMPK/Nrf-2, STAT1/STAT3	Xiao et al. (2019)
Berberine	Colitis	AMPK	Takahara et al. (2019)
BJ-3105	Colitis	AMPK, NOX	Gurung et al. (2020)
6-Gingerol	Colitis	AMPK	Chang and Kuo (2015)
GL-V9	Colitis	AMPK/FOXO3a	Zhao et al. (2015)
Mesalazine	Colitis	AMPK, NF-κB	Park et al. (2019)

Note: Indigo naturalis, also known as Qing-dai, or QD, a traditional Chinese medicine; BJ-3105, a 6-alkoxypyridin-3-ol derivative; GL-V9, a new synthetic flavonoid derivative.

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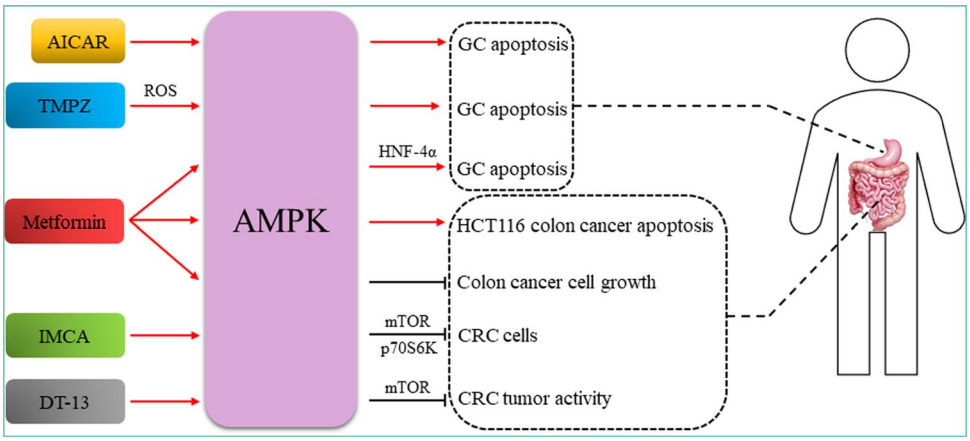

FIGURE 3 Overview of the relationship between AMPK activation and gastrointestinal cancers. CRC, colorectal cancer; DT-13, short lilli saponin monomer 13; GC, gastric cancer; IMCA, 2-imino6-methoxy-2 h-chromene-3-carbothioamide; TMPZ, tetramethylpyrazine.

induced activation of the ROS/AMPK pathway and promoted GC cell apoptosis through mitochondrial apoptosis (B. Yi et al., 2013). Likewise, the benzopyran derivative 2-imino6-methoxy-2 h-chromene-3-carbothioamide (IMCA) has been observed to significantly inhibit the viability of CRC cells by upregulating the activity of AMPK and downregulating the mTOR/p70S6k signaling pathway (Zhang et al., 2020). Moreover, the short lilli saponin monomer 13 (DT-13) inhibited CRC tumor activity both in vivo and in vitro, which seemed to be related to AMPK activation and mTOR inhibition (Wei et al., 2019). It is widely known that mTOR activation is positively related to cancer progress. Thus, AMPK might inhibit the GC and CRC tumor through the inhibition of mTOR. In addition, some preliminary evidence indicate AMPK agonist might inhibit CRC through the binding point in intestinal microbiota (Jones & Molloy, 2021). However, the mechanism by which AMPK regulates CRC still needed to be further elucidated. Overall, there is substantial evidence that AMPK is involved in the regulation of various gastrointestinal diseases, as summarized in Figure 3. Thus, AMPK has broad therapeutic potential for curing intestinal diseases.

5 | CONCLUSION

The intestine is indispensable for nutrition absorption and barrier function. AMPK plays an important role in these functions and serves as a potential therapeutic target for intestinal diseases. The current review focused on intestinal glucose uptake and found that AMPK activation promotes glucose uptake through a seemingly contradictory mechanism: upregulation of GLUT2 and downregulation of SGLT1 expression. In addition, AMPK activation and intestinal AA transport are positively related. Intriguingly, AMPK activation upregulates the expression of CD36 but downregulates the expression of FATP4, which are both dominant intestinal LCFA transporters. It is worth noting that current reports on AMPK regulating intestinal FAT/CD36 and LCFA uptake are relatively lacking, and the mechanism remains to be further explored. Accumulating evidence has demonstrated that AMPK has a positive effect on both the intestinal mechanical barrier and the intestinal immune barrier by promoting TJ, AMP, and SIgA expression. However, these findings are obviously inadequate and incomplete. It would be of interest to deeply reveal the role of AMPK and its upstream and downstream signaling molecules in the regulation of intestinal function. To date, most investigations have indicated that AMPK plays a pivotal role in the prevention and treatment of IBD and GC. Understanding the effects of AMPK on intestinal diseases might provide the therapeutic target for the treatment of intestinal diseases.

AUTHOR CONTRIBUTIONS

Shihai Zhang initiated the idea, the scope, and the outline of this review paper. Zhihui Wu, Chengfei Xu, Siwang Yang, Tenghui Zheng, Qihui Li, and Jiayuan Shao studied and analyzed all of the publications cited in this paper and were involved in the manuscript preparation. Wutai Guan and Shihai Zhang conducted the final editing and proofreading. All authors read and approved the final manuscript. All authors have read and agreed to the published version of the manuscript.

ACKNOWLEDGMENTS

This study was financially supported by the National Natural Science Foundation of China (Nos. 31872364 and 31802067), Guangdong Basic and Applied Basic Research Foundation (No. 2021A1515010440), Science and Technology Program of Guangzhou (No. 202102020056).

CONFLICT OF INTEREST

The authors declare no conflicts of interest.

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How to cite this article: Wu, Z., Xu, C., Zheng, T., Li, Q., Yang, S., Shao, J., Guan, W., & Zhang, S. (2022). A critical role of AMP-activated protein kinase in regulating intestinal nutrient absorption, barrier function, and intestinal diseases. *Journal of Cellular Physiology*, 237, 3705–3716. https://doi.org/10.1002/jcp.30841

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frontiers in Veterinary Science

ORIGINAL RESEARCH
published: 16 August 2021
doi: 10.3389/fvets.2021.714068

Effects of Dietary Monoglyceride and Diglyceride Supplementation on the Performance, Milk Composition, and Immune Status of Sows During Late Gestation and Lactation

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Edited by:
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Specialty section:
This article was submitted to Animal Reproduction - Theriogenology, a section of the journal Frontiers in Veterinary Science

Received: 24 May 2021
Accepted: 23 July 2021
Published: 16 August 2021

Citation:
Song H, Chai W, Yang F, Ren M, Chen F, Guan W and Zhang S (2021) Effects of Dietary Monoglyceride and Diglyceride Supplementation on the Performance, Milk Composition, and Immune Status of Sows During Late Gestation and Lactation. Front. Vet. Sci. 8:714068. doi: 10.3389/fvets.2021.714068

Monoglyceride and diglyceride (MGDG) have antiviral and antibacterial properties and act as emulsifiers to increase dietary lipid digestibility. The primary aim of this trial was to investigate the effects of dietary MGDG supplementation on the reproductive performance and health status of sows during late gestation and lactation. One hundred sows (Landrace × Large White, mean parity of 4.59) were randomly allocated to groups receiving two different diets with 4% soybean lipids or 4% MGDG from day 85 of gestation to day 21 of lactation. Milk samples were collected on the day of farrowing (colostrum) and on day 14 of lactation, and blood samples were collected from the sows on days 0, 14, and 21 of lactation. Compared with control sows, sows fed MGDG showed no significant differences in reproductive performance ($P > 0.05$), but sow back fat thickness loss decreased during lactation ($P < 0.05$). There was a significant decrease in TNF-α concentrations in colostrum in the MGDG-supplemented sows compared with that in the soybean lipid-supplemented sows ($P < 0.05$). Dietary MGDG supplementation decreased sow plasma IL-8 concentrations on day 0 of lactation and IL-18 concentrations on days 14 and 21 of lactation ($P < 0.05$). Administration of MGDG increased the glucose and total cholesterol concentrations in sow plasma on day 14 and day 21, respectively ($P < 0.05$). The findings in this study suggest that MGDG supplementation could be effective in reducing back fat loss, decreasing inflammatory factor levels, and controlling total cholesterol (TCHO) concentrations during lactation.

Keywords: sow, monoglyceride and diglyceride, reproductive performance, milk composition, soybean lipids

INTRODUCTION

Monoglyceride (MG) and diglyceride (DG) are critical hydrolyzed products of dietary triglycerides. Under normal physiological conditions, the hydrolysis of dietary triglycerides starts from the stomach, and 5–40% of triglycerides are hydrolyzed by gastric lipase (1). These undigested triglycerides will be further hydrolyzed in the duodenum, which produces glycerols, MGs, DGs,

and free fatty acids (2, 3). After digestion, MGs, free fatty acids, and other lipids are associated with bile salts to form micelles for absorption.

MG and DG not only act as intermediate metabolites during triglyceride digestion but are also considered excellent water-in-oil (W/O)-type emulsifiers due to their specific amphiphilic molecular structures (hydrophilic glyceryl radicals and lipophilic alkyl radicals). Emulsifying agents have been found to promote the incorporation of fatty acids into micelles and to increase the digestibility of fat when added to the diets of rats and chicks (4, 5). The carbon chain length of fatty acids has been proposed to affect the formation of micelles (6). It is more difficult for long-chain fatty acids (LCFAs) to form micelles than medium-chain fatty acids (MCFAs) and short-chain fatty acids (SCFAs). Soybean lipids are widely used in animal diets and are mainly composed of LCFAs. To enhance the digestibility of dietary lipids, supplementation with emulsifiers has been reported as an efficient way to enhance the formation of micelles (7). Currently, a cocktail of monoglycerides and diglycerides (MGDG) is widely used as an emulsifier and/or stabilizer in the food industry (8, 9). Therefore, monoglycerides and diglycerides (MGDGs) may be efficiently utilized as energy sources to relieve weight loss and improve animal production performance.

In addition, monoglycerides have been known for a long time to have strong antiviral and antibacterial properties (10, 11). Monolinolein is reported as a more efficient antibacterial compound than its corresponding fatty acids (12). The sites of action on bacteria are the cell wall and cell membrane. Monoglycerides are reported to be incorporated into the lipid membrane and cause destabilization of the bilayer (13). For instance, monoglycerides have antibacterial activities against *Helicobacter pylori* (14). Both monolinolein and monolinolenin have antibacterial activity against Group B *Streptococcus* (12). Intriguingly, the antimicrobial and antiviral activities of MG and DG are partially dependent on the nature of fatty acids (15).

Sufficient energy intake and less bacterial infection are critical for sows to maintain their productive performance during late gestation and lactation. Previously, it has been reported that lipids can be used to increase the energy available to the sow during gestation and lactation. The addition of lipids to sow diets attenuates weight loss and improves the weight gain and survival of piglets (16, 17). Because MGDG is easily digested and exhibits antibacterial properties, we propose that MGDGs may have beneficial effects on the health status of sows. The aim of this study was to investigate the effects of dietary MGDG supplementation on sow performance, back fat thickness, milk composition, and inflammatory factors during late pregnancy and lactation.

MATERIALS AND METHODS

Animals and Experimental Design

The experimental procedures followed a protocol approved by the South China Agricultural University Animal Care and Use Committee (No. 20110107-1, Guangzhou, China). One hundred multiparous sows (Large White × Landrace, mean parity of 4.59) were selected for the study. The sows were categorized by parity, backfat thickness, and historical reproductive performance before being randomly assigned to two dietary treatment groups, a control group ($n = 50$) and an MGDG group ($n = 50$), to ensure that these characteristics were balanced between treatments.

This trial was conducted from gestation day (G) 85 to lactation day (L) 21 at a commercial pig farm. The control group diet included 4% soybean lipids, and the MGDG group diet included 4% MGDGs (containing 40% monoglycerides and 50% diglycerides; Guangzhou Jiadele Nutrition Technology Co., Ltd., Guangzhou, China). From days 85 to 109 of gestation, all sows were managed in the same gestation facility, which was a semi-open building. On G109, all sows were moved to farrowing crates. During the entire experimental period, the sows were assigned to a pad/fan-cooled farrowing house (for active cooling, AC), and the sows within each dietary treatment were fed the same experimental diet during both late gestation and lactation.

Diets and Management

Each diet was formulated to meet or exceed the nutrient requirements of late gestating and lactating sows (18). **Table 1** shows the diet compositions and nutrient levels. In the experimental diets, the 4% lipid source was either soy lipids or MGDGs. The fatty acid composition of the supplemental fat sources was analyzed by gas chromatography (GC-2010 Pro, SHIMADZU Corporation, Kyoto, Japan). Mono- and diglycerides were measured according to the AOAC official method [(19), 993.18]. The fatty acid profiles of soy lipids and MGDGs are described in **Table 2**.

The experiment began on day 85 of pregnancy. At this time, the sows were individually housed in gestation crates with a solid concrete floor and had free access to drinking water. Approximately 1 week before parturition, the sows were moved into the environmentally controlled farrowing house and individually housed in farrowing crates ($2.2 \times 2.4 \times 1.5\ m^3$) with a solid concrete floor and a piglet creep area with a heating lamp. The sows and the piglets had free access to water.

During gestion, the sows were fed 3.0 kg/d. The sows were not fed on the day of farrowing. After farrowing, the feed supply was increased by 1 kg/d until day 3 and then by 0.5 kg/d until day 6 of lactation. Afterward, the animals were allowed *ad libitum* consumption of the lactation diet, which was adjusted for each sow depending on daily intake. No creep feed was offered to piglets during the experiment. Piglets were cross fostered in the same dietary treatment within 24 h of birth so that piglets of similar weight were brought together. The litter size was adjusted and maintained at 10–12 piglets per sow.

Data and Sample Collection

Sow and Litter Performance

At farrowing, the total number of piglets [the numbers of live, weak (BW <0.8 kg), stillborn, and mummified piglets] and the litter weights were recorded. At weaning, the number of pigs weaned and the litter weight were measured.

TABLE 1 | Ingredients and chemical compositions of the diets (as-fed basis).

Item	Control diet	MGDG diet
Ingredient (%)		
Corn	43.8	43.8
Soybean meal, 43.0% CP	24.00	24.00
Barley, 11.33% CP	10.00	10.00
Wheat bran, 15.7% CP	6.00	6.00
Wheat flour, 15.3% CP	5.00	5.00
Fish meal, 62.8% CP	2.00	2.00
Soy lipids	4.00	
MGDG		4.00
Dicalcium phosphate	1.00	1.00
Vitamin and mineral premix[a]	4.00	4.00
Mold inhibitor	0.20	0.20
Total	100.00	100.00
Nutrient composition, unit		
DE, Mcal/kg	14.27	14.65
ME, Mcal /kg	13.72	14.27
NE, Mcal /kg	10.31	10.63
CP, %	17.93	17.93
EE, %	6.54	6.54
CF, %	3.31	3.31
Ca, %	1.00	1.00
Total P, %	0.74	0.74
Available P, %	0.50	0.50
Lys	1.04	1.04
Met + Cys	0.62	0.62
Thr	0.68	0.68
Trp	0.22	0.22
Digestible Lys, %	0.91	0.91
Digestible Met + Cys, %	0.51	0.51
Digestible Thr, %	0.57	0.57
Digestible Trp, %	0.19	0.19

[a]*Per kilogram of complete diet, the vitamin and mineral premix supplied the following: vitamin A, 10,000 IU; vitamin D3, 3,600 IU; vitamin E, 100 IU; vitamin K_3, 7.2 mg; vitamin B_1, 3 mg; riboflavin, 10.8 mg; vitamin B_6 5.4 mg; vitamin B_{12}, 0.06 mg; D-pantothenic acid, 36.0 mg; niacin, 60.0 mg; folic acid, 6 mg; biotin, 0.6 mg; copper, 10 mg (as $CuSO_4.5H_2O$); iron, 80 mg (as $FeSO_4$. H_2O); manganese, 30.0 mg (as $MnSO_4$); zinc, 80.0 mg (as $ZnSO_4$); selenium, 0.15 mg (as Na_2SeO_3); iodine, 0.14 mg [as Ca $(IO_3)_2$]; and cobalt, 0.1 mg (as $CoCl_2.6H_2O$).*

TABLE 2 | Fatty acid composition of the Soybean Lipids MGDGs.

Composition	Soybean lipids	MGDGs
C16:0	11.96	4.16
C18:1	23.83	2.54
C18:2	52.71	25.17
C18:3	4.81	0.76
C18:0	4.02	6.37
C16:0&C16:0	–	0.76
C16:0&C18:0	–	4.02
C16:0&C18:2	–	6.49
C18:1&C18:1-2	–	16.42
C18:2&C18:2-3	–	22.43
Others	2.67	0.98

Sow Back Fat Thickness

The back fat thickness of the sow at the P2 point (6.5 cm from the middle line of the last rib) was measured using a real-time ultrasound facility (Renco Lean-meter, Renco Corporation, Minnesota, USA) on days 0 and 14 of lactation.

Blood Sampling

At farrowing, on L14 and at weaning, 10 mL blood samples were taken from 10 sows (near the average BW and parity in each treatment group) by ear venipuncture using heparinized Vacutainer tubes (Sanli Medical Technology Development Co., Ltd., Hunan, China). Plasma was harvested after centrifugation at 3,000 × g for 10 min. After collection, each plasma sample was divided into three 0.5 mL samples (pipetted into 1 mL frozen tubes and immediately frozen in liquid nitrogen for immunoglobulin analysis) and a 2 mL sample (transferred to a 4 mL centrifuge tube and then stored at −80°C for cytokine and blood parameter analysis).

Colostrum and Milk Sampling

Colostrum and milk samples were taken from 10 sows (near the average BW and parity in each treatment group). Colostrum was sampled by hand expression from functional glands within 12 h post-partum without oxytocin injection. Fourteen-day milk was sampled after intramuscular injection of 20 IU oxytocin (Jiangxi Huiqifeng Bio-Technique Co., Ltd., Jiangxi, China).

Approximately 30 mL of sample was collected each time. After collection, the colostrum or milk was divided into two 15 mL centrifuge tubes and stored at −80°C for nutritional composition, immunoglobulin, and cytokine analyses.

Chemical Analysis

Colostrum and Milk Composition

The colostrum and milk samples were tested for solids-not-fat, fat, protein, and lactose using a fully automated milk analyzer (ULTRAMILER-UL40AC, Hangzhou Ultrasun Technologies Co., Ltd., Zhejiang, China).

Immunoglobulin Concentrations

The immunoglobulin A (IgA), immunoglobulin G (IgG), and immunoglobulin M (IgM) concentrations in colostrum and milk were analyzed by ELISA using pig immunoglobulin-specific kits (CUSABIO Biotech Company, Wuhan, China). Prior to analysis, the lipids in colostrum and milk were removed by centrifugation at 3,000 × g and 4°C for 20 min according to a method described previously (20). The ELISA procedure was as follows: (1) A blank well was prepared without any solution. (2) Fifty microliters of a standard or sample was added to each well. (3) Fifty microliters of HRP-conjugate was added to each well (not to the blank well). (4) The wells were incubated for 40 min at 37°C. (5) The wells were aspirated and washed 5 times. (6) Ninety microliters of TMB substrate was added to each well. The wells were incubated for 20 min at 37°C. (7) Fifty microliters of stop solution was added to each well. The wells were measured with a plate reader at 450 nm within 5 min.

Cytokine Concentrations

Tumor necrosis factor α (TNF-α), interleukin (IL)-8, and IL-18 levels in sow plasma, colostrum and milk were determined with ELISA kits (CUSABIO Biological Engineering Co., Ltd., Wuhan, China). The ELISA procedure was as follows: (1) One hundred microliters of a standard or sample was added to each well and incubated for 2 h at 37°C. (2) The liquid of each well was removed without washing. (3) Then, 100 μL of biotin-antibody (1x) was added to each well and incubated for 1 h at 37°C. (4) The wells were aspirated and washed 3 times. (5) Then, 100 μl of HRP-avidin was added to each well and incubated for 1 h at 37°C. (6) The wells were aspirated and washed 5 times. (7) Ninety microliters of TMB substrate was added to each well and incubated for 20 min at 37°C. (8) Fifty microliters of stop solution was added to each well. The wells were measured with a plate reader at 450 nm within 5 min.

Blood Parameter Concentrations

The concentrations of glucose (GLU), plasma urea nitrogen (PUN), triglycerides (TGs), total cholesterol (TCHO), high-density lipoprotein cholesterol (HDL-C), and low-density lipoprotein cholesterol (LDL-C) in plasma were determined with commercial kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, China). The procedure was as follows: (1) First, 2.5 μl of distilled water was added to the blank well. (2) Then, 2.5 μl of a standard or sample was added to each well. (3) A total of 250 μl of TMB substrate was added to each well. (4) The wells were incubated for 10 min at 37°C. (5) The wells were measured with a plate reader at 510 within 5 min.

Statistical Analysis

Reproductive and lactation performance, back fat thickness, and colostrum and milk composition were analyzed by independent-sample Student's *t*-tests using SPSS 22.0 (IBM-SPSS Inc., Chicago, Illinois, USA) after checking for normality and homogeneity of variance according to the Shapiro–Wilk and Levene tests, respectively. Plasma cytokine concentration and plasma constituent concentration data were analyzed using the mixed procedure of SAS with repeated measures. The model included diet (control vs. MGDG), parity number, and timepoint (defined as a repeated measure) and their interaction as fixed effects and a random effect. The results are expressed as the means and standard error means (SEMs). The effects of treatment on the presence or absence of estrus after weaning were evaluated using chi-square analysis (21). Probability values <0.05 were considered to indicate significance, and probability values <0.10 were considered to indicate tendencies toward differences between treatments.

RESULTS

Reproductive and Lactation Performance

The effects of dietary MGDG supplementation during late gestation and lactation on sow reproductive and lactation performance are shown in **Table 3**. The total number of piglets born, the numbers of stillborn and live-born piglets, the litter birth weights, and the individual piglet birth weights were unaffected by the treatment ($P > 0.05$; **Table 3**). The average daily feed intake (ADFI) and the estrus rate were not affected by MGDG supplementation ($P > 0.05$). The average daily gain (ADG), litter weight, average piglet weight, and preweaning survival during lactation were unaffected by the MGDG-supplemented diet compared with the control diet ($P > 0.05$).

TABLE 3 | Effects of dietary MGDG supplementation during late gestation and lactation on sow reproductive and lactation performance.

Parameter	Control	MGDG	*P*-value
No. of sows	50	50	
ADFI (day 0–day 21 of lactation), kg	5.34 ± 0.08	5.26 ± 0.10	0.634
Reproductive performance			
Total No. of pigs born/litter	11.58 ± 0.37	12.20 ± 0.36	0.771
No. of pigs born alive/litter	10.92 ± 0.37	11.64 ± 0.36	0.133
No. of stillbirths/litter	0.46 ± 0.05	0.35 ± 0.07	0.152
No. of weak pigs/litter	0.43 ± 0.12	0.70 ± 0.13	0.134
No. of mummies/litter	0.07 ± 0.01	0.11 ± 0.003	0.122
Litter birth weight, kg	16.78 ± 0.53	16.89 ± 0.50	0.442
Individual piglet weight, kg	1.56 ± 0.13	1.47 ± 0.12	0.335
Lactation performance			
No. of pigs after cross-foster	10.34 ± 0.17	10.41 ± 0.15	0.686
No. of weaning pigs	9.44 ± 0.20	9.22 ± 0.21	0.481
Preweaning survival, %	91.58 ± 1.35	88.81 ± 0.98	0.270
Individual pig weight after cross-foster, kg	1.96 ± 0.55	1.90 ± 0.54	0.907
Individual pig weight at weaning, kg	6.54 ± 0.13	6.67 ± 0.12	0.853
Litter weight after cross-foster, kg	20.02 ± 0.50	19.69 ± 0.57	0.211
Litter weight at weaning, kg	61.88 ± 1.88	61.43 ± 1.69	0.842
ADG, g/day	212.09 ± 5.80	223.31 ± 4.88	0.435

ADFI, average daily feed in take during lactation; ADG, average daily gain. A P < 0.05 indicates a significant difference among treatments.

Back Fat Thickness and Estrus Interval

The effects of dietary MGDG supplementation during late gestation and lactation on sow colostrum and milk composition are shown in **Table 4**. The loss of backfat thickness was significantly decreased after MGDG supplementation compared with the control group during lactation ($P < 0.05$; **Table 4**).

Colostrum and Milk Composition

The effects of dietary MGDG supplementation during late gestation and lactation on sow colostrum and milk composition are shown in **Table 5**. No differences between the treatment and control groups were found with regard to the protein and lactose levels in colostrum and milk ($P > 0.05$; **Table 5**). However, the sows with dietary MGDG supplementation tended to have higher fat concentrations in their colostrum than sows fed the control diet ($P < 0.1$).

TABLE 4 | Effects of dietary MGDG supplementation during late gestation and lactation on sow back fat thickness and estrus interval.

Parameter	Control	MGDG	*P*-value
n	50	50	
Sow back fat			
Back fat at farrowing	18.33 ± 1.23	18.50 ± 0.72	0.905
Back fat at weaning	14.92[b] ± 0.53	16.77[a] ± 0.42	0.023
Back fat loss during lactation	3.41[a] ± 0.43	2.18[b] ± 0.29	0.029
Estrus rate within 7 days after weaning, %	91.49	91.30	0.795

A P < 0.05 indicates the existence of a significant difference among treatments.
[a,b]means values within a row with different letters superscripts means significantly different (P < 0.05).

TABLE 5 | Effects of dietary MGDG supplementation during late gestation and lactation on sow colostrum and milk composition.

Parameter	Control	MGDG	*P*-value
n	10	10	
Colostrum (%)			
Non-fat-solids	20.90 ± 0.46	19.26 ± 0.92	0.109
Protein	11.33 ± 0.23	10.27 ± 0.62	0.102
Fat	4.46 ± 0.40	5.42 ± 0.32	0.087
Lactose	7.89 ± 0.19	7.23 ± 0.37	0.115
Milk (%)			
Non-fat-solids	10.35 ± 0.15	10.63 ± 0.12	0.147
Protein	3.80 ± 0.06	3.92 ± 0.04	0.123
Fat	6.93 ± 0.23	7.09 ± 0.32	0.695
Lactose	5.85 ± 0.12	5.91 ± 0.07	0.652

A P < 0.05 indicates the existence of a significant difference among treatments.

Immunoglobulin Concentrations

The effects of dietary MGDG supplementation during late gestation and lactation on the immunoglobulin concentrations in sow colostrum and milk are shown in **Table 6**. No significant treatment differences were observed in terms of IgG, IgM, and IgA levels in colostrum and milk ($P > 0.05$; **Table 6**).

Cytokine Concentrations

The effects of dietary MGDG supplementation during late gestation and lactation on the cytokine concentrations in sow colostrum, milk, and plasma are shown in **Table 7**. Compared with the sows fed the control diet, those fed the MGDG-supplemented diet had a lower TNF-α concentration in their colostrum ($P < 0.05$; **Table 7**). During the lactation period, lower cytokine concentrations in sow plasma were observed on L0 (IL-8), L14 (IL-18), and L21 (IL-18) ($P < 0.05$).

Blood Parameter Concentrations

The effects of dietary MGDG supplementation during late gestation and lactation on blood parameters in the plasma of sows are shown in **Table 8**. On L14, the GLU levels in plasma were higher in MGDG-fed sows than in sows in the control group. On the day of weaning, the TCHO content in plasma was lower in the MGDG group than in the control group ($P < 0.05$; **Table 8**). The PUN, HDL-C, LDL-C, and TG levels were not affected by MGDG supplementation ($P > 0.05$).

TABLE 6 | Effects of dietary MGDG supplementation during late gestation and lactation on immunoglobulin levels in sow colostrum and milk.

Parameter	Control	MGDG	*P*-value
n	10	10	
Colostrum (mg/mL)			
IgG	11.03 ± 1.45	11.11 ± 1.06	0.858
IgA	3.12 ± 0.44	3.61 ± 0.58	0.531
IgM	2.48 ± 0.23	2.78 ± 0.31	0.326
Milk (day 14) (mg/mL)			
IgG	1.25 ± 0.12	0.91 ± 0.19	0.405
IgA	2.25 ± 0.24	2.61 ± 0.34	0.490
IgM	1.01 ± 0.09	1.18 ± 0.12	0.561

IgG, immunoglobulin G; IgA, immunoglobulin A; IgM, immunoglobulin M. A P-threshold <0.05 indicates the existence of a significant difference among treatments.

TABLE 7 | Effects of dietary MGDG supplementation during late gestation and lactation on cytokine concentrations in sow colostrum, milk, and plasma.

Parameter	Control	MGDG	*P*-value
n	10	10	
Colostrum			
IL-8, pg/mL	3592.14 ± 363.37	3142.93 ± 307.79	0.519
IL-18, pg/mL	1469.73 ± 86.98	1259.80 ± 243.93	0.332
TNF-α, ng/mL	1.37 ± 0.13[a]	0.36 ± 0.08[b]	0.026
Milk			
IL-8, pg/mL	793.09 ± 64.47	622.91 ± 80.47	0.118
IL-18, pg/mL	398.56 ± 31.12	335.89 ± 38.42	0.399
TNF-α, ng/mL	1.04 ± 0.15	0.91 ± 0.02	0.768
L0			
IL-8, pg/mL	2039.56 ± 364.63	1138.16 ± 117.11	0.047
IL-18, pg/mL	273.48 ± 38.90	238.96 ± 17.13	0.256
TNF-α, pg/mL	251.67 ± 59.46	182.16 ± 10.95	0.122
L14			
IL-8, pg/mL	975.61 ± 31.80	873.92 ± 95.87	0.615
IL-18, pg/mL	183.10 ± 26.19[a]	22.03 ± 3.00[b]	0.014
TNF-α, pg/mL	311.66 ± 79.34	266.58 ± 7.69	0.068
L21			
IL-8, pg/mL	914.05 ± 140.15	875.85 ± 89.01	0.602
IL-18, pg/mL	242.43 ± 36.31[a]	45.87 ± 5.35[b]	0.019
TNF-α, pg/mL	336.38 ± 10.93	269.62 ± 8.77	0.298

IL-8, interleukin-8; IL-18, interleukin-18; TNF-α, tumor necrosis factor α; L0, lactation day 0; L14, lactation day 14; L21, lactation day 21. A P < 0.05 indicates the existence of a significant difference among treatments.
[a,b]means values within a row with different letters superscripts means significantly different (P < 0.05).

DISCUSSION

In this study, supplementation with MGDG in a sow diet reduced back fat loss during the lactation period. To date, the effects of backfat on sow reproductive performance are still

TABLE 8 | Effects of dietary MGDG supplementation during late gestation and lactation on sow plasma constituent concentrations.

Item	Control	MGDG	*P*-value
n	10	10	
Day 85 of gestation (mmol/L)			
GLU	4.77 ± 0.21	4.43 ± 0.24	0.312
TG	1.87 ± 0.29	1.69 ± 0.11	0.558
TCHO	7.26 ± 0.52	8.43 ± 1.03	0.323
HDL-C	0.77 ± 0.12	0.80 ± 0.07	0.794
LDL-C	1.11 ± 0.07	0.92 ± 0.09	0.104
PUN	7.96 ± 0.33	7.71 ± 0.51	0.684
Day of farrowing (mmol/L)			
GLU	4.80 ± 0.33	5.40 ± 0.32	0.207
TG	1.87 ± 0.41	1.81 ± 0.11	0.727
TCHO	6.07 ± 0.41	5.23 ± 0.81	0.366
HDL-C	0.86 ± 0.07	0.61 ± 0.10	0.054
LDL-C	1.30 ± 0.10	1.11 ± 0.15	0.340
PUN	7.34 ± 0.65	7.89 ± 0.41	0.444
Day 14 of lactation (mmol/L)			
GLU	4.81^{b} ± 0.32	5.93^{a} ± 0.21	0.010
TG	1.71 ± 0.14	1.52 ± 0.11	0.306
TCHO	11.59 ± 0.77	10.29 ± 1.23	0.386
HDL-C	1.60 ± 0.36	1.07 ± 0.05	0.179
LDL-C	1.99 ± 0.65	1.04 ± 0.08	0.164
PUN	11.48 ± 0.37	11.13 ± 0.0.54	0.599
Day of weaning (mmol/L)			
GLU	5.88 ± 0.35	5.15 ± 0.36	0.162
TG	1.77 ± 0.20	1.45 ± 0.21	0.288
TCHO	12.15^{a} ± 1.08	9.05^{b} ± 0.58	0.021
HDL-C	1.41 ± 0.04	1.13 ± 0.08	0.111
LDL-C	1.66 ± 0.15	1.39 ± 0.12	0.127
PUN	10.21 ± 0.61	9.14 ± 0.83	0.176

GLU, glucose; TG, triglyceride; TCHO, total cholesterol; HDL-C, high density lipoprotein cholesterol; LDL-C, low density lipoprotein cholesterol; PUN, plasma urea nitrogen. A P-threshold <0.05 indicates the existence of significant difference among treatments.
[a,b]means values within a row with different letters superscripts means significantly different (P < 0.05).

inconsistent. Some contend that backfat is not a reliable predictor of subsequent sow reproductive performance (22), but other scientists argue that there is an optimal range of backfat (15–16 mm or even at least 20 mm) for maintaining subsequent reproduction (23, 24). Increasing dietary energy levels is an effective way to reduce back fat thickness loss in sows because the requirement to mobilize their body lipids is decreased. In our experiment, the MGDG diet contained a higher level of net energy (calculated value), which could directly alleviate the loss of back fat thickness. Another possible reason is that MGDG acts as an emulsifier in the sow diet, which increases the ileal apparent digestibility of dry matter, organic matter, crude protein, and gross energy of the base diet (25). This hypothesis still needs to be confirmed in the future.

Sow milk directly affects the growth and development of piglets during lactation. Protein, lactose, and fat are major nutritional components in sow milk, accounting for 4.9, 5.1, and 7.1%, respectively (26). Amino acids are a basal component of sow milk synthesis and stimulate milk synthesis through the activation of mTORC1 (27). The addition of lipids to sow diets during late pregnancy and lactation significantly increases the amount of fat in the milk of the sows (28). In addition, dietary supplementation with emulsifiers (lysophospholipids or sodium stearoyl-2-lactylate) is also reported to increase milk fat concentration (29). However, in this study, no significant difference in milk composition was observed when sows were fed the MGDG diet.

Immunoglobulins in colostrum and milk can decrease the susceptibility of newborns to infection (26). Until now, the results regarding the effects of dietary emulsifier supplementation on milk secretary immunoglobulins have not been consistent. Wang et al. (30) found that dietary emulsifier levels linearly increased IgA and IgG concentrations in colostrum and milk. Zhao et al. (29) reported that the concentration of milk IgG was not affected by emulsifier supplementation (24, 29). In this experiment, no significant difference was observed in IgG, IgA, and IgM levels. The discrepancy in the effects of emulsifiers in different studies might be due to the different efficiencies of different studies.

In pathogen invasion, bacterial endotoxin (lipopolysaccharide, LPS) can trigger the secretion of proinflammatory cytokines, such as tumor necrosis factor-α (TNF-α) and interleukin-1β (IL-1), through the activation of the myeloid differentiation factor 88 (MyD88)/nuclear factor-kappa B (NF-κB) signaling pathway (31). These inflammatory factors can cause fever and activate T and B lymphocytes, which further trigger the secretion of other inflammatory mediators (32–34). In our trial, supplementation with MGDG in a sow diet decreased the plasma cytokine concentrations (IL-18 and IL-8). Both IL-18 and IL-8 are identified as proinflammatory cytokines. IL-18 participates in natural killer cell activation and T helper 1 (Th1) cell responses, and IL-8 mainly regulates the recruitment and activation of neutrophils during inflammation (35). The potential underlying mechanisms for MGDG-inhibited secretion of IL-18 and IL-8 are listed as follows. First, MGDG has strong antiviral and antibacterial ability, which could decrease the amount of pathogenic bacteria in the intestine and decrease intestinal inflammation (20). Second, MGDG could directly bind to GPR119 (a G protein coupled to Gs) and decrease the secretion of IL-18. Previously, activation of the GPR109A (Gi) signaling pathway was found to increase the secretion of IL-18 (36). Gs and Gi signaling inversely regulate cell biological function through the modulation of adenyl cyclase (AC) (37). Thus, it is highly possible that MGDG could decease the secretion of IL-18. Further research is still required to verify this hypothesis.

At weaning, the present study found that the concentration of TCHO in sow plasma decreased, which is in agreement with the data of Jones et al. (38). Wang et al. (30) also reported that the level of serum TCHO decreased linearly in lactating sows as dietary emulsifier supplementation increased. Cholesterol is an abundant and indispensable substance in animals that can directly reflect lipid metabolism. It plays an important role in the formation of cell membranes and in the synthesis of bile acids and vitamin D (39). During lactation, excessive metabolism of adipose tissue leads to increases in plasma lactate cholesterol levels in sows. Therefore, the lower plasma concentrations of TCHO in the MGDG group suggest that the sows fed the MGDG-supplemented diet might have used less stored body tissues to supply energy for maintenance requirements and milk

production during lactation. Therefore, MGDG supplementation might have enhanced the digestion and absorption of lipids, thus increasing the energy supply to lactating sows in this study.

Collectively, this study found that dietary MGDG supplementation during late gestation and lactation improves sow body condition and alleviates inflammation. Further research is warranted to examine whether dietary MGDGs regulate the inflammation of sows through the modification of intestinal microbes.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The animal study was reviewed and approved by South China Agricultural University Animal Care and Use Committee. Written informed consent was obtained from the owners for the participation of their animals in this study.

AUTHOR CONTRIBUTIONS

HS and WC carried out this experiment collected data and wrote the manuscript. WG and SZ designed the research and gave guidance on writing paper. SZ, FC, MR, and WG reviewed the manuscript. HS, WC, and FY helped to prepare the experiment. All authors read and approved the final manuscript.

FUNDING

This study was financially supported by National Natural Science Foundation of the P. R. of China (Nos. 31872364 and 31802067), Guangdong Basic and Applied Basic Research Foundation (No. 2021A1515010440), and Major Science and Technology Projects in Anhui Province (201903a06020002).

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antioxidants

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Review

Exploring the Benefits of Probiotics in Gut Inflammation and Diarrhea—From an Antioxidant Perspective

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Abstract: Inflammatory bowel disease (IBD), characterized by an abnormal immune response, includes two distinct types: Crohn's disease (CD) and ulcerative colitis (UC). Extensive research has revealed that the pathogeny of IBD encompasses genetic factors, environmental factors, immune dysfunction, dysbiosis, and lifestyle choices. Furthermore, patients with IBD exhibit both local and systemic oxidative damage caused by the excessive presence of reactive oxygen species. This oxidative damage exacerbates immune response imbalances, intestinal mucosal damage, and dysbiosis in IBD patients. Meanwhile, the weaning period represents a crucial phase for pigs, during which they experience pronounced intestinal immune and inflammatory responses, leading to severe diarrhea and increased mortality rates. Pigs are highly similar to humans in terms of physiology and anatomy, making them a potential choice for simulating human IBD. Although the exact mechanism behind IBD and post-weaning diarrhea remains unclear, the oxidative damage, in its progression and pathogenesis, is well acknowledged. Besides conventional anti-inflammatory drugs, certain probiotics, particularly *Lactobacillus* and *Bifidobacteria* strains, have been found to possess antioxidant properties. These include the scavenging of reactive oxygen species, chelating metal ions to inhibit the Fenton reaction, and the regulation of host antioxidant enzymes. Consequently, numerous studies in the last two decades have committed to exploring the role of probiotics in alleviating IBD. Here, we sequentially discuss the oxidative damage in IBD and post-weaning diarrhea pathogenesis, the negative consequences of oxidative stress on IBD, the effectiveness of probiotics in IBD treatment, the application of probiotics in weaned piglets, and the potential antioxidant mechanisms of probiotics.

Keywords: inflammatory bowel disease; oxidative stress; probiotics; weaned piglets

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Citation: Li, Q.; Zheng, T.; Ding, H.; Chen, J.; Li, B.; Zhang, Q.; Yang, S.; Zhang, S.; Guan, W. Exploring the Benefits of Probiotics in Gut Inflammation and Diarrhea—From an Antioxidant Perspective. *Antioxidants* **2023**, *12*, 1342. https://doi.org/10.3390/antiox12071342

Academic Editor: Sunmin Park

Received: 30 May 2023
Revised: 17 June 2023
Accepted: 19 June 2023
Published: 26 June 2023

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1. Introduce

Inflammatory bowel disease (IBD) is an incurable chronic inflammatory gastrointestinal disease that primarily includes Crohn's disease (CD) and ulcerative colitis (UC). The global prevalence of IBD has rapidly increased in recent years, with approximately 6.8 million people suffering from IBD in 2022 [1]. IBD types are typically distinguished by the location of inflammation and the histopathological characteristics of the gastrointestinal tract. Clinically, CD can occur in any region of the gastrointestinal tract, including the ileum and colon, characterized by transmural inflammation. On the other hand, UC specifically appears in the colon and rectum, with inflammation limited to the mucosa [2]. As an inappropriate immune response, the causes of IBD are considered multifaceted, involving genetic predisposition, environmental factors (Western diet, poor sanitation, and

smoking), damage to intestinal epithelial integrity, and dysbiosis in the gut microbiome [3]. The exact underlying mechanisms of IBD remain unknown; however, accumulated data from animal experimental models and clinical studies suggest that oxidative stress (OS) signaling occupies a dominant position in the pathogeny of IBD. In brief, OS leads to damage to the gastrointestinal mucosal layer and dysbiosis, which are important features of IBD patients. This, in turn, stimulates immune responses and triggers IBD [4]. Therefore, relieving systemic oxidative stress becomes a crucial goal in treating IBD. Currently, the supplementation of reactive oxygen species (ROS) production inhibitors, corticosteroids, aminosalicylates, and substances that stimulate endogenous antioxidant enzymes have gradually emerged as complementary and alternative therapies for IBD treatment [5]. In addition, probiotics are being developed as therapeutic strategies for IBD. Studies have shown that probiotics, particularly *Lactobacilli* and *Bifidobacteria*, offer benefits for IBD patients by improving intestinal microecology, protecting intestinal mucosal barrier integrity, and modulating immune responses [6]. These effects are associated with their ability to scavenge ROS, chelate metals, and regulate the levels of host antioxidant enzymes [7]. Moreover, evidence from clinical studies suggests that the transplantation of healthy donor-derived microbiota to IBD patients promotes the recovery of their gut microbiota and the resolution of inflammation [8]. Therefore, approaches such as gut microbiota transplantation or the oral administration of probiotics hold potential as therapeutic interventions with which to alleviate clinical symptoms in IBD patients.

In pork production, weaning and feed transition often lead to intestinal barrier damage, intestinal villus atrophy, and an overload of proinflammatory factors (TNF-α, IL-6), resulting in diarrhea, decreased feed intake, and compromised growth [9]. Post-weaning diarrhea (PWD), caused by intestinal inflammation and oxidative stress, contributes to significant economic losses. Traditionally, antibiotics have been used as a means to alleviate diarrhea and promote growth [10]; however, due to the rise in antibiotic resistance among intestinal pathogens and concerns about drug residues, antibiotics are gradually being banned in many countries. Currently, certain probiotics have been suggested as alternatives to antibiotics in weaned piglets, exerting anti-inflammatory and antioxidant effects, modulating the microbiome, enhancing intestinal epithelial barrier function, and alleviating diarrhea [11,12].

In clinical research, difficulty in sampling, environmental limitations, and ethics often hinder direct research on human diseases. For a long time, small rodents have been important model animals for basic medical research, making significant contributions to the understanding of the pathogenesis and treatment of human diseases; however, pigs are more similar to humans in terms of physiology and anatomy than rodents, making them a potential choice for simulating human diseases [13]. At present, there are pig models for cardiovascular diseases, metabolic disorders, and neurological diseases, which provide considerable support for the analysis and treatment of human diseases [14]. Therefore, we take the intestinal inflammation of weaned piglets as an example with which to discuss the following points sequentially: the relationship between oxidative stress and IBD, the potential of probiotics in IBD treatment, the application of probiotics in weaned piglets, and the possible mechanisms of probiotics in IBD treatment.

We conducted a thorough search using PubMed, Medline, and Web of Science databases from 2010 to 2023, and found a total of 41 papers to include in this review. These papers included in vitro cell tests, small rodents (mainly induced with dextran sodium sulphate or 2,4,6-trinitrobenzenesulfonic acid), and piglets (induced with weaning stress) as IBD models. In addition, we also searched for double-blind, placebo-controlled trials of adults and children with active or quiescent CD or UC within the past 20 years. This review provides valuable insights into the potential of probiotics, particularly lactobacillus, in alleviating IBD symptoms.

2. Oxidative Stress in Inflammatory Bowel Disease

OS is an imbalance between oxidants and antioxidants, with reactive oxygen species (ROS) being the most common highly reactive molecules in organisms. ROS, including superoxide ($O_2\cdot^-$), peroxy radical ($RO_2\cdot$), hydroxyl radical ($HO\cdot$), and hydroperoxy radical ($HO_2\cdot$), are natural byproducts of metabolism [15]. They are primarily produced by organelles such as the endoplasmic reticulum, mitochondria, and peroxisome, as well as by enzymes, such as peroxidase, NADPH oxidase, xanthine oxidase, lipoxygenase, glucose oxidase, and epoxidase [16]. Antioxidant systems, on the other hand, consist of enzymatic and nonenzymatic defenses. Enzymatic defenses, including catalase, superoxide dismutase (SOD), and glutathione peroxidase (GP-x), are present in all cells. Nonenzymatic defenses typically involve substances such as glutathione, ascorbic acid, vitamin E, C, A, and metal elements (zinc, copper, manganese, and iron) [2]. At homeostatic levels, ROS have numerous physiological functions, such as cell signal transmission, growth, differentiation, apoptosis, and inflammation; however, under OS, overloaded ROS would damage cell biomacromolecules, especially membrane lipids, DNA, and proteins [2].

2.1. Oxidative Stress Is the Trigger of IBD

Currently, OS is receiving increasing attention as a potential etiology or trigger of IBD. Numerous studies have compared the levels of OS markers between healthy individuals and IBD patients. The results have shown that the levels of antioxidant enzymes (including PON1, SOD, CAT, and GP-x) and nonenzymatic antioxidant substances (vitamins A, C, E, and β-carotene) are higher in healthy individuals compared to IBD patients [4,17]. Additionally, the most commonly evaluated index is the total antioxidant status (TAS) or total antioxidant capacity (TAC), which reflects the overall antioxidant capacity of an individual [18]. The study found that the TAS/TAC in the serum or plasma of adult patients with IBD (including CD and UC) uniformly decreased [3]. Conversely, pro-oxidases/agents, such as MPO, NO, spermine oxidase, COX2, NOX2, and NOS2, were all raised in the intestinal mucosa and serum of IBD patients [19]. Moreover, the concentrations of lipid peroxidation products (4-hydroxynonenal and malondialdehyde), DNA oxidation products (8-OHdG), and oxidative protein products (hydroxylated or carbonylated proteins) with proinflammatory properties have been shown to be positively correlated with the severity of IBD [20–22]. Additionally, oxidative-damage-induced DNA strand breakage, pyrimidine/purine loss, or abnormal pyrimidine and purine modification are considered key factors in the occurrence of IBD [3,23]. Similarly, another index, the "Oxidative Stress Index", obtained by dividing the total oxidative capacity by the total antioxidant status, is significantly higher in IBD patients compared to the healthy population [3].

OS is closely associated with the main pathological feature of IBD, which is inflammation. Studies have found a positive correlation between indices of OS and levels of C-reactive protein, an inflammatory marker in CD patients [24]. Similarly, clinical evidence suggests that plasma free thiols, the main substrates of ROS, are inversely correlated with inflammatory biomarkers [24,25]. Mechanistically, redox signaling stimulates NF-κB signaling, which is intimately involved in the upregulation of inflammatory cytokines (IL-1, IL-8) and inflammatory cell infiltration. During the immune response, polymorphonuclear leukocytes and monocytes infiltrate massively into the injured intestinal mucosa, stimulating the ROS/RN-generating system to increase oxidative stress [24] (Figure 1).

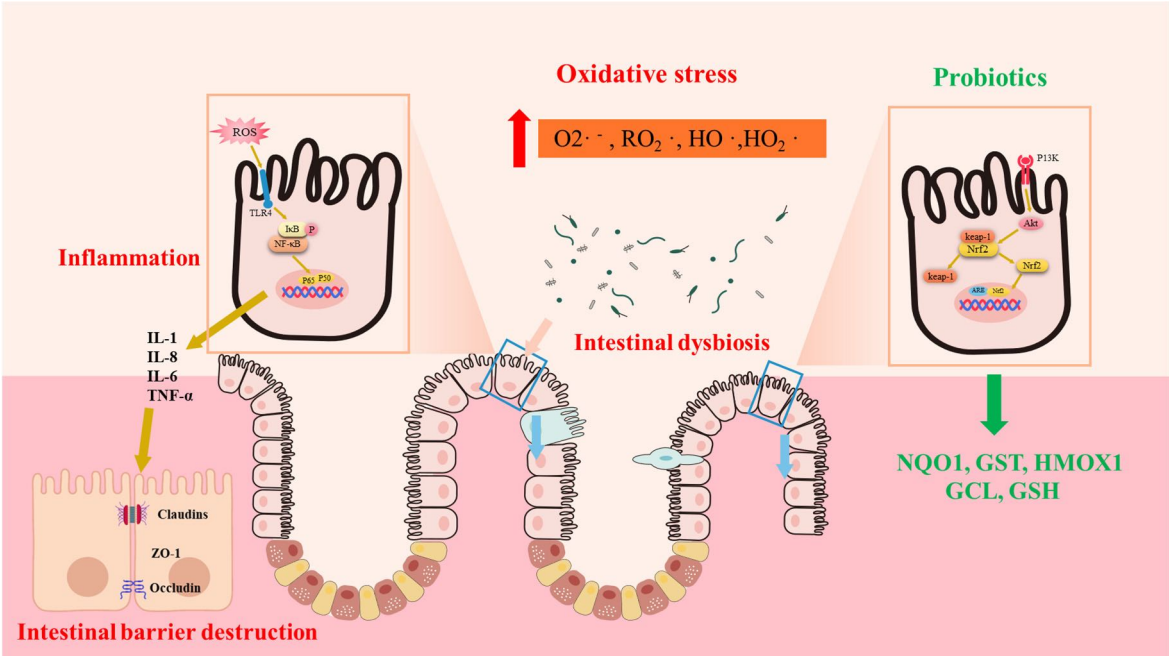

Figure 1. Oxidative stress is associated with the pathogenesis of IBD. Oxidative stress leads to the overexpression of inflammatory factors, which destroy the intestinal barrier and cause opportunistic pathogens to invade the mucosa, exacerbating the vicious cycle. Probiotics stimulate the expression of antioxidant enzymes and relieve IBD by activating the Nrf2 signaling pathway. Superoxide ($O_2\cdot^-$), peroxy radical ($RO_2\cdot$), hydroxyl radical (HO·), hydroperoxy radical ($HO_2\cdot$), NAD(P)H dehydrogenase quinone 1 (NQO1), glutathione S-transferase (GST), heme oxygenase 1 (HMOX1), glutamate cysteine ligase (GCL), and glutathione (GSH).

2.2. Oxidative Stress Leads to Intestinal Dysbiosis

Notably, intestinal dysbiosis, marked by microbial diversity and a decrease in beneficial bacteria alongside an increase in pathogenic bacteria, is another prominent feature of IBD patients [26]. Accumulating evidence suggests that disharmony between the intestinal flora and the immune response of the intestinal mucosa occupies a central role in IBD pathogenesis [27]. Some opportunistic pathogenic bacteria (*E. coli* and *Helicobacter pylor*) have been identified as the main sources of intestinal redox signals, which directly or indirectly (stimulate neutrophils) produce ROS, leading to the development of IBD exacerbations [28,29]. Additionally, the differential structure of the gut microbiota in patients with IBD compared to healthy individuals has been extensively studied [30]. The abundance of beneficial bacteria normally present in the gut of healthy individuals, such as *Bacteroidetes* and *Firmicutes*, is significantly reduced in patients with IBD, while harmful populations, such as *Proteobacteria* and *Actinobacteria*, are increased [31]. Among the beneficial bacteria, *Faecalibacterium*, producing butyrate, with anti-inflammatory effects, is one of the most abundant species in the human gut [26]; however, the abundance of *Faecalibacterium prausnitzii* is decreased in the gut of IBD patients [32]. Similarly, levels of *Roseburia* spp., another butyrate-producing bacteria, are remarkably lower in populations at a high genetic risk for IBD [26]. Additionally, the abundance of *Bifidobacterium* is also decreased. In terms of pathogenic bacteria, the relative abundance of *Proteobacteria* (mainly *Escherichia coli)* and proinflammatory properties (*Escherichia* and *Fusobacterium*) is higher in IBD patients [33]. Similarly, a TNBS-induced murine model found raised populations of *E. coli* as well as *Clostridium* spp. and reduced populations of *Bifidobacterium* and *Lactobacillus* [34]. Dysbiosis leads to damage of intestinal mucosal integrity, which causes opportunistic bacteria to invade the mucosa, leading to inflammatory cascades [35].

Additionally, oxidative stress and inflammation caused by ROS overload are tightly intertwined, leading to intestinal mucosal barrier damage in IBD patients. This, in turn,

increases mucosal permeability, allowing pathogen invasion, which further stimulates proinflammatory factor and ROS production, creating a vicious cycle [36].

Currently, the focus of IBD therapy is on reducing inflammation, and mainstream drugs include combinations of immunosuppressive and anti-inflammatory agents, such as anti-TNF-α antibodies and corticosteroids [37]. Furthermore, many antioxidant therapies, such as ROS production inhibitors, dietary interventions, and antioxidants, are being investigated as auxiliary therapies for IBD, exhibiting promising results [5]. Since gut microbiota interfere with both local and systemic immune responses, and their dynamic changes markedly depending on environmental factors and IBD treatment [38], supplementing IBD patients with probiotics that have antioxidant capacity might be a potential new therapy.

3. Probiotics in the Treatment of IBD

The advantages of probiotics in the treatment of IBD have been extensively studied in recent decades. The use of probiotics in patients with IBD has increased by 50% in recent years. Accumulating evidence suggests that certain probiotic strains are beneficial for the treatment and prevention of IBD, both in animal models and humans. The most commonly used probiotics are *Lactobacilli* and *Bifidobacteria*, which have been reported to improve the total antioxidant status of IBD patients. This improvement may be attributed to their ability to scavenge reactive oxygen species (ROS), chelate metals, stimulate host antioxidant enzyme levels (SOD, CAT, and GP-X), and modulate the gut flora [39–41].

3.1. Effect of Probiotics on Alleviating UC

The colon harbors the highest concentration of microbes in the human body. Several probiotics that normalize the composition of the colonic microbiome have shown benefits for patients with ulcerative colitis (UC). Currently, dextran sulfate sodium (DSS) and 2,4,6-trinitrobenzene sulfonic acid (TNBS) are commonly used to induce experimental models of UC and CD, respectively. In mouse model of DSS-induced colitis, *Bifidobacterium lactis A6*, *Bifidobacterium longum. infantis BB-02*, and *Bifidobacterium animalis lactis BB12* have been shown to inhibit OS, reduce colonic inflammation, and improve intestinal permeability [42]. Similarly, *Lactobacillus plantarum 2142* inhibited oxidative-stress-induced proinflammatory cytokine overexpression in the IPEC-J2 cell line [43]. In human studies, *Bifidobacterium* and *Lactobacillus acidophilus* have demonstrated benefits for UC patients, including reduced rectal bleeding symptoms, improved endoscopic scores, and better redox statuses [44–46]. The administration of *Lactobacillus reuteri* enemas to children with ulcerative proctitis effectively improved their clinical scores [47]; however, some studies have not observed significant differences with the supplementation of the same species, suggesting that the combined use of multiple strains may be more effective. The De Simone formulation, consisting of eight lactic-acid-producing species, has been extensively studied and shown to provide relief for pediatric and adult UC patients [35,48]. Another effective probiotic mixture is VSL#3, which includes *Lactobacillus*, *Bifidobacterium*, and *Streptococcus thermophilus* [49]. It has demonstrated efficacy in mouse models of UC and mild to moderate UC patients, reducing rectal bleeding, inflammatory markers, and improving mucosal antioxidant capacity. The alleviating effect of probiotics on IBD has been summarized in Supplementary Table S1. It is important to note that most patients in clinical settings receive anti-inflammatory drugs as part of their routine care, and investigating the synergistic effects between conventional drugs and probiotics is necessary. Combining VSL#3 with probiotics has shown reduced rectal bleeding frequency [50]. Dual treatment with probiotic mixtures and mesalazine has resulted in shorter recovery times and improved endoscopic images in UC patients [51], whereas some studies have shown that probiotics (*E. coli Nissle 1917* and VSL#3) have no significant therapeutic effect on UC patients [48,50,52]. The controversial results might be due to the differences in trial design, evaluation criteria, treatment duration, research scale, and patient characteristics (such as age, disease development stage, geographical location, and intervention type) [53]. Therefore, it is necessary to carry out a unified design experiment on a larger patient group to correctly evaluate the beneficial effects of probiotics

on UC; however, it is generally believed that a longer treatment with the combination of *VSL#3* and *lactobacillus* after surgery may have a better therapeutic effect on UC.

3.2. Effect of Probiotics on Alleviating CD

The effectiveness of probiotics in Crohn's disease (CD) treatment remains disputed. Some studies have shown positive outcomes, such as reduced colonic edema and improved histological scores in CD mice treated with *Bifidobacterium bifidum* in addition to the relief of symptoms in children with CD treated with *Lactobacillus rhamnosus* and *Saccharomyces boulardii* [54,55]; however, other studies have not found significant benefits with the use of probiotics in CD patients, including the effectiveness of *Lactobacillus rhamnosus* and VSL#3 [34,56,57]. Overall, probiotics are not recommended for the treatment of CD patients based on current evidence [47].

Given that IBD is a multifactorial disease, it is not reasonable to use the same probiotic species for all patients to achieve the same efficacy. Personalized medicine should be considered in future studies, taking into account factors such as IBD subtype, the location of the pathology, disease severity, the composition of the patient's microbiota, and environmental as well as genetic background, to determine the appropriate bacterial strains and doses for individual patients.

4. Probiotics Inhibit Intestinal Oxidative Damage in Weaning Piglets

In pig farming, oxidative stress is one of the major causes of disease. The intestine is a main target of ROS attack, which easily leads to intestinal inflammation, barrier disruption, diarrhea, and microbial disorders, ultimately resulting in reduced feed intake and slow weight gain, severely compromising farming benefits. Studies have shown that MDA, protein hydroxyl, and ROS are significantly increased in the liver, intestine, and blood of weaned piglets [58], whereas the activities of GSH-PX and SOD are significantly inhibited [59]. Intestinal oxidative stress induces microbiome dysregulation, leading to post-weaning diarrhea (PWD), and intestinal infections are a major problem facing pig production [9].

Given its antioxidant properties, various strains of *Lactobacillus* have been reported as potentially being able to alleviate piglets' post-weaning diarrhea. Research shows that an increased mean daily weight gain and a significant decrease in the rate of diarrhea were observed when LPS-challenged piglets were fed *Lactobacillus salivarius*, and also accompanied by decreased levels of proinflammatory mediators (IL-6 β, TNF- α, IL-2, and IFN- γ) in serum and mesenteric lymph nodes [9]. Similarly, the alleviating effect of *Lactobacillus gasseri*, *Lactobacillus reuteri*, and *Lactobacillus acidophilus* on diarrhea in piglets was demonstrated in several reports, which was associated with a reduction in enterotoxigenic *Escherichia coli* (ETEC) adhesion [11,60]. In addition, *Lactobacillus acidophilus* and *Lactobacillus casei* alleviated the severity of PWD by decreasing systemic immune responses and intestinal oxidative stress [11].

The intestine is the crucial place of nutrient digestion and absorption; thus, villus health remarkably affects the growth conditions of livestock, and is usually evaluated through villi height (VH), crypt depth (CD), and the villus-height-to-crypt-depth ratio (VCR) [49], whereas the shortening of intestinal villi and increased crypt depth via oxidative stress impede nutrient absorption [61]. On the other hand, intestinal nutrient absorption is mainly performed in a transmembrane or paracellular manner, closely related to tight junction proteins [62]. Tight junctions mainly include occludin, claudin-1, and ZO-1 proteins, and their dynamic changes have a key role in regulating the intestinal barrier and cell survival [63]. In addition to the function of digesting and absorbing nutrients, the intestinal epithelium also serves as a barrier against harmful antigens and pathogens. Intestinal barrier damage caused by oxidative stress is usually manifested by the disruption of tight junction proteins and the release of diamine oxidase (DAO) into the serum [64].

Interestingly, research showed piglets fed with *Lactobacillus plantarum* have higher VH as well as VCR and lower CD [65]. Additionally, dietary supplementation with *Lactobacillus*

delbrueckii successfully reversed the LPS-induced increase in serum DAO and intestinal CD, as well as raised occludin, ZO-1, and Claudin-1 levels in the ileum of piglets [66]. Pretreatment with *L. salivarius* could stimulate the expression levels of SOD, GSH-PX4, and CAT in the intestine of weaned piglets, but inhibited the immune response and oxidative stress caused by LPS infection, thus restoring the intestinal integrity of the weaned piglets [9]. Furthermore, *Lactobacillus delbrueckii* supplementation alleviated an LPS-induced increase in MDA in serum but decreased jejunal mucosa 8-hydroxy-2-deoxyguanosine levels of piglets [67]. These results suggest that *Lactobacillus* maintains the intestinal epithelial barrier integrity by reducing oxidative stress. Other similar reports are summarized in Supplementary Table S2.

5. Potential Signaling Pathways Underlying the Antioxidant Actions of Probiotics

Nuclear factor erythroid 2–related factor 2 (Nrf2) is a member of the cap'n'collar transcription factor family and consists of seven NEH domains. Currently, Nrf2 has emerged as a well established ubiquitin-dependent signaling system in response to OS [68]. High levels of ROS stimulate the separation of Nrf2 from its constitutive inhibitor: Keap1. Subsequently, Nrf2 enters the nucleus and binds to antioxidant response element (ARE) sequences, initiating the transcription of antioxidant genes such as NQO1, GST, HMOX1, GCL, and GSH (Figure 2). A large body of investigation indicates that Nrf2 activation could inhibit OS and inflammation, thereby preventing UC [69]. The activation of the Nrf2 system by probiotics in a host is believed to be one of the important mechanisms through which they exert antioxidant properties. In an in vivo UC rat model, *Lactobacillus delbrueckii* and *Lactobacillus fermentum* play a protective role by upregulating the Nrf2/Ho-1 pathway [70]. Similarly, *Lactobacillus helveticus* has also been shown to activate the Nrf2 pathway, relieving intestinal oxidative stress in mouse models [71]. Furthermore, the remission of LPS-induced intestinal injury by *Bacillus coagulans TL3* is also related to Nrf2 signaling activation [72]. In vitro, the activation of the Nrf2 pathway has also been shown to mediate the antioxidant effect of *Bifidobacterium* infantis, *Clostridium butyricum*, and *Lactobacillus casei Shirota* in intestinal injury [73–75]. The activation of toll-like receptors (TLRs) has been reported to stimulate Nrf2-ARE signaling and HO-1, both in vivo and in vitro [76]. Additionally, numerous studies have reported that the stimulation of TLR-Nrf2 signaling by *Lactobacilli* might also be responsible for their antioxidant benefits in the piglets' guts [77,78].

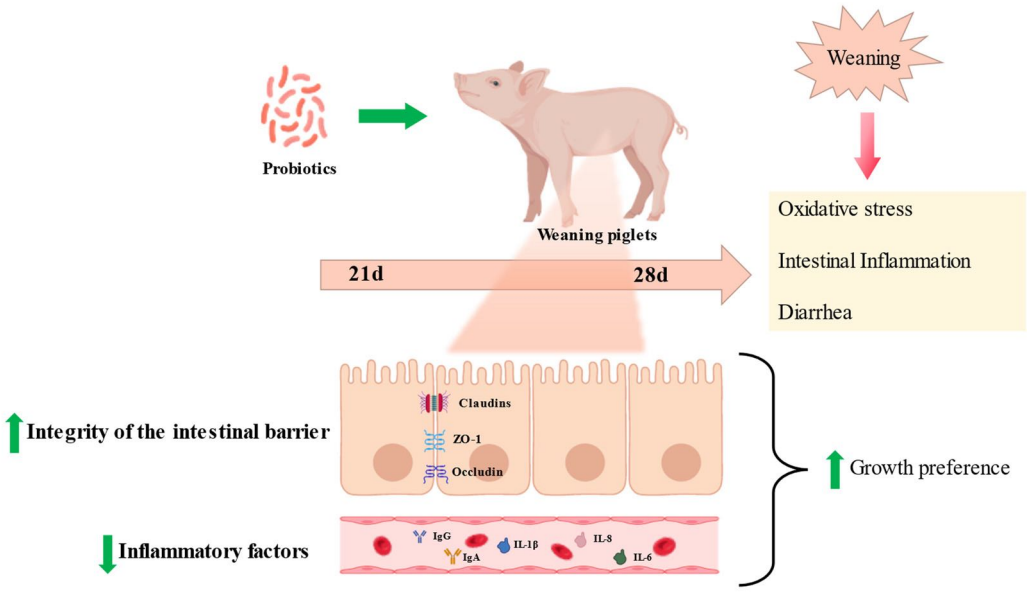

Figure 2. Probiotics relieve weaning stress in piglets. Probiotics can relieve intestinal inflammation, protect intestinal morphology, and reduce the diarrhea rate caused by oxidative stress in weaned piglets.

Nuclear factor kappa-B (NF-κB) is another transcription factor that accounts for the redox mechanism of probiotics. It has been found that NF-κB has a close relationship with Nrf2. Specifically, the loss of Nrf2 can increase NF-κB activity, leading to more serious inflammation, and NF-κB activation can also mediate the transcription of Nrf2 [50]. Lactobacillus species, such as *Lactobacillus johnsonii L531*, *Lactobacillus reuteri*, *Lactobacillus brevis*, and *Lactobacillus fermentans*, have been shown to activate the NF-κB pathway, relieving intestinal OS and inflammation in rats [50,79,80]. Additionally, the artificial modification of *Lactobacillus* expressing SOD showed similar results [81]. Moreover, *Bifidobacterium* downregulates ROS and inhibits NF-κB pathways to modulate the intestinal immune system and protect the intestinal epithelium, as has been addressed in detail [82].

Silent information regulator factor 2-related enzymes (SIRTs) are highly conserved NAD^+-dependent class III histone deacetylases. Currently, there are seven recognized members in the human SIRT family: SIRT1 to SIRT7.

The crosstalk between SIRT1 and Nrf2/ARE plays a key role in antioxidant defense. In brief, SIRT1 prompts the nuclear translocation of Nrf2, thereby upregulating the expression of antioxidant proteins and phase II detoxification enzymes [83]. In rats with aging-induced colitis, *Lactobacillus C29* treatment decreased the plasma levels of ROS, malondialdehyde (MDA), and C-reactive protein, while increasing SIRT1 expression [84]. Furthermore, the alleviation of high-fat-diet-induced UC by *B. longum* and *L. plantarum* is also associated with the activation of SIRT1 [85]. It has also been shown that activated SIRT2 can deacetylate the forkhead box proteins (FOXO1a and FOXO3a) to increase the expression of FoxO-dependent antioxidant enzymes [86]. The activation of manganese superoxide dismutase (Mn-SOD)/SOD2 by *B. longum* and *L. acidophilus* to reduce cellular ROS levels mediated by SIRT2 has been reported [87].

Mitogen-activated protein kinases (MAPKs) pertain to the serine/threonine kinase family and participate in numerous biological processes, including cell inflammation, antioxidation, and cell death [88]. As of now, three MAPK types have been discovered: ERK, JNK, and p38 MAPK. It is generally accepted that Nrf2 activation mediated by ERK1/2, JNK, and p38 accounts for the expression of phase II detoxifying enzymes [52,89]. *Lactobacillus rhamnosus GG* prevents the H2O2-induced disruption of tight junctions in the human intestinal epithelium, which may be mediated through ERK1/2 [90]. Additionally, both heat-killed and active *Lactobacillus brevis* effectively ameliorated subtotal duodenal and colonic injury caused by mercury poisoning or DSS by blocking oxidative stress and inflammation through a p38-MAPK-mediated pathway [91,92].

6. Summary and Outlook

Taken together, IBD and post-weaning diarrhea are complex and multifactorial diseases with unclear direct causes and pathological mechanisms; however, the significant role of oxidative stress in their pathogenesis has been widely recognized. Currently, anti-inflammation and anti-oxidation are important treatment targets for IBD and post-weaning diarrhea.

Studies have shown that probiotics, particularly *Lactobacillus* and *Bifidobacteria*, possess antioxidant properties in mammals. Therefore, providing probiotics appears to be a promising strategy for IBD treatment. Probiotics may improve various pathological aspects of IBD, with mixed-species formulations being more effective than single-species ones. VSL#3 and the De Simone formulation have emerged as the most effective microbial agents for treating UC; however, probiotic formulations seem to be less effective in treating CD compared to UC, potentially due to differences in inflammation location, disease severity, and duration. Similarly, incorporating probiotics into the feed of weaned piglets can significantly reduce diarrhea, intestinal inflammation, and barrier disruption. The antioxidant properties of probiotics mainly involve scavenging free radicals, chelating metal ions, modulating antioxidant enzyme expression, and influencing gut microbiota. At the molecular level, probiotics can impact signaling pathways, such as Nrf-2, NF-κB, MAPK, and SIRTs, to exert antioxidant effects.

It should be noted that animal IBD models do not fully replicate the human immunological profile, particularly in multifactorial diseases. Therefore, the efficacy of probiotics should be tested in various models. Additionally, the lack of standardized evaluation criteria for antioxidant capacity hampers the comparison of results between studies. Moreover, the appropriate dosage, species type, mixing ratio, and treatment duration of probiotic preparations have yet to be determined. Furthermore, chemical drugs and surgical treatments remain preferred in current clinical practices, so the combination therapy of conventional treatment approaches with probiotics should be further investigated. These aforementioned issues should be the focus of future studies.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/antiox12071342/s1. Table S1: The alleviating effect of probiotics on IBD; Table S2: The therapeutic effect of probiotics on intestinal inflammation in weaned piglets. References [93–120] are cited in the supplementary materials.

Author Contributions: Q.L.: investigation, writing—original draft, and visualization. T.Z.: investigation and visualization. H.D.: investigation and writing—original draft. J.C.: investigation and writing—original draft. B.L.: investigation and visualization. Q.Z.: investigation and visualization. S.Y.: writing—original draft. S.Z.: supervision, project administration, and writing—review and editing. W.G.: writing—review and editing. All authors have read and agreed to the published version of the manuscript.

Funding: This study was financially supported by the National Key R&D Program of China (2021YFD1300700), the Guangdong Basic and Applied Basic Research Foundation (2021A1515010440 and 2023A1515012098), and the Science and Technology Program of Guangzhou (202102020056).

Data Availability Statement: Not applicable.

Conflicts of Interest: The authors declare no conflict of interest.

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Wu et al. Cell Communication and Signaling (2022) 20:25
https://doi.org/10.1186/s12964-022-00830-6

Cell Communication and Signaling

RESEARCH

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Energy deprivation-induced AMPK activation inhibits milk synthesis by targeting PrlR and PGC-1α

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Abstract

Background: The mammary gland is responsible for milk production and secretion, which is critical for neonatal health during lactation. Lactation efficiency is largely affected by energy status with unclear mechanism.

Results: In the current study, we found that synthesis of milk fat and protein was significantly inhibited under energy-deficient conditions, which is accompanied with AMP-activated protein kinase (AMPK) activation. Modulating the AMPK signaling pathway directly or indirectly affects the synthesis of milk fat and protein. Besides mammalian target of rapamycin complex 1 (mTORC1) signaling in the regulation of milk synthesis, we discovered that AMPK mainly regulates the synthesis of milk protein through prolactin signaling. Mechanistically, AMPK triggers the ubiquitination of prolactin receptor (PrlR) through regulating the activity of β-transducin repeat-containing protein (β-TrCP, an E3 ligase). Subsequently, PrlR is degraded by the endocytosis process of lysosomes, which further attenuates prolactin signaling. In addition, our results revealed that AMPK activation inhibits milk fat synthesis through decreasing and accelerating de novo synthesis and β-oxidation of fatty acids, respectively. To be precise, AMPK activation inhibits rate limiting enzymes and transcriptional regulatory factors involved in de novo fatty acid synthesis and decreases the acetylation process of peroxisome proliferator-activated receptor gamma coactivator-1 alpha (PGC-1α) to strengthen the oxidation of fatty acids.

Conclusions: Taken together, AMPK regulates the synthesis of milk not only depends on canonical mTORC1 signaling and key rate-limiting enzymes, but also through manipulating the degradation of PrlR and the acetylation of PGC-1α.

Keywords: AMPK, Mammary epithelial cells, Milk protein, Milk fat, PrlR, PGC-1α

Background

The mammary gland is the hallmark of mammals, which is involved in the process of milk synthesis. The nutritional property of maternal milk determines the neonatal growth and health outcomes during breastfeeding. Maternal nutrients not only supply substrates but also provide large amounts of energy for the guarantee of milk synthesis [1]. During early lactation, maternal nutrition intake is usually insufficient to meet the requirement for milk secretion, which leads to a state called 'negative energy balance'. As a crucial sensor for cellular energy level, AMP-activated protein kinase (AMPK) is activated during the decrease of cellular ATP and the increase of ADP and AMP under energy stress. In addition, AMPK is also activated by the absence of fructose-1,6-bisphosphate (FBP), which is a metabolite of glucose [2]. Preliminary evidence indicated that once activated, AMPK attenuates milk synthesis through the inhibition of

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enzymes that directly participate in the synthesis of milk fat [3] and milk protein [4, 5]. However, whether AMPK regulates milk synthesis through other critical enzymes or pathways remains largely unknown.

Mammary epithelial cells control milk fat level by balancing fatty acids synthesis and their degradation through β-oxidation system. Current studies mainly focus on the processes of de novo fatty acid (FA) synthesis, which includes FA uptake, activation, intracellular transport, elongation and desaturation, as well as triacylglycerol (TAG) synthesis and lipid droplet formation [6]. A number of rate-limiting enzymes of de novo fatty acid synthesis have been identified as the downstream targets of AMPK, such as acetyl-coA carboxylase alpha (ACACA) (phosphorylation site is S79) and sterol regulatory element-binding protein 1c (SREBP1c) (phosphorylation site is Ser372). ACACA catalyzes the conversion of acetyl-CoA to malonyl-CoA and initiates de novo FA synthesis [7]. SREBP1c is a transcription factor for lipid homeostasis, which regulates milk fat synthesis in cows [8], goats [9] and swine [10]. Currently, whether AMPK regulates milk fat synthesis through β-oxidation system remains less well understood.

Milk protein synthesis is mainly regulated by the process of gene transcription and protein translation. Mammalian target of rapamycin (mTOR) is a master regulator that promotes anabolic process. Previously, mTOR complex 1 (mTORC1) has been identified to control gene transcription and protein translation through regulating its two downstream molecules including ribosomal protein S6 kinase 1 (S6K1) and 4E binding protein 1 (4EBP1). In the mammary epithelial cells of rats [11] and cows [4], inhibition of mTORC1 activity could downregulate the mRNA expression of genes encoding milk proteins such as alphaS1-casein (CSN1S1) and beta-casein (CSN2) and the process of milk protein translation [5]. Currently, how AMPK regulates milk protein through mTORC1-independent pathway (such as JAK2-STAT5) remains largely unknown.

In our current study, we confirmed that AMPK regulates the synthesis of milk partially through the activation of mTORC1 signaling and expression of rate limiting enzymes. Importantly, we identified prolactin receptor (PrlR) and peroxisome proliferator-activated receptor-C coactivator-1 alpha (PGC-1α) as novel potential targets of AMPK to regulate milk protein and fat synthesis, respectively. We further found that AMPK induces the activity of β-TrCP, which leads to the ubiquitination of PrlR and disruption of the prolactin signaling. AMPK promotes the β-oxidation of milk fatty acids through regulating the acetylation of PGC-1α. Taken together, we identified a novel molecular mechanism that AMPK regulates the synthesis of milk not only depends on canonical mTORC1 signaling and key rate-limiting enzymes, but also through manipulating the degradation of PrlR and the acetylation of PGC-1α.

Materials and methods

Cell cultures

HC11 (mouse mammary epithelial cells) used in this study was first cultured in DMEM/F12 medium (Thermo) containing 10% FBS (fetal bovine serum), 0.4% HS (hydrocortisone), 0.4% EGF (epidermal growth factor), 0.4% ITS (Insulin-Transferrin-Selenium) and 1% PS (penicillin–streptomycin) in a cell incubator at 37 ℃ and 5% CO_2 concentration. When the cells reached to around 80% confluence, they were seeded in culture plates and incubated with the same medium and conditions. When the cells in culture plates reach to approximately 80% confluence again, medium were changed to DMEM/F12 glucose-free medium (Thermo). This step was mainly to deplete the glucose in the cells for subsequent experiments. After 4 h-starvation treatment, cells were assigned into treatment mediums and harvested after 12 h and 24 h for real-time PCR and western-blotting, respectively. The reagents used in this experiment (mainly inhibitors or activators of pathways) are listed in Table 1.

RNA-Seq analysis

Total RNA was extracted using Trizol reagent kit (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. The DNA libraries were sequenced on the Illumina sequencing platform by Genedenovo Biotechnology Co., Ltd (Guangzhou, China).

Table 1 Reagents for this experiment

Reagent	Company	Cat No
AICAR	Sigma	A9978
Phenformin hydrochloride	Sigma	P7045
Compound C	Sigma	171261
Rapamycin	Sigma	V900930
Pitstop	Sigma	SML1169
EIPA	Sigma	3378
MG132	Sigma	M8699
MA	Sigma	M9281
SB203580	Sigma	HY-10256

Lipidomics

After the cells were processed, they were scraped and collected with PBS. The subsequent operations and data processing were completed under the technical guidance and assistance of Novogene Co., Ltd. (Beijing, China).

siRNA transfection

The siRNA and other reagents used in this experiment were provided by RiboBio (China), and our operation was in accordance with the instructions provided by them. We first selected the one with the highest transfection efficiency from the 3 siRNA products provided by the company, and then used it for subsequent formal experiments.

Oil red O staining of lipid droplets

To observe the production of milk fat more intuitively, oil red O staining solution (Sangon Bio, Shanghai) was used to observe the lipid droplets. In this experiment, the oil red dye solution used was diluted with double distilled water in a ratio of 6:4, and then filtered through 45-nm filters for 3 to 5 times before it can be used as a working solution. The cells were seeded into 24-well plates and cultured according to the methods mentioned above. They were harvested 24 h after being treated with the treatment medium for oil red staining. In brief, the cells were rinsed 3 times with PBS and fixed with 4% paraformaldehyde for 30 min at room temperature. Then, fresh working solution was added to cells (200 μL per well) followed by rinse with PBS twice. After 6 h of incubation at room temperature, cells were rinsed with PBS until there were no visible pellets before being observed and photographed under a microscope.

Measurement of triacylglyceride concentration

For quantitative estimation of the synthesis of intracellular and extracellular triacylglyceride, Triglyceride Assay Kit (Nanjing jiancheng, China) was used to detect the concentration of triacylglyceride in this research. The cells were seeded into 12-well plates and cultured as described previously and collected 24 h after being treated with the treatment medium for triacylglyceride determination. Briefly, 100 μL of medium from each well was collected uniformly for the subsequent determination of extracellular triacylglyceride. After rinsing with PBS, 50 μL of RIPA lysis buffer containing 1% PMSF and 1% phosphatase inhibitor was added to each well. After lysis on ice for around 15 min, the mixture in the well was collected as the samples for the subsequent determination of intracellular triacylglyceride. The determination of triacylglyceride concentration was achieved by detecting the OD value of the sample at a wavelength of 510 nm as the instructions suggested.

Table 2 Antibodies for this experiment

Antibody	Company	Cat No	Dilution
FASN	Abcam	ab99539	1:1000
ACACA	Abcam	ab72046	1:1000
FABP3	Abcam	ab231568	1:1000
DGAT1	Abcam	ab181180	1:1000
SREBP1	Abcam	ab3259	1:1000
alpha-S1-casein	Santa Curz	sc365929	1:100
beta-casein	Santa Curz	sc166520	1:100
WAP	Santa Curz	sc398276	1:100
Phospho-AMPK	CST	2535	1:1000
AMPK	CST	2532	1:1000
Phospho-mTOR	CST	5536	1:1000
mTOR	CST	2983	1:1000
Phospho-S6K1	CST	9234	1:1000
S6K1	CST	9202	1:1000
Phospho-4EBP1	CST	9451	1:1000
4EBP1	Abcam	ab32024	1:1000
Phospho-JAK2	CST	3776	1:1000
JAK2	CST	3230	1:1000
Phospho-STAT5	CST	4322	1:1000
STAT5	CST	94205	1:1000
Phospho-p38	CST	4511	1:1000
p38	CST	8690	1:1000
SirT1	CST	8469	1:1000
β-TrcP	CST	4394	1:1000
PrlR	Abcam	ab170935	1:1000
PGC-1α	Santa Curz	sc518025	1:100
Acetylated-lysine	CST	9441	1:1000
Anti-ubiquitin	Abcam	ab140601	1:2000
IgG	Santa Curz	sc2025	1:100
β-actin	Abcam	ab8226	1:2000
Goat anti-mouse IgG	ZENBIO	511103	1:5000
Goat anti-rabbit IgG	ZENBIO	511203	1:5000
Goat anti-mouse IgG	Jackson	115545003	1:500

Western-blotting

As with the previous steps for determining the triglyceride concentration, the HC11 cells were first fully lysed by RIPA (160 μL per well for 6-well plates). After the cell lysate were denatured by high temperature by using a PCR instrument, they were mixed with the 40 μL loading buffer per well and used as the sample for

Table 3 Primer sequences for real-time PCR

Genes	Forward primers	Reverse primers	Size, bp
FASN	AGCACTGCCTTCGGTTCAGTC	AAGAGCTGTGGAGGCCACTTG	94
ACACA	GAAGTCAGAGCCACGGCACA	GGCAATCTCAGTTCAAGCCAGTC	119
FABP3	ACCTGGAAGCTAGTGGACAG	TGATGGTAGTAGGCTTGGTCAT	106
DGAT1	CAGCTGTGGCCTTACTGGTTGA	CGGCACCACAGGTTGACATC	118
SREBP1	AGAAGCTCAAGCAGGAGAACCTGA	ACTTCGGGTTTCATGCCCTCCATA	127
AMPK	AACCTGAGAACGTCCTGCTTGATG	TGACTTCTGGTGCGGCATAATTGG	132
CSNS1	AATTCTTCCAGCTTGGTGCCTCTC	CAATGCCTTCAGGGGTGTCGTAG	105
CSN2	AGGAACAGCAGCAAACAGAGGATG	GGCTGAAGAGGCGGCAGAAAG	136
WAP	ACAGAGTGTATCATCTGCCAAA	ACCAATGTTGACAGGAGTTTTG	102
PCG-1α	CAGAGAGTATGAGAAGCGAGAG	AGCATCACAGGTATAACGGTAG	232
PrlR	CCTGAAATCCACAAATGTCGTT	CATATGGAAGTGTACTGCTTGC	197
PIP	CATCAACAGCACAGGAAAATCA	AGGATGATCATTACAGAGGCAG	158
BCAT	TTTGCTGGAGAAAGAGGATCAG	GCTTCTCTTTTAAGACAGTGGC	91
GPT	CTTCAAGCAGTTTCAAGCAGAG	TTGAGGGAAGGAATACATAGCG	139
GOT	ACAAGAACACACCAATCTACGT	ATAGGGCCGAATGTCCTTAAAA	92
ASCT2	TCCTTTCTAGATCTCGTGAGGA	CTGGACCATGGTTGAATTGATC	150
ASCT1	GCAGGACAGATTTTCACCATTC	CAAGATGATGGCAATTGTGAGG	99
xCT	CTATTTTACCACCATCAGTGCG	ATCGGGACTGCTAATGAGAATT	102
CAT2	CATCGGAATAGTGACGTCCTTA	AAGGCATCATAAGCGTTAAAGC	80
CAT1	CTGTGTTTTGGTCTTACGGTAC	AAAAAGCCTGTCTGTGATTCAC	132
4F2hc	AGGGACTCCTGTTTTTAGCTAC	GTGAAAGATGCTGGACTCATTC	109
LAT2	TGGATCCTTACAAGAACCTTCC	CATTGCAGTGACGTATGCAATA	98
ACTIN	CCACCATGTACCCAGGCATT	CGGACTCATCGTACTCCTGC	190

subsequent experiments. Under the action of electrophoresis, the proteins in the sample were separated by 10% or 12% SDS-PAGE gels. Then, the protein molecules were transferred onto the PVDF membrane in the tris–glycine system. Immediately afterwards, the PVDF membranes were rinsed with TBST 3 times and blocked with 6% skimmed milk for two hours at room temperature. The PVDF membranes were then rinsed 3 times with TBST and incubated under gentle shaking overnight at 4℃ with the primary antibodies. After overnight incubation, the PVDF membranes were rinsed 3 times with TBST and incubated under gentle shaking at room temperature for 1 h with the secondary antibodies. The chemiluminescent signal was detected using super ECL reagents (P1020), and signals obtained were quantified by ImageJ Software (ImageJ 1.52a). All antibodies used in this experiment (including subsequent immunofluorescence and immunoprecipitation experiments) are shown in Table 2.

Immunofluorescence staining

As shown in previous studies, immunofluorescence staining was performed to determine the location and yield of the target protein. We use 96-well plates to culture and process cells for immunofluorescence. The cells were washed, fixed with 4% paraformaldehyde, treated with tiriton X-100, and blocked with 5% BSA, and then incubated with the primary antibodies and the secondary antibodies in turn. After being stained by DAPI, the cells were observed and imaged under a fluorescence microscope.

Immunoprecipitation

In order to detect the degree of acetylation or ubiquitination of a single protein, we introduced immunoprecipitation to separate the target protein from other proteins, and then used the corresponding antibodies to bind to the target protein. The main steps are as follows: (1) fully lyse the cells cultured in the culture dishes with CHAPS

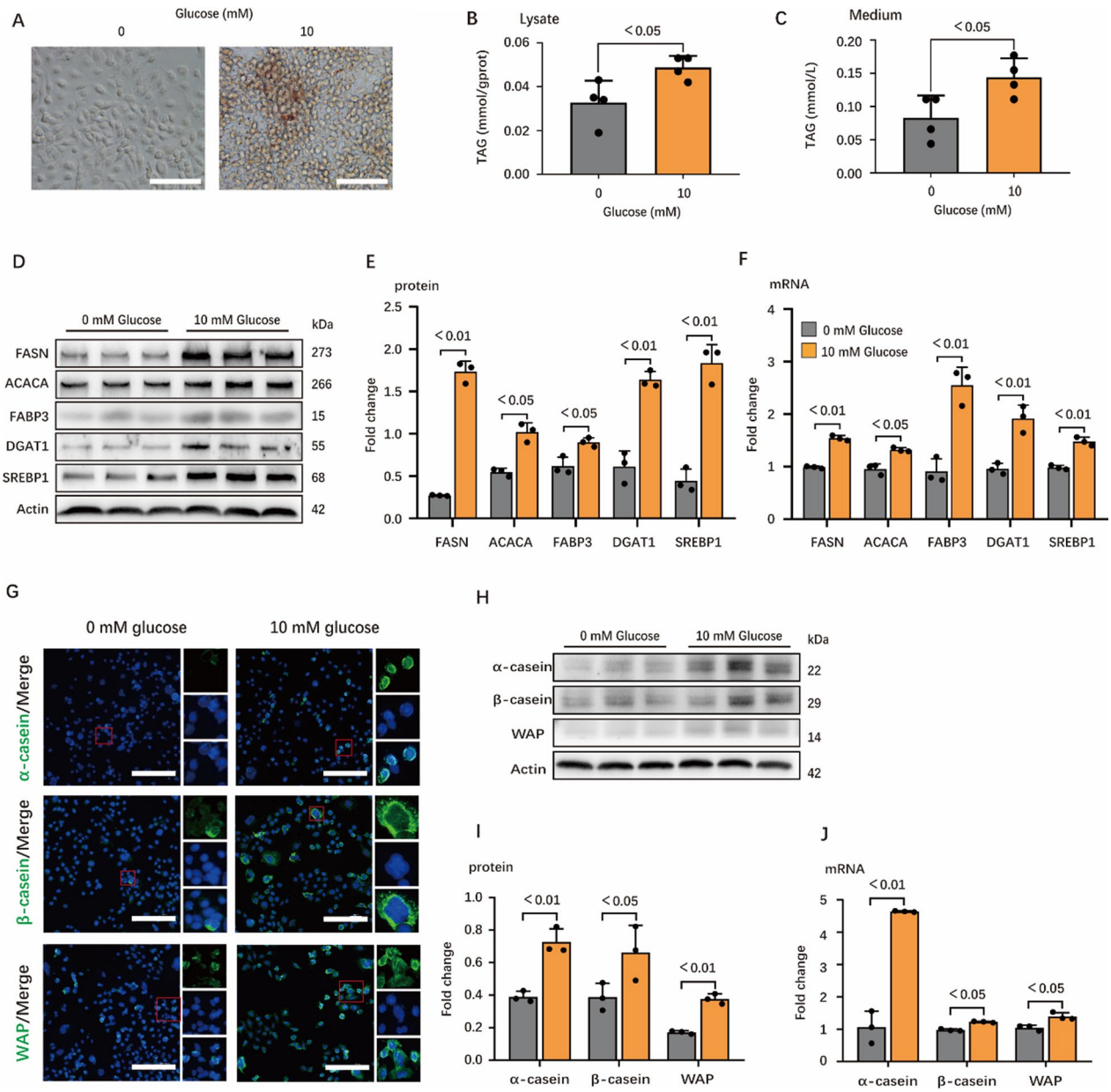


Fig. 1 Energy stress suppresses synthesis of milk fat and protein in HC11. **A** Oil red O staining images of HC11 under 0 mM glucose (left) and 10 mM glucose (right). Scale bars are 100 μm. **B** and **C** TAG concentration in cell (**B**) and medium (**C**), n = 4. **D**–**F** Western blots of HC11 lysates. In (**D**), bands were incubated with target proteins including FASN, ACACA, FABP3, DGAT1, SREBP1 and Actin antibodies, n = 3. Protein expressions of target proteins are shown in (**E**), n = 3. **F** Relative mRNA expression of target proteins, n = 3. **G** Confocal microscopy of HC11 immunostained for α-casein (green), β-casein (green), WAP (green) and DAPI (blue). Scale bars, 100 μm. The small red boxes in the figure are typical areas selected, and the three small images on the right are the enlarged images of the target protein, nucleus and merge images in selected areas, respectively. **H**–**J** Western blots and real time PCR of HC11 lysates of genes critical for protein synthesis. In (**H**), bands were incubated with target proteins including α-casein, β-casein, WAP and Actin antibodies, n = 3. Protein expressions of target proteins are shown in (**I**), n = 3. **J** Relative mRNA expressions of target proteins, n = 3. In this figure, cells for oil red O staining, TAG determination, immunofluorescence staining and WB were collected after 24 h incubation with 0 mM and 10 mM glucose mediums and cells for real-time PCR are 12 h. All data with error bars are averages ± SEM. In histograms, the numbers above the column are the p-values marked as a range if significantly different ($P < 0.05$) and each small black dot represents a test value

lysis buffer (TIANDZ, China), and centrifuge the lysate to collect the supernatant; (2) take a small part of the supernatant and save it for later use, and add IgG and the target protein antibody to the rest and incubate at 4 °C for 2 h; (3) add the A/G beads (washed and blocked in advance) (Santa cruz, sc2003) to the solution in the previous step, and incubate it overnight at 4 °C; (4) discard the supernatant and add chaps buffer, then bath at 100 °C for 5 min; after centrifugation, the supernatant and loading buffer are mixed. The subsequent operations are basically the same as western-blotting.

Real-time PCR

The cells were harvested after 12 h incubation for real-time PCR. Total RNA was isolated from cells by using EZ-press RNA Purification Kit (EZ-Bio, Shanghai) according to the protocol. After being tested the purity and integrity via agarose gel electrophoresis, the RNA was then reverse transcribed into cDNA by Color Reverse Transcription Kit (EZ-Bio, Shanghai) including gDNA removers and colored mix. The diluted cDNA was thoroughly mixed with Color SYBR Green qPCR Mix (EZ-Bio, Shanghai) and the primers of the target gene as well as double distilled water to form a 20 μL system for qPCR. The thermal cycling conditions of qPCR reactions were as follows: 95 °C for 1 min followed by 40 cycles of denaturation at 95 °C for 15 s, annealing at 59 °C for 15 s, and extension at 72 °C for 40 s. Primer suquences we used for real-time PCR were listed in Table 3.

Statistical analysis

Data of western-blotting, real-time PCR and TAG concentration were analyzed by one-way analysis of variance using IBM SPSS Statistics 20.0. The graphs were performed and analyzed with GraphPad Prism software 8.0. All data were demonstrated as (mean + SEM, n). $P < 0.05$ was considered as statistically significant and $P < 0.01$ was considered as highly statistically significant.

Results

Glucose starvation inhibits milk fat and protein synthesis

As a major energy source for epithelial cells of the mammary gland, glucose starvation increases cellular AMP/ATP ratio and triggers energy stress. To understand the effects of energy stress on milk synthesis, we first studied the effects of glucose starvation on milk protein and fat synthesis in mouse mammary epithelial cell line HC11. As expected, glucose starvation dramatically inhibited the milk fat synthesis (Fig. 1A–C). Correspondingly, protein (Fig. 1D, E) and gene (Fig. 1F) levels of critical enzymes (FASN, ACACA, FABP3 and DGAT1) participating in milk fat de novo synthesis and the regulatory factor (SREBP1) were also down-regulated under glucose starvation. We further detected the effects of glucose starvation on milk protein synthesis. Similarly, we observed positive reactions to α-casein, β-casein and WAP in the cytoplasm of HC11 cells treated with 0 mM glucose, while they were greatly inhibited compared with cells treated with 10 mM glucose (Fig. 1G). Milk protein synthesis was significantly inhibited during glucose deficiency, as is shown by the results from proteins (Fig. 1H, I) and genes expression (Fig. 1J) of milk protein (α-casein, β-casein and WAP). In order to confirm the effects of glucose on epithelial cells of the mammary gland, glucose dose-dependent experiment was conducted on porcine mammary gland epithelial cells (pMECs). Similar to the results from HC11 cell line,

(See figure on next page.)

Fig. 2 AMPK activation attenuates synthesis of milk fat and protein in HC11. **A** Oil red O staining images of HC11. Scale bars are 100 μm. **B** and **C** TAG concentration in cells (**B**) and medium (**C**), n = 4. **D** Heatmap analysis of up-regulated (red) or down-regulated genes (green) in HC11, n = 3. The 0 mM glucose group and the Phe group were compared with the 10 mM group respectively, * means $P < 0.05$, ** means $P < 0.01$ and *** means $P < 0.001$. **E**–**G** Western blots and real time PCR of HC11 lysates for critical fat synthesis genes. **E** Representative western-blotting bands hybridized with target proteins including FASN, ACACA, FABP3, DGAT1, SREBP1 and Actin antibodies, n = 3. (**F**) is the result of analyzing the WB bands and (**G**) represents data from real-time PCR, n = 3. **H** Confocal microscopy of HC11 immunostained for α-casein (green), β-casein (green), WAP (green) and DAPI (blue). Scale bars, 100 μm. The small red boxes in the figure are typical areas selected, and the three small images on the right are the enlarged images of the target protein, nucleus and merge images in selected areas, respectively. **I**–**K** Western blots and real time PCR of HC11 lysates for critical protein synthesis genes. **I** Representative western-blotting bands hybridized with target proteins including α-casein, β-casein, WAP and Actin antibodies, n = 3. **J** Result of analyzing the WB bands and (**K**) represents data from real-time PCR, n = 3. In this figure, cells for oil red O staining, TAG determination, lipidomics, immunofluorescence staining and WB were collected after 24 h incubation with 0 mM glucose medium, 10 mM glucose medium, (10 mM glucose + 1.5 mM AICAR) medium and (10 mM glucose + 300 μM Phe) medium, and cells for real-time PCR are 12 h. All data with error bars are averages ± SEM. In histograms, the numbers above the column are the *p*-values marked as a range if significantly different ($P < 0.05$) and each small black dot represents a test value

Fig. 2 (See legend on previous page.)

treating pMECs with different concentration of glucose after glucose starvation dose-dependently increased the milk fat synthesis (Additional file 1: Fig. S1A) with the upregulation of fat de novo synthesis enzymes (Additional file 1: Fig. S1B–C). Collectively, energy stress may inhibit milk fat (also verified in pMECs) and protein synthesis in HC11.

AMPK activation mimics the negative effects of glucose starvation on milk fat and protein synthesis

AMPK is a crucial sensor to detect cellular energy status. Glucose starvation was shown to decrease the cellular ATP/ADP ratio (Additional file 1: Fig. S1D) and activated AMPK in HC11 cells (Additional file 1: Fig. S1E–F). To identify the potential role of AMPK on milk fat and protein synthesis, we used compounds 5-aminoimidazole-4-carboxamide ribonucleotide (AICAR; an AMP analogue) and phenformin to activate AMPK to mimic the effects of glucose starvation. Strikingly, treatment with these compounds both highly inhibited (especially phenformin) milk fat synthesis (Fig. 2A–C). Furthermore, the type of triglyceride (LCFA saturation) that decreased during AMPK activation was detected (Fig. 2D). Significant decrease was observed in the levels of proteins (Fig. 2E, F) and genes (Fig. 2G) of enzymes related to milk fat synthesis. Similarly, synthesis of milk protein was also downregulated to varying degrees when AMPK was activated by AICAR and phenformin (Fig. 2H–K). In general, these results demonstrated that glucose starvation might inhibit milk fat and protein synthesis through AMPK signaling pathway.

Energy stress inhibits milk fat and protein synthesis partially through AMPK

To confirm the role of AMPK in energy stress-induced decrease of milk synthesis, we studied the role of AMPK inhibitor Compound C in milk synthesis under glucose starvation. As fatty acid synthesis might require glucose as substrates, 2 mM glucose was used to establish the slight glucose starvation model (Additional file 1: Fig. S2A), which could both activate AMPK and supply basic level of glucose as substrates. As expected, Compound C alleviated the negative effects of glucose starvation on milk fat synthesis (Fig. 3A–C) and enhanced the expression of proteins and genes of key enzymes (Fig. 3D–F). Immunofluorescent staining also showed that AMPK inhibition stimulated milk protein synthesis (Fig. 3G). Similar tendency was also observed in genes and proteins that related to milk protein synthesis (Fig. 3H–J). To further test whether the AMPK activity regulates milk fat and protein synthesis under energy stress, we knocked down AMPK using small interfering RNA (siRNA). The negative effects of AMPK on milk fat and protein synthesis were partially rescued when AMPK was knocked down (Fig. 3K, L). Intriguingly, milk fat and protein were significantly increased when HC11 was directly treated with compound c under severe energy deprivation (0 mM glucose) (Additional file 1: Fig. S2B–E), which indicates that other substrates might replace glucose for milk synthesis (as we will discuss below). Taken together, energy stress regulates milk fat and protein synthesis partially through AMPK signaling.

(See figure on next page.)

Fig. 3 Inhibition of AMPK promotes the synthesis of milk fat and protein in HC11. **A** Oil red O staining images of HC11. Scale bars are 100 μm. **B** and **C** TAG concentration in cell (**B**) and medium (**C**), n = 6. **D**–**F** Western blots of HC11 lysates. In (**D**), bands were incubated with target proteins including FASN, ACACA, FABP3, DGAT1, SREBP1 and Actin antibodies, n = 3. Protein expressions of target proteins are shown in (**E**), n = 3. **F** Relative mRNA expressions of target proteins, n = 3. **G** Confocal microscopy of HC11 immunostained for α-casein (green), β-casein (green), WAP (green) and DAPI (blue). Scale bars, 100 μm. The small red boxes in the figure are typical areas selected, and the three small images on the right are the enlarged images of the target protein, nucleus and merge images in selected areas, respectively. **H**–**J** Western blots and real time PCR of HC11 lysates for critical protein synthesis genes. In (**H**), bands were incubated with target proteins including α-casein, β-casein, WAP and Actin antibodies, n = 3. Protein expressions of target proteins are shown in (**I**), n = 3. (**J**) shows relative mRNA expressions of target proteins, n = 3. **K** TAG concentration in cell (top) and medium (bottom), n = 6. Cells are first transfected with siRNA and treated with the corresponding medium, the cell lysate and medium are then collected for detection. **L** Western blots of HC11 lysates for AMPK signaling pathway, critical milk protein and fat synthesis genes. In this figure, cells for oil red O staining, TAG determination, immunofluorescence staining and WB were collected after 24 h incubation with 0 mM glucose medium, 2 mM glucose medium and (2 mM glucose + 50 μM Compound C) medium and cells for real-time PCR are 12 h. All datas with error bars are averages ± SEM. In histograms, the numbers above the column are the p-values marked as a range if significantly different ($P < 0.05$) and each small black dot represents a test value

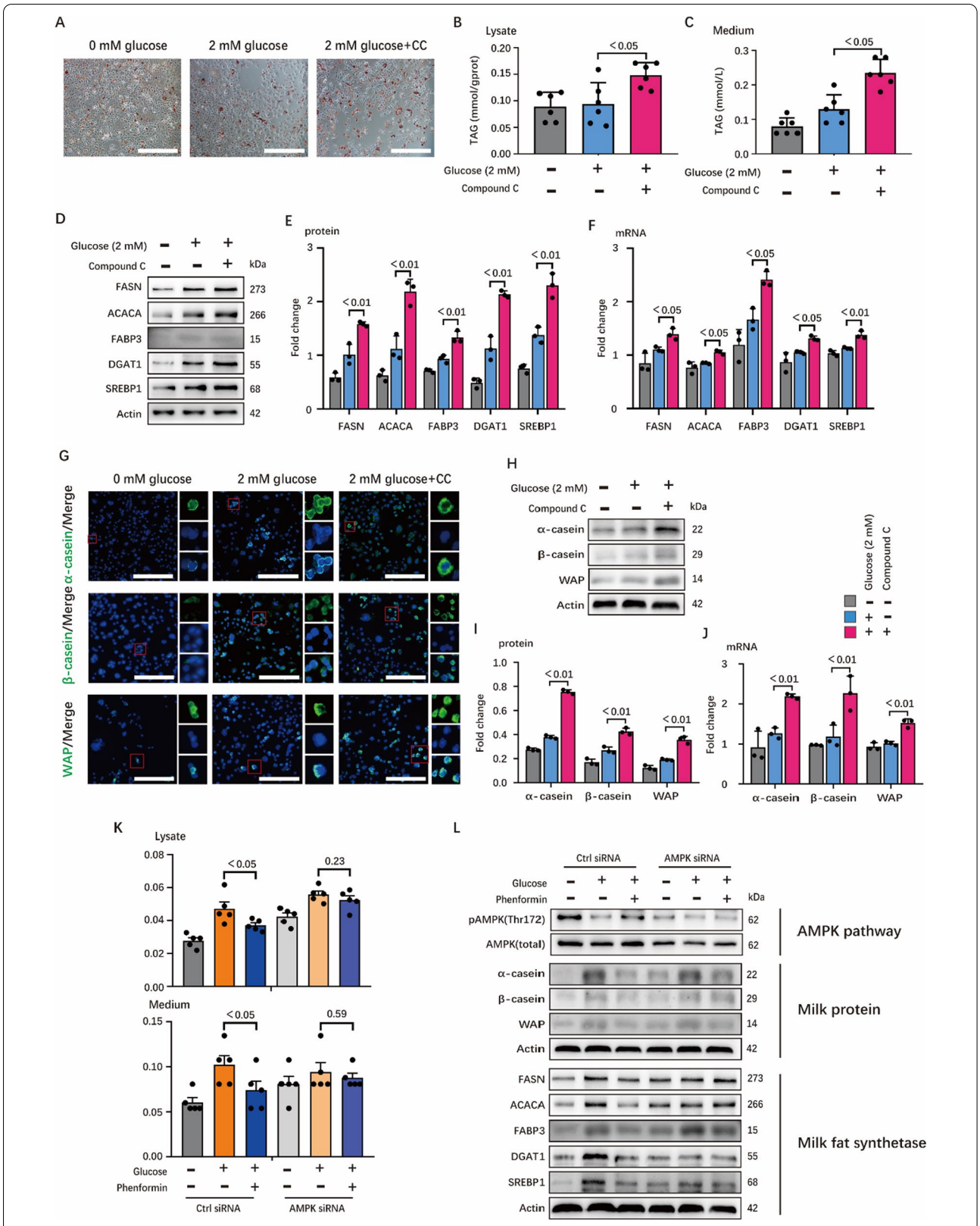


Fig. 3 (See legend on previous page.)

AMPK signaling regulates milk synthesis partially through mTORC1 pathway

mTORC1 is a master regulator of cell growth and metabolism. Activation of mTORC1 further phosphorylates S6K1 and 4EBP1 and promotes protein synthesis, which might partially regulate milk fat and protein synthesis. In HC11 cell line, glucose starvation and/or AMPK agonists (AICAR and Phenformin) both inhibited mTORC1 signaling (phosphorylation of S6K1 and 4EBP1) (Additional file 1: Fig. S3A–B). Besides, glucose starvation also regulates the AMPK/mTORC1 pathway in PMEC (Additional file 1: Fig. S3C–D). In contrast, AMPK antagonist (Compound C) activated the mTORC1 signaling pathway (Additional file 1: Fig. S3E–F). To further investigate the role of mTORC1 on milk fat and protein synthesis, we pretreated the HC11 cell line with rapamycin (an inhibitor of mTORC1) to inhibit mTORC1 pathway (Additional file 1: Fig. S4A-B) before glucose stimulation. Likewise, the positive effects of glucose on the expression of critical enzymes involved in milk fat (Additional file 1: Fig. S4C–H) and protein synthesis (Additional file 1: Fig. S4I–K) was inhibited. Thus, this evidence indicates the critical role of mTORC1 in milk synthesis.

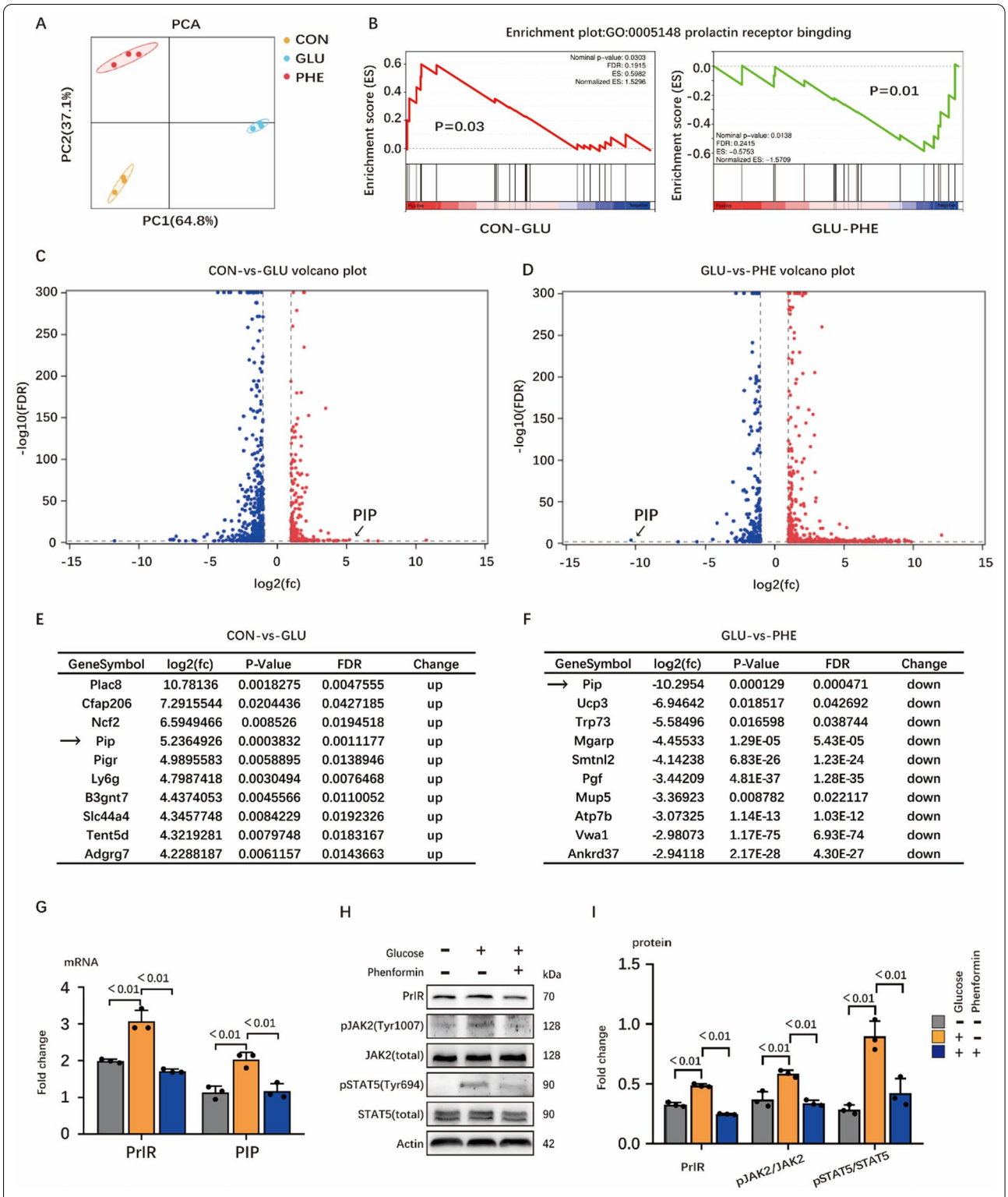
Energy stress might regulate milk synthesis through AMPK/PrlR pathway

In order to discover novel mechanism of how energy stress regulates milk synthesis, RNA-seq was performed to compare the transcriptomes of HC11 cell line starved for glucose or treated with AMPK agonist with those under normal situation (Fig. 4A). GSEA analysis indicated that signaling pathways related to prolactin binding were significantly inhibited in group CON (glucose starvation) and group PHE (AMPK activation) (Fig. 4B). Intriguingly, the fold change of prolactin induced protein (PIP, considered as a downstream of PrlR signaling pathway) was prominent in the volcano map (Fig. 4C, D) and was ranked fourth and first in terms of up- and down-regulated genes in Fig. 4C, D, respectively (Fig. 4E, F). Therefore, we made a hypothesis that AMPK activation might decrease PrlR expression and inhibit its downstream signaling. Real-time PCR confirmed the decrease of PrlR and PIP in group CON and group PHE (Fig. 4G). Furthermore, the phosphorylation of JAK2 and STAT5 (downstream targets of PrlR signaling) were also significantly inhibited under glucose starvation or AMPK activation (Fig. 4H, I). We concluded that energy stress may regulate milk synthesis through PrlR pathway due to the significant change on AMPK/PrlR and its downstream pathways under AMPK activation.

Intriguingly, RNA-seq and GSEA analysis found that genes (4F2hc, xCT, CAT1, ASCT1, ASCT2) involved in amino acid transportation were up-regulated under glucose starvation and stimulation of phenformin (Additional file 1: Fig. S5A–B). These RNA-seq data were then confirmed by RT-PCR (Additional file 1: Fig. S5C). Even though amino acids were largely transported into cells, milk protein synthesis was still inhibited under glucose starvation and stimulation of phenformin, which was shown by our previous results. These evidences indicated that amino acids might not be used for milk synthesis, but stead metabolized for energy supply. Amino acid transaminase catalyzes the transfer of the amino group (-NH2), which converts amino acids into α-keto acid for further metabolism. RNA-seq data and RT-PCR data further confirmed that multiple amino acid transaminases (BCAT2, GPT2, GOT1) were up-regulated with under glucose starvation (Additional file 1: Fig. S5D–E), which indicates that these amino acids could be used as energy supply.

(See figure on next page.)

Fig. 4 Activated AMPK induces the downstream PrlR/JAK2/STAT5/PIP pathway. **A** PCA of RNA-seq, n = 3. **B** GSEA showing enrichment of prolactin receptor binding in HC11 differentially expressed genes. The graph (left) shows the result of the control group-vs-glucose group and another graph (right) shows glucose group-vs-PHE group. **C** and **D** Volcano plots based on the significantly different genes in each comparison group. **C** Volcano plot of the control group-vs-glucose group and **D** Glucose group-vs-PHE group. The dots that the arrows point to are PIP. **E** and **F** (**E**) are top 10 significantly up-regulated 10 genes in the con-vs-glu comparison, and (**F**) are top 10 significantly down-regulated in the glu-vs-PHE comparison. **G** Relative mRNA expressions of PrlP and PIP, n = 3. **H** and **I** Western blots of HC11 lysates for PrlR/JAK2/STAT5 pathway. In (**H**), bands were incubated with target proteins and Actin antibodies, n = 3. Protein expressions of target proteins are shown in (**I**), n = 3. In this figure, cells for WB were collected after 24 h incubation with 0 mM glucose medium, 10 mM glucose medium and (10 mM glucose + 300 μM Phe) medium and cells for real-time PCR and RNA-seq are 12 h. All data with error bars are averages ± SEM. In histograms, the numbers above the column are the p-values marked as a range if significantly different ($P < 0.05$) and each small black dot represents a test value



GeneSymbol	log2(fc)	P-Value	FDR	Change
Plac8	10.78136	0.0018275	0.0047555	up
Cfap206	7.2915544	0.0204436	0.0427185	up
Ncf2	6.5949466	0.008526	0.0194518	up
→ Pip	5.2364926	0.0003832	0.0011177	up
Pigr	4.9895583	0.0058895	0.0138946	up
Ly6g	4.7987418	0.0030494	0.0076468	up
B3gnt7	4.4374053	0.0045566	0.0110052	up
Slc44a4	4.3457748	0.0084229	0.0192326	up
Tent5d	4.3219281	0.0079748	0.0183167	up
Adgrg7	4.2288187	0.0061157	0.0143663	up

GeneSymbol	log2(fc)	P-Value	FDR	Change
→ Pip	-10.2954	0.000129	0.000471	down
Ucp3	-6.94642	0.018517	0.042692	down
Trp73	-5.58496	0.016598	0.038744	down
Mgarp	-4.45533	1.29E-05	5.43E-05	down
Smtnl2	-4.14238	6.83E-26	1.23E-24	down
Pgf	-3.44209	4.81E-37	1.28E-35	down
Mup5	-3.36923	0.008782	0.022117	down
Atp7b	-3.07325	1.14E-13	1.03E-12	down
Vwa1	-2.98073	1.17E-75	6.93E-74	down
Ankrd37	-2.94118	2.17E-28	4.30E-27	down

Fig. 4 (See legend on previous page.)

AMPK activation plays a role in the ubiquitination-induced degradation of PrlR by up-regulating the activity of β-TrCP

In order to confirm whether the PrlR signaling is involved in AMPK-regulated milk fat and protein synthesis, we knocked down PrlR using siRNA. After PrlR knockdown, AMPK activation still inhibited milk fat synthesis (Fig. 5A, B). Nevertheless, the synthesis of milk protein was significantly suppressed after PrlR knockdown as is shown by immunofluorescence images (Fig. 5C). Besides, we also observed similar trends at the protein level (Fig. 5D, E). Thus, PrlR seems to promote the synthesis of milk protein but not milk fat.

PrlR is a transmembrane protein, its degradation could be regulated by a variety of physiological processes (such as ubiquitination) and multiple organelles (such as proteasomes and lysosomes). Thus, we then studied the degradation mechanism of PrlR. The results showed that PrlR seems to be taken up more through endocytosis and finally be degraded by lysosomes (Fig. 6A–C). F-box protein β-transducin repeat-containing protein (β-TrCP) is a component of the ubiquitin ligase complex that triggers ubiquitin-dependent degradation of multiple proteins. We found that β-TrCP was significantly up-regulated under AMPK activation, which is accompanied with the degradation of PrlR (Fig. 6D, E). Importantly, when the β-TrCP was knocked down by siRNA, its induction of PrlR degradation was reduced (Fig. 6D–F). Consistent with this, the expression level of milk protein also decreased with the knockdown of β-TrCP (Fig. 6D–G). In order to confirm that the degradation of PrlR may be due to β-TrCP-induced ubiquitination, we used MA (lysosomal inhibitor) to block the degradation process, and used immunoprecipitation to observe the ubiquitination of PrlR. We found that AMPK activation can significantly promote the ubiquitination of PrlR (Fig. 6H). In subsequent experiments, we found that knockdown of β-TrCP can weaken the ubiquitination of PrlR (Fig. 6I). In summary, AMPK activation can promote the degradation of PrlR under lysosome endocytosis, specifically by up-regulating the activity of β-TrCP to regulate ubiquitination-induced degradation of PrlR.

AMPK activation up-regulates the expression of PGC-1α and enhances the β-oxidation of fatty acids by inhibiting PGC-1α acetylation

In order to identified how AMPK regulates milk fat level, we considered to study the process of fatty acids synthesis and fatty acid β-oxidation. Previously, ACC (critical enzyme involved in fatty acid synthesis) and PGC-1α (critical enzyme involved in fatty acid β-oxidation) were identified as downstream targets of AMPK and found to play significant roles in fat metabolism in hepatocyte [12] and adipocyte [13]. Since a number of evidences have already indicated that ACC can affect fat production in mammary gland. Here, we would illustrate the impact of AMPK on milk fat synthesis from the perspective of fatty acid oxidation, thus, we took PGC-1α as the entry point. Subsequent tests further proved that the negative effect of AMPK on milk fat synthesis was partially rescued when PGC-1α was knocked down (Fig. 7A–C). These evidence clearly indicated that AMPK might regulate milk fat synthesis mainly through PGC-1α in mammary epithelial cells.

Since previous studies have found that AMPK regulates PGC-1α through p38 MAPK [14] or Sirt1 [15] in cancer cells and skeletal muscle, we further investigated the relationship between AMPK and PGC-1α in HC11. We found that AMPK activation inhibits the phosphorylation of p38 pathway. When p38 was inhibited, the expression of PGC-1α was increased independent of the activation

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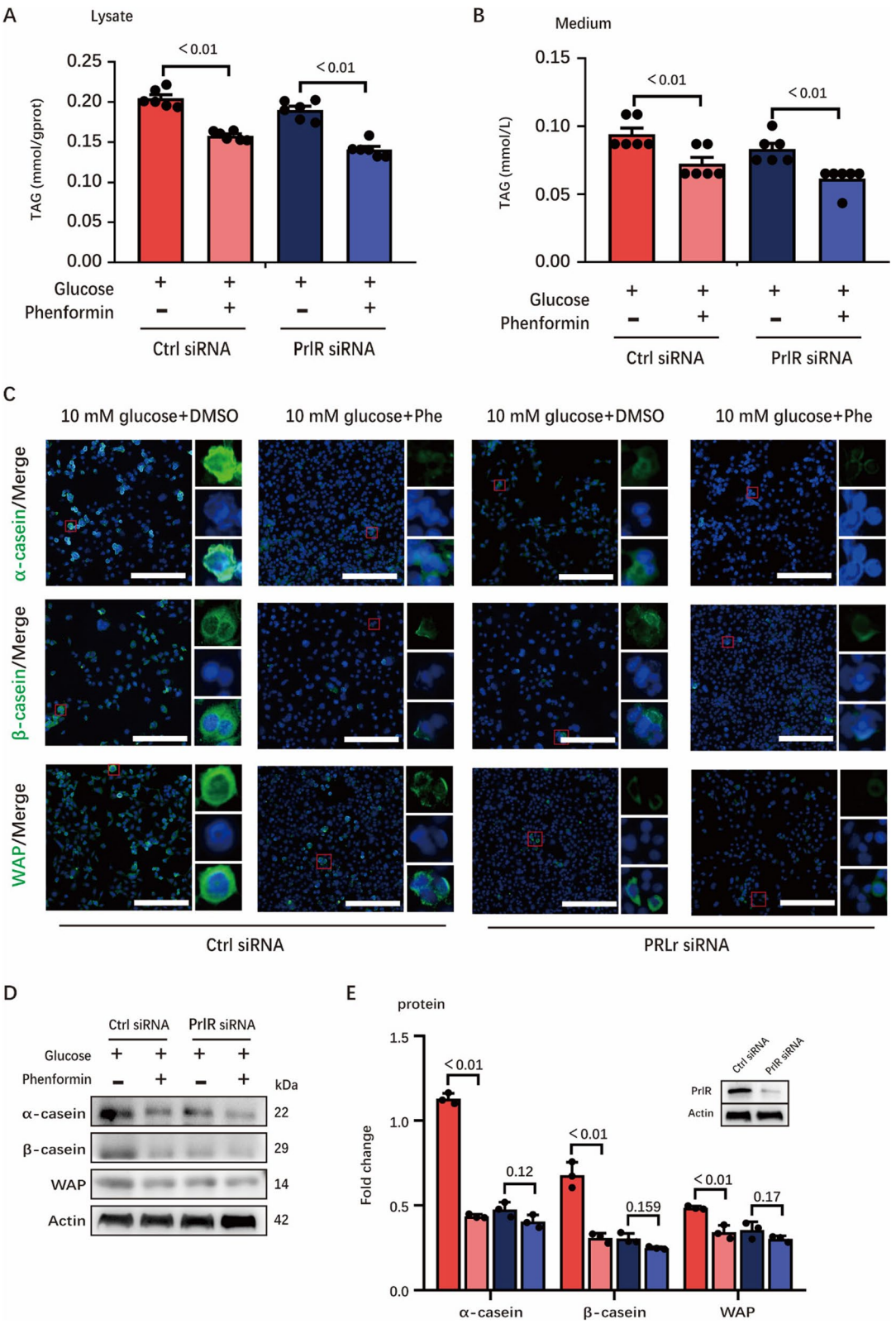
Fig. 5 Knock-down of PrlR lowers synthesis of milk protein but little affects milk fat. **A** and **B** TAG concentration in cell (**A**) and medium (**B**), n = 6. **C** Confocal microscopy of HC11 immunostained for α-casein (green), β-casein (green), WAP (green) and DAPI (blue). Scale bars, 100 μm. The small red boxes in the figure are typical areas selected, and the three small images on the right are the enlarged images of the target protein, nucleus and merge images in selected areas, respectively. **D** and **E** Western blots of HC11 lysates for critical milk protein synthesis genes. **D** Representative western-blotting bands hybridized with target proteins including α-casein, β-casein, WAP and Actin antibodies, n = 3. **E** Result of analyzing the WB bands. In this figure, cells (transfected by siRNA in advance) for TAG determination, immunofluorescence staining and WB were collected after 24 h incubation with 10 mM glucose medium and (10 mM glucose + 300 μM Phe) medium. All datas with error bars are averages ± SEM. In histograms, the numbers above the column are the p-values marked as a range if significantly different ($P < 0.05$) and each small black dot represents a test value



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of AMPK (Fig. 7D, E). Regarding Sirt1, although AMPK activation promotes Sirt1 expression, Sirt1 knockdown has no effect on PGC expression induced by AMPK (Fig. 7F, G). In order to explore its mechanism, we used immunoprecipitation to observe the relationship between AMPK and the acetylation of PGC-1α. We found that glucose starvation or AMPK activation decreased the acetylation level of PGC-1α (Fig. 7H). Collectively, AMPK might regulate milk fat synthesis partially by up-regulating the activity of PGC-1α and inhibiting its acetylation.

Discussion

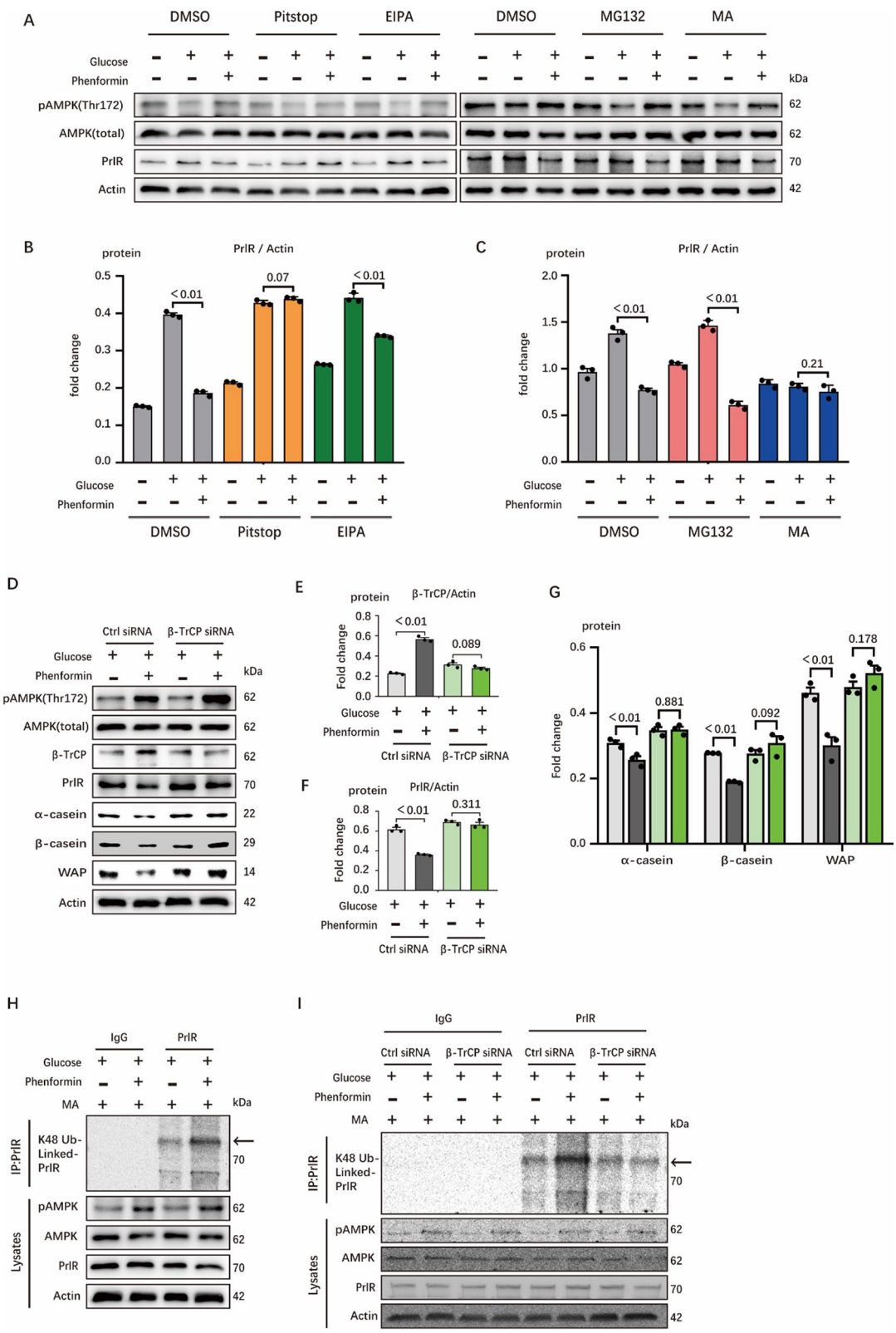
The intimate relationship between milk synthesis and AMPK signaling has been identified for a long time [16], whereas the underlying mechanism is not well characterized. Here, we identified two novel target proteins (PrlR and PGC-1α) of AMPK signaling that are involved in the regulation of milk synthesis. Mechanistically, AMPK triggers the ubiquitination and degradation of PrlR through β-TrCP, which then attenuates the PrlR-JAK2-STAT5 pathway involved in milk protein synthesis. At the same time, AMPK decreases the acetylation process of PGC-1α and inhibits milk fat accumulation.

PrlR signaling regulates the growth and maturation of the mammary gland, which is also critical for the maintenance of lactation [17]. PrlR deficiency significantly inhibits milk protein synthesis. In this study, RNA-seq data revealed that the gene expression of PrlR was down-regulated when AMPK was activated, which indicates AMPK might regulate milk protein synthesis through PrlR. Ubiquitination is one of the crucial mechanism to induce the degradation of hormone receptors [18]. Studies have shown that AMPK could control the uptake of long-chain fatty acids (LCFA) by regulating the expression of the membrane receptor CD36 through Parkin-mediated polyubiquitination in intestinal epithelial cells [19]. Furthermore, the activation of AMPK triggers the interaction between β-TrCP and Glioma-associated oncogenes (GLI1), which induces β-TrCP-mediated GLI1-ubiquitination and degradation in human brain cancer cells [20]. Our results first revealed that PrlR was mainly degraded by lysosome through endocytosis process under the influence of AMPK activation. In the above process, AMPK activation up-regulated the activity of β-TrCP, which induced the ubiquitination degradation of PrlR.

PGC-1α is considered as a transcriptional coactivator of PPARγ [21], which regulates fatty acid β-oxidation in the liver [22] and skeletal muscle [23]. To date, the role of PGC-1α in mammary gland is still unclear. In this study, we found AMPK regulates milk fat production not only through the process of milk fat synthesis, but also the process of β-oxidation. Previously, AMPK activation has been reported to activate CaMKKβ and increase the expression of PGC-1α [24]. In addition, more evidences also indicate AMPK might regulate the expression of PGC-1α through Sirt1 [25] or p38 MAPK dependent signaling [14]. Surprisingly, in this study, we observed that AMPK regulates activity of PGC-1α mainly through the p38 MAPK signaling pathway, but not Sirt1 signaling in mammary epithelial cells. The inconsistent results regarding the regulatory molecules that signal AMPK to PGC-1α could partially be explained by tissue-specific regulation system. To our

(See figure on next page.)

Fig. 6 β-TrCP mediating AMPK is involved in the ubiquitination degradation of PrlR. **A**–**C** Western blots of HC11 lysates for PrlR, n = 3. As shown in the left part of (**A**), the cells were treated with DMSO, Pitstop and EIPA to explore the effect of AMPK activation on the degradation of PrlR to explore the degradation mode of PrlR. In the right part of (**A**), similar methods have also been used to explore the organelles involved in the degradation of PrlR. (**B)** and (**C)** are the quantifications of protein expression in the above two experiments, respectively. (Pitstop: pinocytosis inhibitor, EIPA: endocytosis inhibitor, MG132: proteasome inhibitor, MA: lysosomal inhibitor) **D**–**G** Western blots of HC11 lysates for AMPK/β-TrCP/PrlR pathway, n = 3. In (**D**), bands were incubated with indicated proteins and Actin antibodies, n = 3. Protein expressions of β-TrCP, PrlR and milk proteins are shown in (**E**), (**F**) and (**G**), respectively, n = 3. **H** Immunoblot analysis of PrlR immunoprecipitates in HC11, probed for AMPK/Ub-PrlR pathway. After being cultured in the designated medium (all containing MA), cells were lysed by CHAPS lysis buffer, and lysate were not only used for immunoprecipitation to detect the ubiquitination of PrlR but also for WB to detect AMPK/PrlR pathway. **I** Immunoblot analysis of PrlR immunoprecipitates in HC11, probed AMPK/β-TrCP/Ub-PrlR pathway. After being transinfected by siRNA and cultured in the designated medium (all containing MA), cells were lysed by CHAPS lysis buffer, and lysate were not only used for immunoprecipitation to detect the ubiquitination of PrlR but also for WB to detect AMPK/PrlR pathway. In this figure, cells for WB were collected after 24 h incubation with indicated medium. All datas with error bars are averages ± SEM. In histograms, the numbers above the column are the *p*-values marked as a range if significantly different ($P < 0.05$) and each small black dot represents a test value



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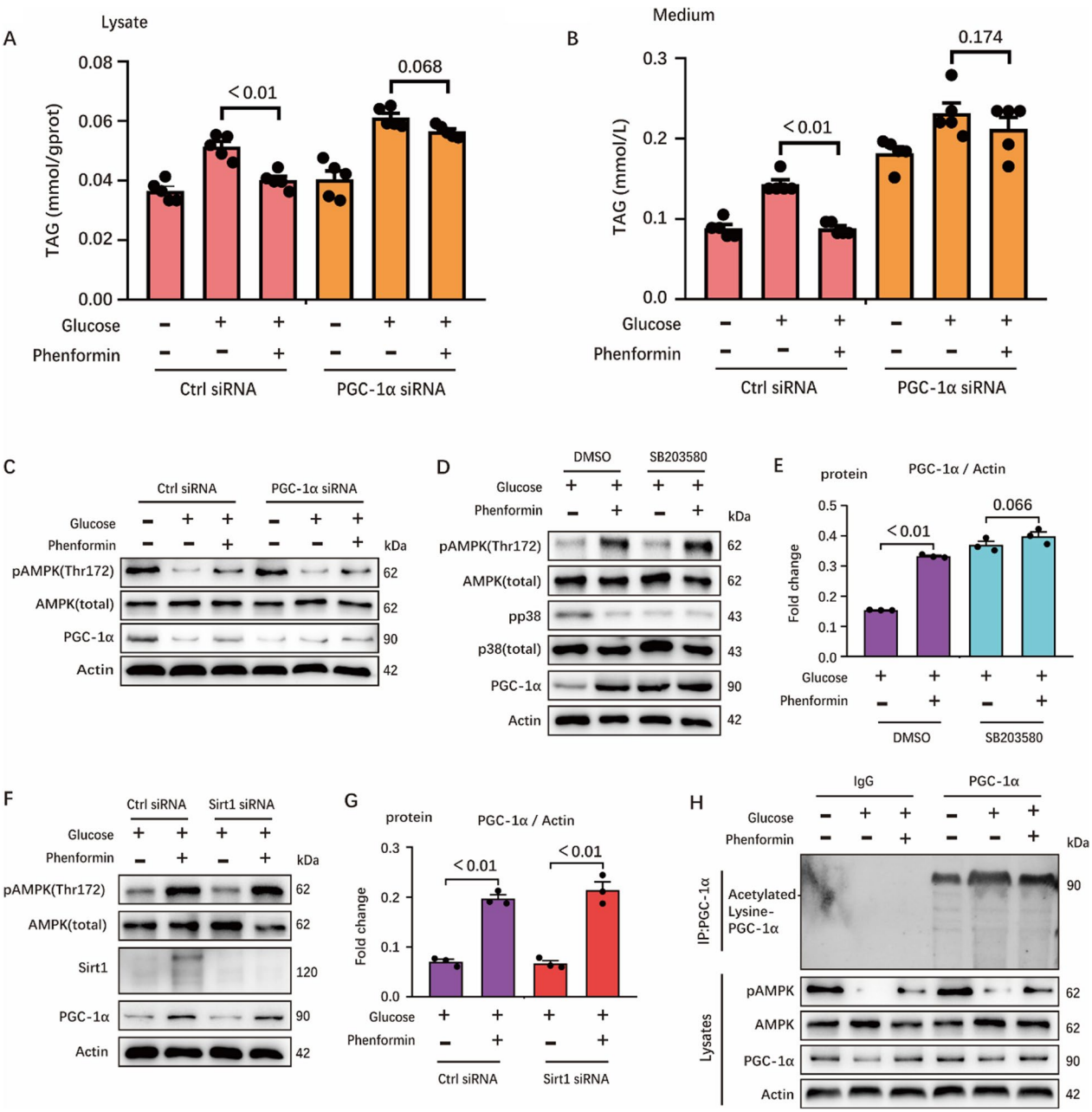

Fig. 7 Loss of PGC-1α facilitates milk fat production due to its acetylation regulated by AMPK. **A** and **B** TAG concentration in cell (**A**) and medium (**B**), n = 5. In the experiment, cells (transfected by siRNA in advance) for TAG determination were collected after 24 h incubation with 0 mM glucose medium, 10 mM glucose medium and (10 mM glucose + 300 μM Phe) medium. **C** Western blots of HC11 lysates for AMPK/PGC-1α signaling pathway, n = 3. **D** and **E** Western blots of HC11 lysates for AMPK/p38/PGC-1α signaling pathway, n = 3. Cells were treated with 10 mM glucose medium and (10 mM glucose + 300 μM Phe) medium in the case of DMSO and SB203580 (p38 inhibitor) for 24 h, respectively. In (**D**), bands were incubated with target proteins and Actin antibodies, n = 3. Protein expressions of target proteins are shown in (**E**), n = 3. **F** and **G** Western blots of HC11 lysates for AMPK/Sirt1/PGC-1α signaling pathway, n = 3. Cells were treated with 10 mM glucose medium and (10 mM glucose + 300 μM Phe) medium in the case of control-knockdown and Sirt1-knockdown for 24 h, respectively. In (**F**), bands were incubated with target proteins and Actin antibodies, n = 3. Protein expressions of target proteins are shown in (**G**), n = 3. **H** Immunoblot analysis of PGC-1α immunoprecipitates in HC11, probed for acetylation of PGC-1α. Cells were lysed by CHAPS lysis buffer, and lysate were not only used for immunoprecipitation to detect the acetylation of PGC-1α but also for WB to detect AMPK/PGC-1α pathway. In this figure, cells for TAG determination and WB were collected after 24 h incubation with indicated medium. All datas with error bars are averages ± SEM. In histograms, the numbers above the column are the *p*-values marked as a range if significantly different ($P < 0.05$) and each small black dot represents a test value

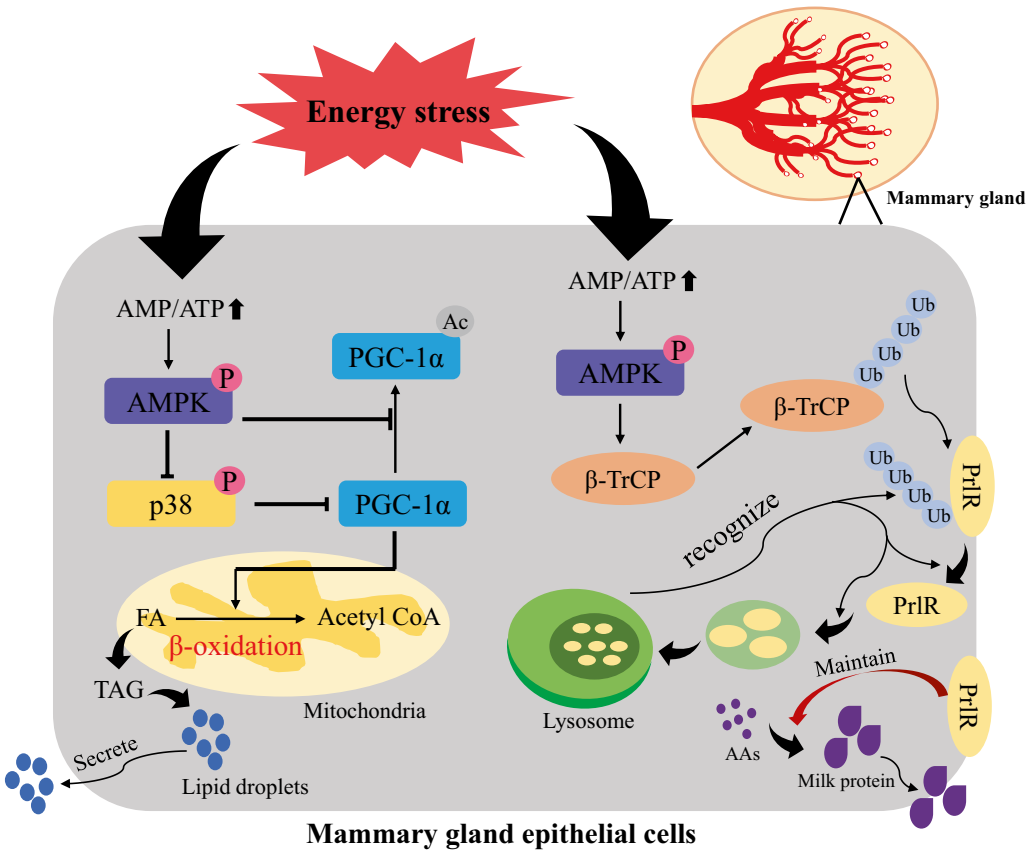

Fig. 8 Overall of AMPK's effect on milk fat and protein in HC11 cell line

knowledge, the activity of PGC-1α is regulated by post-translational modifications, including phosphorylation [26] and acetylation [27]. In subsequent experiments, we provided evidence that AMPK can inhibit the acetylation of PGC-1α. AMPK activation inhibits the activity of ACACA, which makes it difficult for acetyl-CoA to be converted into fatty acids. Thus, acetyl-CoA accumulation might eventually lead to the inhibition of the acetylation reaction [28]. As a non-histone protein, the molecular mechanism of acetylation of PGC-1α warrants additional work.

Conclusions

In summary, we confirmed the hypothesis that AMPK regulates the production of milk protein and fat in the mammary gland by regulating the degradation of PrlR and the acetylation of PGC-1α, respectively (Fig. 8). Although some mechanisms require further verification, it is foreseeable that this mechanism can be utilized to provide new possibilities and inspiration for the regulation of milk synthesis.

Abbreviations

AMPK: AMP-activated protein kinase; mTORC1: Mammalian target of rapamycin complex 1; PrlR: Prolactin receptor; β-TrCP: β-Transducin repeat-containing protein; PGC-1α: Peroxisome proliferator-activated receptor gamma coactivator-1 alpha; FBP: Fructose-1,6-bisphosphate; FA: Fatty acid; TAG: Triacylglycerol; ACACA: Acetyl-coA carboxylase alpha; SREBP1c: Sterol regulatory element-binding protein 1c; S6K1: S6 kinase 1; 4EBP1: 4E binding protein 1; pMECs: Porcine mammary gland epithelial cells; TSC2: Tuberin 2.

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s12964-022-00830-6.

Additional file 1. **Fig. S1.** Glucose increases milk fat production but inhibits AMPK. (A) Oil red O staining images of pMECs. Scale bars are 100 μm. (B and C) Western blots of pMECs lysates for critical fat synthesis genes. (B) are representative western-blotting bands hybridized with target proteins including FASN, ACACA, FABP3, DGAT1, SREBP1 and Actin antibodies, n=3. (C) is the result of analyzing the WB bands, n=3. (D) Ratio of ATP and ADP, n=3. HC11 cells were collected after 24 h incubation with 0 mM and 10 mM glucose mediums. (E and F) Western blots analysis of HC11 lysates for AMPK signaling pathway, n=3. Cells were collected after 24 h incubation with 0 mM and 10 mM glucose mediums. **Fig. S2.** Inhibition of AMPK still promotes the synthesis of milk fat and protein in the absence of glucose. (A) Western blots analysis of HC11 lysates for AMPK signaling pathway. Cells were collected after 24 h incubation with 0 mM glucose medium, 10 mM glucose medium, (10 mM glucose +CC) medium, 2 mM glucose medium and (2 mM glucose +CC) medium. (B and C) TAG TAG

concentration in cell (B) and medium (C), n=6. (D and E) Western blots of HC11 lysates for critical protein synthesis genes. (D) are representative western-blotting bands hybridized with target proteins including α-casein, β-casein, WAP and Actin antibodies, n=3. (E) is the result of analyzing the WB bands, n=3. **Fig. S3.** AMPK activates the mTORC1/S6K1/4EBP1 pathway in both HC11 and pMECs. (A and B) Western blots analysis of HC11 lysates for mTORC1/S6K1/4EBP1 signaling pathway, n=3. Cells were collected after 24 h incubation with 0 mM glucose medium, 10 mM glucose medium, (10 mM glucose +AICAR) medium and (10 mM glucose +Phe) medium. (C and D) Western blots analysis of pMECs lysates for AMPK/mTORC1 signaling pathway, n=3. (E and F) Western blots analysis of HC11 lysates for AMPK/mTORC1 signaling pathway. Cells were collected after 24 h incubation with 0 mM glucose medium, 2 mM glucose medium and (2 mM glucose +CC) medium, n=3. **Fig. S4.** mTORC1 up-regulates synthesis of milk fat and protein in HC11. (A and B) Western blots analysis of HC11 lysates for mTORC1/S6K1/4EBP1 signaling pathway, n=3. (C) Oil red O staining images of HC11. Scale bars are 50 μm. (D and E) TAG concentration in cell (D) and medium (E), n=4. (F-H) Western blots and real time PCR of HC11 lysates for critical fat synthesis genes. (F) are representative western-blotting bands hybridized with target proteins including FASN, ACACA, FABP3, DGAT1, SREBP1 and Actin antibodies, n=3. (G) is the result of analyzing the WB bands and (H) represents datas from real-time PCR, n=3. (I-K) Western blots and real time PCR of HC11 lysates for critical protein synthesis genes. (I) are representative western-blotting bands hybridized with target proteins including α-casein, β-casein, WAP and Actin antibodies, n=3. (J) is the result of analyzing the WB bands and (K) represents datas from real-time PCR, n=3. In this figure, cells for oil red O staining, TAG determination and WB were collected after 24 h incubation with 0 mM glucose medium, 10 mM glucose medium and (10 mM glucose +rapamycin) medium and cells for real-time PCR are 12 h. All datas with error bars are averages ± SEM. In histograms, the numbers above the column are the p-values marked as a range if significantly different ($P < 0.05$) and each small white dot represents a test value. **Fig. S5.** AMPK is involved in regulating the activity of amino acid transporters in HC11. (A) GSEA showing enrichment of amino acid transport across plasma membrane in HC11 differentially expressed genes. The graph (left) shows the result of the control group-vs-glucose group and another graph (right) shows glucose group-vs-PHE group. (B and C) (B) is heatmap analysis of up-regulated (red) or down-regulated genes (blue) in HC11, n=3. The tested genes are amino acid transporters selected from the GSEA gene set (shown in the figure). (C) is the result of real-time PCR, n=3. (D and E) (D) is heatmap analysis of up-regulated (red) or down-regulated genes (blue) in HC11, n=3. The tested genes are typical aminotransferases (shown in the figure). (E) is the result of real-time PCR, n=3. In this figure, cells for RNA-seq and real-time PCR were collected after 12 h incubation with 0 mM glucose medium, 10 mM glucose medium and (10 mM glucose +Phe) medium. All datas with error bars are averages ± SEM. In histograms, the numbers above the column are the p-values marked as a range if significantly different ($P < 0.05$) and each small white dot represents a test value.

Acknowledgements
We thank the National Natural Science Foundation of the P.R. of China, Guangdong Basic and Applied Basic Research Foundation, Science and Technology Program of Guangzhou.

Authors' contributions
SZ and FC designed the research. ZW took the lead in performing all experiments with critical help of QL, SY, TZ and JS, and conducted the statistical analysis. ZW and SZ wrote the manuscript. SZ, FC and WG conducted the final editing. SZ supervised the study throughout. All authors read and approved the final manuscript.

Funding
This study was financially supported by the National Key R&D Program of China (2021YFD1300700), Guangdong Basic and Applied Basic Research Foundation (No. 2021A1515010440), Science and Technology Program of Guangzhou (No. 202102020056).

Availability of data and materials
The data analyzed during the current study are available from the corresponding author on request.

Declarations

Ethics approval and consent to participate
Not applicable.

Consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

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Received: 10 December 2021 Accepted: 9 January 2022
Published online: 05 March 2022

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Lead Article

Novel advances in understanding fatty acid–binding G protein–coupled receptors and their roles in controlling energy balance

Min Tian, Zhihui Wu, Jinghui Heng, Fang Chen, Wutai Guan, and Shihai Zhang

Diabetes, obesity, and other metabolic diseases have been recognized as the main factors that endanger human health worldwide. Most of these metabolic syndromes develop when the energy balance in the body is disrupted. Energy balance depends upon the systemic regulation of food intake, glucose homeostasis, and lipid metabolism. Fatty acid–binding G protein–coupled receptors (GPCRs) are widely expressed in various types of tissues and cells involved in energy homeostasis regulation. In this review, the distribution and biological functions of fatty acid–binding GPCRs are summarized, particularly with respect to the gut, pancreas, and adipose tissue. A systematic understanding of the physiological functions of the fatty acid–binding GPCRs involved in energy homeostasis regulation will help in identifying novel pharmacological targets for metabolic diseases.

INTRODUCTION

Fatty acids (FAs) are critical components of cellular membranes, precursors of lipid hormones, and major sources of energy. Current evidence suggests that free FAs (FFAs) also participate in the regulation of multiple cellular functions through G protein–coupled receptors (GPCRs) (also identified as FFA-sensitive receptors [FFARs]).[1–3] GPCRs are important pharmaceutical targets because they coordinate numerous cellular signaling cascades and regulate many physiological functions.[4] In humans, the GPCR family members are each encoded by 800 genes, with 20 genes encoding the G_α subunit, 5 genes encoding the G_β subunit, and 14 genes encoding the G_γ subunit.[5] To date, approximately 33% of Food and Drug Administration (FDA)-approved drugs target GPCRs.[6] GPCRs contain 7 transmembrane domains that directly bind with heterotrimeric G proteins.[7] Heterotrimeric G proteins consist of G_α (binding to either guanosine triphosphate (GTP) or guanosine diphosphate (GDP)), G_β, and G_γ subunits.[8] The activation of GPCRs results in a conformational change in the α-subunit, which leads to the exchange of GTP for GDP and the dissociation of the $G_{\beta\gamma}$ subunits.[8] Subsequently, free G_α and $G_{\beta\gamma}$ subunits trigger individual downstream targets and modulate pivotal cellular biological functions.[8] The specific downstream targets of GPCRs largely depend on the classification of the G_α subunits, consisting of $G_{\alpha s}$, $G_{\alpha i}$, $G_{\alpha q/11}$, and $G_{\alpha 12/13}$.[8]

Metabolic discord leads to diabetes, obesity, and related diseases worldwide, jeopardizing health around the globe.[9,10] Recent studies have shown that FA-

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Key words: energy balance, GPR40, GPR41, GPR43, GPR120, G protein–coupled receptor

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doi: 10.1093/nutrit/nuab021

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binding GPCRs are involved in the regulation of whole-body energy balance and metabolic functions. Fatty acid-sensing GPCRs are widely expressed in organs involved in the regulation of energy metabolism, including the gut, pancreas, and adipose tissue.[11–14] Gut-derived (peptide YY [PYY], cholecystokinin [CCK], and glucagon-like peptide 1 [GLP-1]) and adiposity-derived (leptin) hormones participate in the short-term and long-term regulation of energy intake, while pancreas-derived hormones (glucagon and insulin) are mainly responsible for glucose homeostasis. In this review, the canonical downstream targets of FFARs coupled to different G_α subunits are summarized. Next, this review provides an overview of the distribution and biological functions of FFARs in tissues and/or organs that are involved in energy homeostasis. The limitations of the current understanding of FFARs are also highlighted in this review. Understanding the physiological functions of FFARs in the regulation of energy homeostasis will help in identifying novel pharmacological targets for metabolic diseases.

FFAR-MEDIATED ACTIVATION OF G PROTEINS

Free fatty acids are generally classified into short-chain FAs (SCFAs, with the number of carbon atoms fewer than 6, $C \leq 6$), medium-chain FAs (MCFAs, $6 < C \leq 12$), and long-chain FAs (LCFAs, $C > 12$).[15] The activation of FFARs is dependent on the number of carbon atoms in the main carbon chain of the FFA,[4] including GPR40 (also named FFAR1, coupling $G_{\alpha q}$), GPR41 (FFAR3, coupling $G_{\alpha i}$), GPR43 (FFAR2, coupling $G_{\alpha q}/G_{\alpha i}$), and GPR120 (FFAR4, coupling $G_{\alpha q}/G_{\alpha i}$).[15] The agonists and antagonists of the various FFARs have been previously summarized.[3,15]

GPR41 and GPR43 have a similar molecular structure (52% similarity and 43% identity) and are activated by SCFAs.[16] The ligand affinity of GPR43 is "acetate = propionate > butyrate", whereas the ligand affinity of GPR41 is "propionate > butyrate > pentanoate".[16–18] GPR40 and GPR120 are both critical for the sensing of MCFAs and LCFAs, although their amino acid homology is only 10%.[19,20] GPR40 is activated by saturated and unsaturated FAs with chain lengths of C8 to C22,[21] while GPR120 is activated by FAs with chain lengths of C14 to C18 (saturated FAs) and C16 to C22 (unsaturated FAs).[20,22] In addition, GPR84 is proposed to be activated by MCFAs, which are highly expressed in immune cells. Although some evidence indicates that GPR84 is also expressed in the intestine and adipose tissue, its function is still unclear and is thus not discussed in this review.[23] In contrast to receptors with FFA-sensing ability, GPR119 (coupled to $G_{\alpha s}$) is mainly activated by the triacylglycerol (TG) metabolite 2-monoacylglycerol (2-MAG) and oleic acid metabolites, including 1-oleoyl-lysophosphatidylcholine, *N*-oleoyl-dopamine, and 2-oleoyl glycerol (2-OG).[24–26] Food-derived FAs can directly activate corresponding FFARs (especially GPR40 and GPR120), depending on their specific FA profiles. The FA profiles of foods consumed for daily sustenance and their potential binding FFARs are listed in Table 1.[27–44] Moreover, dietary fiber can be fermented by microorganisms in the intestine to produce a large amount of SCFAs, which can also activate FFARs (GPR41 and GPR43) in the intestine and are important to a healthy organism.[45,46]

FFARs activate the $G_{\alpha i}$, $G_{\alpha s}$, and/or $G_{\alpha q}$ pathways. These G_α signaling pathways coordinate to regulate cellular biological functions by initiating a cascade of signaling events. To better understand the potential molecular mechanism of FFAR in biological regulation, critical downstream targets of FFAR-induced G_α signaling pathways are summarized (Figure 1). $G_{\alpha i}$ and $G_{\alpha s}$ coordinate to modulate the production of adenosine 3′,5′-cyclic monophosphate (cAMP) with the subsequent regulation of adenylyl cyclase (AC).[47] The activated form of $G_{\alpha i}$ (GTP-bound $G_{\alpha i}$) directly inhibits the activity of AC. In contrast, $G_{\alpha s}$ is critical for the activation of AC. As a crucial secondary messenger, the cAMP regulates numerous cellular functions, largely through protein kinase A (PKA) and an exchange protein directly activated by PKA and cAMP (EPAC). PKA consists of 2 regulatory subunits and 2 catalytic subunits. Once activated, the PKA catalytic subunits phosphorylate and regulate multiple nuclear and cytosolic substrates. EPAC acts as a guanine nucleotide exchange factor for the small GTP-binding protein Rap-1. Rap-1 influences a variety of target proteins that control diverse processes, including integrin activation, vesicle trafficking, and neuron polarity. The direct downstream target of $G_{\alpha q}$ is phospholipase Cβ (PLCβ). Activated PLCβ converts lipid PtdIns(4,5)P_2 (PIP_2) to inositol-1,4,5-trisphosphate (IP_3) and diacylglycerol (DAG).[47] IP_3 induces an increase in cellular Ca^{2+} signaling, which is thought to regulate a number of cell functions, including muscle contraction and hormone secretion.[48] DAG associates with multiple proteins and activates a variety of signaling cascades, including protein kinase C (PKC)-dependent pathways.[49]

SENSING OF FATTY ACIDS BY ENTEROENDOCRINE CELLS IN THE GUT

Intestinal enteroendocrine cells (EECs) produce a number of satiety gut hormones and play crucial roles in the regulation of appetite. EECs comprise 1% of total intestinal cells and are divided into multiple distinct populations, such as L cells, A cells, I cells, K cells, and N

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Table 1 **Fatty acid profiles of food consumed for daily sustenance and their potential binding free fatty acid–sensitive receptors**

Fat sources	Nutritional forms of fatty acid (>5%)	Potential binding FFARs	Reference
Milk			
Human colostrum	Palmitic acid C16:0; stearic acid C18:0; oleic acid C18:1 n-9; linoleic acid C18:2 n-6.	GPR40; GPR120	Azulay Chertok et al (2017)[27], Wu et al (2020)[28]
Human transitional milk	Lauric acid C12:0; myristic acid C14:0; palmitic acid C16:0; stearic acid C18:0; oleic acid C18:1 n-9; linoleic acid C18:2 n-6.	GPR40; GPR84; GPR120	Wu et al (2020)[28]
Human milk	Lauric acid C12:0; myristic acid C14:0; palmitic acid C16:0; stearic acid C18:0; oleic acid C18:1 n-9; linoleic acid C18:2 n-6.	GPR40; GPR84; GPR120	Wu et al (2020)[28], Hageman et al (2019)[29]
Cow colostrum	Myristic acid C14:0; palmitic acid C16:0; stearic acid C18:0; oleic acid C18:1 n-9.	GPR40; GPR84; GPR120	Mann et al (2016)[30]
Cow milk	Myristic acid C14:0; palmitic acid C16:0; stearic acid C18:0; oleic acid C18:1 n-9.	GPR40; GPR84; GPR120	Bernard et al (2018)[31]
Goat colostrum	Capric acid C10:0; lauric acid C12:0; myristic acid C14:0; palmitic acid C16:0; stearic acid C18:0; oleic acid C18:1 n-9.	GPR40; GPR84; GPR120	Lou et al (2018)[32]
Goat milk	Capric acid C10:0; lauric acid C12:0; myristic acid C14:0; palmitic acid C16:0; stearic acid C18:0; oleic acid C18:1 n-9.	GPR40; GPR84; GPR120	Bernard et al (2018)[31]
Sheep milk	Capric acid C10:0; lauric acid C12:0; myristic acid C14:0; palmitic acid C16:0; stearic acid C18:0; oleic acid C18:1 n-9.	GPR40; GPR84; GPR120	Serra et al (2018)[33], Pietrzak-Fiećko et al (2020)[34]
Pig colostrum	Palmitic acid C16:0; oleic acid C18:1 n-9; linoleic acid C18:2 n-6.	GPR40; GPR120	Lv et al (2015)[35]
Pig milk	Palmitic acid C16:0; palmitoleic acid C16:1 n-7; oleic acid C18:1 n-9; linoleic acid C18:2 n-6.	GPR40; GPR120	Lv et al (2015)[35]
Plant-based fat			
Soybean oil	Palmitic acid C16:0; oleic acid C18:1 n-9; linoleic acid C18:2.	GPR40; GPR120	Yang et al (2018)[36]
Corn oil	Palmitic acid C16:0; oleic acid C18:1 n-9; linoleic acid C18:2 n-6.	GPR40; GPR120	Radcliffe et al (2004)[37]
Palm oil	Lauric acid C12:0; palmitic acid C16:0; oleic acid C18:1 n-9; linoleic acid C18:2.	GPR40; GPR84; GPR120	Yang et al (2018)[36]
Coconut oil	Caprylic acid C8:0; capric acid C10:0; lauric acid C12:0; myristic acid C14:0; palmitic acid C16:0; oleic acid C18:1 n-9.	GPR40; GPR84; GPR120	Yang et al (2018)[36]
Cottonseed oil	Lauric acid C12:0; palmitic acid C16:0; oleic acid C18:1 n-9; linoleic acid C18:2.	GPR40; GPR84; GPR120	Radcliffe et al (2004)[37]
Peanut	Palmitic acid C16:0; oleic acid C18:1 n-9; linoleic acid C18:2, n-6.	GPR40; GPR120	Orsavova et al (2015)[38]

(continued)

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Table 1 **Continued**

Fat sources	Nutritional forms of fatty acid (>5%)	Potential binding FFARs	Reference
Rapeseed	Oleic acid C18:1 n-9; linoleic acid C18:2, n-6; C20:1(n-9).	GPR40; GPR120	Orsavova et al (2015)[38]
Sesame	Palmitic acid C16:0; stearic acid C18:0; oleic acid C18:1 n-9; linoleic acid C18:2; n-6.	GPR40; GPR120	Orsavova et al (2015)[38]
Olive	Palmitic acid C16:0; oleic acid C18:1 n-9; linoleic acid C18:2, n-6.	GPR40; GPR120	Orsavova et al (2015)[38]
Sunflower	Palmitic acid C16:0; oleic acid C18:1 n-9; linoleic acid C18:2; n-6.	GPR40; GPR120	Orsavova et al (2015)[38]
Rice bran	Palmitic acid C16:0; oleic acid C18:1 n-9; linoleic acid C18:2, n-6.	GPR40; GPR120	Orsavova et al (2015)[38]
Wheat germ	Palmitic acid C16:0; oleic acid C18:1 n-9; linoleic acid C18:2, n-6; C20:1(n-9).	GPR40; GPR120	Orsavova et al (2015)[38]
Animal-based oils			
Lard	Palmitic acid C16:0; oleic acid C18:1 n-9; linoleic acid C18:2 n-6.	GPR40; GPR120	Azir et al (2017)[39]
Cocoa butter	Palmitic acid C16:0; stearic acid C18:0; oleic acid C18:1 n-9.	GPR40; GPR120	Azir et al (2017)[39]
Butter	Myristic acid C14:0; palmitic acid C16:0; stearic acid C18:0; oleic acid C18:1 n-9.	GPR40; GPR84; GPR120	Bobe et al (2007)[40], O'Callaghan et al (2016)[41]
Animal-based fat			
Subcutaneous fat of calves	Palmitic acid C16:0; stearic acid C18:0; oleic acid C18:1 n-9.	GPR40; GPR120	Dahl et al (2020)[42]
Belly fat quality of finishing pigs	Oleic acid C18:1; linoleic acid C18:2.	GPR40; GPR120	Stephenson et al (2016)[43]
Jowl fat quality of finishing pigs	Oleic acid C18:1; linoleic acid C18:2.	GPR40; GPR120	Stephenson et al (2016)[43]
4-month-old castrated male goat subcutaneous adipose tissue	Palmitic acid C16:0; stearic acid C18:0.	GPR40; GPR120	Wang et al (2019)[44]

cells.[50–52] FFARs, namely, GPR40, GPR41, GPR43, GPR119, and GPR120, are widely expressed in EECs (Table 19,20,53–81,214). FAs trigger the release of neurotransmitters and/or intestinal peptides in EECs through FFAR-mediated signaling cascades. Recently, the current method for classifying EECs has been challenged, because mature EECs are proposed to simultaneously express various peptide hormones.[50] Different physiological responses of EECs are likely dependent on their location within the gastrointestinal tract.[82] However, the current evidence in this field is inconsistent and limited, so further study is required. Thus, in this review, the biological functions of FFARs are summarized based on the traditional classification of various EEC populations (Figure 2).

L cells predominantly regulate energy homeostasis and satiety signaling via the secretion of PYY and GLP-1.[20,83,84] These cells are widely distributed in the distal small intestine and colon.[64] Compared with other non-L-cell populations, both GPR43 ($G_{\alpha q}/G_{\alpha i}$) and GPR41 ($G_{\alpha i}$) are highly expressed in L cells.[65] Fiber-derived SCFAs dramatically increase the secretion of PYY and GLP-1 in human intestinal models.[65,85] GPR43 (but not GPR41) acts as a dominant receptor for SCFA-induced GLP-1 and PYY secretion from L cells. GPR43$^{-/-}$ mice exhibit reduced SCFA-triggered GLP-1 secretion, which was not observed in GPR41$^{-/-}$ mice.[65] Similarly, knocking out GPR43 blocked almost all of the PYY secretion induced by SCFAs in mice and rats.[1] As previously mentioned, GPR41 is coupled to the $G_{\alpha i}$ protein, while GPR43 is coupled to both $G_{\alpha q}$ and $G_{\alpha i}$ proteins.[16–18,66] The inhibition of $G_{\alpha i}$ signaling fails to block the secretion of GLP-1.[65] This result might partially explain why GPR43 specifically regulates PYY and GLP-1 secretion. Mechanistically, SCFAs are thought to induce gut hormone secretion through the activation of $G_{\alpha q}$-dependent GPR43 signaling, with an increase in intracellular Ca^{2+}. Interestingly, some experiments have also revealed the minor downregulation of EEC-derived PYY in GPR41$^{-/-}$ cells.[67] These contradictory results might be due to a slightly decreased level of GPR43 expression upon GPR41 knockout.[65] In addition to

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Table 2 **Distribution and activation of free fatty acid–sensitive receptors involved in energy homeostasis**

Cell types	Main locations	FFARs	Ligands	Reference
A cells	Stomach	GPR41(Gαi), GPR43(Gαq/Gαi)	SCFAs	Date et al (2000),[53] Wang et al (2012)[54]
		GPR40(Gαq), GPR120(Gαq/Gαi)	LCFAs	Gong et al (2014),[55] Lu et al (2012)[56]
I cells	Proximal small intestine	GPR40($G_{\alpha q}$), GPR120($G_{\alpha q}$/$G_{\alpha i}$)	LCFAs	Fried et al (1991),[57] Lieverse et al (1995),[58] Sykaras et al (2012)[59]
K cells	Proximal small intestine	GPR40($G_{\alpha q}$), GPR120($G_{\alpha q}$/$G_{\alpha i}$)	LCFAs	Parker et al (2009),[60] Iwasaki et al (2015),[61] Sankoda et al (2019)[62]
		GPR119($G_{\alpha s}$)	2-MAG	Mandøe et al (2015)[63]
L cells	Distal small intestine and colon	GPR41($G_{\alpha i}$), GPR43($G_{\alpha q}$/$G_{\alpha i}$)	SCFAs	Nøhr et al (2013),[64] Tolhurst et al (2012),[65] Lee et al (2008)[66]
		GPR40($G_{\alpha q}$), GPR120($G_{\alpha q}$/$G_{\alpha i}$)	LCFAs	Hirasawa et al (2005),[20] Samuel et al (2008),[67] Xiong et al (2013)[68]
N cells	Distal small intestine and large intestine	GPR40($G_{\alpha q}$), GPR120($G_{\alpha q}$/$G_{\alpha i}$)	LCFAs	Li et al (2016),[69] Drewe et al (2008),[70] Li et al (2018)[81]
β cells	Pancreas	GPR41($G_{\alpha i}$), GPR43($G_{\alpha q}$/$G_{\alpha i}$)	SCFAs	Tang et al (2015),[71] Pingitore et al (2019)[72]
		GPR40($G_{\alpha q}$)	LCFAs	Itoh et al (2003),[19] Kristinsson et al (2013),[73] Steneberg et al (2005)[74]
		GPR119($G_{\alpha s}$)	2-MAG	Overton et al (2006),[75] Chu et al (2008)[76]
Adipocytes	Adipose tissue	GPR43($G_{\alpha q}$/$G_{\alpha i}$)	SCFAs	Hong et al (2005),[77] Kimura et al (2013)[78]
		GPR120($G_{\alpha q}$/$G_{\alpha i}$)	LCFAs	Ichimura et al (2012),[79] Hilgendorf et al (2019)[80]

Abbreviations: FFARs, free fatty acid–sensitive receptors; LCFAs, long-chain fatty acids; SCFAs, short-chain fatty acids.

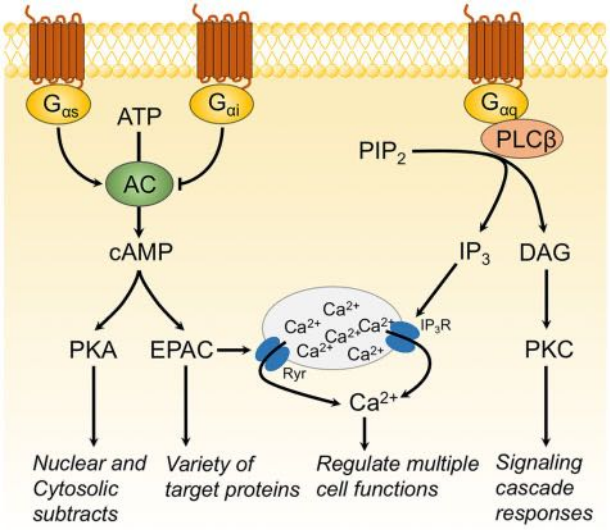

Figure 1 **Canonical G_α signaling pathway of free fatty acid receptors**

SCFAs, LCFAs are also proposed to be sensed by L cells upon the activation of GPR40 ($G_{\alpha q}$) and GPR120 ($G_{\alpha i}$/$G_{\alpha q}$).[20,68] An agonist of GPR40/GPR120, GW9508, has been reported to increase the amount of GLP-1 secreted.[86] Recently, numerous studies have indicated that LCFAs regulate GLP-1 and PYY secretion, largely through the activation of GPR40. Talukdar et al (2010)[87] reported that knocking out GPR120 does not affect GLP-1 secretion. Xiong et al (2013)[68] found that corn oil increases the secretion of GLP-1 in wild-type and GPR120$^{-/-}$ mice, but not in GPR40$^{-/-}$ mice. Only GPR40 agonists (AM-1638 and TAK-875), but not the GPR120 agonist Metabolex-209, stimulate the secretion of GLP-1.[86,88] In addition, the effects of LCFAs and $G_{\alpha q}$-only GPR40 agonists on GLP-1 secretion are almost identical.[89] Taken together, these findings suggest that SCFAs and LCFAs predominantly trigger the secretion of PYY and GLP-1 upon the activation of GPR40 and GPR43 in L cells, respectively. The downstream activation of this pathway is largely dependent on the activation of $G_{\alpha q}$.

A cells, also known as ghrelin cells, are mainly located in the stomach (from the stomach to the colon) and regulate ghrelin secretion.[53] Ghrelin is considered a "hunger" hormone for meal initiation and energy balance regulation.[90] Fatty acids are critical components that inhibit the secretion of ghrelin; for instance, α-linolenic acid inhibits 70% of ghrelin secretion.[55] GPR40 ($G_{\alpha q}$) and GPR120 ($G_{\alpha q}$/$G_{\alpha i}$) are both expressed in ghrelin cells to sense dietary LCFAs. Compared with GPR40, GPR120 is highly expressed in A cells and dramatically inhibits the secretion of ghrelin.[55,56,91] Recent evidence suggests that the secretion of ghrelin is predominantly controlled by a balance between $G_{\alpha s}$ and $G_{\alpha i}$ signaling, but not by $G_{\alpha q}$ signaling.[91] $G_{\alpha s}$ and $G_{\alpha i}$ co-regulate the activity of AC, which triggers the secretion of ghrelin in A cells through the modification of

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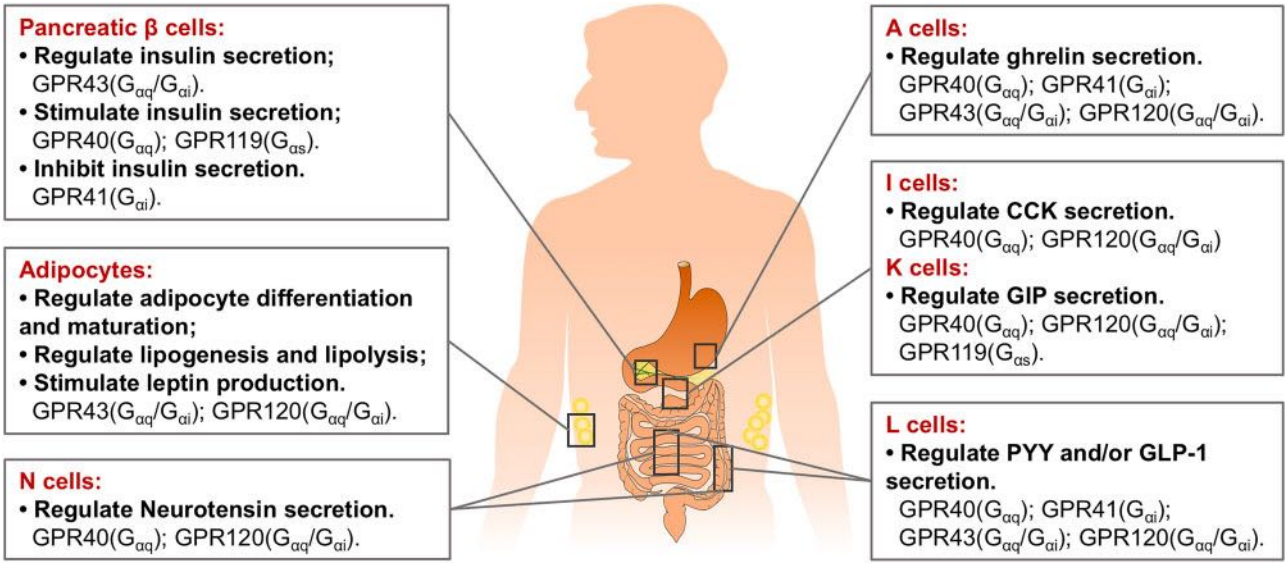

Figure 2 **Physiological functions of free fatty acid–sensitive receptors involved in energy homeostasis**. Enteroendocrine cells are divided into multiple distinct populations, such as L cells, A cells, I cells, K cells, and N cells. Fatty acids trigger the release of neurotransmitters and/or intestinal peptides in enteroendocrine cells through free fatty acid–sensitive receptors–mediated signaling cascades. Furthermore, free fatty acid–sensitive receptors are abundantly expressed in pancreatic β cells and adipocytes and are involved in glucose homeostasis and lipid metabolism

cAMP levels.[91] As sensors of SCFAs, GPR43 ($G_{\alpha q}$/$G_{\alpha i}$) and GPR41 ($G_{\alpha i}$) are also expressed in A cells. In A cells, the expression of GPR43 is 20-fold higher than that of GPR41, which mainly regulates the secretion of ghrelin.[91] The effects of GPR43 and GPR41 on ghrelin secretion might vary among different species. In humans, mice, and rats, SCFA concentrations in the stomach are relatively lower than those in the hindgut, where they do not seem to activate GPR43 or GPR41. However, in ruminants, SCFAs are largely produced in the rumen and have been reported to regulate ghrelin secretion through the activation of GPR43 in A cells.[54] Taken together, these findings show that FAs inhibit the secretion of ghrelin in A cells through GPR120 and GPR43, which might be attributed to the activation of a $G_{\alpha i}$-dependent pathway.

I cells are mainly located in the proximal small intestine, where they regulate the secretion of CCK based on the amount of sensed dietary LCFAs. CCK is a peptide hormone that mainly regulates the secretion of digestive enzymes from the pancreas and the stimulation of gallbladder contraction.[57,58,92] As receptors for LCFAs, GPR40 ($G_{\alpha q}$) and GPR120 ($G_{\alpha q}$/$G_{\alpha i}$) are both expressed in I cells.[59] Notably, the activation of GPR40 in I cells increases the secretion of CCK.[93] Compared with the levels in GPR40$^{+/+}$ mice, linoleic acid–induced intracellular Ca^{2+} concentration and CCK secretion are decreased by 50% in GPR40$^{-/-}$ mice.[93] The change in intracellular Ca^{2+} level indicates that the $G_{\alpha q}$ pathway might play a role in the control of CCK secretion.[94] To date, no data are available regarding the effect of SCFAs on CCK secretion from I cells. Collectively, these findings suggest that in I cells, dietary FAs mainly trigger CCK secretion through the activation of GPR40 by an unclear mechanism.

Similar to I cells, K cells are primarily located in the small intestine. These cells respond to dietary FAs and regulate the secretion of the gastric inhibitory peptide (GIP).[60] The dominant function of GIP is the enhancement of glucose-dependent insulin secretion.[95] K cells with high levels of GPR120 expression are mostly located in the upper small intestine, while those expressing high levels of GPR40 are mainly found in the lower small intestine.[61] Recently, increasing evidence has indicated that LCFAs regulate the secretion of GIP through GPR40 ($G_{\alpha q}$) and GPR120 ($G_{\alpha q}$/$G_{\alpha i}$). For example, FA-induced GIP expression is greatly inhibited in GPR40$^{-/-}$, GPR120$^{-/-}$, and GPR40$^{-/-}$ and GPR120$^{-/-}$ double-knockout mice.[61,62] Interestingly, 2-OG has been reported to activate GPR119 and increase the secretion of GIP.[63] Altogether, this evidence indicates that GIP secretion from K cells is regulated through the activation of GPR40, GPR119, and GPR120.

N cells are mainly located in the distal small intestine and large intestine, where they regulate the secretion of neurotensin (NT). NT is reported to regulate gastrointestinal motility, fat absorption, and glucose homeostasis.[69,96,97] The secretion of NT depends on the ingestion of nutrients.[70] LCFAs are reported to trigger NT secretion through GPCRs in N cells.[70,81] Knocking out both GPR40 and GPR120 decreases the secretion of

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NT from N cells.[81] Intriguingly, the overexpression of GPR120, but not of GPR40, reverses the decrease in NT secretion in double-knockout mice.[81] In addition, the specific agonist of GPR120, but not that of GPR40, stimulates the secretion of NT.[81] Recently, NT was demonstrated to be profoundly integrated with the secretion of GLP-1 and PYY.[98] However, the secretory granules consisting of NT were different than those consisting of GLP-1 and PYY.[98] As already mentioned in this review, the secretion of GLP-1 and PYY is mainly regulated through GPR40. Thus, the activation of different FFARs might regulate the secretion of different hormones from the same EECs. To date, no evidence has been presented to indicate whether the activation of GPR41 and GPR43 leads to the secretion of NT. Thus, these findings indicate that GPR120 is the most critical FA-binding GPCR for NT secretion.

SENSING OF FATTY ACIDS BY β CELLS IN PANCREATIC ISLETS

The pancreas is one of the most critical organs involved in the regulation of energy homeostasis. Pancreatic islet dysfunction is a potential inducer of type 2 diabetes, which is commonly characterized by decreased insulin secretion, increased glucagon secretion, and reduced β-cell mass. Multiple GPCRs have been identified in pancreatic β cells and act as potential targets for the regulation of insulin and glucagon secretion. Glucose is the most crucial nutrient that triggers the secretion of insulin from pancreatic β cells. It increases the intracellular Ca^{2+} concentration in pancreatic β cells and promotes the exocytosis of insulin vesicles.[99] Similarly, the activation of the GPCR $G_{\alpha q/11}$ subunit also increases the amount of Ca^{2+} released from intracellular storage via IP_3. The other potential cellular pathway to enhance the exocytosis of insulin vesicles is induced through the activation of PKA and PKC, which is triggered by cyclic adenosine 3', 5'-monophosphate (AMP) and DAG, respectively. In addition, the activation of $G_{\alpha s}$ was also reported to increase β-cell mass.[100,101] Thus, both the $G_{\alpha s}$ and $G_{\alpha q}$ pathways seem to positively regulate the secretion of insulin.

Fatty acids play important roles in maintaining basal insulin secretion and enhancing glucose-stimulated insulin secretion.[102,103] Previously, FAs were thought to regulate insulin secretion as a form of long-chain coenzyme A (CoA) ester through the regulation of ion channels located in β cells.[104] However, recent findings suggest that FAs regulate insulin secretion through the activation of GPCRs in pancreatic β cells. Intriguingly, the LCFAs sensed by pancreatic β cells are mainly derived from the hydrolysis of circulating chylomicrons with lipoprotein lipase secreted by islets.[105,106] GPR40 ($G_{\alpha q}$) is abundantly expressed in pancreatic β cells and is activated by LCFAs to increase glucose-stimulated insulin secretion (Figure 2).[19,107,108] GPR40 seems to be the primary GPCR for sensing FAs in the pancreas.[109] LCFAs reduce the voltage-gated K^+ current through GPR40 activation, leading to an increase in intracellular Ca^{2+} concentration and the amount of insulin released.[99] It has also been proposed that phospholipase C (PLC) and cAMP-PKA signaling is involved in the process of LCFA-induced insulin secretion.[99,109] GPR40 contributes to both acute and chronic insulin secretory responses to FAs in mice.[73,110] GPR40 knockdown by siRNA decreased LCFA-induced insulin secretion from pancreatic β cells.[19,107] In GPR40-knockout mice, insulin secretion was impaired in response to LCFA stimulation.[74,110,111] Correspondingly, the overexpression of GPR40 in the pancreatic β cells of obese mice (induced by a high-fat diet) increased mouse insulin sensitivity.[112]

Notably, long-term exposure of islets to LCFAs can be lipotoxic, which further reduces insulin secretion.[74,113] To date, it is still not clear whether this negative effect is due to the excessive activation of GPR40. A study showed that the lipotoxicity induced by palmitate in an insulinoma cell line was independent of GPR40 activation level.[114] However, another study showed that GW9508 (an GPR40 agonist) inhibited insulin secretion.[115] More experiments are required to clarify this mechanism in the future. Recently, a number of GPR40 agonists have been identified, and they may offer alternative ways to assess the lipotoxicity induced by FFAs.[116] The development of an GPR40 agonist would make GPR40 a potential therapeutic target for diabetes. A few studies have shown that GPR120 is also expressed in pancreatic β cells,[117,118] but whether GPR120 regulates insulin secretion remains to be determined.

GPR119 is an orphan GPCR and is proposed to be activated by lipid amides (such as oleoylethanolamide [OEA]) and 2-MAG.[75,105] The Activation of GPR119 triggers the secretion of insulin through the induction of intracellular cAMP (in the $G_{\alpha s}$ signaling pathway).[119,120] Knocking down GPR119 by siRNA decreased insulin secretion in pancreatic β cells.[119] However, no difference was observed in the insulin secretion of GPR119-knockout mice compared with that of wild-type mice.[76] Nevertheless, the activation of GPR119 can enhance glucose-induced insulin secretion, which indicates GPR119 is a promising therapeutic target for diabetes.

Originally, the SCFA receptors GPR43 ($G_{\alpha q}/G_{\alpha i}$) and GPR41 ($G_{\alpha i}$) were found to be mainly expressed in extra-islet tissues. However, recent studies indicate that these 2 receptors are also expressed in pancreatic β cells and might regulate insulin secretion through $G_{\alpha i}$ and

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$G_{\alpha q}$ (Figure 2).[71,72] Knocking out both GPR43 and GPR41 led to greater insulin secretion and a profound enhancement of glucose tolerance in mice fed a high-fat diet.[71] However, the circulating levels of SCFAs were insufficient to trigger the activation of FFARs. This evidence suggests that GPR43 and GPR41 might act in a cell-autonomous manner in β cells to inhibit insulin secretion.[71,72] To date, evidence regarding the effects of GPR43 and GPR41 on insulin secretion is still limited, and further study is required.

SENSING OF FATTY ACIDS BY ADIPOCYTES

Traditionally, adipose tissue was considered as only a unique container for energy deposition. Over the past 30 years, this tissue has been found to participate in metabolic processes and act as a critical regulator of energy homeostasis. Adipocytes are classified into white adipocytes and brown adipocytes. In contrast to the wide range of species that have white adipocytes, only mammals have brown adipocytes, and they mainly surround the heart and great vessels during infancy. Brown adipocytes uniquely express mitochondrial uncoupling protein-1 (UCP-1), which promotes proton leakage through the inner membrane of mitochondria, so that the proton motive force is dissipated, releasing heat.[121] Adipose tissues are proposed to regulate energy balance through endocrine and nonendocrine pathways. On the one hand, adipocytes secrete multiple adipokines (leptin, adiponectin, omentin, and visfatin) to regulate multiple systemic effects, such as food intake, energy expenditure, and lipid metabolism.[11,122] On the other hand, FAs derived from adipocytes also directly regulate energy homeostasis by targeting the FFARs located in the pancreas, muscles, and the liver.

In adipose tissue, $G_{\alpha s}$ and $G_{\alpha i}$ signaling is proposed to cooperatively regulate fat metabolism. Activation of $G_{\alpha s}$ triggers cAMP/PKA and stimulates lipolysis by activating hormone-sensitive lipase (HSL) and perilipin.[4,123] Consistent with the findings regarding $G_{\alpha s}$ signaling, the blockade of $G_{\alpha i}$ signaling also promotes lipolysis. Mechanistically, adipo-G_i-KO adipocytes increased cAMP/PKA signaling and activated PTPase 1B (PTP1B).[124] Moreover, adipocyte $G_{\alpha i}$ signaling has been demonstrated to regulate glucose homeostasis and insulin secretion. Intriguingly, the activation of $G_{\alpha q}$-coupled GPCRs is also promoted to stimulate the process of lipolysis, although the underlying mechanism is still not clear.[4] In brown adipocytes, the activation of $G_{\alpha q}$ decreases the expression of UCP-1 and inhibits brown adipogenesis through the RhoA/ROCK pathway. This evidence indicates that the inhibition of $G_{\alpha q}$ may be a novel way to treat obesity.[125] A recent study proposed that $G_{\beta\gamma}$ might also participate in the regulation of adipose function. Knocking out β-arrestin-2 (a critical signaling molecule downstream of $G_{\beta\gamma}$) in adipocytes reduces adiposity and induces the burning of additional calories.[126] Thus, all downstream GPCRs ($G_{\alpha i}$, $G_{\alpha s}$, $G_{\alpha q}$, and $G_{\beta\gamma}$) seem to participate in the regulation of fat metabolism.

FFARs are abundantly expressed in adipocytes and are involved in lipid metabolism.[77,127] Currently, increasing evidence suggests that SCFAs inhibit lipolysis through GPR43 but not GPR41.[77,78] GPR43 is likely to be a sensor of excessive dietary energy in adipose tissue.[78] An initial study suggested that high-fat-diet-induced fat deposition in mice might be due to the high expression of GPR43 in their adipose tissue.[77] The inhibitory effect of SCFAs on lipolysis is eliminated in GPR43$^{-/-}$ adipose cells.[128] In differentiated adipocytes, knock-in GPR43 exerts antilipolytic effects.[129] Although GPR43 couples to both $G_{\alpha i}$ and $G_{\alpha q}$ proteins, the process of lipolysis seems to be regulated by the cAMP signaling pathway (which is dependent on $G_{\alpha i}$ but not on $G_{\alpha q}$).[129] However, this result still needs to be confirmed. Additionally, the activation of GPR43 regulates energy homeostasis through the inhibition of insulin signaling and the secretion of leptin.[78,130] Taken together, these findings indicate that SCFAs inhibit the process of lipolysis upon the activation of GPR43 ($G_{\alpha i}$).[78,128]

As a sensor for long-chain fatty acids (LCFAs), only GPR120 (binding $G_{\alpha q}$/$G_{\alpha i}$) is expressed in adipocytes. GPR120 is proposed to regulate adipocyte differentiation and maturation in animals. Obese individuals have higher levels of GPR120 expression in adipose tissue than lean individuals.[79] Simultaneously, the inhibition of lipogenesis and decreased adipocyte differentiation are observed in GPR120-deficient mice.[79] Additionally, recent findings suggest that GPR120 regulates the function of preadipocytes and initiates adipogenesis.[80] Preadipocytes are located near the vasculature in fat tissue and can be converted into mature adipocytes upon the sensing of dietary FA changes. Mechanistically, LCFAs activate ciliary GPR120 and stimulate adipogenesis through the ciliary cAMP/EPAC pathway.[80] Thus, activation of GPR120 enhances adipogenesis and FA synthesis in adipose cells (Figure 2). In addition, GPR120 is expressed in the brown adipose tissue (BAT), and the activation of GPR120 enhances BAT activity and induces the browning of white fat.[131] To date, there is no evidence indicating whether GPR120 is involved in the secretion of adipokines.

THE REGULATION OF SYSTEMIC ENERGY HOMEOSTASIS WITH FATTY ACIDS

As mentioned previously, the gut, pancreas, and adipose tissue regulate whole-body energy homeostasis by

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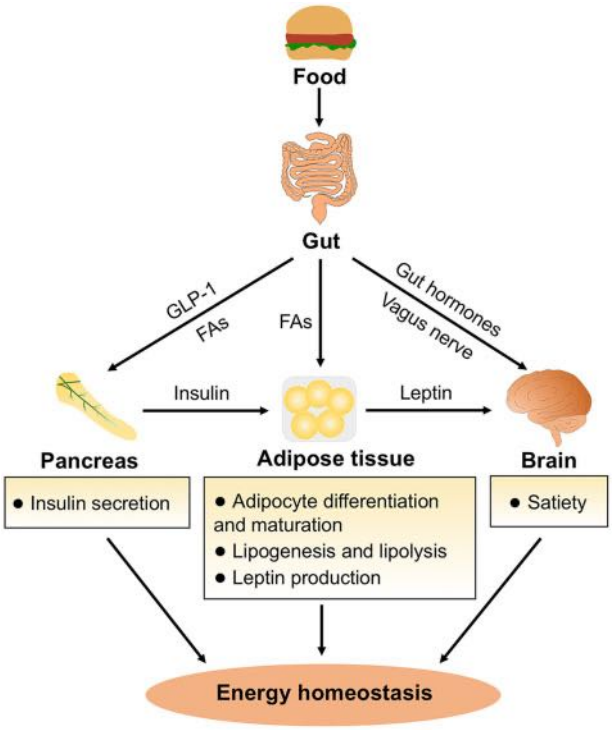

Figure 3 **Regulation of systemic energy homeostasis with fatty acids**. Dietary fats are digested and absorbed in the intestine and stimulate the release of gastrointestinal hormones. Circulating fatty acids and gastrointestinal hormones (such as GLP-1) stimulate the secretion of insulin in pancreatic β cells. Simultaneously, the gastrointestinal hormone also directly activates the receptor on the afferent vagus nerve in the intestine and transmits the satiety signal to the brain. Insulin secreted by the pancreas can activate insulin receptors on the adipocyte membrane, thereby regulating adipogenesis and lipolysis. In addition, circulating fatty acids also directly activate fatty acid receptors on adipocytes and stimulate the secretion of leptin, which leads to satiety. These physiological responses together maintain the energy homeostasis mediated by fatty acid receptors. *Abbreviations*: FAs, fatty acids.

modifying food intake, glucose homeostasis, and lipid metabolism (Figure 3).[13,14] Importantly, cross talk is initiated among these tissues upon the activation of FFARs. The gut is critical for nutrient absorption and energy intake. The fluctuation of food-derived and microbe-derived FAs regulates the secretion of gastrointestinal hormones.[25,132,133] Food-derived LCFAs and microbe-derived SCFAs can induce the secretion of PYY, GLP-1, and CCK, but can inhibit the secretion of ghrelin upon the activation of FFARs (GPR40, GPR41, GPR43, GPR119, and GPR120).[24–26,91,93,132–134] Gastrointestinal hormones (PYY and CCK) directly stimulate the afferent vagus nerve in the lamina propria of the intestine, thereby transmitting signals to the nerve center, suppressing appetite, and controlling energy source intake.[12,135,136] The most critical incretins that regulate glucose homeostasis are GLP-1 and GIP, which are dominant hormone bridges connecting signals from the gut to the pancreas. However, a recent study found that the specific deletion of GPR43 and GPR41 in intestinal cells did not change the glucose tolerance of diabetic animals.[71] The contradictory nature of these results needs to be resolved in the future.

The pancreas belongs to both the digestive system and the endocrine system, which control digestion and blood sugar levels, respectively.[13] GLP-1 triggers insulin secretion and decreases glucagon secretion from β cells, which regulates glucose homeostasis. Long-term treatment of islets with GLP-1 increases β-cell mass through the activation of protein kinase B (PKB).[100,101] Interestingly, this process is also dependent on the activation of GPCRs ($G_{\alpha s}$).[137] Similar to GLP-1, the GIP receptor is located in β cells and couples to $G_{\alpha s}$.[138,139] GIP triggers the activation of insulin and promotes the proliferation of β cells. Thus, FAs not only directly trigger the secretion of insulin through pancreatic FFARs but also indirectly affect their release through gut-derived GLP-1 and GIP upon the activation of intestinal FFARs.[19,82,105,140,141] The predominant target organs of insulin are the liver and adipose tissue. Insulin stimulates lipogenesis and gluconeogenesis in the liver through the Akt/mTORC1 and Akt/FOXO1 pathways, respectively.[142] Furthermore, circulating insulin binds to the insulin receptor in adipocytes, which results in the inhibition of lipolysis with the decrease in adipose triglyceride lipase (ATGL) and HSL.[143–145] Therefore, insulin functions together with circulating FAs to inhibit lipolysis in adipose tissue through insulin receptors and FFARs. The activation of GPR120 also induces the secretion of leptin (an adipocyte-derived hormone), which suppresses appetite and inhibits energy expenditure.

Collectively, dietary LCFAs directly activate FFARs located in the gut, pancreas, and adipose tissue. These organs function together to promote increased energy deposition and decreased energy intake. Similarly, dietary fiber–fermented SCFAs have been demonstrated to regulate the secretion of gut hormones. After absorption, these SCFAs can inhibit FA deposition and the secretion of leptin with the activation of GPR43. Understanding the role of the FFARs involved in metabolic regulation is critical for the future development of new therapeutic approaches for metabolic diseases.

FUTURE RESEARCH DIRECTIONS

Where are FFARs located in EECs?

The luminal concentrations of fiber-fermented SCFAs are approximately 50–100 mM, which is a thousand-fold higher than the EC_{50} of GPR43 (259–537 μM) and

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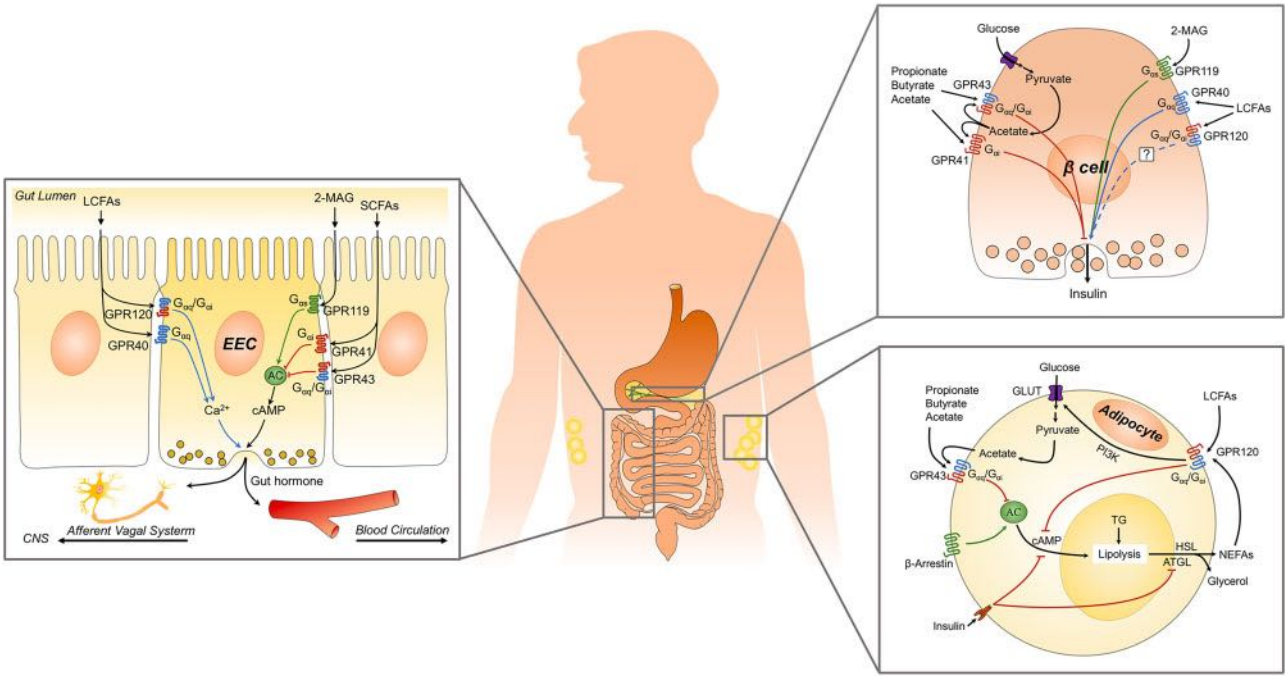

Figure 4 **Free fatty acid–sensitive receptor signaling pathways systematically regulate whole-body energy homeostasis.** *Abbreviations*: ATGL, adipose triglyceride lipase; cAMP, adenosine 3′5′-cyclic monophosphate; CNS, central nervous system; HSL, hormone-sensitive lipase; LCFAs, long-chain fatty acids; NEFAs, non-essential fatty acids; SCFAs, short-chain fatty acids; TG, triacylglycerol.

GPR41 (12–274 μM).[146,147] Luminal SCFAs are less likely to trigger the secretion of gut hormones through GPR43 and GPR41, which are located on the apical side of EECs, because fluctuations in SCFA concentration is much greater than that of the pharmacological dose used to trigger GPR43 and GPR41 activation. Furthermore, the continuous activation of GPCRs would lead to the internalization of these receptors. Thus, GPR43 and GPR41 might be located on the basolateral side of the EECs and only sense the SCFAs absorbed from the gut. This hypothesis might partially explain why only vascular, but not luminal, stimulation with agonists induces the secretion of GLP-1.[148] It is worth noting that internalization of GPCRs is also reported to regulate related signaling pathways.[149] However, the details of this alternative pathway still need to be ascertained in the future.

Is FFAR signaling biased?

GPCRs stimulate multiple intracellular signaling pathways at the same time, as they can couple more than one G_α protein. Recent studies found that the activation of GPCRs may be biased. Suomivuori et al (2020)[150] determined the underlying mechanism regarding arrestin bias and G protein bias with respect to the angiotensin II type 1 receptor. Qiao et al (2020)[151] deciphered the structural basis of $G_{\alpha s}$ and $G_{\alpha i}$ recognition of the glucagon receptor. GPR43 and GPR120 are both coupled to both $G_{\alpha q}$ and $G_{\alpha i}$. As we previously summarized, in EECs, GPR43 and GPR120 prefer to regulate biological functions through the $G_{\alpha q}$ pathway, instead of the $G_{\alpha i}$ pathway, with an unknown mechanism. Thus, it is possible that FFAs might regulate the $G_{\alpha q}$ pathway through a biased signaling pathway.

Does the $G_{\beta\gamma}$ signaling pathway contribute to FFAR signaling regulation?

In addition to the G_α subunit, $G_{\beta\gamma}$ subunits are also reported to regulate a number of effector targets. Once GPCR is activated, the dissociation of G_α exposes the binding sites of $G_{\beta\gamma}$ to its downstream targets.[152,153] Although $G_{\beta\gamma}$ binds with different subtypes of G_α, $G_{\beta\gamma}$ signaling is more likely to be triggered by the activation of $G_{\alpha i}$.[154] G protein-coupled receptor kinase 2 (GRK2) is one of the downstream targets of $G_{\beta\gamma}$ signaling, and it is involved in a negative feedback loop for $G_{\beta\gamma}$ activation.[155] Additionally, the downstream targets of $G_{\beta\gamma}$ are tissue specific. For example, in cardiomyocytes, $G_{\beta\gamma}$ directly binds to and activates ERK (extracellular signal-regulated kinase) and phospholipase Cε (PLCε) and regulates the corresponding pathways.[156] Intriguingly, GPR41, GPR43, and GPR120 are all coupled to $G_{\alpha i}$, and activation of these receptors might trigger the $G_{\beta\gamma}$ pathway. Future research also needs to ascertain the

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specific downstream targets of $G_{\beta\gamma}$ in intestinal EECs, pancreatic β cells, and adipocytes.[157]

CONCLUSION

In summary, energy homeostasis is predominantly regulated by the intestine, pancreas, and adipose tissue. FFARs are abundantly expressed in these tissues and act as sensors to detect fluctuations in food-derived and microbe-derived FAs. The activation of FFARs regulates downstream proteins and organ-specific functions, mainly through the activation of the $G_{\alpha q}$ and $G_{\alpha i}$ signaling pathways (Figure 4). Future in-depth research on FFARs and their agonists and antagonists will contribute to the development of new therapeutic approaches for diseases such as obesity and diabetes.

Acknowledgments

Author contributions. T.M. and S.Z. initiated the idea, envisioned the scope, and prepared the outline of this review paper. M.T., S.Z., Z.W., J.H., and F.C. studied and analyzed all of the publications cited in this paper and were involved in the manuscript preparation. S.Z. and W.G. conducted the final editing and proofreading. All authors read and approved the final manuscript.

Funding. This work was supported by National Natural Science Foundation of the P. R. of China (no. 31802067 and 31872364) and the Natural Science Foundation of Guangdong Province (No. 2018A030310201).

Declaration of interest: The authors have no relevant interests to declare.

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frontiers | Frontiers in Nutrition

TYPE Original Research
PUBLISHED 16 February 2023
DOI 10.3389/fnut.2023.1098715

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OPEN ACCESS

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SPECIALTY SECTION
This article was submitted to
Nutrition and Food Science Technology,
a section of the journal
Frontiers in Nutrition

RECEIVED 15 November 2022
ACCEPTED 26 January 2023
PUBLISHED 16 February 2023

CITATION
Qi Y, Zheng T, Liu X, Yang S, Li Q, Shao J, Zeng X, Guan W and Zhang S (2023) Sodium acetate regulates milk fat synthesis through the activation of GPR41/GPR43 signaling pathway. *Front. Nutr.* 10:1098715.
doi: 10.3389/fnut.2023.1098715

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Sodium acetate regulates milk fat synthesis through the activation of GPR41/GPR43 signaling pathway

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Background: Fat is a critical component in milk, which provided energy for the early growth and development of mammals. Milk fat is positively related to the concentration of acetate in the blood, while the underlying mechanism is still unclear.

Objective: This study is to investigate the effects of sodium acetate (NaAc) on milk fat synthesis in the mammary gland, and explored the underlying mechanism.

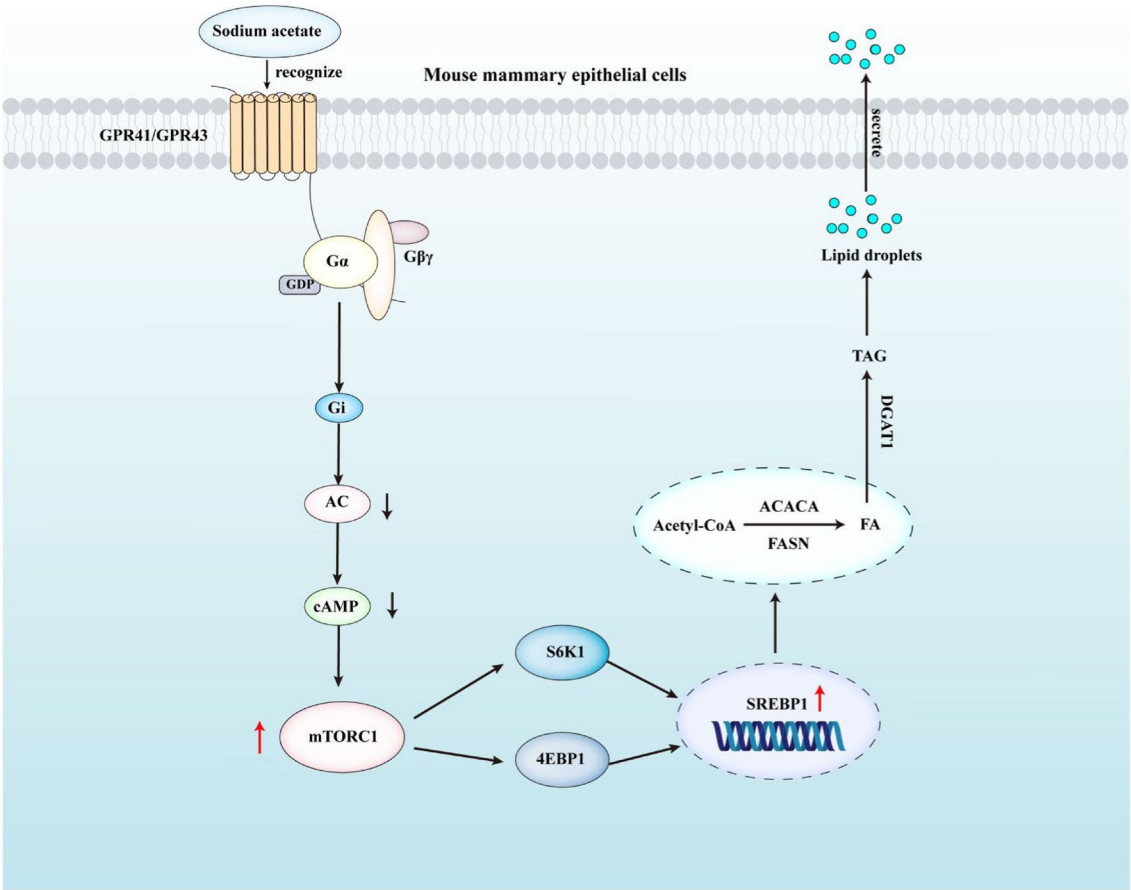
Methods: *In vitro* experiments were carried out in mouse mammary epithelial cell line (HC11) cells cultured with NaAc to explore the potential pathway of NaAc on milk fat synthesis. Furthermore, 24 pregnant mice (from d 18.5 of gestation to d 7 of lactation, exposed to 200mM NaAc drinking water) were used as an *in vivo* model to verify the results.

Results: In this study, we found that NaAc promoted milk fat synthesis and the expression of related genes and proteins in HC11 mammary epithelial cells with the activation of GPCR and mTORC1 signaling pathways ($p<0.05$). Pretreatment with the mTORC1 inhibitors and G protein inhibitors attenuated the NaAc-induced milk fat synthesis in HC11 mammary epithelial cells ($p<0.05$). Importantly, the effect of NaAc on milk synthesis was attenuated in GPR41 and GPR43 knockdown HC11 mammary epithelial cells ($p<0.05$). This evidence indicates that NaAc might regulate milk fat synthesis through the GPR41/GPR43-mTORC1 pathway. Consistently, in *in vivo* experiment, dietary supplementation with NaAc significantly increased milk fat content and fat synthesis-related proteins in mice mammary glands with the activation of mTORC1 and GPCR signaling pathways at peak lactation ($p<0.05$).

Conclusion: The addition of NaAc promoted the increase of milk fat synthesis in HC11 mammary epithelial cells and mice mammary glands at peak lactation. Mechanistically, NaAc activates GPR41 and GPR43 receptors, leading to the activation of the mTORC1 signaling pathway to promote the synthesis of milk fat.

KEYWORDS

sodium acetate, milk fat, GPR41, GPR43, mTORC1, mammary epithelial cells



AC = adenylyl cyclase; cAMP = cyclic adenosine monophosphate; mTORC1 = mammalian target of rapamycin complex C1; S6K1 = ribosomal protein S6 kinase 1; 4EBP1 = 4E-binding protein 1; SREBP1 = sterol-regulatory element binding protein1; ACACA = acetyl-CoA carboxylα; FASN = fatty acid synthase; DGAT1 = diacylglycerol O-Acyltransferase 1; TAG = Triacylglycerol.

GRAPHICAL ABSTRACT

Introduction

Milk is a critical nutrient source during mammalian offspring's early life, which mainly includes fat, protein, lactose and small amounts of vitamins and minerals (1). Milk fat provided energy and essential fatty acids for neonatal growth and development, which is one of the most important components in milk (2). Milk fat could be affected by dietary energy and nutrients. Energy deficiency induces AMPK activation, which further inhibits milk synthesis by targeting PrlR and PGC-1α (3). Amino acids, such as branched-chain amino acids, methionine, arginine and lysine are also found to be involved in the regulation of milk synthesis in mammary epithelial cells (4). In addition, 18-Carbon fatty acids promote cytosolic triglycerides (TAG) accumulation by promoting genes and proteins related to milk synthesis (5).

In ruminants, short-chain fatty acids (SCFA) are largely produced by Rumen microbial fermentation of carbohydrates (6). High concentrations of SCFA not only act as energy substrates, but are also used to produce animal products, such as milk (7, 8). Compared with ruminants, monogastric animals are not able to efficiently digest dietary fiber, which leads to fewer SCFA in the stomach and the small intestine (9). Thus, due to the comparatively lower concentration of SCFA in monogastric animals, SCFA might not only act as a substrate but also function as a signaling molecule in the mammary gland.

G protein-coupled receptors (GPCR) are the largest family of cell membrane receptors, which are involved in the regulation of multiple cellular and physiological functions (10). GPCR contains 7 transmembrane protein structural domains which directly bind to G proteins in the membrane. G proteins are composed of three subunits, including G_α (bound to GTP or GDP), G_β and G_γ (11, 12). GPR41 and GPR43 have been identified as SCFA receptors in recent years. GPR43 initiates signal transduction by activating $G_{\alpha i}/G_{\alpha q}$ pathway, while GPR41 initiates signal transduction by activating the $G_{\alpha i}$ pathway (13, 14). One of the crucial downstream of GPCR is the mammalian target of rapamycin complex 1 (mTORC1). mTORC1 is a central coordinator of cell metabolism, which senses the nutritional status and regulates cell

growth and physiological homeostasis (15–17). Importantly, mTORC1 promotes the generation of lipid droplets and the formation of TAG in mammary epithelial cells *via* regulating the activity of sterol regulatory element binding proteins (SREBP1), which is an important transcriptional regulator and nuclear receptor in *de novo* milk fat synthesis and changes in its mRNA abundance and protein expression affect milk fat synthesis (18, 19).

Previously, GPR41 and GPR43 have been reported to participate in the regulation of lipid metabolism (20, 21). However, it is still not clear the biological role of GPR41 and GPR43 in milk fat synthesis. Therefore, the primary objective of this study is to determine the role of GPR41 and GPR43 agonists (SCFA) on milk synthesis and identify the potential signaling pathway involved in this process. Understanding the mechanism of nutrients to promote milk fat synthesis is crucial to increase milk quality and beneficial to the health and development of neonates.

Materials and methods

Animals and experimental design

All of the procedures performed in animal feeding and sample harvesting during this study were approved by the South China Agricultural University Animal Care and Use Committee (no. 20110107-1, Guangzhou, China). Mice were fed a standard chow diet (provided by Guangdong Medical Experiment Center). And the major composition of raw materials is: Northeast corn, wheat, soybean meal, fish meal, chicken meal, multivitamins, vitamin K, vitamin E, vitamin D, animal fat, calcium hydrogen phosphate, trace elements, etc. As is shown in Supplementary Figure S1, mice were housed under a 12:12 light–dark cycle during the duration of study at 25°C and 50–60% humidity. Mice were provided *ad libitum* access to basal diet fed and fresh water. Twenty-four C57BL/6 mice (8 weeks old) were randomly divided into the control group and NaAc group and adapted 7–10 d. Male mice: female mice were mated in a ratio of 1:3. Treatment begins at d 18.5 of gestation to d 7 of lactation: The control group was fed with normal drinking water, and the treatment group was fed with water containing 200 mM NaAc (Sigma Aldrich, St. Louis, MO, United States). On d 7 of lactation, record the offspring's weight, dam body weight and feed intake.

Mammary gland sampling

On the morning of d 7 of lactation, 7 mice were euthanized after fasting for 12 h, and mammary gland tissue was collected using the method mentioned by Cheng et al. (22). Samples were quickly placed in liquid nitrogen after collection, and then stored in the −80°C refrigerator for testing.

Milk sampling and analysis

On the morning of d 7 of lactation, mice's milk was collected according to the method of DePeters et al. (23). Briefly, nursing pups were moved for 3 h, dams were ip-injected with oxytocin (4 IU per mouse) and anesthetized 5 min later with Avertin (Sigma Aldrich, St. Louis, MO, United States). Droplets of milk were aspirated by massaging the mammary gland and collected in a 2 mL siliconized microcentrifuge tube with the use of a vacuum system attached to a human breast pump (Evenflo, Miamisburg, OH, United States). Milk samples were stored at −20°C until analyzed. Two internal standards were used to determine the milk fat content (17:0 triacylglycerol and 19:0 FA methyl ester) (24). The content of prolactin in plasma was determined by ELISA (Huamei, Wuhan, China).

Cell culture and treatment

HC11, mouse mammary gland cells, were cultured in complete medium (DMEM/F12 medium [Thermo]), mainly including 10% FBS (fetal bovine serum), 5 μg/mL IGF-1 (insulin-like growth factor-1) 10 ng/mL EGF (epidermal growth factor), 5 μg/mL ITS (Insulin-Transferrin-Selenium) and 10 μg/mL PS (penicillin–streptomycin) in cells incubator at 37°C and 5% CO_2 concentration. To induce differentiation, cells were incubated in medium deprived of epidermal growth factor for 24 h and then cultured in DIP medium (1 μM dexamethasone, 5 μg/mL insulin, and 5 μg/mL prolactin) for the indicated time according to the experimental design. The reagents used in this experiment (sodium acetate, sodium propionate, and sodium butyrate and inhibitors) were provided by Sigma-Aldrich (St. Louis, MO, United States).

Experiment I

HC11 cells were cultured in different concentrations (0, 0.1, 0.75, and 1.5 mM) of NaAc, sodium propionate and sodium butyrate medium, $n = 3$. After 24 h incubation, HC11 cells were collected to examine lipid droplet formation with Oil red O staining, TAG content and the phosphorylation levels of ERK and CREB.

Experiment II

HC11 cells were cultured with different concentrations (0, 0.25, 0.5, 0.75, 1.0, and 1.5 mM) of NaAc, $n = 3$. After 24 h incubation, cells were collected to examine lipid droplet formation with Oil red O staining, TAG content and the genes and proteins expression of *FASN, ACACA, FABP3, DGAT, SREBP1* and the phosphorylation levels of mTOR, S6K1, and 4EBP1.

Experiment III

Pretreat HC11 cells with 50 nM Rapamycin for 2 h and then cells were cultured in 0.75 mM NaAc, $n = 3$. After 24 h incubation, cells were collected to examine lipid droplet formation with Oil red O staining, triglyceride content and the genes and proteins expression of *FASN*, *ACACA*, *FABP3*, *DGAT*, *SREBP1* and the phosphorylation levels of mTOR, S6K1, and 4EBP1.

Experiment IV

Pretreat HC11 cells with PTX, Gallein and NF023 for 2 h and then cells were cultured in 0.75 mM NaAc, $n = 3$. After 24 h incubation, cells were collected to examine proteins expression of FASN, ACACA, FABP3, DGAT, and SREBP1.

Experiment V

Pretreat HC11 cells with GPR41 siRNA and GPR43 siRNA (RiboBio, Guangzhou, China) for 6 h and then cells were cultured in 0.75 mM NaAc, $n = 3$. After 24 h incubation, cells were collected to examine TAG content.

RNA isolation and real-time PCR

According to the instructions, total RNA was isolated from tissue and cell samples using an EZ-press RNA purification kit (EZ-Bio, Shanghai, China). The RNA samples were subjected to electrophoresis in agarose gel electrophoresis to verify their integrity. cDNA was prepared using an RNA reverse transcription kit (EZ-Bio, Shanghai, China) according to instructions. Real-time PCR was performed by ABI StepOnePlusTM real-time PCR Systems. The reaction system consists of 10 μL Real-Time PCR Master Mix, 2 μL cDNA, 0.8 μL of each PCR primer and 7.2 μL DEPC water. The following thermal profile was used for RT-PCR: 95°C for 1 min, followed by 40 cycles of denaturation at 95°C for 15 s, annealing at 59°C for 15 s, and extension at 72°C for 40 s. Set 40 repetitions per set. Relative gene expression was calculated according to the $2^{-\Delta\Delta Ct}$ method and β-actin was used to normalize the data. Primer sequences we used for real-time PCR are shown in Supplementary Table S1.

Western blotting

Mammary tissue and HC11 cells samples were homogenized in RIPA lysis buffer supplemented with 1% PMSF. Total protein concentration was determined by BCA Protein Assay Kit (Biyuntian, Shanghai, China). Finally, the protein samples were boiled and denatured under 100°C and stored at −20°C for subsequent tests. The proteins were separated by electrophoresis on 10% or 12% SDS-PAGE gels and transferred to PVDF membranes (Sigma Aldrich, St. Louis, MO, United States). The membranes were blocked with 6% skimmed milk for 2 h after washing with Tris-buffered saline with Tween (TBST), followed by overnight incubation under gentle shaking with primary antibodies at 4°C. After washing with TBST, PVDF membranes were incubated with secondary antibodies for 1 h. The chemiluminescent signal was detected by using ECL reagents (P1020), and bands were quantified by ImageJ Software (ImageJ 1.52a). The antibodies used in this experiment are as follows. Primary antibodies: CREB (1:1000, 9197S) and P-CREB (1:1000, 9198S) purchased from Cell Signaling Technology (Danvers, MA, United States). FASN (1:2000, ab99359), ACACA (1:1000, ab72046), FABP3 (1:1000, ab231568), DGAT1 (1:500, ab100982), SREBP1 (1:1000, ab28481), 4EBP1 (1:1000, ab2606) and S6K1 (1:1000, ab9366) all purchased from Abcam (Cambridge, MA, United States); mTOR (1:1000, #2983), P-mTOR (1:1000, #5356), ERK (1:3000, 9102S), P-ERK (1:3000, 9101S), P-4EBP1 (1:1000, 9451S) and P-S6K1 (1:1000, #9234) all purchased from Cell Signaling Technology(Danvers, MA, United States). beta-actin (1:2000, bs-0061R) purchased from Beijing Bo Osen Biotechnology Co., Ltd. (Beijing, China); Secondary Antibodies: Goat anti-rabbit IgG (1:5000, 511,203) and Goat Anti-mouse IgG (1:5000, 511,103) purchased from ZenBio (Chengdu, China).

Oil red O staining of lipid droplets and tag content analysis

The oil red O staining method was used to detect lipid droplets in HC11 cells. Cells were washed with PBS (Sigma Aldrich, St. Louis, MO, United States) for 3 times, then added with 4% paraformaldehyde and fixed at room temperature for 40 min. Subsequently, HC11 cells were washed twice with PBS, then added oil red O staining solution (Sangon Bio, Shanghai, China), and incubated at room temperature for 3 h. Discard the staining solution and rinse HC11 cells with 65% isopropanol once. Then HC11 cells were washed with PBS 5 times and observed under an inverted microscope (400×). In this study, Triglyceride Assay Kit (Jiancheng, Nanjing, China) was used to detect the content of TAG.

Statistical analysis

Excel software was used to collect and sort out all data in this study, and SPSS 25.0 software was used for analysis. According to the experiment design, *T*-test and one-way ANOVA were used to analyze the results. All analysis results were expressed in the form of mean ± SEM, and $p < 0.05$ was used as the criterion of significance of difference.

Results

Short-chain fatty acids promoted the milk fat synthesis in HC11 cells

To detect the effect of SCFA on milk fat synthesis, HC11 cells were treated with different concentrations of NaAc, sodium propionate and sodium butyrate. As shown in Figure 1A, compared with sodium propionate and sodium butyrate, NaAc efficiently promotes the synthesis of cells' lipid droplets. The most effective concentration of NaAc on milk synthesis in HC11 cells is 0.75 mM, which significantly increases TAG content (Figure 1B; $p < 0.05$). While 0.75 mM sodium propionate and sodium butyrate had no significant effect on TAG synthesis in HC11 cells (Figure 1B).

Differential regulation of ERK and CREB phosphorylation by short-chain fatty acids

Activation of the GPCR signaling pathway is characterized by the stimulation of the ERK/MAPK cascade (25). To test whether SCFA regulates milk fat synthesis through the GPCR signaling pathway, we detected the phosphorylation of ERK in HC11 cells treated with different concentrations of SCFA. NaAc (Figures 1C,D), sodium propionate (Figures 1F,G), but not sodium butyrate (Figures 1I,J) significantly increased the phosphorylation level of ERK at the concentration of 0.75 mM ($p < 0.05$). Previously study found GPCR-regulated cellular cAMP involved in the modification of mTORC1 activity, which is a crucial pathway for milk synthesis (26). Activation of $G_{\alpha i}$ protein could inhibit AC (adenylate cyclase) from converting ATP into cAMP, then inhibit the phosphorylation of CREB. Thus, the phosphorylation level of CREB is used as an indicator of G_i activation. As shown in the results, 0.75 mM NaAc (Figures 1C,E) significantly inhibited the phosphorylation level of CREB ($p < 0.05$), while sodium propionate (Figures 1F,H) and sodium butyrate (Figures 1I,K) had no effect on CREB phosphorylation. Thus, NaAc is selected for subsequent research.

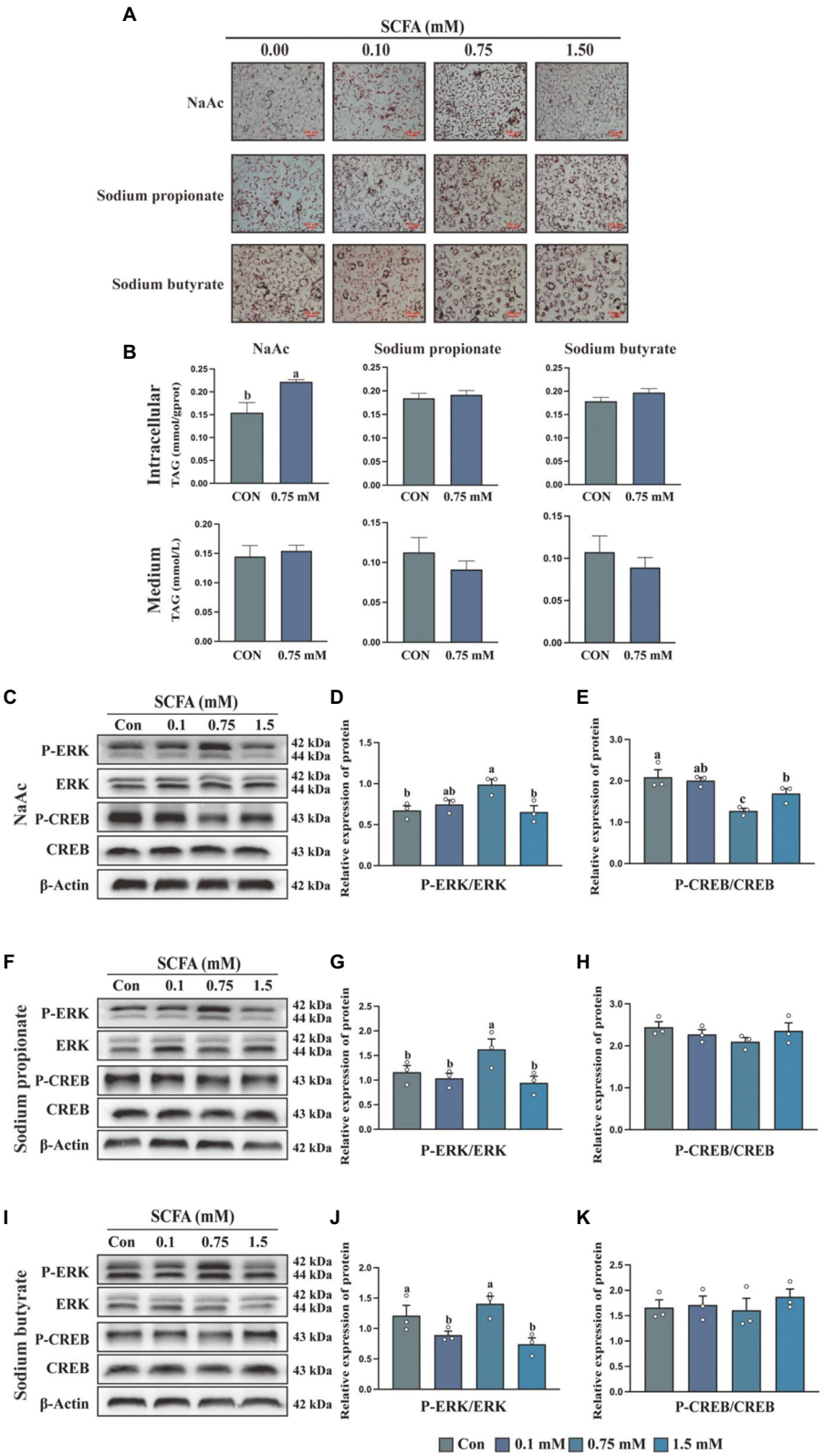


FIGURE 1
Short-chain fatty acids (SCFA) regulate milk fat synthesis in HC11 cells through the $G_{\alpha i}$ pathway **(A)** Oil red O staining images of HC11 cells treated with different concentrations of sodium acetate (NaAc), sodium propionate and sodium butyrate. Scale bars are 100μm. **(B)** TAG content in intracellular and medium. The relative protein expression level of P-ERK/ERK and P-CREB/CREB were treated with different concentrations of NaAc **(C–E)**, sodium propionate **(F–H)**, and sodium butyrate **(I–K)**. All data with error bars are averages ± SEM (n=3). In histograms, no letter or the same letter above the bar graph indicates no significant difference (p>0.05), and different letters indicate a significant difference (p<0.05). Each small white dot represents a test value.

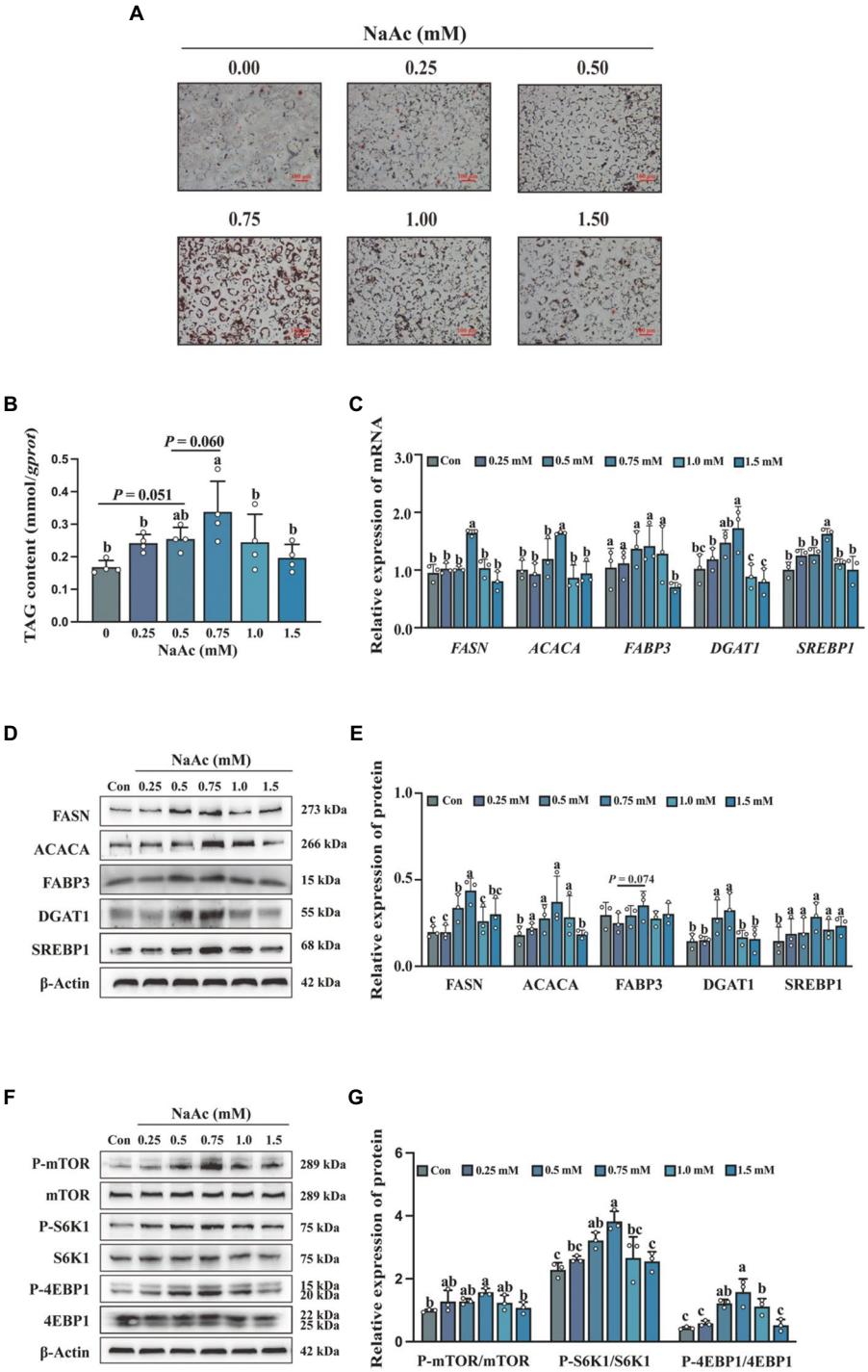


FIGURE 2
Sodium acetate (NaAc) promoted milk fat synthesis and activated the mTORC1 signaling pathway in HC11 cells. HC11 cells were exposed to different concentrations (0.00, 0.25, 0.50, 0.75, 1.00, and 1.50mM) of NaAc. **(A)** Oil red O staining images of HC11 cells. Scale bars are 100μm. **(B)** TAG content of HC11 cells. **(C)** Relative mRNA expression abundance of genes related to milk fat synthesis. **(D–G)** Relative expression abundance of proteins related to milk fat synthesis and mTORC1 pathway. All data with error bars are averages ± SEM (n=3). In histograms, no letter or the same letter above the bar graph indicates no significant difference (p>0.05), and different letters indicate a significant difference (p<0.05). Each small white dot represents a test value.

Sodium acetate promotes milk fat synthesis in HC11 cells with the activation of the mTORC1 signaling pathway

To detect the effects of NaAc on milk fat synthesis, HC11 cells were treated with various concentrations of NaAc (0, 0.25, 0.5, 0.75, 1.0, and 1.5 mM) for 24 h. 0.75 mM NaAc significantly promoted lipid droplet formation (Figure 2A) and synthesis of TAG content (Figure 2B) in HC11 cells (p < 0.05). Consistently, 0.75 mM NaAc also up-regulated the expression of milk fat synthesis-related genes (*FASN, ACACA, DGAT1*, and *SREBP1*; Figure 2C) and proteins (FASN, ACACA, DGAT1, and SREBP1; Figures 2D,E) in HC11 cells (p < 0.05). In addition, NaAc

activated the mTORC1 signaling pathway (as indicated by phosphorylation of mTOR, S6K1, and 4EBP1) in HC11 cells (Figures 2F,G; $p < 0.05$).

The inhibition of the mTORC1 pathway in HC11 cells resulted in the weakened of sodium acetate-induced milk fat synthesis

To confirm the relationship between mTORC1 activation and milk fat synthesis, we analyzed the effect of NaAc on rapamycin pretreated HC11 cells. NaAc-induced lipid droplet formation (Figure 3A) and synthesis of TAG content (Figures 3B,C) were attenuated in rapamycin pretreated HC11 cells ($p < 0.05$). Consistently, mTORC1 inhibition also down-regulated the expression of milk fat synthesis-related genes (*FASN, ACACA, FABP3, DGAT1*, and *SREBP1*; Figure 3D) and proteins (FASN, ACACA, FABP3, DGAT1, and SREBP1; Figures 3E,F) induced by NaAc in HC11 cells ($p < 0.05$). As expected, rapamycin also inhibited NaAc-induced activation of mTORC1 signaling, as indicated by the phosphorylation levels of mTOR, S6K1, and 4EBP1 (Figures 3G,H; $p < 0.05$).

Sodium acetate-induced milk fat synthesis is regulated through GPR41/GPR43-Gi pathways

To further clarify whether GPR41 and GPR43 were involved in the regulation of NaAc-induced milk fat synthesis, a small interfering RNA (siRNA) knock-down approach was used to inhibit the expression of GPR41 and GPR43 in HC11 cells. As shown in Figure 4A, siRNA, PTX, NF023, and Gallein inhibited the GPCR receptors, G_i, $G_{\alpha i}$, and $G_{\beta\gamma}$, respectively. Intriguingly, NaAc-induced milk fat synthesis was significantly decreased both in GPR41 and GPR43 knockdown cells (Figure 4B; $p < 0.05$). This evidence indicated NaAc regulated milk synthesis through GPR41 and GPR43. To further clarify the downstream molecular mechanisms of GPR41 and GPR43, we used PTX, Gallein, and NF023, respectively. As shown in Figure 4C, PTX, Gallein, and NF023 treatments all inhibit NaAc-induced TAG synthesis in HC11 cells ($p < 0.05$). Furthermore, the effect of related inhibitors on G protein is shown in Figures 4D,E, according to the phosphorylation degree of ERK and CREB, PTX significantly inhibited G_i, Gallein significantly inhibited $G_{\beta\gamma}$, and NF023 significantly inhibited $G_{\alpha i}$ ($p < 0.05$). Consistently, NaAc-induced expression of the proteins related to milk fat synthesis (FASN, ACACA, DGAT, and SREBP1) was also significantly down-regulated when G_i, $G_{\beta\gamma}$, and $G_{\alpha i}$ were inhibited in HC11 cells (Figures 4F,G; $p < 0.05$).

Sodium acetate regulates mouse offspring weight, milk fat content, G protein-coupled receptors and mTORC1 signaling pathways

Dietary supplementation of NaAc significantly increased milk in pups born (Figure 5A) and offspring weight (Figure 5B) by mothers from NaAc treatment is observed, when compared to the control group ($p < 0.05$). Consistently, compared with the control group, dietary supplementation of NaAc significantly increased milk fat content (Figure 5C), but not dam body weight, dam feed intake and prolactin (Figures 5D–F). The expression of genes (*FASN, ACACA, DGAT1*, and *SREBP1*) and proteins (FASN, ACACA, DGAT1, and SREBP1) related to milk fat synthesis in the mammary gland tissues of mice were significantly increased in NaAc group (Figures 5G–I). In addition, NaAc supplementation activated the GPCR (judged by phosphorylation level of ERK) and mTORC1 (judged by phosphorylation levels of mTOR, S6K1, 4EBP1) signaling pathways (Figures 5J,K).

Discussion

In recent years, the potential link between SCFA and milk fat synthesis has attracted wide attention (27). A large number of studies have shown that SCFA not only serves as signal molecules to promote fatty acid transport from adipose tissue to the mammary gland (28) but also directly participates in *de novo* milk fat synthesis as substrates (9). To date, the findings regarding SCFA on milk synthesis are mainly focused on ruminants, especially dairy cows. SCFA promoted the synthesis of intracellular TAG and significantly increased the mRNA abundance of genes related to milk fat synthesis (ACACA and FASN) and transcriptional regulation genes involved in lipid synthesis (SREBP1) in bovine mammary epithelial cells (BMEC) (7). Furthermore, SCFA also reduced the inflammatory response of BMEC by inhibiting histone deacetylase (29). However, the role of SCFA on the lactation performance of monogastric animals is not well elucidated. In this study, we found that NaAc efficiently promoted the lipid droplets in HC11 cells with the activation of GPCR and mTORC1 signaling pathways. Importantly, dietary NaAc supplementation enhances the milk fat content in lactating mice. However, it is worth noting that high concentration of acetic acid could be toxic to the cells and further inhibits milk fat synthesis (30).

GPR41 and GPR43 participated in the regulation of multiple biochemical and physiologic processes. For instance, activation of the GPR41/43 pathway inhibited the development of atherosclerosis (31). In addition, GPR41 activation resisted neuroinflammation, which could be used as a therapeutic strategy for treating Alzheimer's disease (32). Importantly, both GPR41 and GPR43 are largely expressed in adipocytes and are involved in the regulation of lipid metabolism (33). High expression of GPR43, but not GPR41 promoted fat deposition (33, 34). The difference between GPR41 and GPR43 activation on fat synthesis might attribute to the difference of G proteins coupled to GPR41 and GPR43. GPR43 activates signal transduction by activating $G_{\alpha i}/G_{\alpha q}$ pathway, while GPR41 activates signal transduction by activating the $G_{\alpha i}$ pathway (13). Intriguingly, in this experiment, knockdown of either GPR41 or GPR43 by siRNA both decreased NaAc-induced TAG synthesis in HC11 cells, which indicated $G_{\alpha i}$ signaling pathway might play a vital role in this process. Activation of both GPR41 and GPR43 leads to increased intracellular Ca^{2+} concentration, activation of extracellular regulated protein kinase ERK1/2, and decreased cAMP concentration (35). When HC11 cells were treated with PTX (G_i inhibitor), Gallein ($G_{\beta\gamma}$ inhibitor), and NF023 ($G_{\alpha i}$ inhibitor), the effect of NaAc on TAG synthesis was weakened, and PTX had the most obvious inhibitory effect on TAG synthesis and milk fat synthesis related protein. These results suggest that NaAc binding with GPR41 and GPR43 can promote the increase of milk fat synthesis in HC11 cells mainly through the activation of the downstream $G_{\alpha i}$ pathway.

mTORC1 is widely known as a critical regulator of protein synthesis through its downstream S6K1 and 4EBP1. Previous studies have pointed out that the mTORC1 signaling pathway is not only involved in the regulation of protein translation but also acted as an

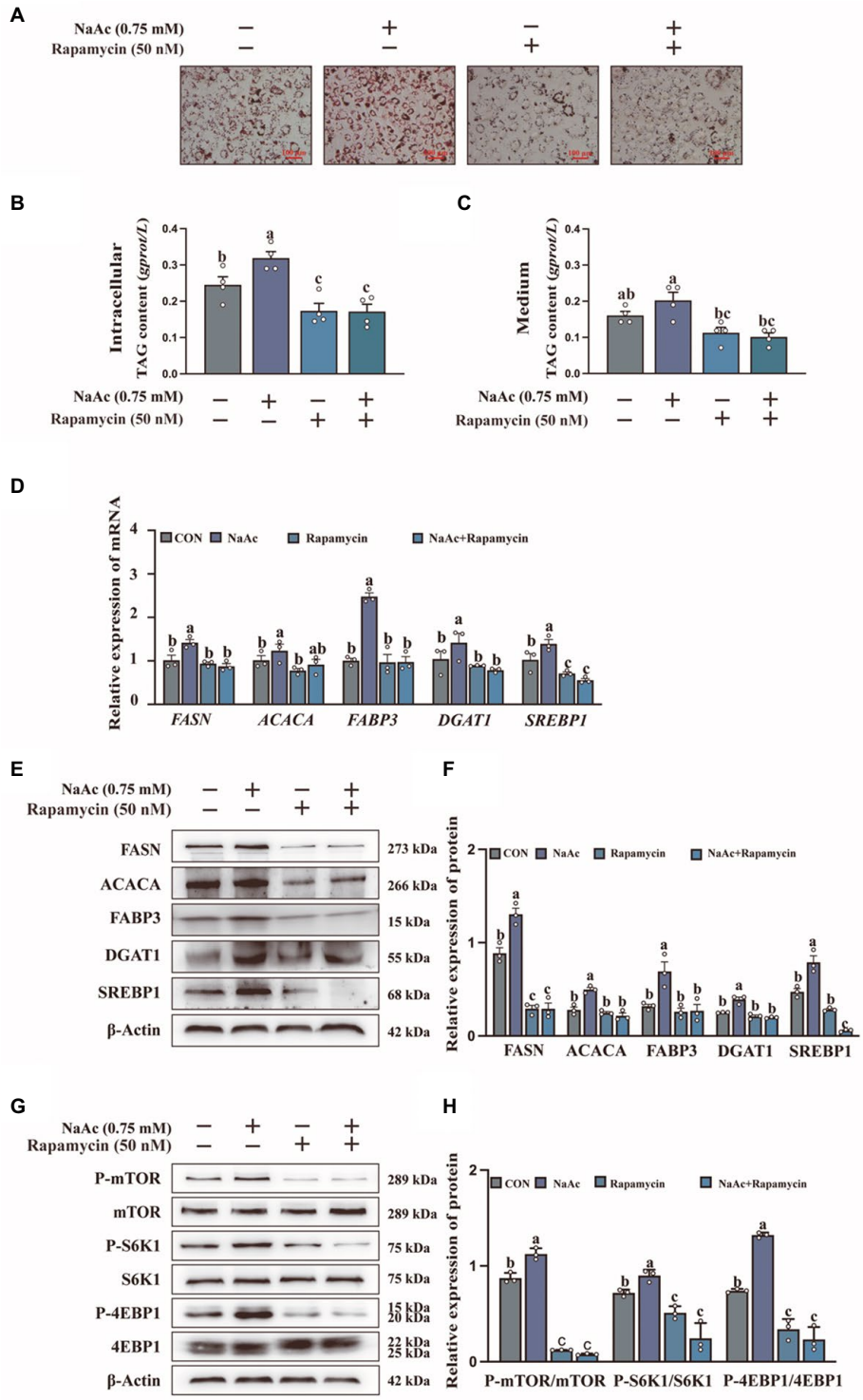


FIGURE 3
Effect of sodium acetate (NaAc) on milk fat synthesis and mTORC1 activation after pretreated with rapamycin. HC11 cells used in this figure were pre-treated for 2h with 50nM rapamycin (mTOR inhibitor) before incubation with 0.75mM NaAc for 24h. **(A)** Oil red O staining images of HC11 cells. Scale bars are 100μm. TAG content in intracellular **(B)** and medium **(C)**. **(D)** Relative mRNA expression abundance of genes related to milk fat synthesis. **(E–H)** Relative expression abundance of proteins related to milk fat synthesis and mTORC1 pathway. All data with error bars are averages ± SEM (n=3). In histograms, no letter or the same letter above the bar graph indicates no significant difference (p>0.05), and different letters indicate a significant difference (p<0.05). Each small white dot represents a test value.

important regulator of lipid anabolism (36). In dairy cows, mTORC1 activation of regulates gene expression of key enzymes in mammary gland development and milk fat synthesis (37, 38). Furthermore, methionine can protect the mammary gland from oxidative stress by activating the mTORC1 pathway, thus promoting mammary gland development (39). In this experiment, NaAc-induced milk fat synthesis was significantly reduced when mTORC1 activity is inhibited by rapamycin. Recently, the relationship between GPCR and

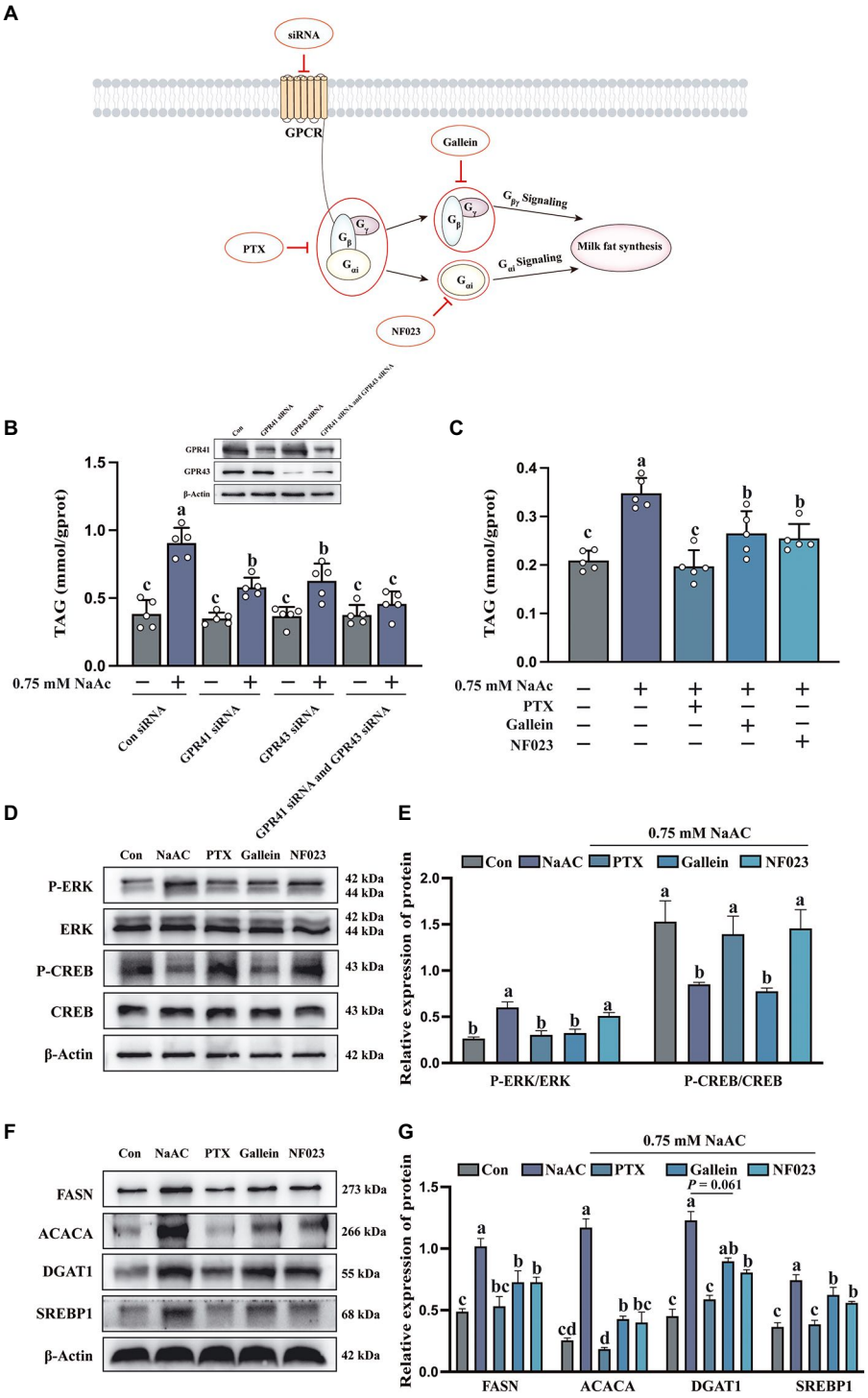


FIGURE 4
The inhibition of G protein attenuated the promoting effect of sodium acetate (NaAc) on milk fat synthesis. Cells used in this figure were pre-treated with PTX, Gallein, and NF023 for 2h or GPR41 siRNA and GPR43 siRNA for 6h, respectively, and then incubated with 0.75mM NaAc for 24h. **(A)** Targets of G protein-related inhibitors and siRNA. **(B,C)** TAG content in HC11 cells with different treatments, n=5. **(D,E)** Phosphorylation degree of ERK and CREB in different treatments, n=3. **(F,G)** Relative expression abundance of proteins related to milk fat synthesis, n=3. All data with error bars are averages ± SEM. In histograms, no letter or the same letter above the bar graph indicates no significant difference (p>0.05), and different letters indicate a significant difference (p<0.05). Each small white dot represents a test value.

mTORC1 signaling are uncovered. Activation of $G_{\alpha s}$ could induce the cellular cAMP level which further inhibited the mTORC1 activity through PKA phosphorylation of raptor on ser 791 with the help of AKAP13 (40). Conversely, activation of $G_{\alpha i}$ could decrease the cellular level of cAMP, which might further promote the activation of mTORC1 (17). However, this hypothesis still needs further confirmation. In addition, it is still unclear how $G_{\beta\gamma}$ regulates milk synthesis, which requires more future research.

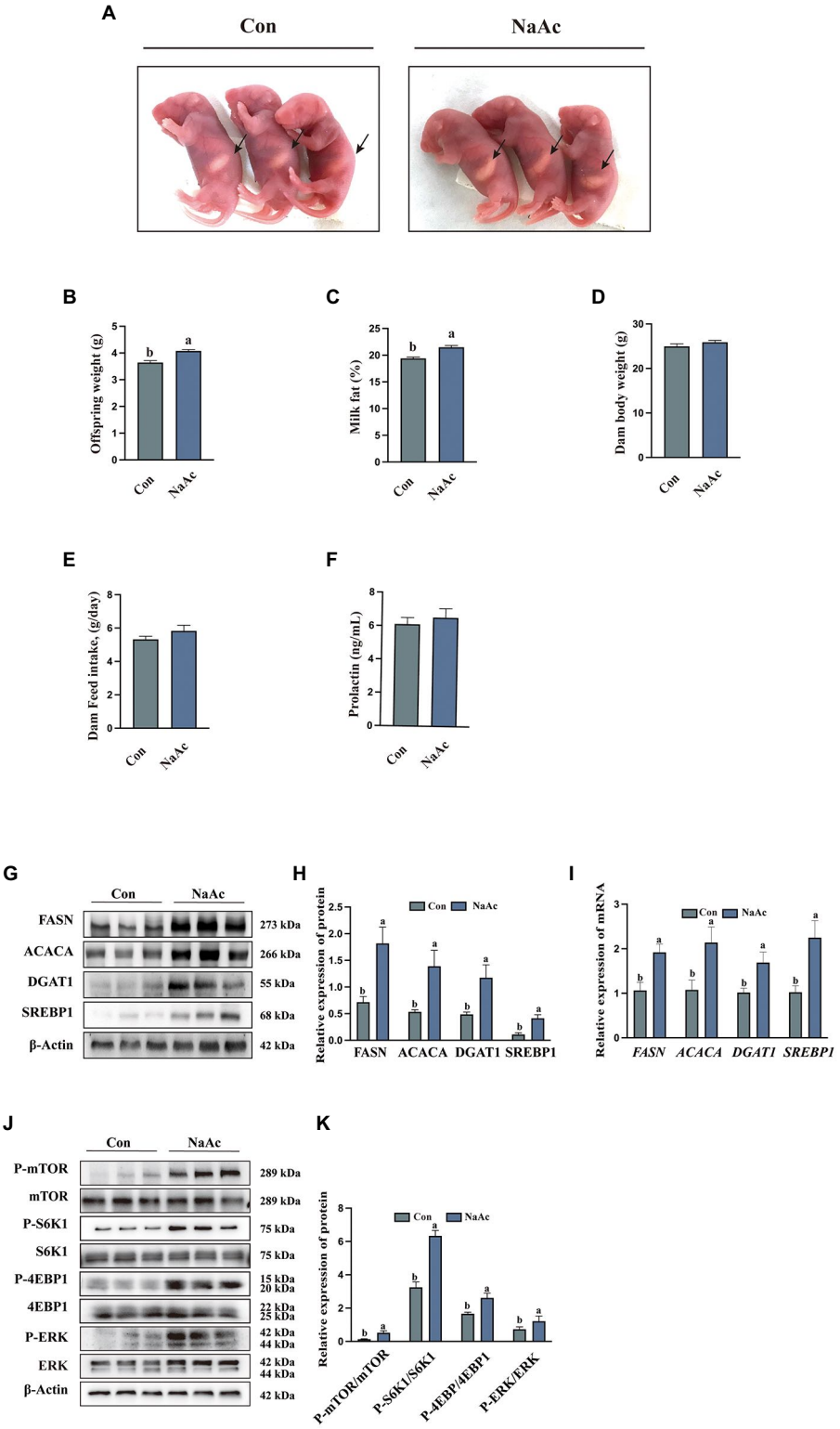


FIGURE 5
Effect of dietary supplementation of sodium acetate (NaAc) on milk fat synthesis and mTORC1 signaling in mice. **(A)** Milk in pups born by mothers, n=3. **(B)** Offspring weight on d 7 of lactation, n=7. **(C)** Milk fat content on d 7 of lactation, n=7. **(D)** Dam baby weight on d 7 of lactation, n=7. **(E)** Dam feed intake during lactation, n=7. **(F)** Prolactin content in plasma of mice on d 7 of lactation, n=7. **(G,H)** Relative expression abundance of proteins related to milk fat synthesis in mammary gland tissue, n=3. **(I)** Relative mRNA expression abundance of target genes, n=3. **(J,K)** Relative expression abundance of proteins related to GPCR and mTORC1 signaling pathways in mammary gland tissue, n=3. All data with error bars are averages ± SEM. In histograms, no letter or the same letter above the bar graph indicates no significant difference (p>0.05), and different letters indicate a significant difference (p<0.05).

Conclusion

We have found that the addition of NaAc promoted the increase of milk fat synthesis in HC11 cells and mammary gland tissue of mice at peak lactation. Mechanistically, we believe that NaAc activated GPR41 and GPR43, leading to the activation of mTORC1 to promote the synthesis of milk fat.

Data availability statement

The original contributions presented in the study are publicly available. This data can be found here: https://figshare.com/search?q=10.6084%2Fm9.figshare.22086329.

Ethics statement

The animal study was reviewed and approved by South China Agricultural University Animal Care and Use Committee (no. 20110107-1, Guangzhou, China).

Author contributions

YQ, SZ, XZ, and WG: designed the study. YQ, SZ, and JS: conducted research. YQ, SY, and QL: analyzed the data. YQ, TZ, and XL: wrote the manuscript. SZ: critically reviewed the manuscript. YQ had primary responsibility for the final content. All authors contributed to the article and approved the submitted version.

Funding

This study was financially supported by the National Key R&D Program of China (2021YFD1300700), Guangdong Basic and Applied Basic Research Foundation (No. 2021A1515010440), Science and Technology Program of Guangzhou (No. 202102020056), and National Natural Science Foundation of China (Nos. 31802067 and 31872364).

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnut.2023.1098715/full#supplementary-material

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Dietary Supplementation with Lysozyme–Cinnamaldehyde Conjugates Enhances Feed Conversion Efficiency by Improving Intestinal Health and Modulating the Gut Microbiota in Weaned Piglets Infected with Enterotoxigenic *Escherichia coli*

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Citation: Tian, Z.; Chen, J.; Lin, T.; Zhu, J.; Gan, H.; Chen, F.; Zhang, S.; Guan, W. Dietary Supplementation with Lysozyme–Cinnamaldehyde Conjugates Enhances Feed Conversion Efficiency by Improving Intestinal Health and Modulating the Gut Microbiota in Weaned Piglets Infected with Enterotoxigenic *Escherichia coli*. *Animals* **2023**, *13*, 3497. https://doi.org/10.3390/ani13223497

Academic Editor: Yong Su

Received: 25 September 2023
Revised: 17 October 2023
Accepted: 8 November 2023
Published: 13 November 2023

Correction Statement: This article has been republished with a minor change. The change does not affect the scientific content of the article and further details are available within the backmatter of the website version of this article.

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Simple Summary: Intestinal health is an important indicator of the healthy growth of piglets. As the agricultural industry eliminates the use of antibiotics in animal feed, alternatives to antibiotics will be needed. This study aims to evaluate the efficacy of lysozyme–cinnamaldehyde conjugates (LC) as a potential alternative to antibiotics in treating piglets infected with enterotoxigenic *Escherichia coli* (ETEC). The result shows that the LC-supplemented diet effectively mitigated the adverse effects of *E. coli* K88, including intestinal barrier damage and inflammation. Furthermore, it improved the structure of the intestinal flora, ultimately contributing to better growth performance in piglets. LC could be a useful, safe, and natural anti-inflammatory feed additive to prevent the decline in piglets' intestinal health.

Abstract: This study aims to evaluate the efficacy of lysozyme–cinnamaldehyde conjugates (LC) as a potential alternative to antibiotics in treating piglets infected with enterotoxigenic *Escherichia coli* (ETEC). The results demonstrated that piglets fed with the LC diet exhibited lower rectal temperature and fecal scores at 9 h, 24 h, and 48 h post-ETEC challenge. Furthermore, LC supplementation led to significant improvements in the mechanical and immune barriers of the jejunum and ileum, as indicated by an increased villi-height-to-crypt-depth ratio (VCR) and the expression of tight junction proteins, mucin, and β-defensins. Furthermore, the LC diet lowered the levels of pro-inflammatory cytokines TNF-α and IL-1β in the plasma. Further analyses showed that the LC diet downregulated genes (specifically TLR4 and MyD88) linked to the TLRs/MyD88/NF-κB signaling pathway in the small intestine. Additionally, 16SrDNA sequencing data revealed that LC supplementation increased the α diversity of intestinal microorganisms and the relative abundance of *Lactobacillus*. In summary, the LC-supplemented diet effectively mitigated the adverse effects of *E. coli* K88, including intestinal barrier damage and inflammation. Furthermore, it improved the structure of the intestinal flora, ultimately contributing to better growth performance in piglets.

Keywords: lysozyme; cinnamaldehyde; *Escherichia coli* K88; piglet; intestinal health

1. Introduction

Enterotoxigenic *Escherichia coli* (ETEC) is the primary cause of piglet post-weaning diarrhea (PPWD), a condition that results in high mortality rates. PPWD can result in decreased production performance, leading to high mortality rates [1]. Traditionally, antibiotics have been used in the diet to prevent PPWD and improve growth performance [2]. However, the overuse of antibiotics has led to the spread of drug-resistant bacteria [3,4]. Research has also shown the worldwide emergence of pathogens that are resistant to colistin and methicillin-resistant *Staphylococcus aureus*, posing a considerable threat to public health [5]. To address this issue, many countries have enacted laws restricting the use of antibiotics in animal feed [6]. As a result, there is an urgent need to explore alternatives to antibiotics in the form of functional feed additives.

Lysozyme, also known as N-acetyl cell wall glycan hydrolase or cell wall enzyme, is a natural anti-inflammatory, antibacterial, antiviral, and immune-enhancing agent [7]. In animal husbandry, lysozyme is a common feed additive due to its beneficial effects on growth and feed efficiency [8]. Research indicates that lysozyme supplementation significantly improves the apparent total tract digestibility of dry matter and gross energy [9], increases the villus height/crypt depth ratio [10], and reduces the serum levels of IL-1 and IL-6 in ETEC-infected weaned piglets [11]. However, the practical application of lysozyme is limited due to its instability and relatively narrow spectrum of antibacterial activity [12]. As a result, it is often combined with other antimicrobial agents to enhance its effectiveness [13].

Cinnamaldehyde, a bioactive compound found in Cinnamomum plants, is known for its distinctive aromatic odor. Pharmacological studies have demonstrated that Cinnamomum exhibits numerous beneficial activities, including antioxidant, antiviral, anti-inflammatory, antibacterial, and immunomodulatory effects [14]. Furthermore, recent research has revealed that the dietary inclusion of cinnamaldehyde effectively inhibits the NF-κB signaling pathway, thereby enhancing intestinal health in early weaned rats [15].

Lysozyme–cinnamaldehyde conjugates (LC) are formed through the reduction coupling effect of cinnamaldehyde on lysozyme. This process involves the combination of the aldehyde group in cinnamaldehyde with the amino acid residue of lysozyme, resulting in the formation of an imine bond. The conjugate has the potential to enhance the antibacterial capability of lysozyme and improve its stability. Therefore, the objective of this study was to investigate the effects of dietary LC on protecting weaning piglets challenged with ETEC, and to investigate the underlying mechanisms involved.

2. Materials and Methods

2.1. Animal Experimental Design

The animal test programs in this study strictly comply with the Protection and Use of Experimental Animals guidelines issued by the Animal Protection and Use Committee of South China Agricultural University (2022g028, Guangzhou, China). Thirty weaned piglets (Duroc × Landrace × Yorkshire, half male and half female, 24 ± 1 d, 7 ± 0.5 kg) were selected and divided into five groups based on similar body weight, with six replications in each group. The experimental groups were designed as follows: (1) basal diet (CON); (2) basic diet with ETEC (CON + ETEC); (3) basal diet with antibiotics and ETEC (50 mg/kg of quinenone, 75 mg/kg of chlortetracycline, 50 mg/kg of kitasamycin; ATB + ETEC); (4) basal diet with 0.05% LC (lysozyme/cinnamaldehyde = 1:3; Beijing Agrobeta Biotech Co., Ltd. (Beijing, China); effective content of 50,000 U/g; LC); (5) basal diet with 0.05% LC and ETEC (LC + ETEC). The basic diet formula of the experiment was prepared according to the recommendations of the National Research Council (NRC, 2012). The basal diets were mash, and the diet composition and nutrition level were shown in Table 1. Prior to the experiment, the pig house and the metabolic cage underwent thorough cleaning and disinfection using 20% concentrated glutaraldehyde. The piglets were placed in a metabolic cage, provided with free access to food and water, and the temperature was maintained at 30 ± 2 °C with a humidity level below 80%. The design of the animal experiment is shown in Figure S1.

Table 1. Compositions of basal diet and nutrient profile.

Ingredient Composition	Content (%)	Calculated Nutrient Profile [b]	Content
corn (7.4%, CP)	22.16	Digestible energy (MJ/kg)	14.83
broken rice (7.6%, CP)	31.09	Crude protein (%) [c]	17.98
soybean meal (43%, CP)	16.0	Crude fat (%) [c]	5.18
extruded soybean (35%, CP)	3.00	Dry matter (%) [c]	89.51
fermented soybean (50%, CP)	5.00	Crude fiber (%) [c]	2.17
Peruvian fish meal (65%, CP)	3.50	Ash (%) [c]	5.55
whey (3%, CP)	7.50	Calcium (%) [c]	0.80
glucose	1.25	Phosphorus (%) [c]	0.68
sucrose	3.50	Available phosphorus (%) [c]	0.48
soybean oil	2.60	Lysine (%) [c]	1.39
calcium hydrophosphate	1.20	Digestible lysine (%) [c]	1.25
acidifier	0.20	Digestible methionine + Cystine (%) [c]	0.69
limestone	0.60	Digestible threonine (%) [c]	0.74
salt	0.30	Digestible tryptophan (%) [c]	0.21
zinc oxide (78%, Zn)	0.20		
choline chloride (50%)	0.10		
L-lysine HCl (98.5%)	0.25		
DL-methionine (99%)	0.28		
L-threonine (98.5%)	0.22		
L-tryptophan (98.5%)	0.05		
premix [a]	1.00		

[a] Supplied per kilogram of diet: 12,000 IU of vitamin A, 3500 IU of vitamin D_3, 40 IU of vitamin E, 3.5 mg of vitamin K3, 18 mg of vitamin B, 10 mg of vitamin B2, 12.8 mg of vitamin B6, 0.034 mg of vitamin B12, 60 mg of nicotinic acid, 25 mg of D-pantothenic acid, 2.0 mg of folic acid, 0.26 mg of D-biotin, 180 mg of iron, 75.0 mg of copper, 1500 mg of zinc, 50.0 mg of manganese, 0.64 mg of iodine, and 0.3 mg of Selenium. [b] Calculated nutrient profiles were the calculated values. [c] Analyzed values.

2.2. Bacteria Preparation and Oral Challenge

Enterotoxigenic *Escherichia coli* (CVCC225, serotype O149: K91: K88ac; toxin LT and ST) was purchased from the China Veterinary Preservation Center. The ETEC was cultured overnight in a nutrient broth (NB, Huankai, Guangzhou, China) and subsequently diluted to a final concentration of 5×10^9 CFU/mL for the challenge piglets. After a three-day adaptation period, each group of piglets was fed with corresponding diets for seven days. On the eighth day, the CON + ETEC group, ATB + ETEC group, and LC + ETEC group were fed with 10 mL (5×10^9 CFU/mL) of the *E. coli* K88 solution. The CON group and LC group were fed with the same amount of liquid medium and continued to feed for seven days.

2.3. Fecal Score and Rectal Temperature

All piglets' rectal temperatures and fecal scores were measured using an electronic rectal thermometer (Omron MC-347, Omron, Dalian, China) before and 9 h, 24 h, 48 h, 96 h, and 168 h after the challenge with ETEC according to the fecal scoring system reported by Marquardt et al. [16]. (0: normal, hard form; 1: soft feces, soft form; 2: mild diarrhea, no form, loose, puddles; 3: severe diarrhea, watery).

2.4. Production Performance

The piglets were weighed on days 0, 8, and 15, and their daily feed intake was consistently recorded throughout the trial. These measurements were used to calculate the average daily gain (ADG), average daily feed intake (ADFI), and feed conversion rate (F/G) of the piglets.

2.5. Sample Collection

Blood samples were collected from each piglet's anterior vena cava on the morning of the 15th day of the trial period. A volume of 5 mL of blood was collected from each

piglet using an EDTA anticoagulant tube. The collected blood samples were centrifuged at 1000× *g* for 15 min to obtain plasma samples. Simultaneously, fresh feces from all piglets were collected and stored at −80 °C in a 5 mL aseptic EP tube for testing. Well-shaped areas (2 cm) of the duodenum, jejunum, and ileum were cut off, washed with normal saline, and placed in transparent plastic bottles containing 4% paraformaldehyde at room temperature. Additionally, 2 cm tissue samples from the duodenum, jejunum, and ileum were carefully washed with normal saline. The mucous membrane was then scraped off with a glass plate and placed in a 5 mL cryopreservation tube. The samples were quickly frozen using liquid nitrogen and stored at −80 °C. It is important to note that all samples were taken from the same part of each piglet.

2.6. Measurement of Inflammatory Factor

The concentrations of IL-1β (CSB-E06782p, Jiancheng, Nanjing, China), TNF-α (CSB-E16980p, Jiancheng, Nanjing, China), IL-6 (CSB-E06786p, Jiancheng, Nanjing, China), and IL-10 (CSB-E06779p, Jiancheng, Nanjing, China) in plasma were measured using an ELISA kit. The measurements were conducted following the instructions provided by the kit manufacturer.

2.7. Intestinal Section

The fixed intestinal tissue samples were extracted from a 4% paraformaldehyde solution. They were then graded, dehydrated in a specific order, embedded, and sliced to a thickness of 5 μm. The slices were stained with hematoxylin–eosin staining solution, dehydrated, and sealed. For each sample, two slices were prepared, and three typical visual fields (40 times magnification) were selected. The height of the intestinal villi and the depth of the crypt were determined using image processing software.

2.8. Real-Time Fluorescence Quantitative PCR Detection

RNA extraction from the intestinal tissue was performed using the tissue RNA purification kit (EZB-RN001-plus, EZBioscience, Roseville, MN, USA). The concentration and quality of the extracted RNA were determined using a NanoDrop ND-1000 spectrophotometer (Thermo Scientific, Wilmington, NC, USA). The RNA was then reverse transcribed into cDNA using a reverse transcriptase kit (A0010CGQ, EZBioscience, Roseville, MN, USA). Finally, the expression of the target gene was analyzed using the 2 × Color SYBR Green qPCR Master Mix (ROX2 plus) kit (A0012-R2, EZBioscience, Roseville, MN, USA). Real-time quantitative PCR detection was carried out using the 7500 fast real-time PCR system with a reaction condition of 95 °C for 5 min. The qPCR reaction system consisted of a 20 μL volume with 9.2 μL of the cDNA and ddH2O premixture, 2× Color SYBR Green qPCR Master Mix, and primer pair premixture, and 10.8 μL for each reaction well. The amplification was performed for 40 cycles at 95 °C for 10 s, 60 °C for 30 s, and 72 °C for 30 s. The primer sequences we used for the real-time PCR are listed in Table 2.

2.9. Western Blotting Analysis

The level of protein expression of the tight junction proteins (Claudin-1, Occludin, and ZO-1) was measured using Western blotting. The total protein was isolated using RIPA lysate buffer (Beyotime, Shanghai, China). A total of 10 μg of protein was separated using a 10% SDS-PAGE (P0012AC, Beyotime, Shanghai, China) and transferred to a PVDF membrane for Western blotting. The membrane was then incubated with a specific antibody for 12 h at 4 °C, followed by incubation with a secondary antibody for 1 h at room temperature. Protein bands were detected using an ECL chemiluminescence reagent (P1020 ApplyGen, Beijing, China). The test antibody information is shown in Table 3.

Table 2. Primers used for q-PCR analysis.

Genes	Accession	Direction	Sequences (5′–3′)
TLR4	NM_001113039.1	Forward	GCCATCGCTGCTAACATCATC
		Reverse	CTCATACTCAAAGATACACCATCGG
MyD88	NM_001099923.1	Forward	TGGTAGTGGTTGTCTCTGATGA
		Reverse	TGGAGAGAGGCTGAGTGCAA
IKKα	NM_001114279.1	Forward	TCTTGATCCTCGGAAACCAG
		Reverse	TGCTTCGGCCCATACTTTAC
IKKβ	NM_001099935.1	Forward	CCTCACCTTGCTGAGTGACA
		Reverse	TCCCCACAAAGGAGGTACAG
IκB	EU399817.1	Forward	CTGCTCGGCAATAACACTGA
		Reverse	GAGAGGAGACCGTTGGTGAG
TRAF-6	XM_013990069.2	Forward	GCTGCATCTATGGCATTTGAAG
		Reverse	CCACAGATAACATTTGCCAAAGG
TAK1	NM_001114280.1	Forward	CATGTGGGCTGTTCATAACG
		Reverse	GAGTTGCTCTGGCCTTCATC
SIGIRR	*NM_001315689.1*	Forward	ACCTGGGCTCCCGAAACTAC
		Reverse	GTCATCTTCTGACACCAGGCAAT
TOLLIP	NM_001315800.1	Forward	CCCGCGCTGGAATAAGG
		Reverse	CATCAAAGATCTCCAGGTAGAAGGA
PBD-1	NM_213838.1	Forward	ACCGCCTCCTCCTTGTATTC
		Reverse	CACAGGTGCCGATCTGTTTC
pBD-2	NM_214442.2	Forward	ATTAACCTGCTTACGGGTCTTGGC
		Reverse	CCACTGTAACAGGTCCCTTCAATCC
pBD-3	XM_021074698.1	Forward	TCTTCTTGTTCCTGATGCCTCTTCC
		Reverse	GCCACTCACAGAACAGCTACCTATC
MUC1	XM_021089730.1	Forward	TTCTTCGGGCTGTTGCTACT
		Reverse	ACTGTCTTGGAAGGCCAGAA
MUC2	NM_010843.1	Forward	CTCCATCCGCTTCAGAAAAG
		Reverse	ACTACCTGGGGCCTCTGAAT
MUC4	XM_021068274.1	Forward	CCTCCCAAGCAGATGTCAAT
		Reverse	CTGGGATAAGAATGCCTCCA
β-Actin	XM_021086047.1	Forward	GATCTGGCACCACACCTTCTACAAC
		Reverse	TCATCTTCTCACGGTTGGCTTTGG

TLR4, Toll-like receptor 4; *MyD88*, myeloid differentiation factor 88; *IKKα*, inhibitor of NF-κB kinase α; *IKKβ*, inhibitor of NF-κB kinase β; *IκB*, Inhibitor of κ light chain gene; *TRAF-6*, TNF receptor associated factor 6; *TAK1*, transforming growth factor β activated kinase 1; *SIGIRR*, Single Ig IL-1-related receptor; *TOLLIP*, Toll-interacting protein; *pBD*, porcine beta-defensin; and *MUC*, mucin.

Table 3. Antibodies used for Western blotting analysis.

Names	Company	Cat. No.	Dilution Multiple
Claudin-1	Abcam (Cambridge, UK)	ab15098	1:1000
Occludin	Abcam (Cambridge, UK)	ab31721	1:1000
ZO-1	Abcam (Cambridge, UK)	ab31721	1:1000
β-actin	Bioss (Beijing, China)	bs-0061R	1:5000
Second antibody (Goat anti-rabbit)	ZenBio (Chengdu, China)	511203	1:5000

2.10. 16S rDNA Sequencing

The intestinal microflora of the piglet feces was analyzed using a high-throughput 16s rDNA sequencing technique. The sequencing was conducted by Novogene Denovo in Guangzhou, China. Genomic DNA was extracted from the samples, and the V3 + V4 region of 16s rDNA was amplified using specific primers with a barcode. The primer sequence used was 341F: CCTACGGGNGGCWGCAGTATCTAAT. The purified amplified products were then connected to the sequencing connector, and the sequencing library was constructed and sequenced using Illumina. Finally, the 16s rDNA sequencing data were analyzed using Omicsmart, an online tool provided by Gene Denovo in Guangzhou, China.

2.11. Statistical Analysis

By using the SPSS 26.0 software (IBM Inc., Armonk, NY, USA) to analyze the one-way ANOVA variance of all data and using Duncan's method to make multiple comparisons, the results were expressed as means ± SEM, and the statistical significance standard was $p < 0.05$, It was considered as a trend when $0.05 < p < 0.10$. Graphs were drawn and analyzed using the GraphPad Prism 8.0 software.

3. Results

3.1. Production Performance, Fecal Scores, and Rectal Temperature

As illustrated in Table 4, the CON + ETEC group showed a significant decrease in average daily gain (ADG) and an increase in the feed-to-gain ratio (F/G) when compared to the CON group ($p < 0.05$). No significant difference was observed among the other groups. As depicted in Figure 1A, the fecal score of the CON + ETEC group rose significantly at 9 h, 24 h, 48 h, and 96 h post-challenge in comparison to the CON group ($p < 0.05$). At 24 h post-challenge, piglets in the ATB + ETEC, LC + ETEC, and LC treatments had lower fecal scores compared with those from the CON + ETEC ($p < 0.05$) (Figure 1A). Furthermore, Figure 1B indicates that the rectal temperature of the CON + ETEC group significantly increased at 9 h, 24 h, and 48 h post-challenge relative to the CON group ($p < 0.05$). Compared with the CON + ETEC group, the rectal temperature of the ATB + ETEC group and LC + ETEC group decreased significantly at 24 h after the challenge ($p < 0.05$) (Figure 1B). And there was no significant change between the LC + ETEC group and the ATB + ETEC group.

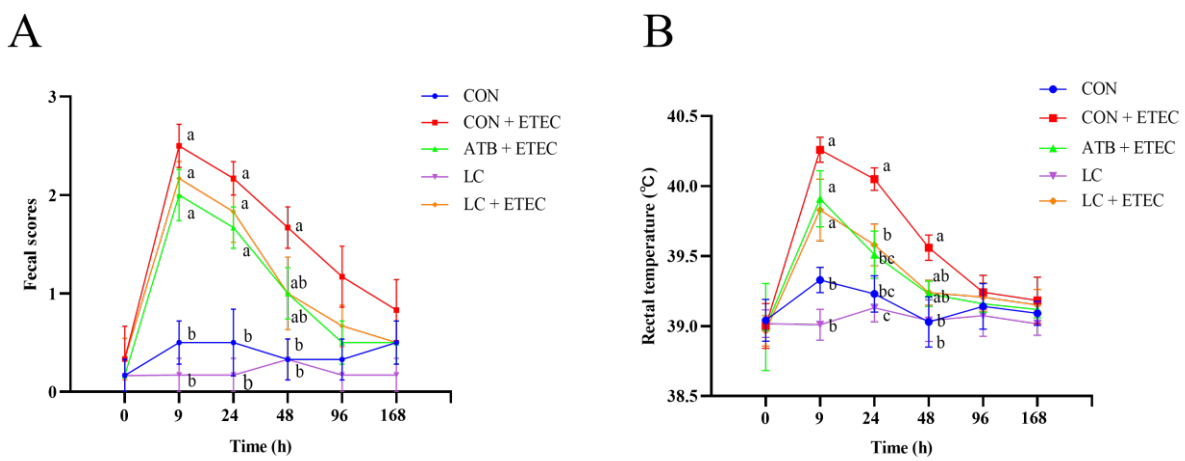

Figure 1. Effects of dietary LC on the fecal scores and rectal temperature in ETEC-challenged piglets. (**A**) The fecal scores were evaluated at 0 h, 9 h, 24 h, 48 h, 96 h, and 168 h after challenge with ETEC according to the fecal scoring system reported by Marquardt et al. (0: normal, hard form; 1: soft feces, soft form; 2: mild diarrhea, no form, loose, puddles; 3: severe diarrhea, watery). (**B**) Rectal temperatures were measured using an electronic rectal thermometer (Omron MC-347) at 0 h, 9 h, 24 h, 48 h, 96 h, and 168 h in ETEC-challenged piglets. Data are expressed as the mean ± SE ($n = 6$). Different letters (a, b, and c) indicate a significant difference among the different groups ($p < 0.05$).

Table 4. Effect of LC on the growth performance of weaned piglets.

Items	Treatments					SEM	*p*-Value
	CON	CON + ETEC	ATB + ETEC	LC	LC + ETEC		
Average body weight (kg)							
0 d	7.10	7.09	7.08	7.06	7.11	0.06	1.00
7 d	8.75	8.71	8.81	8.80	8.85	0.05	0.91
14 d	10.97	10.58	10.85	11.05	10.86	0.10	0.40

Table 4. *Cont.*

Items	Treatments					SEM	*p*-Value
	CON	CON + ETEC	ATB + ETEC	LC	LC + ETEC		
Pre-challenge (0–7 d)							
ADFI (g)	322.27	318.19	339.28	339.17	341.74	7.48	0.81
ADG (g)	235.24	231.43	246.43	248.57	248.33	5.42	0.80
F/G	1.37	1.38	1.38	1.36	1.38	0.03	0.44
Post-challenge (7–14 d)							
ADFI (g)	481.97	460.28	452.41	486.27	444.69	11.24	0.74
ADG (g)	319.24	267.03	290.95	321.91	284.12	7.75	0.06
F/G	1.51 [b]	1.72 [a]	1.55 [b]	1.50 [b]	1.57 [b]	0.02	0.00

$n = 6$. Different letters mean a statistically significant difference among the groups ($p < 0.05$).

3.2. Inflammatory Cytokines

As depicted in Figure 2, compared to the CON group, the plasma levels of TNF-α, IL-1β, and IL-6 in the CON + ETEC group increased significantly post-challenge ($p < 0.05$). Conversely, no significant changes were observed in the ATB + ETEC group or LC + ETEC group. Importantly, Figure 2D indicates that the TNF-α levels in the LC group decreased significantly relative to other groups ($p < 0.05$).

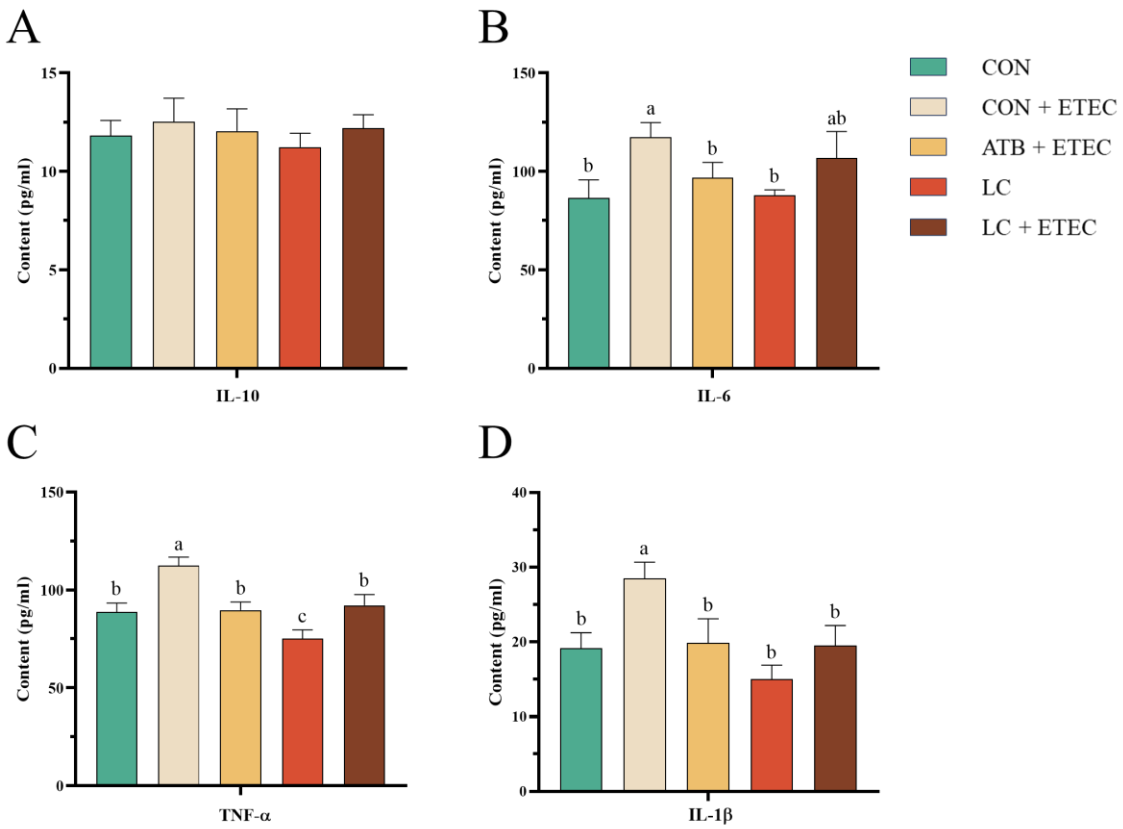

Figure 2. Effects of dietary LC on plasma inflammatory factors in ETEC-challenged piglets. Concentrations of IL-10 (**A**), IL-6 (**B**), TNF-α (**C**), and IL-1β (**D**) were assessed. Data are expressed as the mean ± SE ($n = 6$). Different letters (a, b, and c) indicate a significant difference among the different groups ($p < 0.05$).

3.3. Gut Morphology

Intestinal morphology was examined using H&E staining in the weaned piglets (Figure 3A). Compared to the CON group, the depth of the duodenal recess increased in the CON + ETEC group, while the villus height of the jejunum and ileum, as well as the VCR of each intestinal segment, decreased significantly ($p < 0.05$) (Figure 3B–D). Additionally, the duodenal VCR increased significantly in the ATB + ETEC group compared to the CON + ETEC group ($p < 0.05$) (Figure 3D). And the duodenal VCR of the LC + ETEC group showed an upward trend compared to the CON + ETEC group ($p = 0.061$) (Figure 3D). Furthermore, the jejunum and ileum VCR increased significantly in both the ATB + ETEC group and the LC + ETEC group ($p < 0.05$) (Figure 3D).

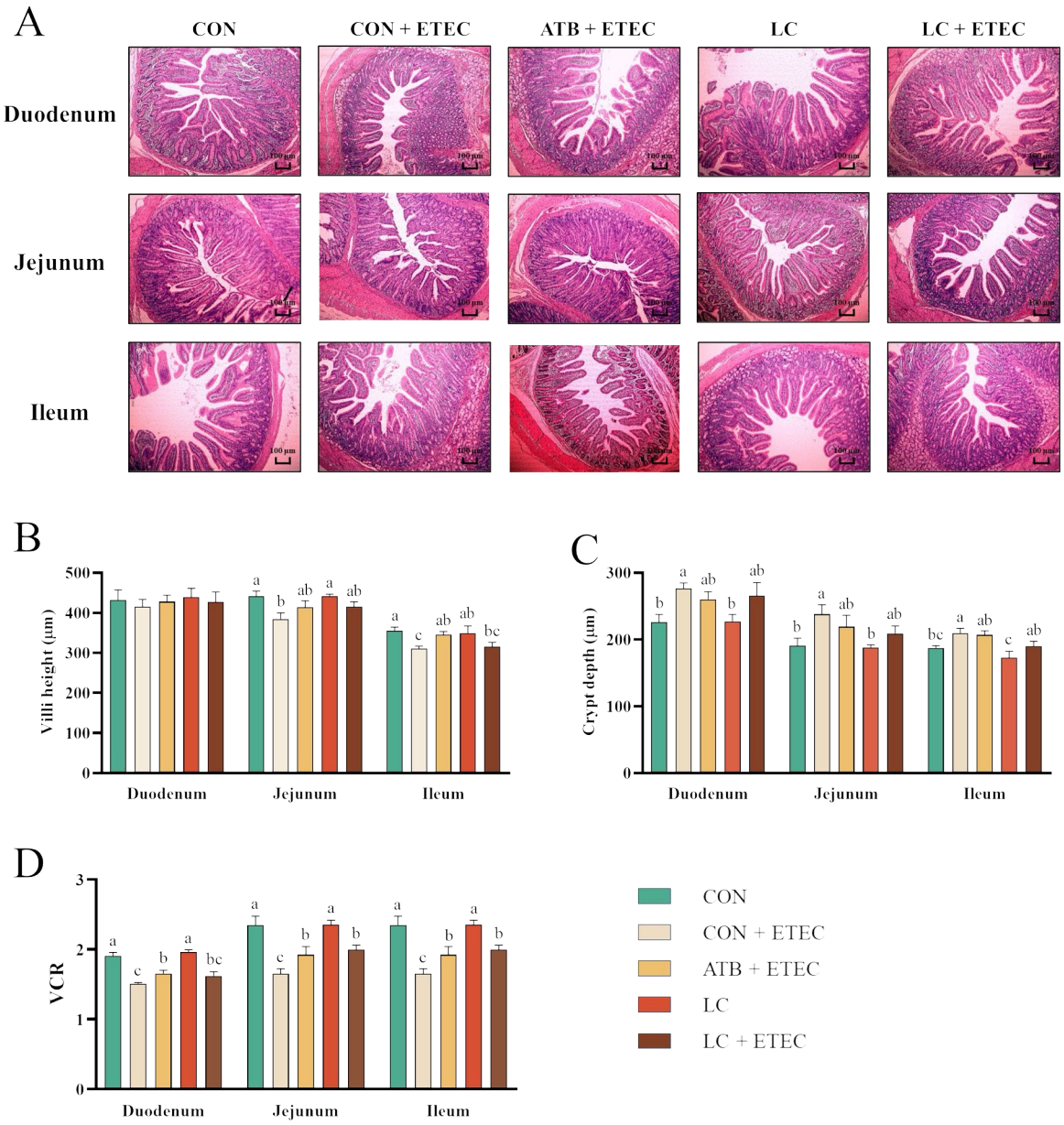

Figure 3. Effects of dietary LC supplementation on the intestinal morphology in ETEC-challenged piglets. (**A**) The hematoxylin and eosin (H&E)-stained intestinal cross-sections in piglets are shown. The scale bar represents 100 μm. (**B**) Villi height, (**C**) crypt depth, (**D**) ratio of villous height to crypt depth (VCR). Data are expressed as the mean ± SE ($n = 6$). Different letters (a, b, and c) indicate a significant difference among the different groups ($p < 0.05$).

3.4. Intestinal Tight Junctions

Similarly, the CON + ETEC group showed a significant decrease in tight junctions across all intestinal segments compared to the CON group ($p < 0.05$) (Figure 4A–F). Notably, the supplementation of LC into the diet led to an increase in Occludin expression throughout the intestinal tract relative to the CON treatment ($p < 0.05$) (Figure 4A–F). In addition, the protein expressions of Occludin, Claudin-1, and ZO-1 in the duodenum, Occludin and Claudin-1 in the jejunum, and Occludin in the ileum were significantly higher in the ATB + ETEC and LC + ETEC groups compared to the CON + ETEC group ($p < 0.05$) (Figure 4A–F). And there were no significant differences in intestinal tight junction protein expression between the ATB + ETEC and the LC + ETEC groups. Full original Western blot (WB) images of Figure 4 are shown in Figure S2.

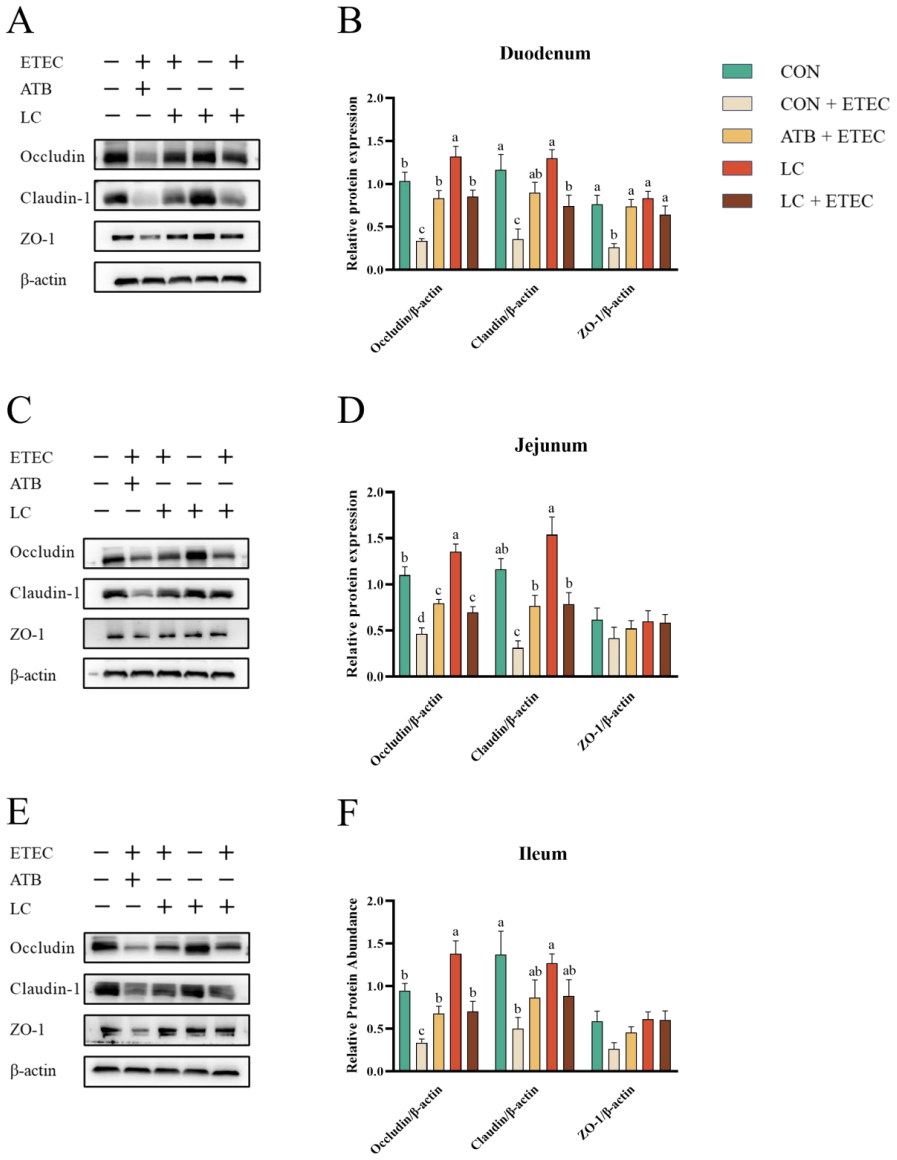

Figure 4. Effects of dietary LC supplementation on the expression of intestinal tight junction proteins in ETEC-challenged piglets. The expression and abundance of tight junction proteins (Occludin, Claudin-1, and ZO-1) and β-actin in the duodenum (**A**,**B**), jejunum (**C**,**D**), and ileum (**E**,**F**) of piglets were analyzed. Data are expressed as the mean ± SE ($n = 3$). Different letters (a, b, and c) indicate a significant difference among the different groups ($p < 0.05$).

3.5. Mucin and β-Defensin

Compared to the CON group, the CON + ETEC group exhibited a significant reduction in the expression levels of *pBD2* in the duodenum, as well as *MUC-1*, *MUC-2*, *pBD1*, and *pBD2* in the jejunum, and *MUC-2*, *pBD1*, *pBD2*, and *pBD3* mRNA in the ileum ($p < 0.05$) (Figure 5A–F). In contrast, the ATB + ETEC and LC + ETEC groups showed no significant down-regulation in the expression of mucin and β-defensin genes (Figure 5A–F). Furthermore, the LC group displayed a marked increase in the mRNA expression levels of *pBD2* in the jejunum, as well as *MUC-2*, *pBD1*, and *pBD2* in the ileum, when compared to the CON group ($p < 0.05$) (Figure 5E,F).

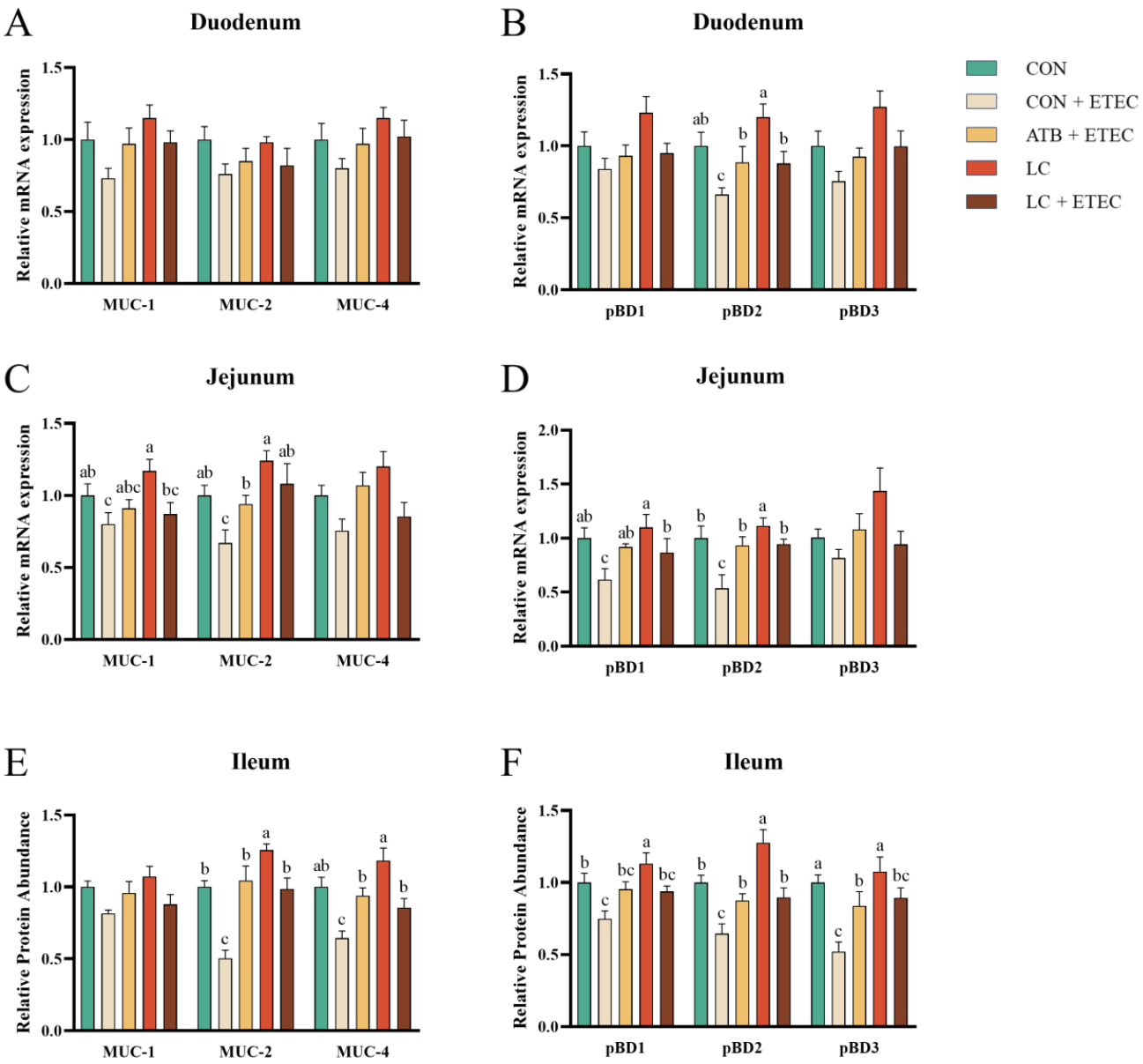

Figure 5. Effects of dietary LC supplementation on mRNA expression levels of mucin and β-defensin of piglets challenged with ETEC. The mRNA expression levels of mucin in the duodenum are presented in (**A**), while the expression levels of β-defensin are shown in (**B**). Similarly, (**C**) represents the expression of mucin mRNA in the jejunum, and (**D**) represents β-defensin expression. Additionally, (**E**,**F**) display the expression levels of mucin and β-defensin mRNA in the ileum. The data is presented as the mean ± SE ($n = 6$). Different letters (a, b, and c) indicate a significant difference among the different groups ($p < 0.05$). MUC, mucin; *pBD*, porcine beta-defensin.

3.6. Abundance of Genes Related to NF-κB/MAPK Signal Pathway

As illustrated in Figure 6, the CON + ETEC group showed a significant up-regulation in the mRNA expression of TLR4 and MyD88 in the duodenum, jejunum, and ileum when compared with the CON group ($p < 0.05$) (Figure 6A–C). Furthermore, the mRNA expression of IKKα and TAK1 in the duodenum, TAK1 in the jejunum, and TRAF6 in the ileum were also significantly up-regulated in the CON + ETEC group ($p < 0.05$) (Figure 6A–C). In contrast, the ATB + ETEC and LC + ETEC groups did not display a significant increase in the expression of NF-κB pathway-related genes. However, the mRNA expression of SIGTRR was significantly up-regulated ($p < 0.05$) in both of these groups (Figure 6A–C). Simultaneously, the LC group showed a significant down-regulation in the mRNA levels of TLR4, IκB, and TRAF6 in the duodenum, as well as TLR4 and TAK1 in the jejunum, and MyD88, TAK1, and TRAF6 in the ileum ($p < 0.05$) (Figure 6A–C). No significant difference in gene expression was observed between the ATB + ETEC and LC + ETEC groups.

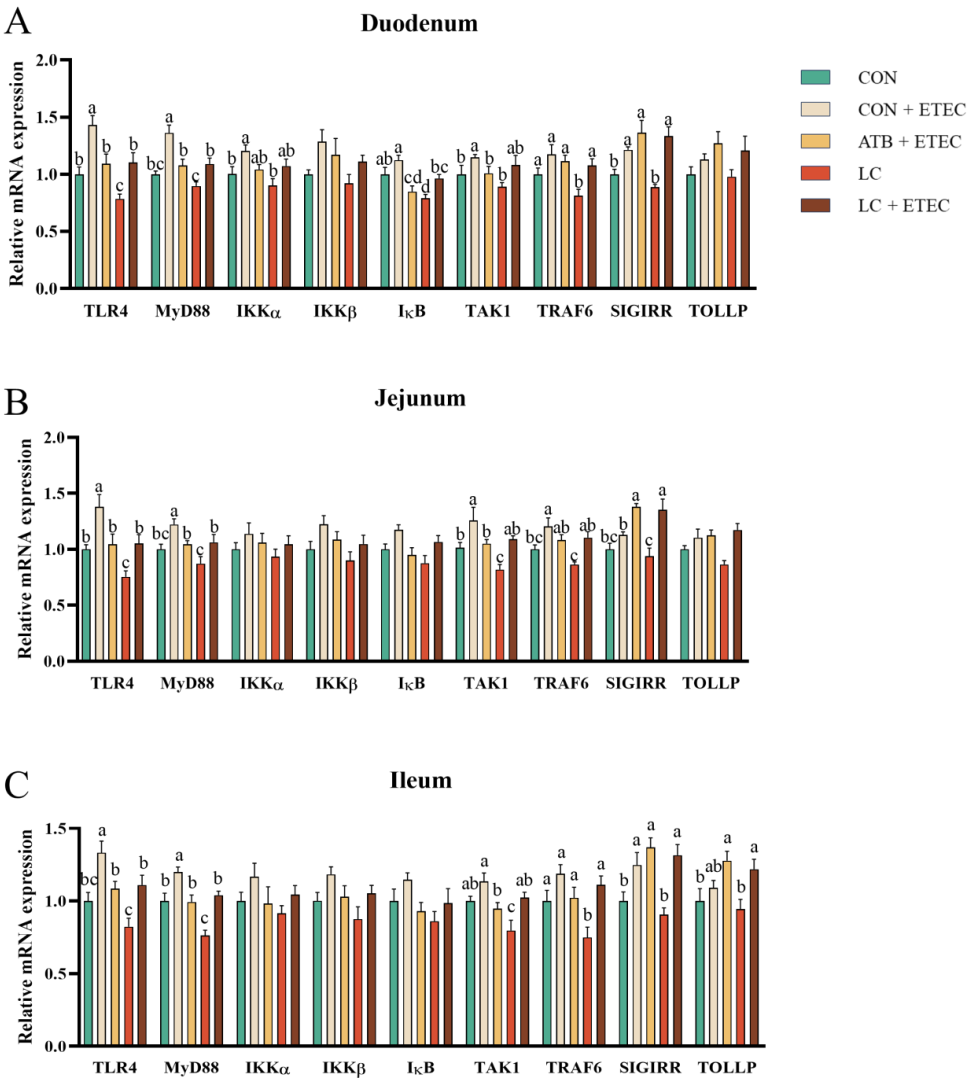

Figure 6. Effects of dietary LC supplementation on the concentration of relative mRNA expression of genes related to inflammation in ETEC-challenged piglets. (**A**) Duodenum, (**B**) jejunum, (**C**) ileum. The data are presented as the mean ± SE ($n = 6$). Different letters (a, b, and c) indicate a significant difference among the different groups ($p < 0.05$). TLR4, Toll-like receptor 4; NF-κB, nuclear factor kappa B; MyD88, myeloid differentiation factor 88; IKKα, inhibitor of NF-κB kinase α; IKKβ, inhibitor of NF-κB kinase β; TAK1, transforming growth factor β (TGF-β)-activated kinase 1; TRAF6, TNF receptor-associated factor 6; SIGTRR, Single Ig IL-1-related receptor; TOLLP, Toll-interacting protein.

3.7. Gut Microbiome

A total of 3,672,934 high-quality sequences were generated from 30 fecal samples (five treatments; $n = 6$); with an average of 122,431 sequences per sample. After removing the noise sequence, 2,956,668 valid tags remained for further analysis. These tags were clustered into operational taxons (OTUs) with 97% sequence similarity. These OTUs were then classified to 22 phyla; 29 classes; 47 orders; 68 families; 170 genera; and 1395 OTUs (97% similarity) for further analysis. The alpha diversity of the ileal microbiota, as indicated by Sob, Chao1, ACE, Shannon, Simpson, and Pielou, is presented in Figure 7A–F. Notably, the CON + ETEC treatment group showed a significant reduction in Chao1 and ACE indices compared to the other groups ($p < 0.05$). Furthermore, no significant differences were found in the beta diversity of ileal microbiota among the treatment groups (Figure 7G,H). The microbial composition of the piglets' ileal contents predominantly consisted of five major bacterial phyla—Firmicutes; Bacteroidetes; Proteobacteria; Cyanobacteria; and Epsilonbacteraeota—which collectively made up over 95% of the total ileal bacterial community (Figure 8A). However; the proportions of these phyla varied among the different treatments. At the genus level, the relative abundance of *Lactobacillus* in piglets was observed to decrease following the ETEC challenge. Conversely, piglets supplemented with LC and ATB exhibited an increase in *Lactobacillus* abundance ($p < 0.05$) (Figure 8B).

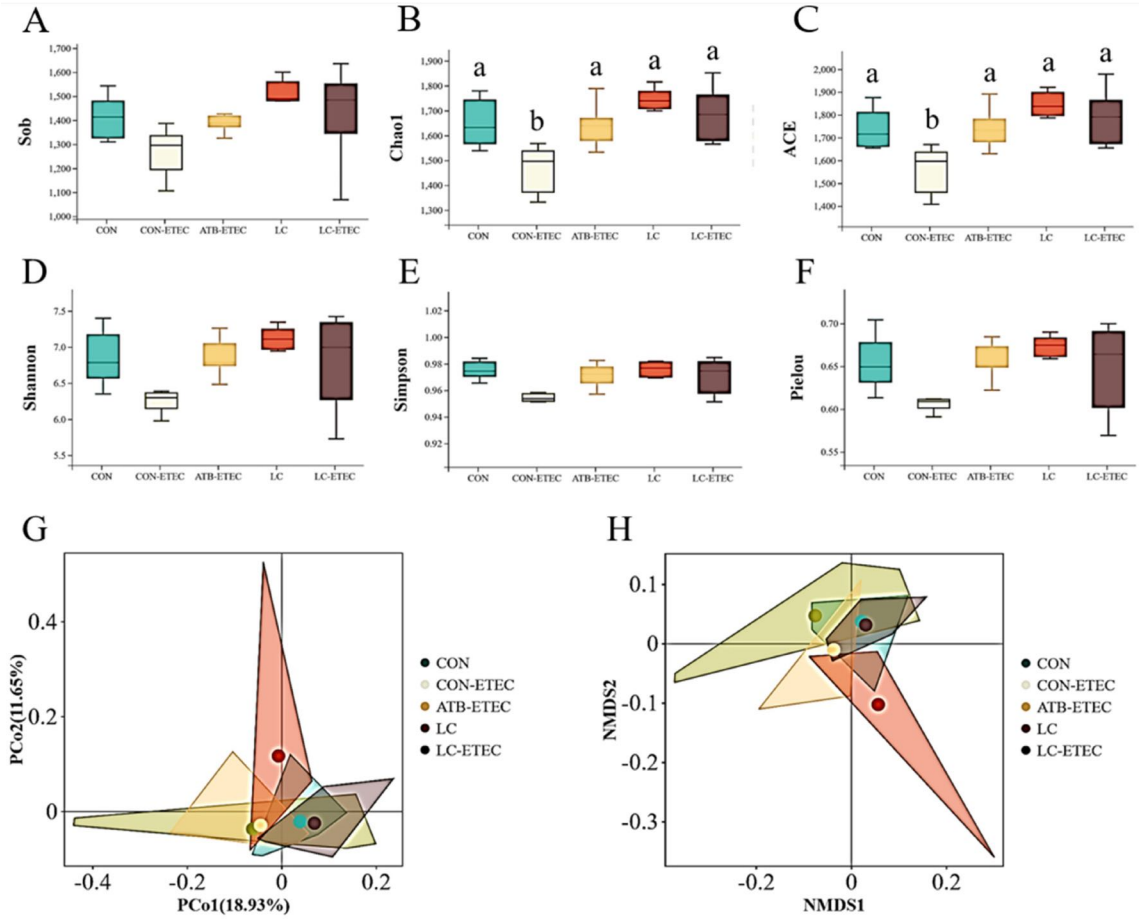

Figure 7. Effects of dietary on alpha diversity and beta diversity of ileal microbiota in ETEC−challenged piglets. (**A**–**F**) Alpha diversity index: Sob, Chao1, ACE, Shannon, Simpson, and Pielou. (**G**) Principal coordinates analysis based on Bray−Curtis distance. (**H**) Plot of NMDS analysis of Bray. Data are expressed as the mean ± SE ($n = 6$). Different letters (a, and b indicate a significant difference among the different groups ($p < 0.05$).

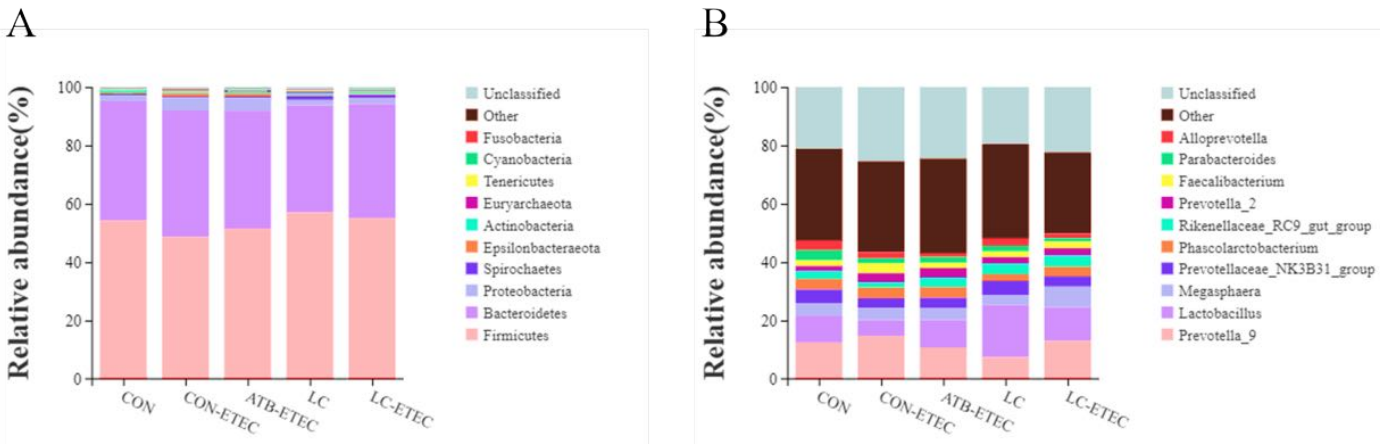

Figure 8. Effects of dietary LC supplementation on the microbial composition of ileal microbiota in ETEC-challenged Piglets. (**A**) Relative abundance in genus. (**B**) Relative abundance in species. Data are expressed as the mean ± SE (n = 6).

4. Discussion

ETEC is a major contributor to diarrhea and intestinal injury in weaned piglets, as mentioned in previous studies [17]. Specifically, the heat instability (LT) and heat-stable (ST) enterotoxins produced by ETEC increase the levels of cyclic adenosine monophosphate (cAMP) and cyclic guanosine monophosphate (cGMP). This leads to an increased secretion of water and electrolytes into the intestinal cavity, culminating in diarrhea in piglets [18]. In this study, we observed a significant rise in both rectal temperature and fecal score in piglets 9 h post-ETEC challenge, followed by a gradual decline. These observations highlight the successful establishment of the ETEC challenge model.

The present study demonstrates that piglets in the dietary LC group displayed lower rectal temperatures and fecal scores, mimicking the beneficial effects observed in the ATB group. These results suggest that LC effectively mitigates ETEC-induced diarrhea in piglets. Previous studies have shown that cinnamaldehyde and lysozyme are known to exert potent bactericidal effects against both Gram-negative and Gram-positive bacteria [19]. Additionally, lysozyme–ZnO nanoparticle coupling [20] and cinnamaldehyde [21] have been found to possess significant antibacterial properties against *E. coli*. These findings strongly suggest that LC has the capacity to inhibit *E. coli* colonization in the intestinal tract. Furthermore, it was observed that the feed-to-gain ratio (F/G) of piglets significantly increased after the challenge, but LC was able to effectively alleviate this result. There is growing evidence that the improvement in the F/G in piglets by LC is closely related to their intestinal health. Garas observed that feeding goat milk rich in lysozyme to pigs infected with ETEC F4 can reduce the incidence of diarrhea and improve their production performance [22].

To investigate the impact of LC on the intestinal health of piglets, we initially assessed the intestinal morphology across all treatment groups. The investigation revealed that different intestinal segments suffered varying degrees of damage after the ETEC challenge, with the jejunum and ileum exhibiting more severe damage. This could be attributed to the fact that ETEC primarily colonizes the posterior intestinal segment [23]. Both LC and ATB were found to effectively alleviate the damage caused by ETEC. As previous research mentioned, lysozyme has been identified as a viable alternative to antibiotics for improving small intestine morphology in pigs, which aligns with the results of our study. Notably, our results suggest an even greater protective effect provided by lysozyme when modified with cinnamaldehyde. It is important to note that intestinal morphology serves as the foundation for intestinal structure and function. Any damage to it not only leads to reduced nutrient absorption in piglets but also serves as an indicator of inflammation and impairment of the intestinal barrier [24].

In this study, we observed a marked down-regulation in the protein expression levels of Occludin, Claudin-1, and ZO-1, as well as in the mRNA expression levels of mucin and defensin genes, in various intestinal segments of ETEC-challenged piglets. Importantly, supplementation with LC mitigated these adverse effects. Several studies have also demonstrated the beneficial effects of lysozyme and cinnamaldehyde in improving the function of the intestinal physical barrier in weaned piglets. For example, Huang et al. reported that human lysozyme positively influenced intestinal morphology and preserved the integrity of tight junctions, especially in the jejunum and ileum [13]. Similarly, Mo demonstrated that daily supplementation with microencapsulated compounds of thymol, carvol, and cinnamaldehyde improved tight junction protein mRNA expression and thereby enhanced intestinal barrier function in weaned piglets [25]. Consistent with these studies, our results also show that LC significantly increased the mRNA expression of *pBD-2* in the duodenum, jejunum, and ileum of piglets. *pBD-2* possesses a broad antibacterial spectrum that is effective against *E. coli*, *Salmonella typhimurium*, and *Staphylococcus aureus*. Additionally, the expression of *pBD-2* contributes to elevating the levels of intestinal tight junction proteins and mucin genes or proteins [26].

In order to further clarify the molecular mechanisms underlying LC's mitigating effects on intestinal inflammation, we examined the TLRs/MyD88/NF-κB signal pathway [27], a classical inflammatory cascade. We also assessed its negative regulatory factors, specifically the Toll-interacting protein and Single Ig IL-1-related receptor (TOLLIP and SIGIRR) [28]. The activation of the TLRs pathway is known to be linked to multi-layered inflammatory intestinal diseases [29]. Our study observed a significant increase in the levels of IL-1β and TNF-α in plasma, as well as the expression of certain genes related to the TLRs/MyD88/NF-κB inflammatory pathway, and the mRNA expression of SIGIRR in the small intestine after the challenge. However, in piglets supplemented with either ATB or LC, these inflammatory pathway genes remained largely unaltered. These observations indicate LC's potential in attenuating intestinal inflammation.

It is widely recognized that the intestinal tracts of animals contain a high density and diverse range of microorganisms [30]. These microorganisms play a crucial role in various aspects such as intestinal development, immune defense, and the maintenance of the intestinal mucosal barrier. These microbial communities substantially shape the production performance and intestinal well-being of weaned piglets [31]. In this study, the analysis of 16S rDNA sequencing revealed a significant decrease in fecal microbial alpha diversity in piglets infected with ETEC. In contrast, no such shift was detected in the microbial diversity of piglets subjected to diets enriched with LC and ATB. This suggests that dietary LC potentially mirrors the microbial diversity-protective effect of antibiotics. Crucially, we identified a decrease in the relative abundance of *Lactobacillus* in piglets after the ETEC challenge, but this increased in those supplemented with LC and ATB. *Lactobacillus*, which constitutes the largest number of beneficial bacteria in the intestine, plays a crucial role in fermenting carbohydrates to produce lactic acid. This helps to reduce the intestinal pH and effectively prevent the reproduction of harmful bacteria [32]. Previous research has also shown that *Lactobacillus* can fine-tune the composition of gut flora [33], boost the expression of intestinal tight junction proteins and mucins [34,35], modulate the intestinal immune responses [36], and trigger the expression of *pBD* [37]. These findings Fprovide significant evidence that LC not only decreases intestinal inflammation but also fortifies the intestinal barrier function.

5. Conclusions

Dietary LC could alleviate the inflammatory reaction and intestinal villus damage caused by *E. coli* K88 in weaned piglets. These benefits are conferred through multiple mechanisms, including the inhibition of the NF-κB signaling pathway, the upregulation of β-defensin gene expression, and the enhancement of the structure and growth performance of the intestinal microbiota. These findings strongly suggest that LC could enhance the

growth performance and intestinal health of weaned piglets and serve as an effective alternative to antibiotics in the future.

Supplementary Materials: The following supporting information can be downloaded at https://www.mdpi.com/article/10.3390/ani13223497/s1, Figure S1. The animal experiment design. Figure S2. Full original Western blot (WB) images.

Author Contributions: S.Z., F.C. and W.G. conceived and designed the experiments. Z.T., T.L. and J.C. participated in the animal experiment. Z.T., J.C., J.Z. and H.G. analyzed the data and conducted the research. Z.T. and J.C. discussed the results and wrote the paper. S.Z. and W.G. conducted the final editing. All authors have read and agreed to the published version of the manuscript.

Funding: This study was financially supported by the National Key R&D Program of China (No. 2021YFD1300700), the Guangdong Basic and Applied Basic Research Foundation (No. 2021A1515010440), the Science and Technology Program of Guangzhou (No. 202102020056), the National Natural Science Foundation of China (Nos. 31802067 and 31872364), and project of Swine Innovation Team in Guangdong Modern Agricultural Research System (No. 2023KJ126).

Institutional Review Board Statement: This study was conducted according to the guidelines of the Declaration of Helsinki and approved by the Ethics Committee of South China Agricultural University.

Informed Consent Statement: Not Applicable.

Data Availability Statement: Data are contained within the article and Supplementary Materials; The datasets generated for this study are available on request from the corresponding author.

Conflicts of Interest: The authors declare no conflict of interest.

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European Journal of Nutrition (2023) 62:2873–2890
https://doi.org/10.1007/s00394-023-03197-7

ORIGINAL CONTRIBUTION

Leucine and arginine enhance milk fat and milk protein synthesis via the CaSR/G_i/mTORC1 and CaSR/G_q/mTORC1 pathways

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Received: 4 May 2022 / Accepted: 23 June 2023 / Published online: 1 July 2023
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Abstract

Background and aims Amino acids (AAs) not only constitute milk protein but also stimulate milk synthesis through the activation of mTORC1 signaling, but which amino acids that have the greatest impact on milk fat and protein synthesis is still very limited. In this study, we aimed to identify the most critical AAs involved in the regulation of milk synthesis and clarify how these AAs regulate milk synthesis through the G-protein-coupled receptors (GPCRs) signaling pathway.

Methods In this study, a mouse mammary epithelial cell line (HC11) and porcine mammary epithelial cells (PMECs) were selected as study subjects. After treatment with different AAs, the amount of milk protein and milk fat synthesis were detected. Activation of mTORC1 and GPCRs signaling induced by AAs was also investigated.

Results In this study, we demonstrate that essential amino acids (EAAs) are crucial to promote lactation by increasing the expression of genes and proteins related to milk synthesis, such as ACACA, FABP4, DGAT1, SREBP1, α-casein, β-casein, and WAP in HC11 cells and PMECs. In addition to activating mTORC1, EAAs uniquely regulate the expression of calcium-sensing receptor (CaSR) among all amino-acid-responsive GPCRs, which indicates a potential link between CaSR and the mTORC1 pathway in mammary gland epithelial cells. Compared with other EAAs, leucine and arginine had the greatest capacity to trigger GPCRs (p-ERK) and mTORC1 (p-S6K1) signaling in HC11 cells. In addition, CaSR and its downstream *G* proteins G_i, G_q, and $G_{\beta\gamma}$ are involved in the regulation of leucine- and arginine-induced milk synthesis and mTORC1 activation. Taken together, our data suggest that leucine and arginine can efficiently trigger milk synthesis through the CaSR/G_i/mTORC1 and CaSR/G_q/mTORC1 pathways.

Conclusion We found that the *G*-protein-coupled receptor CaSR is an important amino acid sensor in mammary epithelial cells. Leucine and arginine promote milk synthesis partially through the CaSR/G_i/mTORC1 and CaSR/G_q/mTORC1 signaling systems in mammary gland epithelial cells. Although this mechanism needs further verification, it is foreseeable that this mechanism may provide new insights into the regulation of milk synthesis.

Keywords Milk synthesis · CaSR · Leucine · Arginine · G_i · G_q

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Introduction

Milk is a unique nutrient source for neonates [1], and its output and composition directly affect the health and growth of mammalian infants [2, 3]. However, insufficient milk production frequently occurs during the lactation period, which is detrimental to neonatal development [4]. During lactation, the intake of AAs by the mammary gland increases, and these AAs not only serve as substrates for milk protein synthesis but also play an important role in mammary gland development [4–8]. Among them, arginine is considered as an essential amino acid in the regulation of mammary gland development and milk secretion by stimulating the extension of mammary ducts and mammary cell proliferation [9]. Besides, arginine is a substrate for the synthesis of NO and polyamines, which promote blood vessel growth and flow in the mammary gland [10]. In addition, the critical role of branched chain amino acids, such as isoleucine and leucine, in mammary protein synthesis and in mammary cell proliferation has also been elaborated [11, 12]. Milk fat is composed of 98% triglycerides (TAGs) and small amounts of monoglycerides, diacylglycerols, cholesterol, and other lipids [13]. Mammary gland de novo lipogenesis contributes to medium-chain fatty acids (< C16), and these fatty acids can be further synthesized into TAGs and secreted into milk [14]. In addition to regulating milk protein synthesis, AAs have been reported to participate in the regulation of milk fat synthesis by manipulating the expression of enzymes and transcription factors, such as fatty acid synthase (FASN), acetyl-CoA carboxylase A (ACACA), and sterol regulatory element-binding protein 1 (SREBP1) [15–17]. However, studies regarding which amino acids that have the greatest impact on milk fat and protein synthesis is still unknown, and additional research is warranted.

As an evolutionarily conserved protein, mTORC1 is a major regulator of anabolism and autophagy [18]. Activation of mTORC1 promotes protein and lipid synthesis in mammary epithelial cells via its downstream effectors, such as ribosomal protein S6 kinase 1 (S6K1) and 4E-binding protein 1 (4EBP1) [19, 20]. AAs deficiency causes a dramatic decrease in the phosphorylation of S6K1 and 4E-BP1, which cannot be compensated by other stimulate such as growth factors or energy, indicating that AAs are required for mTORC1 activation [21]. Correspondingly, numerous studies have reported that AAs (L-arginine, leucine, isoleucine, and phenylalanine) regulate lactation by activating the mammalian target of rapamycin complex 1 (mTORC1) signaling pathway [22–28]. However, how AAs are sensed and signal to mTORC1 is not fully understood, especially, the extracellular amino-acid-sensing mechanism that activates mTORC1.

Classically, activation of mTORC1 by AAs is mediated by the Rag GTPases, which recruits mTORC1 to the lysosome, where AAs signaling initiates [29]. Other research shows the lysosomal AA sensors (PAT1 and SLC38A9), cytoplasmic AA sensors (LRS, Sestrin2 and CASTOR1) and Golgi-localized AA transporter PAT4 are also responsible for mTORC1 activation [29]. Importantly, recent studies have shown that AAs can be sensed by membrane GPCRs receptors [28, 30]. G-protein-coupled receptors (GPCRs) are the most ubiquitous cell surface receptors, with more than 800 members [31]. Activated GPCRs exert intracellular regulation through G-protein-mediated effects, which are characterized by ERK activation [32]. G protein is a heterotrimer composed of α, β, and γ subunits, where G_α binds to GDP and G_β is associated with G_γ. Current research has found that β and γ subunits are very similar in most G proteins, but G_α proteins are divided into six categories: G_s, G_i, $G_{\alpha i,}$ G_q, G_{12}, and G_{13}. In general, the activated GPCRs act as guanine nucleotide exchange factor, promotes the exchange of GDP and GTP in the G_α subunit, and induces the decomposition of the heterotrimer complex into the active G_α and $G_{\beta\gamma}$ complexes, thereby regulating the activity of effector molecules, such as PLCβ, adenylate cyclase (AC), GIRK channels, etc. [32]. Based on sequence homology, GPCRs are subdivided into three classes of A, B, and C [33]. Of note, class C GPCRs include broad-spectrum AA-sensing receptors such as taste receptors (T1Rs), GPRC6A and the calcium-sensing receptor (CaSR), characterized by a nutrient sensitive Venus Fly Trap domain (VFT) [33]. In terms of lactation, lysine regulates milk fat synthesis through the GPRC6A pathway in bovine mammary epithelial cells [34]. In addition, T1R1/T1R3 trigger the activation of mTORC1 signaling and stimulate milk protein synthesis in mouse mammary cells [35, 36]. However, whether other GPCRs receptors mediate the stimulatory effects of AAs on lactation is still unclear and requires further investigation.

In this study, we aimed to identify the most critical AAs involved in the regulation of milk synthesis and clarify how these AAs regulate milk synthesis through the GPCRs signaling pathway.

Materials and methods

Cell cultures

A mouse mammary epithelial cell line (HC11) and porcine mammary epithelial cells (PMECs) were grown in complete DMEM/F12 containing 10% fetal bovine serum (FBS), 5 μg/mL insulin-like growth factor-1 (IGF-1), 5 μg/mL hydrocortisone, 10 ng/mL epidermal growth factor (EGF),

5 μg/mL insulin-transferrin-selenium (ITS), and 10 μg/mL penicillin–streptomycin (PS). Cells were cultured in a cell incubator at 37 °C with 5% CO_2. The medium was changed every day. When the cells reached 50% confluence, they were cultured in DMEM/F12 containing 10% FBS, 1 μg/mL hydrocortisone, 10 μg/mL PS, 0.5 μg/mL insulin, and 2 μg/mL prolactin [37] (Supplemental Fig. 1). The culture medium was changed every day. Cell differentiation was performed for approximately 2 days until the cells reached 80% confluency. Then the cells were starved for 2 h in amino-acid-deprived medium and incubated at 37 °C in a humidified atmosphere containing 5% CO_2. After starvation, the cells were used for the following experiments.

Experiment I

HC11 cells and PMECs were subjected to different treatments: AAF, amino-acid-free medium; AAs, medium with all amino acids; EAAs, medium with only essential amino acids; and NEAAs, medium with only non-essential amino acids. $n = 4$. To induce milk fat and protein synthesis, 2 μg/mL prolactin is supplemented during the treatment period. After 24 h of incubation, the cells were collected to examine lipid droplet formation via oil red O staining, triglyceride content assessment, and determination of α-casein, β-casein, WAP, ACACA, FABP4, DGAT1, SREBP1, FASN, p-mTOR/mTOR, p-S6K1/S6K1, p-4EBP1/4EBP1, and p-ERK/ERK expression levels.

Experiment II

HC11 cells and PMECs were cultured in the AAF, AAs, EAAs, and NEAAs groups. $n = 4$. To induce milk fat and protein synthesis, 2 μg/mL prolactin is supplemented during the treatment period. After 24 h of incubation, the cells were collected to examine the expression levels of T1R1, T1R3, CaSR, GPRC6A, GPR35, and GPR142.

Experiment III

HC11 cells were cultured in the presence of Met, Thr, Trp, Leu, Val, Lys, Ile, Phe, and Arg at concentrations of 0, 1, 10, 100, 500, and 1000 μM, respectively. $n = 1$. To induce milk fat and protein synthesis, 2 μg/mL prolactin is supplemented during the treatment period. After 24 h of incubation, the cells were collected to examine the phosphorylation level of S6K1 and ERK.

Experiment IV

HC11 cells were cultured in AAF, Met, Thr, Trp, Leu, Val, and Arg groups. In the amino acid group, an individual amino acid was added at a concentration of 500 μM on the basis of AAF. $n = 3$. To induce milk fat and protein synthesis, 2 μg/mL prolactin is supplemented during the treatment period. After 24 h of incubation, the cells were collected to examine the phosphorylation level of S6K1 and ERK.

Experiment V

HC11 cells were pretreated with 10 nM NPS-2143 hydrochloride (NPS2143) (Sigma, cat: SML0362) (for 2 h) or CaSR siRNA (for 6 h) and then cultured in the presence of 500 μM leucine or arginine. $n = 4$. To induce milk fat and protein synthesis, 2 μg/mL prolactin is supplemented when cells were treated with amino acids. After 24 h of incubation, the cells were collected to examine the triglyceride content and the mRNA and protein expression levels of CaSR, α-casein, β-casein, WAP, S6K1, p-S6K1, p-ERK, and ERK.

Experiment VI

To confirm the specific mechanistic links downstream of CaSR, HC11 cells were pretreated for 2 h with 10 nM Gp2A (GP Antagonist-2A—Calbiochem), an inhibitor of G_q (Sigma, cat: 371,780); 10 nM NF023 (NF023—Calbiochem), an inhibitor of $G_{\alpha i}$ (Sigma, cat: 480,415); 10 nM PTX (Pertussis toxin), an inhibitor of G_i (Sigma, cat: P7208); or 10 nM gallein (Gallein-CAS 2103-64-2-Calbiochem), an inhibitor of $G_{\beta\gamma}$ (Sigma, cat: 371,708). $n = 4$. Then, the HC11 cells were cultured in the presence of 500 μM leucine or arginine. To induce milk fat and protein synthesis, 2 μg/mL prolactin is supplemented when cells were treated with amino acids. After 24 h of incubation, the cells were collected to examine the triglyceride content and the mRNA and protein expression levels of CaSR, α-casein, β-casein, WAP, S6K1, p-S6K1, p-ERK, and ERK.

Oil red O staining of lipid droplets

Lipid droplets were detected using oil red O staining solution (Sangon Biotechnology, Shanghai). The oil red O dye solution was diluted with double distilled water at a ratio of 6:4 and filtered through a 45 nm filter three to five times to produce a working solution. The cells were cultured in a 24-well plate (500 μL of cells were added to each well), and then the culture plate was placed in a 5% CO_2 incubator at 37 °C for 24 h. The medium was discarded, and the cells were washed twice with PBS. Then the cells were starved in amino-acid-free medium for 2 h and finally transferred to treatment medium for 24 h, with four replicates in each group. After the treatment was completed, the medium was discarded. After three washes with PBS, 1 mL of 4% paraformaldehyde was added to each well, and the cells were fixed at 37 °C for 45 min. Then the cells were again washed

three times with PBS, 220 μL of oil red O was slowly added, and the cells were stained at room temperature for 2.5 h. The staining solution was discarded, and the cells were washed with PBS six times and placed on an inverted microscope (400×) to observe the cells and obtain images.

Measurement of triglyceride concentration

In this study, a triglyceride detection kit (Jiancheng, Nanjing, China) was used to detect the concentration of triglycerides. Cells were seeded into 24-well plates (500 μL of cells were added to each well) and harvested after 24 h of treatment. Briefly, after three washes with PBS, 130 μL of RIPA lysis buffer containing 1% PMSF and 1% phosphatase inhibitor was added to each well. After approximately 30 min of lysis on ice, the liquid was transferred to centrifuge tubes and centrifuged at 200 g at 4 °C for 5 min. Then the supernatant was collected for determination of intracellular triglycerides. Specifically, 250 μL of working solution was added into each well, followed by 2.5 μL of sample, which was mixed evenly with shaking. After incubating the plate at 37 °C for 10 min, the OD value of the sample was measured at a wavelength of 510 nm. Finally, calculate the triglyceride content according to the OD value.

$$\text{Triglyceride content (mmol/gprot)} = \frac{(\text{Sample OD value} - \text{blank OD value})}{(\text{Calibrate OD} - \text{blank OD value})} \times \frac{\text{Calibrator concentration (mmol/L)}}{\text{Sample protein concentration(gprot/L)}}$$

siRNA transfection

The siRNA and other reagents used in this experiment were provided by RiboBio (China), and our protocol was in accordance with the instructions provided by the manufacturer.

Western blotting

First, cells were completely lysed with RIPA buffer (160 μL per well of a 6-well plate). The cell lysate was denatured at 100 °C, mixed with 40 μL of loading buffer per well and then was used as a sample for subsequent experiments. SDS-PAGE was performed with 10% or 12% gels. After electrophoresis, the separated protein molecules were transferred to PVDF membranes. The PVDF membrane was immediately rinsed three times with TBST and blocked with 6% nonfat milk for 2 h at room temperature. The PVDF membrane was then washed three times with TBST and incubated with primary antibody overnight at 4 °C with gentle shaking. After overnight incubation, PVDF membranes were washed three times with TBST and incubated with secondary antibodies for 1 h at room temperature on a shaker. Chemiluminescent signals were detected using Super ECL reagent (P1020), and we scan the bands using Image J software (ImageJ 1.52a) and calculate the grayscale values to quantify the bands. All antibodies used in this experiment are shown in Supplemental Table 1.

Real-time PCR

Cells were harvested for real-time PCR after 24 h of treatment. Total RNA was isolated from cells using an EZ-press RNA purification kit (EZ-Bio, Shanghai) according to the product instructions. The RNA to be tested was first tested for purity and integrity via agarose gel electrophoresis, and then the RNA was reverse transcribed into cDNA using a Color Reverse Transcription Kit (EZ-Bio, Shanghai) containing gDNA remover and a color mixture. The diluted cDNA was thoroughly mixed with Color SYBR Green qPCR Mix (EZ-Bio, Shanghai), target gene primers, and double distilled water to form a 20 μL qPCR system. The thermal cycling conditions for qPCR were as follows: 95 °C for 1 min, followed by 40 cycles of denaturation at 95 °C for 15 s, annealing at 59 °C for 15 s, and extension at 72 °C for 40 s. Four repetitions were set up per set. The primer sequences we used for real-time PCR are listed in Supplemental Tables 2 and 3. The expression stability of housekeeping genes was verified by geNorm [38].

Statistical analysis

All data in this experiment were first collected and organized using Excel software, and then the western blotting, real-time PCR, and TAG concentration data were analyzed via one-way ANOVA between groups using IBM SPSS Statistics 20.0 software. Graphs were drawn and analyzed using GraphPad Prism 8.0 software. All data are shown as the mean ± SEM. $P < 0.05$ was considered statistically significant.

Results

Effects of EAAs on milk fat and milk protein synthesis in HC11 cells and PMECs

We first investigated the effects of EAAs (medium with only essential amino acids) and NEAAs (medium with only non-essential amino acids) on milk protein and milk fat synthesis in HC11 cells and PMECs. As expected, EAAs, rather than NEAAs, were sufficient to promote lipid droplet and triglyceride (TAG) synthesis in HC11 cells (Fig. 1A–C) and PMECs (Fig. 2A–C). Correspondingly, EAAs significantly increased the expression of genes and proteins related to de novo fatty acid synthesis, such as ACACA, fatty acid-binding protein 4 (FABP4), diacylglycerol acyltransferase 1 (DGAT1) and SREBP1 ($P < 0.05$), in HC11 cells (Fig. 1D–F) and PMECs (Fig. 2D–F). Similarly, EAAs significantly increased the expression of milk-synthesis-related genes and proteins (α-casein, β-casein and WAP) compared to NEAAs ($P < 0.05$) in HC11 cells (Fig. 1G–I) and PMECs (Fig. 2G–I). In addition, compared with NEAAs, EAAs significantly triggered mTORC1 signaling (as indicated by the phosphorylation levels of mTOR, S6K1, and 4EBP1) and GPCR signaling (as indicated by the phosphorylation level of ERK) in HC11 cells ($P < 0.05$) (Fig. 1J, K) and PMECs (Fig. 2J, K). The addition of appropriate amounts of EAAs is crucial to improve nitrogen efficiency in dairy production [39, 40], while insufficient EAAs inhibits lactation and reproductive performance in dairy cows [40]. Similarly, our research shows EAAs, but not NEAAs, are sufficient to trigger milk protein and milk fat synthesis in HC11 cells and PMECs. The effect of EAAs on milk synthesis does not seem species specific.

EAAs regulate the expression of CaSR in HC11 cells and PMECs

T1R1, T1R3, CaSR, GPRC6A, GPR35, and GPR142 have been identified as amino-acid-responsive GPCRs in the cell membrane. To identify whether EAAs and NEAAs regulate the expression of GPCRs, amino-acid-responsive GPCRs were analyzed under EAAs and NEAAs treatments in HC11 cells and PMECs. EAAs specifically regulated CaSR mRNA and protein expression but not other amino-acid-responsive GPCRs in HC11 cells (Fig. 3A–C). Consistently, the same results were observed in PMECs (Fig. 3D–F). CaSR, a regulator of calcium metabolism, is located in mammary gland, parathyroid glands, and bones [41]. Studies have shown that mammary CaSR coordinates calcium metabolism to promote calcium transport into milk and calcium accumulation in newborns during lactation [42]. Whereas, decreased expression of CaSR in the mammary gland leads to lactation inhibition in mice [43]. Our results indicate EAAs seems to regulate the function of mammary epithelial cells through regulation of CaSR expression

Leu and Arg are important activators of mTORC1 (p-S6K1) and GPCRs (p-ERK) signaling pathways

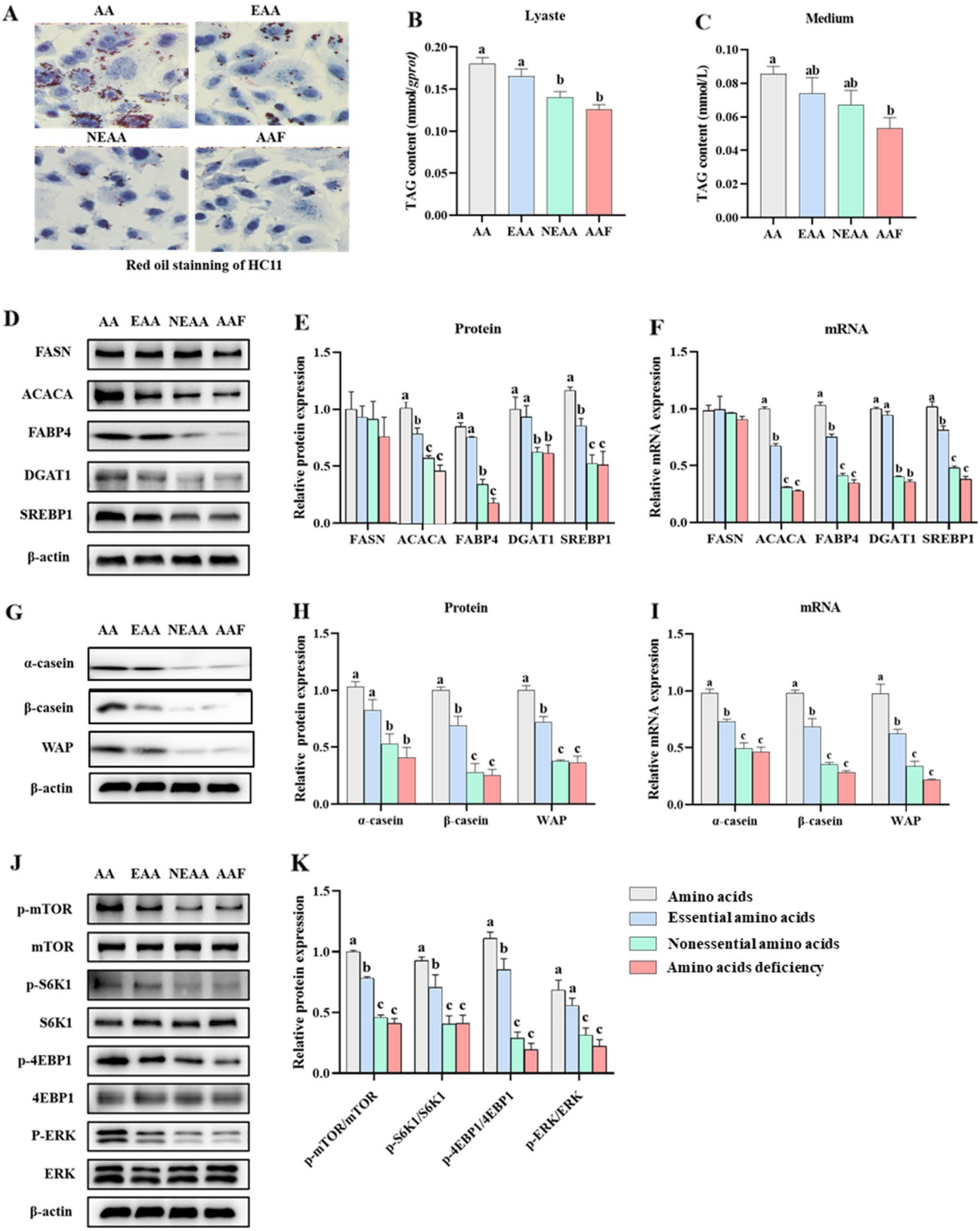
To identify the effect of individual EAAs on mTORC1 and GPCR signaling, we treated HC11 cells with nine individual amino acids. As shown in Supplemental Fig. 2, Met, Thr, Trp, Leu, Val, and Arg activated both mTORC1 signaling (judged by phosphorylation of S6K1) and GPCRs signaling (judged by phosphorylation of ERK). We further compared the effect of Met, Thr, Trp, Leu, Val, and Arg on the activation of mTORC1 and GPCRs signaling and found that Leu and Arg had stronger activation effects on both GPCRs and mTORC1 signaling ($P < 0.01$) (Fig. 4A, B). Consistent with our results, numerous studies indicate that dietary supplementation with Arg and Leu increases milk production via mTORC1 signaling in mammals, such as sows [44], rabbits, and cows [37, 45, 46]. Based on these results, Leu and Arg were selected to further study the precise molecular mechanism underlying how individual amino acids regulate milk synthesis.

Leu- and Arg-induced milk synthesis is attenuated in CaSR-knockdown and CaSR-inhibited HC11 cells

To further investigate the effect of CaSR on milk synthesis, CaSR siRNA and NPS 2143 (a CaSR inhibitor) were used to interfere with the expression or function of CaSR. Leu- or Arg-induced TAG synthesis in HC11 cells was significantly inhibited ($P < 0.05$) when cells were pretreated with NPS 2143 (Figs. 5A, B, 6A, B) or with CaSR siRNA interference ($P < 0.05$) (Fig. 7A, B). Similarly, Leu- or Arg-induced milk protein synthesis and the expression of CaSR in HC11 cells were inhibited to a certain extent after pretreatment with NPS 2143 ($P < 0.1$) (Figs. 5C–E, 6C–E) or CaSR siRNA interference (Fig. 7C–E). When CaSR was inhibited or knocked down, the promotion of the S6K1 and ERK phosphorylation levels induced by leucine and arginine was suppressed in HC11 cells (Figs. 5F, G, 6F, G, 7F, G). These results suggest that Leu and Arg might regulate milk synthesis in part through the CaSR/mTORC1 pathway.

Leu- and Arg-induced CaSR activation regulates milk synthesis through the G_i and G_q pathways

To further elucidate the downstream molecular mechanisms of CaSR, we inhibited G proteins coupled with CaSR, such


A
AA
EAA
NEAA
AAF
Red oil stainning of HC11
B
Lyaste
TAG content (mmol/gprot)
C
Medium
TAG content (mmol/L)
D
FASN
ACACA
FABP4
DGAT1
SREBP1
β-actin
E
Protein
Relative protein expression
F
mRNA
Relative mRNA expression
G
α-casein
β-casein
WAP
H
I
J
p-mTOR
mTOR
p-S6K1
S6K1
p-4EBP1
4EBP1
P-ERK
ERK
K
p-mTOR/mTOR
p-S6K1/S6K1
p-4EBP1/4EBP1
p-ERK/ERK
Amino acids
Essential amino acids
Nonessential amino acids
Amino acids deficiency

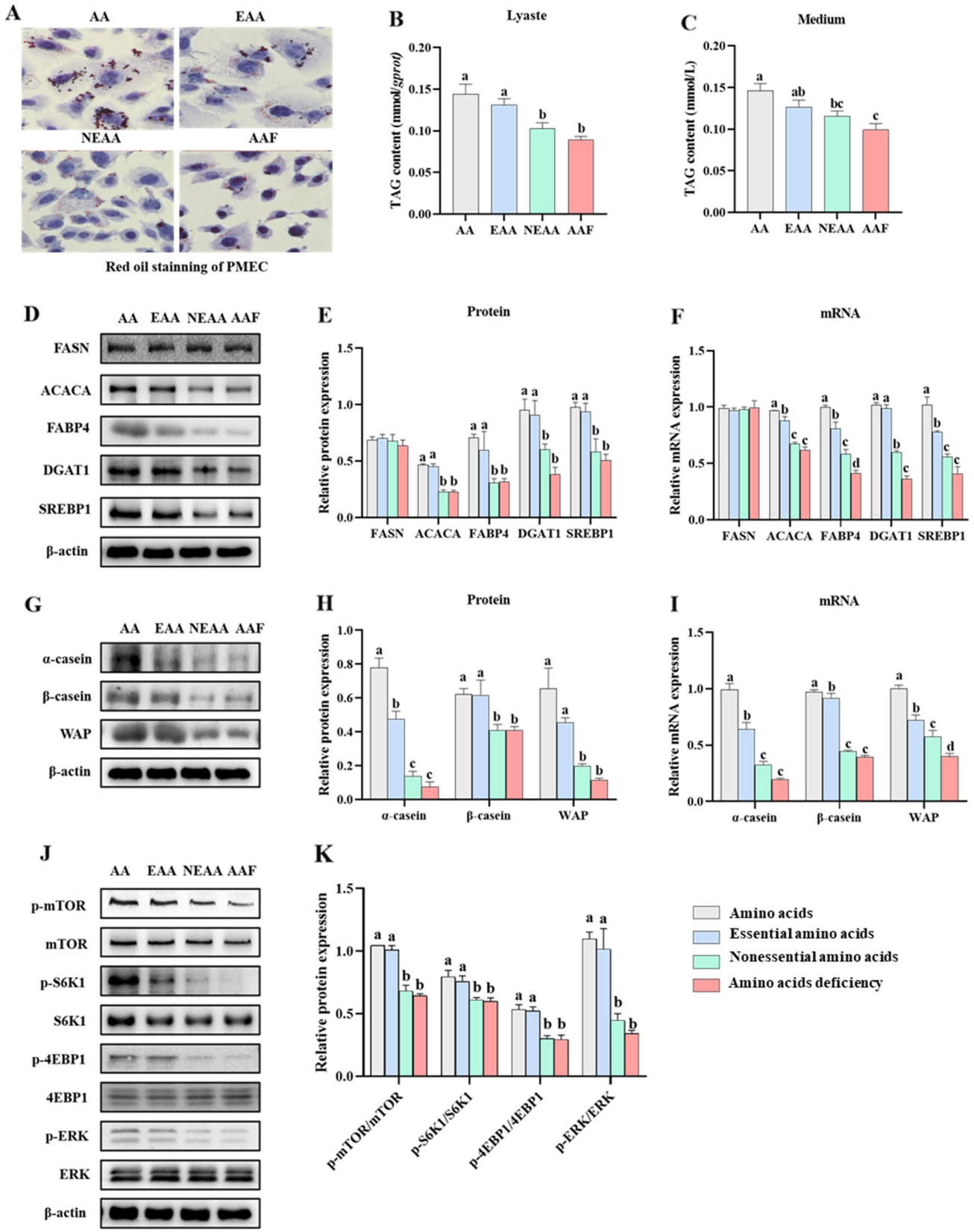
◂**Fig. 1** Effects of essential and non-essential amino acids on milk fat and milk protein synthesis in HC11 cells. **A** Images of oil red O staining of HC11 cells in the AA (top left), EAA (top right), NEAA (bottom left), and AAF (bottom right) groups. **B** and **C** TAG concentration in cells (**B**) and medium (**C**) (scale bar: 100 μm). **D–F** Western blots of pivotal proteins related to milk fat synthesis in HC11 cells. In **D**, the bands show FASN, ACACA, FABP4, DGAT1, SREBP1 and β-actin protein expression levels. Protein and relative mRNA expression levels are shown in **E**, **F**. **G–I** Western blots of pivotal proteins related to milk protein synthesis in HC11 cells. In **G**, the bands show α-casein, β-casein, WAP, and β-actin protein expression levels. Protein and relative mRNA expression levels are shown in **H**, **I**. **J** and **K** Western blots of critical proteins in the mTORC1 pathway in HC11 cells. In **J**, the bands show p-mTOR, mTOR, p-S6K1, S6K1, p-4EBP1, 4EBP1, p-ERK, ERK, and β-actin protein expression levels. The proportion of target protein activated by phosphorylation is shown in **K**. In this figure, cells for oil red O staining, TAG determination, real-time PCR and WB were collected after 24 h of incubation in AAF, amino-acid-free medium; AA, medium with all the amino acids; EAA, medium with only essential amino acids; and NEAA, medium with only non-essential amino acids. $n=4$. All data with error bars are the average ± SEM. In column diagrams, no letter or the same letter above the bar graph indicates no significant difference ($P>0.05$), and different lowercase letters indicate a significant difference ($P<0.05$)

as G_q, $G_{\beta\gamma}$, and G_i. The results showed that inhibition of G_q, $G_{\beta\gamma}$, and G_i all attenuated Leu- (Fig. 8A, B) and Arg-induced (Fig. 9A, B) TAG synthesis in HC11 cells ($P<0.05$), among which, the G_i and G_q inhibition groups had the most significant effect. In addition, inhibition of G_q, $G_{\beta\gamma}$, and G_i significantly reduced the protein content and mRNA expression of CaSR, α-casein, β-casein, and WAP induced by Leu (Fig. 8C–E) and Arg (Fig. 9C–E) in HC11 cells ($P<0.05$), among which, the G_i and G_q inhibition groups had the most significant effect. Similarly, inhibition of G_q, $G_{\beta\gamma}$, and G_i significantly inhibited Leu- (Fig. 8F, G) and Arg-induced (Fig. 9F, G) phosphorylation of S6K1 and ERK ($P<0.05$), among which, the G_i and G_q inhibition groups had the most significant effect. These results suggest that Leu and Arg regulate milk synthesis through the CaSR/G_i/mTORC1 and CaSR/G_q/mTORC1 pathways.

Discussion

An intimate relationship between amino acids and milk synthesis has long been established, but the underlying mechanisms have not been well characterized. mTORC1 signaling is not only an important regulator in protein translation but also involved in the process of fat synthesis and metabolism [47]. Our experimental data showed that EAAs can promote milk synthesis by activating mTORC1 signaling. Previous studies have shown that amino acids such as Leu [48], Lys, and Ile [49] can stimulate milk synthesis by activating the mTORC1 signaling pathway, which is in agreement with our data. Studies have shown that de novo synthesized fatty acids are an important source for milk fat synthesis. Multiple enzymes participate in this process. ACACA is involved in catalyzing the conversion of acetyl-CoA to malonyl-CoA [15]. SREBP1 is a key transcriptional regulator that affects the expression of various genes involved in milk fat synthesis [50]. FABP4 influences fatty acid uptake in mammary epithelial cells [51], and DGAT1 catalyzes the formation of triglycerides from diacylglycerols and fatty acids [52]. Our results showed that EAAs increased the expression of ACACA, DGAT1, FABP4, and SREBP1 in HC11 cells and PMECs. Previously, Met and Leu were reported to increase SREBP1 protein expression in bovine mammary epithelial cells [53, 54]. Another study demonstrated that amino acids activated SREBP1 through the CRTC2-mTOR pathway [55]. Furthermore, it is worth noting that prolactin and amino acids have synergistic effect on lactation. Studies have shown that prolactin can increase the abundance of amino acid transporters (LAT1 and SNAT2) in the mammary glands of rats[56] and cows [57] during lactation, thus improve the utilization of mammary amino acids and milk protein synthesis. Another study demonstrated prolactin stimulates adipophilin synthesis in HC11 cells via the PI3-kinase/Akt/mTOR pathway[58]. However, our study suggests that the addition of prolactin to culture media is not sufficient to compensate for the inhibitory effect of EAAs deficiency on lactation. Altogether, we demonstrate that EAAs are crucial to promotion of milk synthesis by activating mTORC1 signaling and regulating the expression of genes encoding enzymes involved in milk fat synthesis. Currently, it is worth noting that Leu and Arg might also increase milk protein level in HC11 and PMECs partially by inhibiting protein degradation, and additional research is warranted in the future.

Calcium-sensing receptor (CaSR) belongs to the extracellular amino acid sensor family, which can be triggered by extracellular Ca^{2+}, L-amino acids, and short-chain peptides to stimulate subsequent signaling cascades [59]. Many studies have shown that various physiological functions of L-amino acids are mediated by CaSR, such as anti-inflammatory activity in intestinal epithelial cells [60], calcitonin release in human C-cells [61], and cholecystokinin production [62]. In the mammary glands, CaSR activation triggers mammary parathyroid hormone production and calcium transport in milk [42, 63, 64]. In addition, the expression of CaSR peaks during lactation and gradually decreases with the involution of the mammary gland [63]. Our results suggest that Leu and Arg regulate milk synthesis in part through the CaSR/mTORC1 pathway. Consistent with our findings, previous studies have also reported a potential link between CaSR and mTORC1 signaling. For example, CaSR activation positively affects mTOR activity in cystic renal epithelial cells [65]. Furthermore, CaSR promoted IL-6 secretion in breast cancer MDA-MB-231


A
AA
EAA
NEAA
AAF
Red oil stainning of PMEC
B
Lyaste
TAG content (mmol/gprot)
C
Medium
TAG content (mmol/L)
D
FASN
ACACA
FABP4
DGAT1
SREBP1
β-actin
E
Protein
Relative protein expression
F
mRNA
Relative mRNA expression
G
α-casein
β-casein
WAP
H
I
J
p-mTOR
mTOR
p-S6K1
S6K1
p-4EBP1
4EBP1
p-ERK
ERK
K
p-mTOR/mTOR
p-S6K1/S6K1
p-4EBP1/4EBP1
p-ERK/ERK
Amino acids
Essential amino acids
Nonessential amino acids
Amino acids deficiency

◂**Fig. 2** Effects of essential and non-essential amino acids on milk fat and milk protein synthesis in PMECs. **A** Images of oil red O staining of PMECs in the AA (top left), EAA (top right), NEAA (bottom left), and AAF (bottom right) groups. **B** and **C** TAG concentration in cells (**B**) and medium (**C**) (scale bar: 100 μm). **D-F** Western blots of pivotal proteins related to milk fat synthesis in PMECs. In **D**, the bands show FASN, ACACA, FABP4, DGAT1, SREBP1, and β-actin protein expression levels. Protein and relative mRNA expression levels are shown in **E, F**. **G–I** Western blots of pivotal proteins related to milk protein synthesis in PMECs. In **G,** the bands show α-casein, β-casein, WAP, and β-actin protein expression levels. Protein and relative mRNA expression levels are shown in **H**, **I**. **J** and **K** Western blots of critical proteins in the mTORC1 pathway in PMECs. In **J**, the bands show p-mTOR, mTOR, p-S6K1, S6K1, p-4EBP1, 4EBP1, p-ERK, ERK, and β-actin protein expression levels. The proportion of target protein activated by phosphorylation is shown in **K**. In this figure, cells for oil red O staining, TAG determination, real-time PCR and WB were collected after 24 h of incubation in AAF, amino-acid-free medium; AA, medium with all the amino acids; EAA, medium with only essential amino acids; and NEAA, medium with only non-essential amino acids. $n=4$. All data with error bars are the average ± SEM. In column diagrams, no letter or the same letter above the bar graph indicates no significant difference ($P>0.05$), and different lowercase letters indicate a significant difference ($P<0.05$)

cells with activation of the $G_{\alpha s}$/PKC, MEK1/2, and mTORC1 signaling pathways [66]. Another study showed that CaSR activation promotes the release of G_i and $G_{\beta\gamma}$ subunits and stimulates the secretion of cytokines and growth factors in MDA-MB-231 cells through the PI3K/mTOR pathway [67]. Taken together, these findings suggest that activation of CaSR by amino acids further activates mTORC1 signaling.

CaSR is mainly coupled with three heterotrimeric G proteins (G_i, G_q and $G_{12/13}$) to complete its downstream signaling. Our data show that inhibition of G_q, $G_{\alpha i}$, $G_{\beta\gamma}$, and G_i all attenuated Leu- and Arg-induced milk synthesis in HC11 cells. Previous studies have shown a link between Gq and the mTORC1 pathway[68]. For example, mTORC1 and CaSR-G_q signaling pathways synergistically promote the stimulating effect of hyperaminoacidemia on the proliferation of pancreatic α cells [69]. Moreover, G_q activates the AMPK/mTORC1 pathway, enhancing the migration and invasion of prostate cancer cells[70]. Mechanically, G_q activates phospholipase C and increases Ca^{2+} content, which in turn triggers mTORC1 activation[71, 72]. In addition, activation of G_i inhibits adenylate cyclase to hinder the synthesis of cyclic adenosine monophosphate (cAMP) [73–75]. cAMP is an important second messenger in the GPCRs signaling system, and activation of GPCRs coupled to G_i results in decreased production of intracellular cAMP [76]. High levels of cAMP promote complex dissociation and inhibit mTORC1 activity [77, 78]. Thus, activation of G_i can decrease the production of intracellular cAMP, which further promotes activation of the mTORC1 signaling pathway. These results suggest that Leu and Arg regulate milk synthesis through the CaSR/Gi/mTORC1 and CaSR/Gq/mTORC1 pathways. In addition, $G_{\beta\gamma}$/$G_{\alpha i}$ has also been found to be involved in the regulation of milk synthesis, but the mechanism remains unclear.

In addition, pigs are very similar to humans in terms of anatomy, physiology, protein function, and even body size, and have become one of the best model animals for studying the basic laws of human life, major human diseases, and new drug development. Therefore, our elaboration of lactation formulations using a porcine mammary gland cell model also provides a reference for human lactation studies.

Conclusion

Our experimental results showed that EAAs significantly increased lipid droplets and milk protein synthesis in HC11 cells and PMECs. EAAs enhance the level of critical genes related to milk synthesis, such as ACACA, FABP4, DGAT1, SREBP1, α-casein, β-casein, and WAP, with activation of mTORC1 signaling. Among all the EAAs, Leu and Arg had the highest GPCRs and mTORC1 signaling activation efficiency. Thus, Leu and Arg promote milk synthesis in part

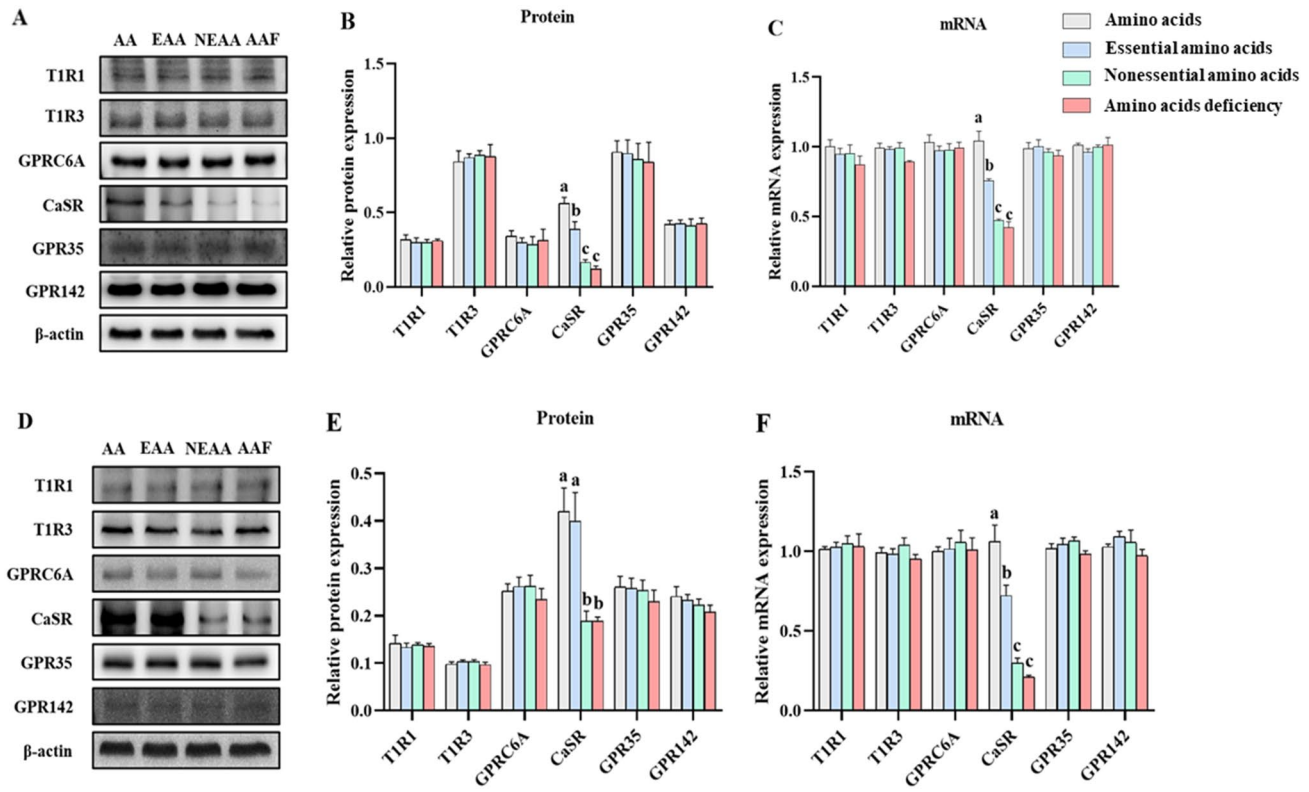


Fig. 3 Effects of essential and non-essential amino acids on the expression of amino-acid-responsive GPCRs in HC11 cells and PMECs. **A-C** Western blots and mRNA expression levels of target genes of amino-acid-responsive GPCRs in HC11 cells. In **A**, the bands show T1R1, T1R3, CaSR, GPRC6A, GPR35, GPR142, and β-actin protein expression levels. The expression levels of target proteins are shown in **B**. **C** Relative mRNA expression levels of target genes. **D–F** Western blots and mRNA expression levels of target genes of amino-acid-responsive GPCRs in PMECs. In **D**, the bands show T1R1, T1R3, CaSR, GPRC6A, GPR35, GPR142, and β-actin protein expression levels. The expression levels of target proteins are shown in **E**. **F** Relative mRNA expression levels of target genes. The cells analyzed in this figure were grown in the following types of medium: AAF, amino-acid-free medium; AA, medium with all amino acids; EAA, medium with only essential amino acids; and NEAA, medium with only non-essential amino acids. $n=4$. All data with error bars are the average ± SEM. In column diagrams, no letter or the same letter above the bar graph indicates no significant difference ($P>0.05$), and different lowercase letters indicate a significant difference ($P<0.05$)

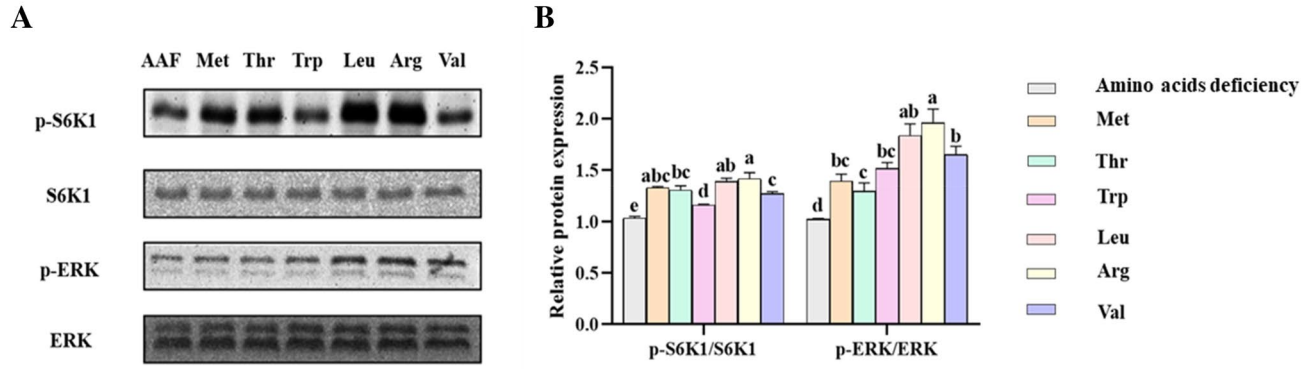


Fig. 4 Effects of Met, Thr, Trp, Leu, Arg, and Val on activation of GPCR (p-ERK) and mTORC1 (p-S6K1) signaling in HC11 cells. In **A**, the bands show S6K1, p-S6K1, ERK, and p-ERK protein expression levels. The ratios of p-S6K1/S6K1 and p-ERK/ERK are shown in **B**. Note: AAF is the control group (without amino acid addition), and in the amino acid group, a single amino acid was added at a concentration of 500 μM based on AAF. All data with error bars are the average ± SEM. In column diagrams, no letter or the same letter above the bar graph indicates no significant difference ($P>0.05$), and different lowercase letters indicate a significant difference ($P<0.05$). *Met* methionine, *Thr* threonine, *Trp* tryptophan, *Leu* leucine, *Arg* arginine, *Val* valine. $n=3$; inhibitor treatment was conducted for 2 h

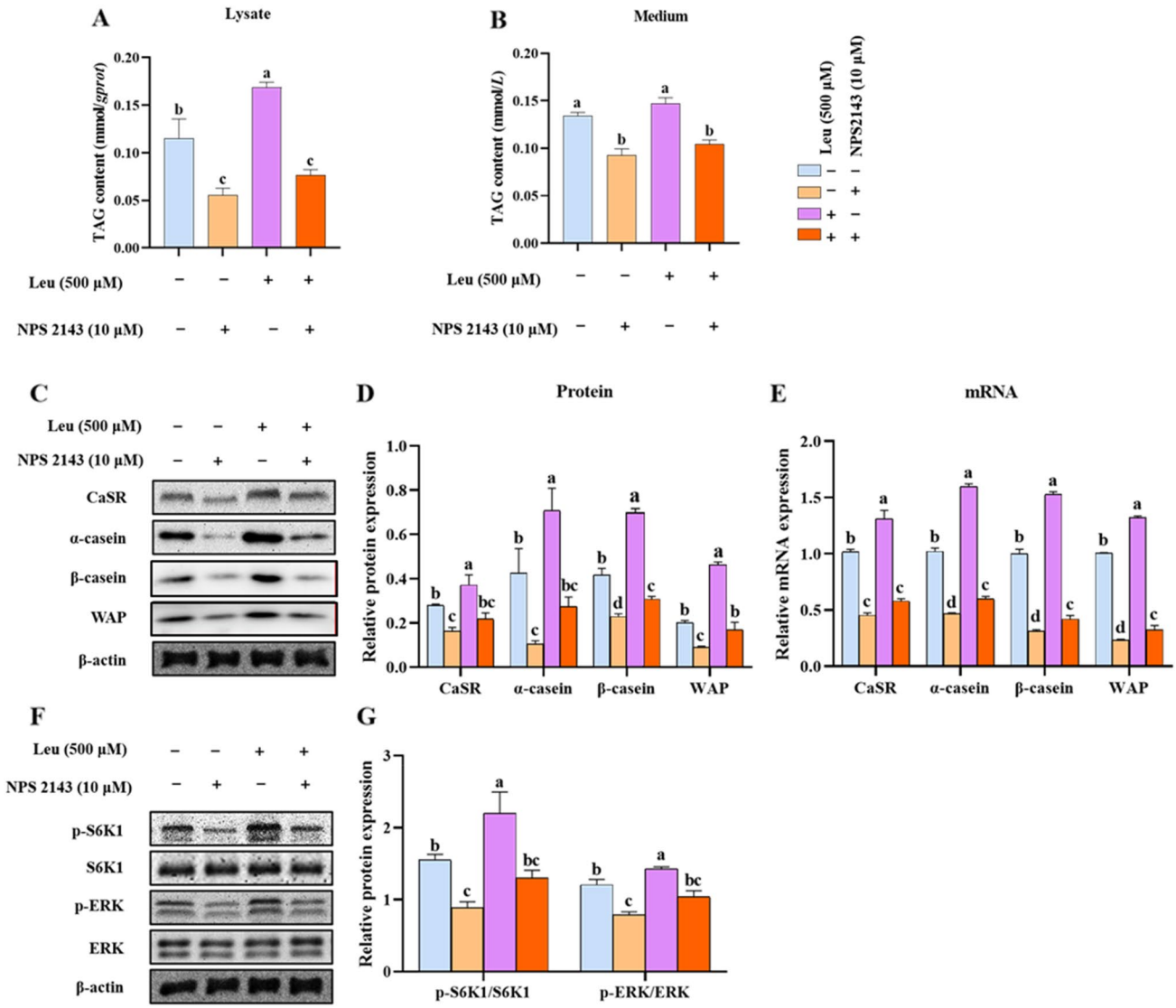


Fig. 5 Effects of leucine on milk synthesis in CaSR-inhibited HC11 cells. **A**, **B** Effects of leucine on TAG content in CaSR-inhibited HC11 cells (**A**) and culture medium (**B**). **C**–**E** Effects of leucine on CaSR, α-casein, β-casein, and WAP protein and mRNA expression levels in CaSR-inhibited HC11 cells. In **C**, the bands show CaSR, α-casein, β-casein, WAP, and β-actin protein expression levels. Protein and relative mRNA expression levels of CaSR, α-casein, β-casein, and WAP are shown in **D**, **E**. **F**, **G** Effects of leucine on the phosphorylation levels of S6K1 and ERK in CaSR-inhibited HC11 cells. In **F**, the bands show S6K1, P-S6K1, P-ERK, ERK, and β-actin protein expression levels. The ratios of p-S6K1/S6K1 and p-ERK/ERK after arginine treatment at 500 μM and NPS 2143 treatment at 10 μM are shown in **G**. All data with error bars are the average ± SEM. In column diagrams, no letter or the same letter above the bar graph indicates no significant difference ($P > 0.05$), and different lowercase letters indicate a significant difference ($P < 0.05$). *Arg* arginine, *NPS 2143* CaSR inhibitor. $n = 4$; inhibitor treatment was conducted for 2 h

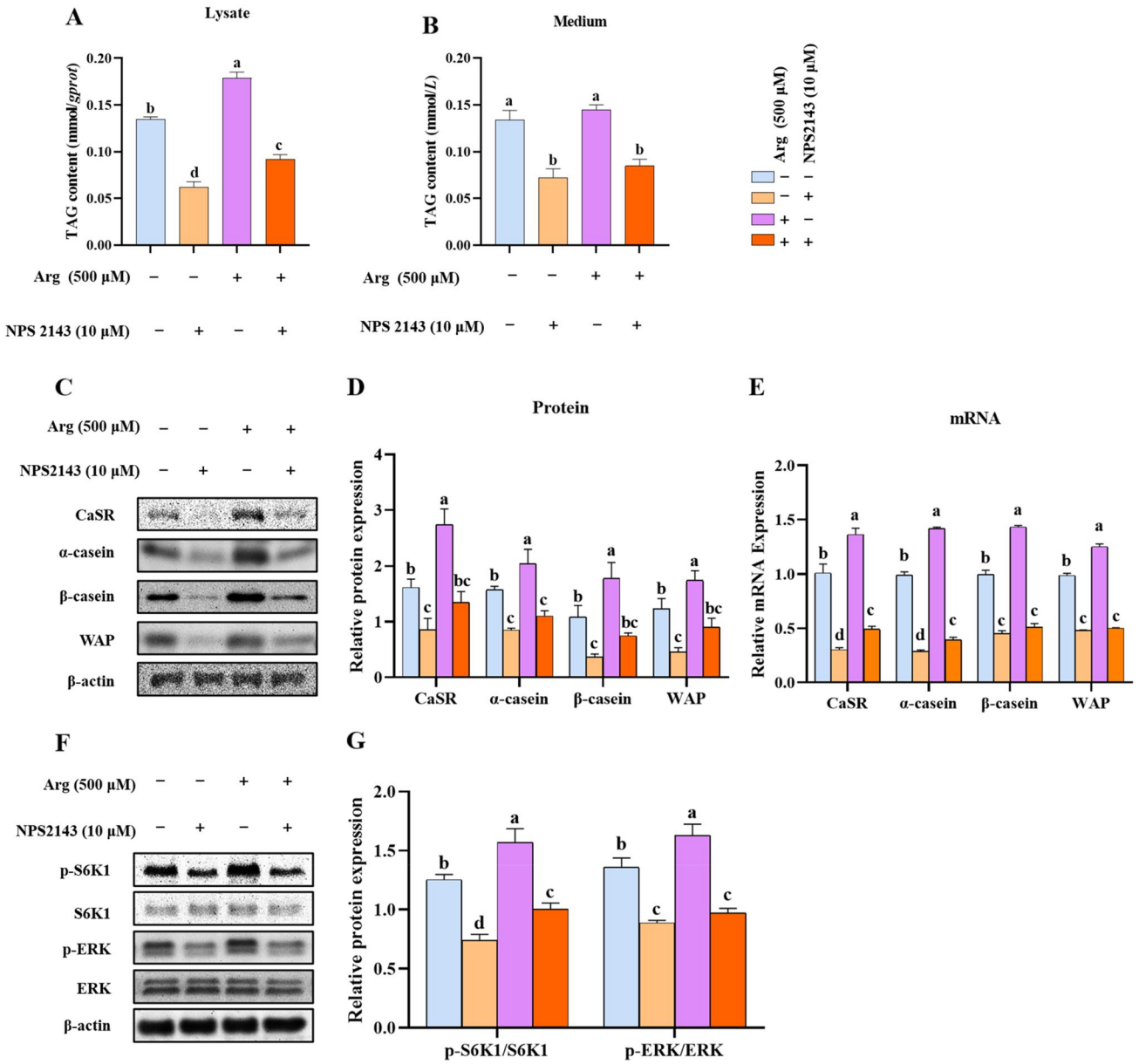


Fig. 6 Effects of arginine on milk synthesis in CaSR-inhibited HC11 cells. **A-B** Effects of arginine on TAG content in CaSR-inhibited HC11 cells (**A**) and culture medium (**B**). **C–E** Effects of arginine on CaSR, α-casein, β-casein, and WAP protein and mRNA expression levels in CaSR-inhibited HC11 cells. In **C**, the bands show CaSR, α-casein, β-casein, WAP, and β-actin protein expression levels. Protein and relative mRNA expression levels of CaSR, α-casein, β-casein, and WAP are shown in **D**, **E**. **F**, **G** Effects of arginine on the phosphorylation levels of S6K1 and ERK in CaSR-inhibited HC11 cells. In **F**, the bands show S6K1, P-S6K1, P-ERK, ERK, and β-actin protein expression levels. The ratios of p-S6K1/S6K1 and p-ERK/ERK after arginine treatment at 500 μM and NPS 2143 treatment at 10 μM are shown in **G**. All data with error bars are the average ± SEM. In column diagrams, no letter or the same letter above the bar graph indicates no significant difference ($P > 0.05$), and different lowercase letters indicate a significant difference ($P < 0.05$). Arg: arginine; NPS 2143: CaSR inhibitor. $n = 4$; inhibitor treatment was conducted for 2 h

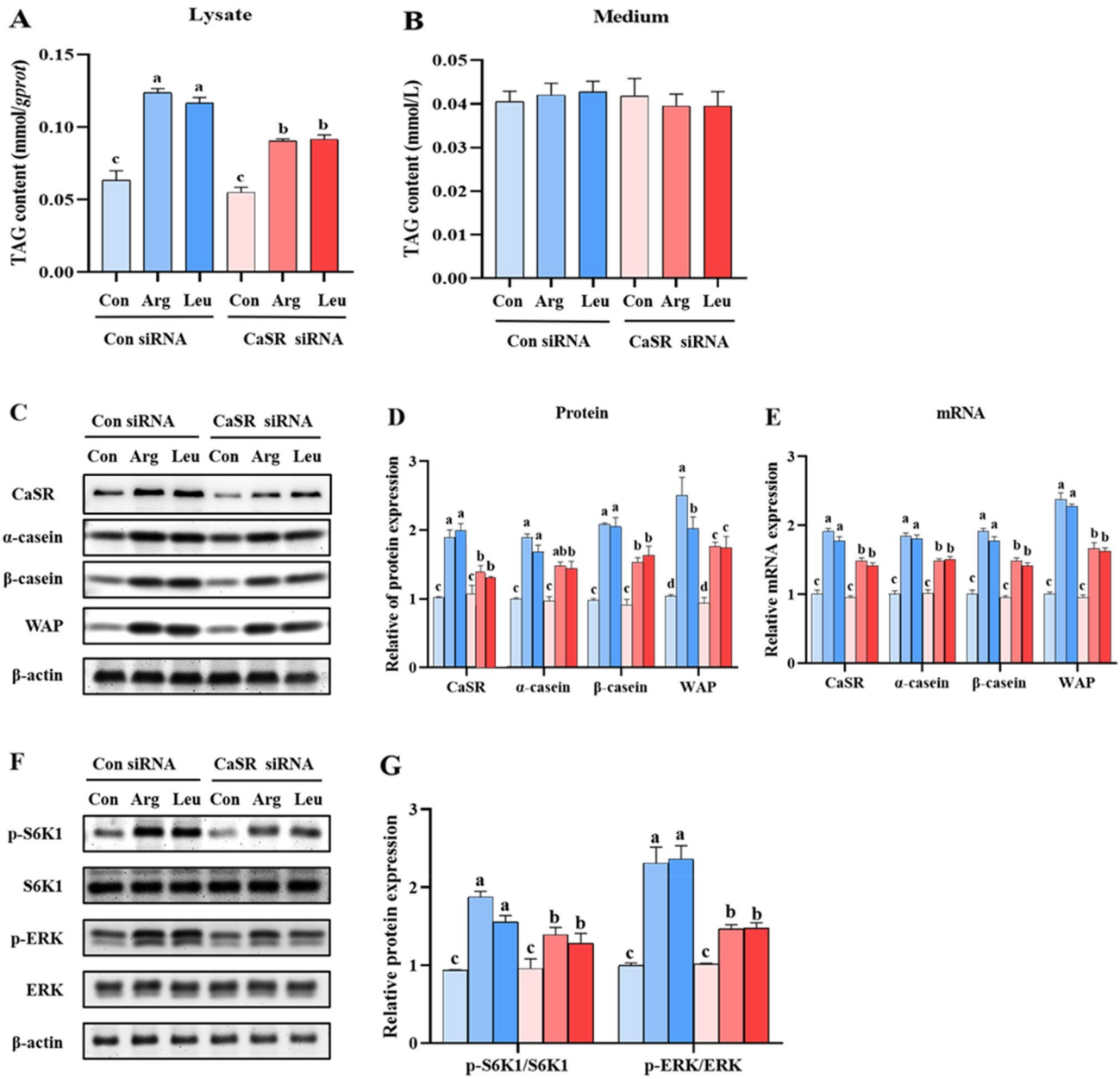


Fig. 7 Effects of Leu and Arg on milk synthesis in CaSR-knockdown HC11 cells. **A**, **B** Effects of Leu and Arg on TAG content in CaSR-knockdown HC11 cells (**A**) and medium (**B**). **C**–**E** Effects of Leu and Arg on milk protein content and related mRNA expression in CaSR-knockdown HC11 cells. In **C**, the bands show CaSR, α-casein, β-casein, WAP, and β-actin protein expression levels. Protein and relative mRNA expression levels of CaSR, α-casein, β-casein, and WAP are shown in **D**, **E**. **F**, **G** Effects of Leu and Arg on the ratio of p-S6K1/S6K1 and p-ERK/ERK in CaSR-knockdown HC11 cells. In **F**, the bands show S6K1, p-S6K1, p-ERK, ERK, and β-actin protein expression levels. The ratio of p-S6K1/S6K1 and p-ERK/ERK is shown in **G**. In the figure, the Con group was treated with medium lacking Leu and Arg. In the Arg/Leu group, 500 μM Arg/Leu was added based on the Con group. All data with error bars are the average ± SEM. In column diagrams, no letter or the same letter above the bar graph indicates no significant difference ($P > 0.05$), and different lowercase letters indicate a significant difference ($P < 0.05$). *Arg* arginine, *Leu* leucine. $n = 4$; CaSR siRNA treatment was conducted for 6 h

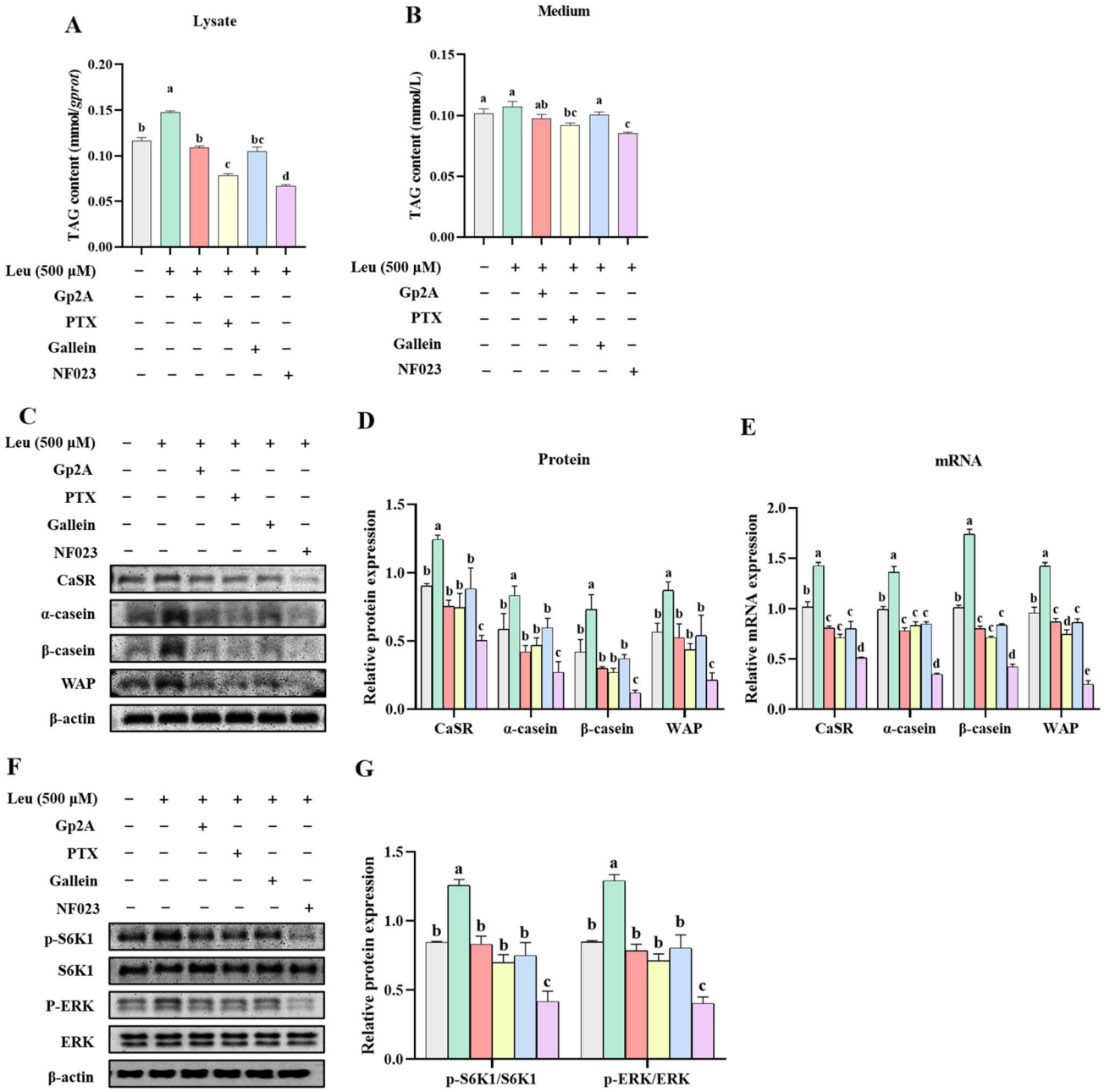


Fig. 8 The addition of Gp2A, PTX, gallein, and NF023 attenuates the leucine-induced promotion of milk TAG (**A**, **B**) and milk protein (**C**–**E**) synthesis in HC11 cells. In C, the bands show CaSR, α-casein, β-casein, WAP, and β-actin protein expression levels. Protein and relative mRNA expression levels of CaSR, α-casein, β-casein, and WAP are shown in **D**, **E**. **F**, **G** The addition of Gp2A, PTX, gallein, and NF023 attenuates the leucine-induced phosphorylation-mediated activation of S6K1 and ERK in HC11 cells. In **F**, the bands show S6K1, p-S6K1, p-ERK, ERK, and β-actin protein expression levels. The ratios of p-S6K1/S6K1 and p-ERK/ERK after leucine treatment at 500 μM are shown in G. *Gp2A* G_q inhibitor, *PTX* G_i inhibitor, *gallein* $G_{\beta\gamma}$ inhibitor, *NF023* $G_{\alpha i}$ inhibitor. All data with error bars are the average ± SEM. In column diagrams, no letter or the same letter above the bar graph indicates no significant difference ($P > 0.05$), and different lowercase letters indicate a significant difference ($P < 0.05$). $n = 4$; the inhibitor concentration was 10 nM; inhibitor treatment was conducted for 2 h

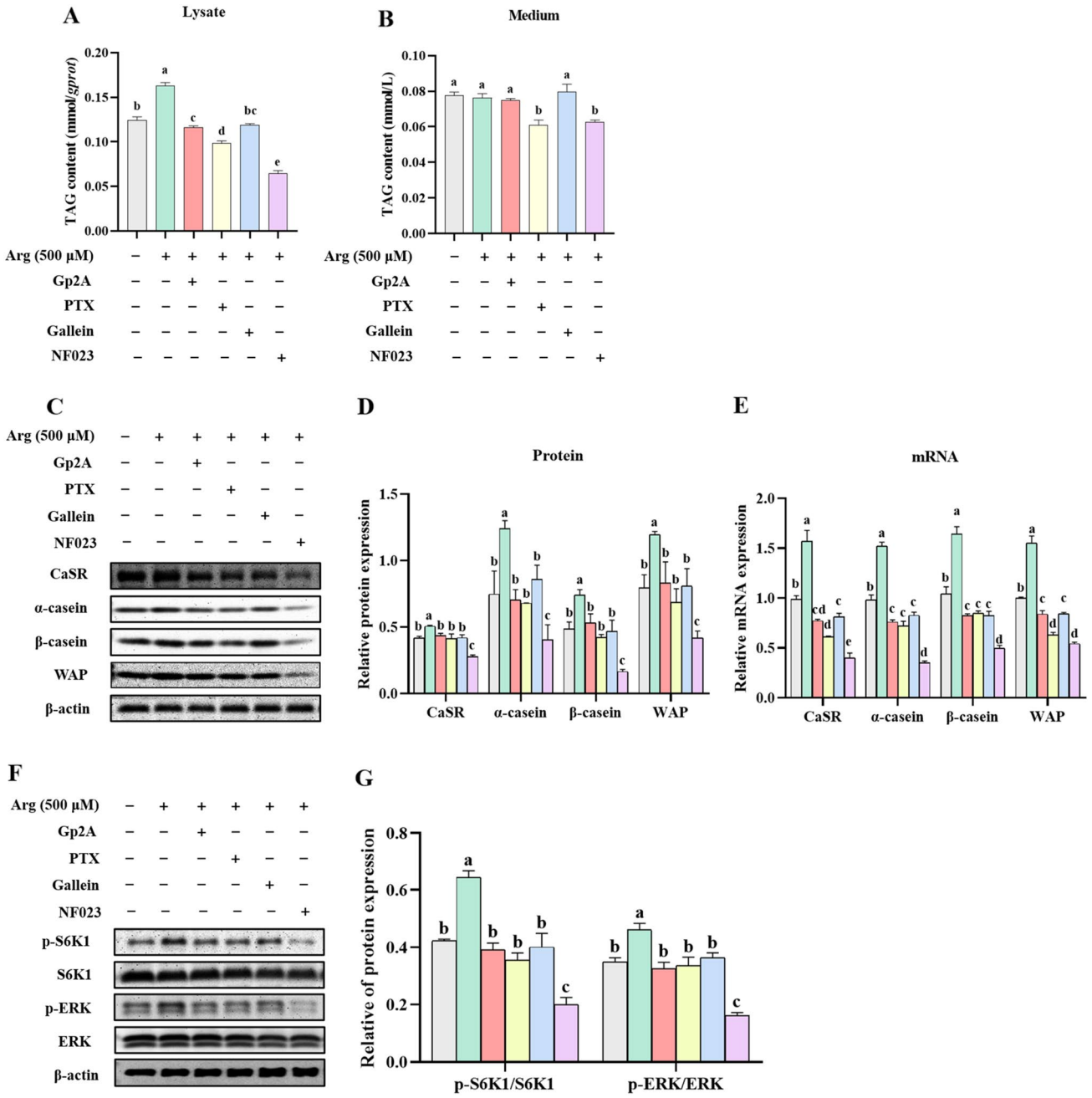


Fig. 9 The addition of Gp2A, PTX, gallein, and NF023 attenuates the arginine-induced promotion of TAG (**A**, **B**) and milk protein (**C–E**) synthesis in HC11 cells. In **C**, the bands show CaSR, α-casein, β-casein, WAP, and β-actin protein expression levels. Protein and relative mRNA expression levels of CaSR, α-casein, β-casein, and WAP are shown in **D**, **E**. **F**, **G** The addition of Gp2A, PTX, gallein, and NF023 attenuates the arginine-induced phosphorylation-mediated activation of S6K1 and ERK in HC11 cells. In **F**, the bands show S6K1, p-S6K1, p-ERK, ERK, and β-actin protein expression levels. The ratios of p-S6K1/S6K1 and p-ERK/ERK after arginine treatment at 500 μM are shown in **G**. *Gp2A* G_q inhibitor, *PTX* G_i inhibitor, *gallein* $G_{\beta\gamma}$ inhibitor, *NF023* $G_{\alpha i}$ inhibitor. All data with error bars are the average ± SEM. In column diagrams, no letter or the same letter above the bar graph indicates no significant difference ($P > 0.05$), and different lowercase letters indicate a significant difference ($P < 0.05$). $n = 4$; the inhibitor concentration was 10 nM; inhibitor treatment was conducted for 2 h

through the CaSR/Gi/mTORC1 and CaSR/Gq/mTORC1 signaling systems in mammary gland epithelial cells.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s00394-023-03197-7.

Funding The research was funded by the following funds: National Key R&D Program of China (No. 2021YFD1300700), the Guangdong Basic and Applied Basic Research Foundation (No. 2021A1515010440 and 2023A1515012098), the Science and Technology Program of Guangzhou (No. 202102020056), the National Natural Science Foundation of China (No. 31802067 and 31872364), and the Anhui Province Key Laboratory of Animal Nutrition Regulation and Health (No. APKLANRH202105).

Availability of data and materials The data that support the findings of this study are available from the corresponding author upon reasonable request.

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Maternal Selenium-Enriched Yeast Supplementation in Sows Enhances Offspring Growth and Antioxidant Status through the Nrf2/Keap1 Pathway

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Abstract: This study evaluated the effects of maternal selenium-enriched yeast (SeY) supplementation during late gestation and lactation on sow performance, transfer of selenium (Se) and redox status, and gut microbiota community, as well as on the gut health of offspring. Seventy pregnant sows on day 85 of gestation were randomly allocated to the following two treatments: (1) sows who were fed a basal diet (basal diet contained 0.3 mg/kg Se as Na_2SeO_3, n = 35); (2) and sows who were fed a SeY-supplemented diet (basal diet with 0.2 mg/kg Se as SeY, n = 35). The offspring piglets were only cross-fostered within the group on day 3 of lactation (L3) according to the pig farm epidemic prevention policy. The plasma, milk, and feces samples from 10 sows, as well as plasma and intestinal samples per treatment, were collected on L1 and L21, respectively. Our results showed that maternal SeY supplementation increased the first week average weight and ADG of piglets ($p < 0.05$). Compared with the CON group, the SeY supplementation increased the Se content in the plasma and milk of sows and the plasma of piglets on L1 and L21 ($p < 0.05$). In addition, in sows, the levels of fat in the milk on L21, the level of IgA, T-AOC, and GSH-Px in the plasma on L21, and the level of T-AOC and GSH-Px in the colostrum were increased, while the MDA content was decreased in the plasma on L1 and in the colostrum and milk on L14 ($p < 0.05$). In the piglet plasma, the levels of IgA on L1 and L21, GSH-Px on L1, and GSH on L21 were increased, while the MDA content was decreased on L1 ($p < 0.05$). Maternal SeY supplementation up-regulated the small intestinal protein abundances of MUC1, E-cadherin, ZO-1, occludin, and claudin and activated the Nrf2/Keap1 signaling pathway in weaned offspring piglets. The 16S rRNA sequencing results showed that fecal microbiota had distinct separations during lactation, and the relative abundances of *unclassified_f_Lachnospiraceae*, *Prevotaceae_UCG-001*, and *Lachnospiraceae_NK4A136_group* were increased on L1. Collectively, the current findings suggest that maternal SeY supplementation during late gestation and lactation could improve the piglet's growth performance, Se status, antioxidant capacity and immunoglobulins transfer at the first week of lactation, as well as alter the fecal microbiota composition by increasing antioxidative-related and SCFA-producing microbiota in sows. These changes contributed to enhancing the small intestinal barrier function and activating the Nrf2/Keap1 pathway in offspring.

Keywords: redox status; sows; selenium-enriched yeast; piglets; small intestine; Nrf2/Keap1

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Citation: Xiong, L.; Lin, T.; Yue, X.; Zhang, S.; Liu, X.; Chen, F.; Zhang, S.; Guan, W. Maternal Selenium-Enriched Yeast Supplementation in Sows Enhances Offspring Growth and Antioxidant Status through the Nrf2/Keap1 Pathway. *Antioxidants* **2023**, *12*, 2064. https://doi.org/10.3390/antiox12122064

Academic Editor: Evangelos Zoidis

Received: 7 November 2023
Revised: 24 November 2023
Accepted: 29 November 2023
Published: 1 December 2023

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1. Introduction

Genetic selection for hyperprolific sows suffers from highly stressful conditions including increased milk production, limited nutrient intake, and increased oxidative stress [1]. Studies have shown that reactive oxygen species (ROS) are increased in late pregnancy and lactation, and maternal oxidative stress may transmit to offspring [2,3]. Early-weaning piglets are met with continuing external stressors, including changing dietary habits, gut microbiota, and environmental factors, leading to intestinal oxidative injury and dysfunction [4]. Intestinal oxidative injury commonly results in a low feeding efficiency and growth performance in piglets [5]. Thus, the oxidative stress during the neonatal and weaning phases may continuously lead to a long-term negative effect on the growth performance, which can persist until adulthood [6]. Previous studies have indicated that antioxidant protection and immune agents, achieved via nutrients, might be transferred from the mother to the newborn during pregnancy or via the maternal colostrum and milk supply [7,8]. Our previous studies have shown that maternal feed additives could affect the offspring's intestinal function through regulation of signal pathways [9]. Therefore, the redox status homeostasis of sows during late gestation and lactation would be of great significance for their reproductive performance and for offspring gut development.

Selenium (Se) is an indispensable trace element to sows and exists in nature in both inorganic and organic forms [10]. Se is a metalloid that is incorporated into polypeptide chains as part of the selenocysteine to fulfil a biofunction. The proteins that contain selenocysteine in their poly-peptide chain are identified as selenoproteins [11]. The major selenoprotein glutathione peroxidase (GSH-Px) enzyme is well known due to its role in the detoxification of hydrogen peroxide and lipid hydroperoxides inside the cellular membrane against ROS damage [12]. The Se content in a sow's blood continues to decrease as the pregnancy and lactation progresses [13]. A Se deficiency during gestation leads to miscarriage, pre-eclampsia, preterm birth, and intrauterine growth retardation [11]. Compared with inorganic forms of Se, selenium-enriched yeast (SeY) mainly consists of selenoamino acids (i.e., selenomethionine) and their analogues [14]. As the organic form of Se, SeY is primarily used on account of the more efficient deposition in maternal tissues and transfer to offspring [15–17]. Hence, it is crucial to supplement SeY during late gestation and lactation in sow diets for the redox status of sow and progeny.

Previous studies showed that when Se was supplied in an organic instead of inorganic form in a sow diet during gestation and lactation, it could improve the reproductive performance and the Se and redox status in the serum, milk, and offspring [13,18,19]. However, other studies have reported that different dietary Se sources or levels when fed during gestation and lactation had no effect on the reproductive performance, including lactation feed intakes, parturition performance, and litter performances of sows [15,16,20]. Our previous study also reported that increasing the SeY supply during late gestation did not affect the reproductive performance of sows [21]. The studies that refer to the effect of SeY supplementation during late gestation and lactation on the reproductive performance of sows are inconsistent [22]. Moreover, the effect of maternal SeY supplementation during late gestation and lactation on the sow's reproductive performance, transfer of selenium and redox status, and gut microbiota community, as well as on the intestinal health of the offspring, has not been extensively studied. Therefore, we investigated the effect of maternal SeY supplementation during late pregnancy and lactation on the sow performance, transfer of redox status, and the intestinal expression of the nuclear factor erythroid2-related factor (Nrf2) and Kelch-like ECH-associated protein 1 (Keap1) pathway in the offspring. We hypothesized that maternal SeY supplementation during late gestation and lactation could improve the lactation performance, antioxidant capacities in sows and their offspring, and gut health through the Nrf2/Keap1 signal pathway. The current study was conducted to verify this hypothesis.

2. Materials and Methods

2.1. Animals, Treatments, and Diets

A total of 70 multiparous (3 to 4 parities) Landrace × Large White sows were randomly selected from a commercial herd located in Guangdong Foodstuffs IMP. & EXP. Group Co., Ltd. in Heyuan City, Guangdong Province, China. Subsequently, the sows were randomly allocated to 2 treatments (35 sows per group) by parity, body condition, and historical reproductive performance as follows: (1) CON group, basal diet contained 0.3 mg/kg Se as Na_2SeO_3, n = 35 (this level is industry level, above recommendation level); (2) SeY group, basal diet with 0.2 mg/kg Se as Se-enriched yeast, n = 35 (Se-enriched yeast with 2000 mg/kg Se; Sel-PlexTM 2000, Alltech Inc., Lexington, KY, USA). The Se level in SeY group did not exceed the upper limit level (0.5 mg/kg) of Se in Food and Drug Administration (FDA) standards in the USA and standards in China. The nutrition levels of the basal diets met and exceeded the nutritional requirements of pregnant and lactating sows according to the National Research Council (NRC) (2012). No antibiotics were used during the entire trial. The feed composition and nutrient level of the basal diet are listed in Table S1.

All sows were housed in individual pregnancy crates (2.2 × 0.8 m) from day 85 until 106 of gestation; then, they were transferred into the parturition rooms on day 107 of gestation until day 23 of lactation, and the total trial period was 52 days. Sows were fed twice per day at 700 and 1600 h. Sows were restricted to feed and received a total of 3.0–3.5 kg/d diets per sow. The farrowing facility where the sows were placed in individual delivery crates (2.5 × 2.5 m) had environment-controlled systems. The room temperature of farrowing rooms was always kept at 21–23 °C. On the farrowing day, sows were fed 1.0 kg of diet and the amount of feed gradually increased by 1.0 kg/d until feeding ad libitum. At farrowing, the total number of piglets born, born alive, stillborn, as well as neonatal piglets' weight (before colostrum intake) was recorded. According to the pig farm epidemic prevention policy, piglets were only cross-fostered within the group on day 3 of lactation; then, the litter size was standardized to 10–12 piglets per litter. Animals had access to water ad libitum throughout the trial. Piglets were weaned on day 23 of lactation and had no access to creep feed. During lactation, feed intake of each sow was recorded to calculate ADFI. In addition, the individual piglet's body weight (BW) and litter size were recorded after being cross-fostered on day 3 of lactation and at weaning on day 23 of lactation. Using these data, the average daily gain (ADG) and survival rate during lactation of piglets were calculated. The weaning-to-estrus interval of sows was tracked and recorded after the weaning of offspring. Backfat thickness was determined at the P2 position (6 cm away from the spine and at the head of the last rib) using an ultrasonic device (Renco Lean-Meater®, Renco Corporation, Minneapolis, MN, USA) on day 110 of gestation and day 23 of lactation to assess the backfat loss of sows during lactation.

2.2. Sample Collection

2.2.1. Collection of Feed and Feces of Sows

The 10 sows per group that were selected for sample collection on day 1 and 21 of lactation were always the same. Approximately 1 kg of pregnancy and lactation diets were collected and stored at −20 °C weekly. On day 1 and 21 of lactation, 10 sows per group were selected for collection of fresh fecal samples through the rectal palpation method, and samples were frozen at −80 °C immediately.

2.2.2. Collection of Plasma Samples of Sows and Piglets

The 10 sows and 6 offspring piglets per group that were selected for sample collection on day 1 and 21 of lactation were always the same. Blood samples (5 mL) were obtained from 10 sows per group via the ear vein and 6 offspring piglets per group via the precaval vein using heparinized vacutainer tubes on day 1 and 21 of lactation. The blood samples were centrifugated at 3000× g for 15 min to acquire plasma samples, which were deposited at −20 °C for subsequent analysis.

2.2.3. Collection of Colostrum and Milk of Sows

After delivery, approximately 20 mL of colostrum samples were collected immediately from every functional teat (10 sows per group) on day 1 of lactation. After injection of 3 mL oxytocin, approximately 20 mL milk samples were collected on day 14 and 21 of lactation. The colostrum and milk samples were immediately frozen at −80 °C for further analysis.

2.2.4. Collection of Small Intestinal Mucosa of Piglets

On day 23 of lactation, one piglet with close to the average BW of each litter (n = 6 per treatment) was selected and euthanized humanely after weaning. The entire intestine was removed from the abdominal cavity. After removal of digesta, the segments (2 cm) were collected from the middle duodenum, jejunum, and ileum, and then fixed in 20% paraformaldehyde. And then, the mucosa samples of small intestine were scraped using a piece of glass, then quickly snap-frozen in liquid nitrogen and stored at −80 °C for subsequent protein extraction.

2.3. Laboratory Analysis

2.3.1. Analysis of Selenium Content in Feed, Plasma, and Milk

The measurement of Se content in feed, plasma, and milk was conducted using fluorometric method outlined by the "National Food Safety Standard Determination of Selenium in Food" (GB5009.93-2017) [23], which was referred to in our previous studies [21]. The Se standard was purchased from SCP Science (Cat#: 140-051-340, Montreal Island, QC, Canada). First, 1 g diet or 1 mL plasma and milk samples were digested using complex acids (the ratio of nitric acid and perchloric acid was 4 to 1) for overnight digestion at room temperature. Se complexes in diet and blood samples were oxidized to be Se^{4+}, which immediately reacted with 2,3-diaminonaphthalene to form 4,5-benzo piaselenol in acidic medium, and then was extracted using cyclohexane. Subsequently, the fluorescence of the organic phase was analyzed via fluorescence spectrometry (RF-5301, shimadzu corporation, Kyoto, Japan). The Se content has a linear correlation with fluorescence degree when Se content in samples is less than 0.5 μg.

2.3.2. Analysis of Colostrum and Milk Composition

Colostrum and milk samples (5 mL per sow) were assessed to determine the contents of solids-not-fat, fat, protein, and lactose using a fully automatic milk composition analyzer (MilkoScan® FTp Analyzer, Foss, Hillerød, Denmark).

2.3.3. Analysis of Immune and Redox Status in Plasma and Milk

Concentrations of immunoglobulin A (IgA), IgG, IgM in plasma, colostrum, and milk were determined using ELISA kits according to the manufacturer's protocols (Huamei Biotechnology, Wuhan, China). In addition, the levels of the total antioxidant capacity (T-AOC), total superoxide dismutase (T-SOD), glutathione (GSH), GSH-Px, and malondialdehyde (MDA) in plasma, colostrum, and milk were measured using commercial ELISA kits according to the manufacturer's instructions (Nanjing Institute of Jiancheng Biological Engineering, Nanjing, China).

2.3.4. Analysis of Relative Protein Abundances

Small intestine tissue proteins were extracted via RIPA Lysis buffer (Beyotime, Jiangsu, China), which added a 1% protease inhibitors PMSF and 1% phosphatase inhibitors cocktail. The concentration of total protein was measured using the BCA assay kit (Beyotime, Jiangsu, China). Resolution of protein was determined via sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gel, followed by a transfer to polyvinylidene difluoride (PVDF) membranes and nonspecific bind blocking with 5% BSA. The membranes were incubated with the probed primary antibodies against β-actin (#4970, CST, Hong Kong, China), Claudin1 (#ab211737, Abcam, Cambridge, UK), Occludin (#ab216327, Abcam), Zonula Occludens (ZO)-1 (#21773-1-AP, Proteintech, Rosemont, IL, USA), Mucin (MUC)1

(#14161, CST), Nrf2 (#ab62352, Abcam), p-Nrf2 (bs-2013R, Bioss, Woburn, MA, USA), and Keap1 (#8047, CST) at 4 °C overnight. After that, membranes were washed with TBST (phosphate-buffered saline with Twen-20) buffer and incubated with a suitable secondary antibody for 1.5 h at room temperature. Finally, target bands were visualized using the Chemiluminescence Image Analysis System (Tanon, Shanghai, China). The relative abundances of target proteins were normalized against β-actin and quantitated using the ImageJ software 1.33 (National Institutes of Health, Bethesda, MD, USA).

2.3.5. Immunofluorescence Analysis of Small Intestine

The distribution and abundance of MUC1 protein in the small intestine of the weaned offspring piglets were determined using immunofluorescence. The small intestine tissues were immobilized and fixed with paraffin wax, and 5 μm sections were prepared. Subsequently, the sections were blocked with 3% bovine serum albumin and incubated overnight at 4 °C with rabbit anti-MUC1 primary antibody (#ab451671, 1: 200; Abcam, Cambridge, UK). Then, the sections were washed five times with phosphate-buffered salt solution (PBS) and incubated with goat antirabbit fluorescein isothiocyanate (FITC)-conjugated secondary antibody (#bs-0295G, Bioss, Beijing, China) for 40 min at room temperature in the dark. Finally, the sections were washed five times with PBS again, and the cell nuclei were stained with 4′-6-diamidino-2-phenylindole (DAPI, #0100-01, SouthernBiotech, Birmingham, AL, USA) for 30 min at room temperature in the dark. Fluorescence imaging of each section was performed using a fluorescent scanning microscope (Nikon Eclipse TI-SR, Tokyo, Japan), and images were captured with the NIKON DS-U3 software 3.0.

2.3.6. Analysis of Fecal Microbiota of Sows

Total bacterial genomic DNA was extracted from 100 mg fecal samples of sows on day 1 and 21 of lactation using the E.Z.N.A.® soil DNA Kit (Omega Bio-tek, Norcross, GA, USA) according to manufacturer's instructions. The quality and content of DNA were detected using 1.0% agarose gel electrophoresis, quantitated via NanoDrop2000 spectrophotometer (Thermo Scientific, Waltham, MA, USA), and then stored at −80 °C for subsequent analysis. The V3–V4 region of the microbial 16S rRNA gene was amplified with primer 338F (5′-ACTCCTACGGGAGGCAGCAG-3′) and 806R(5′-GGACTACHVGGGTWTCTAAT-3′) pairs using a T100 Thermal Cycler PCR thermocycler (BIO-RAD, Hercules, CA, USA). The final volume of 20 μL PCR reaction mixture consisted of 4 μL 5× Fast Pfu buffer, 2 μL 2.5 mM dNTPs, 0.8 μL of each primer (5 μM), 0.4 μL Fast Pfu polymerase, 10 ng of template DNA, and ddH_2O. The amplicon libraries were sequenced on the Illumina MiSeq platform (Illumina, San Diego, CA, USA) using the MiSeq Reagent Kit v3 600 cycles. The sequencing was performed according to the standard protocols of Shanghai Majorbio Biotechnology Co., Ltd. (Shanghai, China).

Pan analysis at the operational taxonomic unit (OTU) level of fecal samples between two groups was performed, evaluating whether the sequencing depth was sufficient to cover all OTUs according to whether the Pan species curve is flat or not. Alpha diversity indices (Ace and Chao1 richness; Shannon and Simpson) of all OTUs were measured using the OTU table and the QIIME2 (2019.4) software. The beta diversity analysis was performed to evaluate the structural variation in fecal microbiota communities. Principal component analysis (PCA) and partial least squares discriminant analysis (PLS-DA) were jointly carried out to determine the significant differences in microbial communities between the two groups. The differences in the relative abundances of fecal microbiota at the phylum and genus levels were simultaneously compared using STAMP 2.1.3 software. The biomarkers of the microbial taxa were identified using the linear discriminant analysis (LDA) effect size (LEfSe) (https://huttenhower.sph.harvard.edu/galaxy/ (accessed on 5 September 2023)), and the graph was plotted with a default parameter LDA score > 2.0.

2.4. Statistical Analyses

Statistical differences of two groups were assessed via Student's *t*-test using the SPSS 22.0 software (Chicago, IL, USA). Each sow (or litter), for reproductive performance analysis, and individual piglets (per litter) for other parameter analyses were used as the experimental unit, respectively. All data are expressed as means ± standard error of the mean (SEM). Statistical difference value between the two treatments was considered significant with $p < 0.05$, and $0.05 < p < 0.10$ was set as a trend toward difference. The differences in alpha diversity indices and the relative microbial abundances were analyzed using the Mann–Whitney U-test. A corrected p value < 0.05 was considered statistically significant.

3. Results

3.1. Se Transfer in Diets, Sows, and Offspring

The Se content in pregnancy and lactation experimental diets as well as the Se transfer from sows to offspring piglets are shown in Figure 1. The pregnancy diets were determined to contain 0.34 and 0.56 mg Se/kg diet in the CON and SeY groups, respectively. The lactation diets were determined to contain 0.37 and 0.55 mg Se/kg diet in the CON and SeY groups, respectively. A higher Se content in sows' and offspring's plasma and sow milk on day 1 and 21 of lactation was detected in the SeY group compared with those in the CON group ($p < 0.05$).

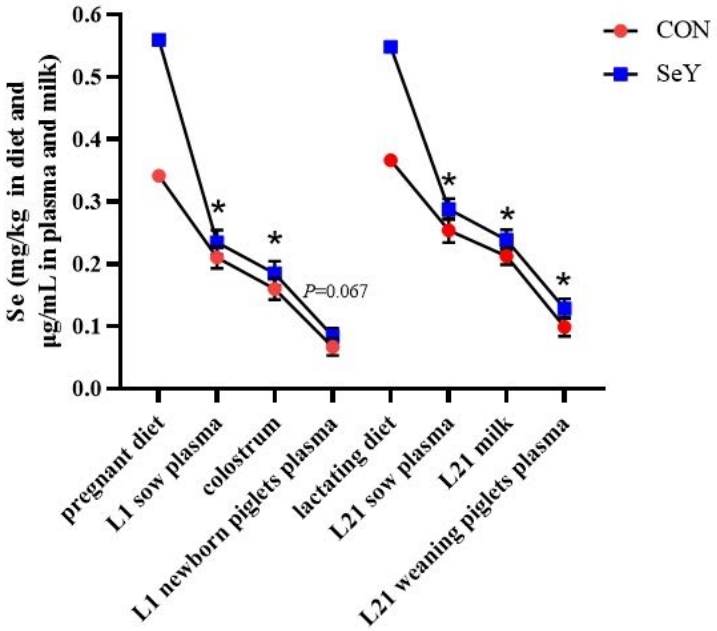

Figure 1. Effect of maternal SeY supplementation during late gestation and lactation on Se transfer from sow diets to offspring piglets. Data are represented as means ± SEM, $n = 6$ per group, * $p < 0.05$. CON = control group, sows fed basal diet containing 0.3 mg/kg Se as Na_2SeO_3; SeY = SeY group, sows fed basal diet with 0.2 mg/kg Se as Se-enriched yeast (Se-yeast with 2000 mg/kg Se).

3.2. The Reproductive Performance of Sows

The sows' reproductive performance is presented in Table 1. The results showed that SeY supplementation during late gestation and lactation showed no effect on the number of piglets born in total, born alive, stillbirths, mummies, and born healthy of sows ($p > 0.05$). In addition, there was no evidence of differences between the two groups in terms of litter weight at birth, litter birth weight, individual piglet weight, weight when born healthy, stillborn weight, and number of pigs weaned, for which the differences were small and insignificant ($p > 0.05$). No significant treatment differences were observed for placental weight, placental weight per piglet, or placental efficiency ($p > 0.05$).

Table 1. Effect of maternal SeY addition during late gestation and lactation on reproductive performance of sows (n = 35).

Item	Sow		*p*-Value
	CON	SeY	
Litters, n	35	35	
Total no. born per litter	11.22 ± 0.36	11.28 ± 0.29	0.906
No. of piglets born alive per litter	10.36 ± 0.33	10.53 ± 0.29	0.704
No. of stillbirths per litter	0.86 ± 0.23	0.75 ± 0.21	0.723
No. of mummies per litter	0.06 ± 0.04	0.08 ± 0.06	0.703
No. of piglets born healthy per litter	10.00 ± 0.31	10.19 ± 0.28	0.641
Litter birth weight, kg	17.51 ± 0.62	17.26 ± 0.54	0.762
Individual piglet weight, kg	1.69 ± 0.05	1.65 ± 0.03	0.473
Weight of piglets born healthy, kg	1.71 ± 0.04	1.67 ± 0.03	0.394
Stillborn weight, kg	1.25 ± 0.11	1.17 ± 0.11	0.625
Placental weight, kg	1.60 ± 0.14	1.72 ± 0.17	0.585
Placental efficiency	12.84 ± 1.18	11.71 ± 0.97	0.482
Placental weight per piglet, kg	0.11 ± 0.01	0.10 ± 0.02	0.508

CON, control group, basal diet containing 0.3 mg/kg Se as Na_2SeO_3; SeY, SeY group, basal diet with 0.2 mg/kg Se as Se-enriched yeast.

3.3. Lactation and Litter Performance of Sows

The effect of maternal SeY addition during late gestation and lactation on the growth performance of nursing piglets is presented in Table 2. The results showed that maternal SeY supplementation during late gestation and lactation had no effect on the litter size and litter weight during the lactation ($p > 0.05$). Maternal SeY supplementation during late gestation and lactation significantly increased the final average weight and mean ADG of offspring piglets in the first week ($p < 0.05$).

Table 2. Effect of maternal SeY addition during late gestation and lactation on growth performance of nursing piglets (n = 35).

Item	Sow		*p*-Value
	CON	SeY	
Litters, n	35	35	
Final litter size			
After day 3 of cross-fostering	9.49 ± 0.23	9.41 ± 0.27	0.835
First week	9.03 ± 0.24	8.94 ± 0.25	0.799
Second week	8.74 ± 0.25	8.74 ± 0.24	0.983
Third week	8.66 ± 0.27	8.68 ± 0.23	0.957
Preweaning survival, %	91.44 ± 2.03	92.48 ± 1.27	0.666
Final litter weight, kg			
After day 3 of cross-fostering	19.33 ± 0.67	19.38 ± 0.71	0.961
First week	28.70 ± 0.91	30.38 ± 0.98	0.213
Second week	42.49 ± 1.53	45.21 ± 1.38	0.192
Third week	58.51 ± 2.06	61.76 ± 1.78	0.283
Final average piglet weight, kg			
After day 3 of cross-fostering	2.04 ± 0.06	2.09 ± 0.08	0.622
First week	3.20 ± 0.08	3.41 ± 0.07 *	0.049
Second week	4.91 ± 0.15	5.21 ± 0.12	0.123
Third week	6.84 ± 0.21	7.18 ± 0.15	0.723
Piglet mean ADG of lactation, g/d			
First week	165.62 ± 7.22	188.31 ± 8.18 *	0.041
Second week	244.87 ± 14.08	256.88 ± 9.70	0.487
Third week	275.54 ± 12.24	280.66 ± 10.13	0.749
Mean of first to third week	228.31 ± 9.29	241.95 ± 6.64	0.239

CON, control group, basal diet containing 0.3 mg/kg Se as Na_2SeO_3; SeY, SeY group, basal diet with 0.2 mg/kg Se as Se-enriched yeast; * $p < 0.05$.

3.4. Feed Intake, Backfat Thickness, and Weaning-to-Estrus Interval of Sows

The feed intake, backfat thickness, and weaning-to-estrus interval of sows are shown in Table 3. No significant treatment differences for ADFI or backfat thickness were observed between the two treatments of sows during the entire lactation ($p > 0.05$). Compared with the CON group, the weaning-to-estrus interval was significantly declined in the SeY group ($p < 0.05$).

Table 3. Effects of SeY addition during late gestation and lactation on feed intake, backfat thickness, and weaning-to-estrus interval of sows ($n = 35$).

Item	Sow		*p*-Value
	CON	SeY	
ADFI of lactation, kg/d			
First week	2.98 ± 0.11	2.99 ± 0.15	0.924
Second week	4.41 ± 0.13	4.42 ± 0.16	0.967
Third week	5.13 ± 0.09	5.06 ± 0.10	0.591
Overall	4.34 ± 0.07	4.27 ± 0.09	0.572
Sow backfat thickness, mm			
Day 110 of gestation	19.54 ± 0.63	18.79 ± 0.43	0.336
Weaning	16.13 ± 0.6	15.64 ± 0.43	0.515
Loss during lactation	3.42 ± 0.28	3.15 ± 0.23	0.478
Weaning-to-estrus interval, d	5.90 ± 0.15	5.66 ± 0.11 *	0.048

CON, control group, basal diet containing 0.3 mg/kg Se as Na_2SeO_3; SeY, SeY group, basal diet with 0.2 mg/kg Se as Se-enriched yeast; * $p < 0.05$.

3.5. Colostrum and Milk Composition

The compositions of the colostrum and milk are presented in Table 4. There were insignificant differences between the two groups in the composition of colostrum with regard to fat, lactose, protein, and solids-not-fat in the colostrum and milk on day 14 of lactation ($p > 0.05$). Compared with the CON group, the SeY supplementation increased the fat content in the milk on day 21 of lactation ($p < 0.05$).

Table 4. Effect of SeY addition during late gestation and lactation on the colostrum and milk composition ($n = 6$).

Item	Sow		*p*-Value
	CON	SeY	
Colostrum, day 1 of lactation, %			
Fat	4.12 ± 0.31	4.20 ± 0.12	0.817
Lactose	4.92 ± 0.1	5.01 ± 0.14	0.610
Protein	7.16 ± 0.27	7.42 ± 0.54	0.682
Solids-not-fat	19.09 ± 0.73	19.71 ± 1.43	0.706
Milk, day 14 of lactation, %			
Fat	5.99 ± 0.27	6.33 ± 0.28	0.392
Lactose	6.55 ± 0.08	6.68 ± 0.23	0.615
Protein	4.73 ± 0.08	4.96 ± 0.14	0.168
Solids-not-fat	12.15 ± 0.15	12.13 ± 0.22	0.941
Milk, day 21 of lactation, %			
Fat	6.24 ± 0.09	6.91 ± 0.19 *	0.005
Lactose	6.44 ± 0.28	6.75 ± 0.21	0.392
Protein	4.63 ± 0.06	4.76 ± 0.1	0.274
Solids-not-fat	11.68 ± 0.1	11.86 ± 0.18	0.373

CON, control group, basal diet containing 0.3 mg/kg Se as Na_2SeO_3; SeY, SeY group, basal diet with 0.2 mg/kg Se as Se-enriched yeast; * $p < 0.05$.

3.6. Immune Indicators in the Plasma and Milk of Sows

The immunoglobulin concentrations in the plasma and milk of sows are shown in Table 5. In the plasma, the IgA contents on day 1 and 21 of lactation were significantly higher in the SeY group than those in the CON group ($p < 0.05$). In the milk, the IgG contents on day 14 and 21 of lactation also showed an up-regulated trend in the SeY group ($p = 0.076$, $p = 0.059$).

Table 5. Effects of SeY addition during late gestation and lactation on the immune indicators of the plasma and milk of sows ($n = 6$).

Item, μg/mL	Sow		*p*-Value
	CON	SeY	
Plasma, day 1 of lactation			
IgA	658.78 ± 33.41	770.36 ± 27.08	0.022
IgG	1431.96 ± 143.16	1481.26 ± 110.87	0.789
IgM	117.79 ± 13.12	151.16 ± 23.42	0.234
Plasma, day 21 of lactation			
IgA	204.81 ± 23.96	379.31 ± 34.09 *	0.001
IgG	344.63 ± 68.34	349.58 ± 29.25	0.945
IgM	78.74 ± 10.24	79.66 ± 5.76	0.939
Colostrum, day 1 of lactation			
IgA	1002.99 ± 115.7	1246.24 ± 142.96	0.211
IgG	9793.69 ± 634.17	10,838.69 ± 576.81	0.243
IgM	973.49 ± 72.46	1098.67 ± 90.79	0.302
Milk, day 14 of lactation			
IgA	102.9 ± 6.18	121.14 ± 17.13	0.336
IgG	915.54 ± 45.43	1054.41 ± 55.30	0.076
IgM	64.53 ± 5.82	74.02 ± 7.59	0.341
Milk, day 21 of lactation			
IgA	84.89 ± 7.58	95.08 ± 7.11	0.346
IgG	801.81 ± 38.27	945.91 ± 57.60	0.059
IgM	72.42 ± 8.31	98.74 ± 12.14	0.105

CON, control group, basal diet containing 0.3 mg/kg Se as Na_2SeO_3; SeY, SeY group, basal diet with 0.2 mg/kg Se as Se-enriched yeast; IgA, immunoglobulin A; * $p < 0.05$.

3.7. Immune Indicators in the Plasma of Offspring Piglets

The immunoglobulin concentrations in the plasma of the offspring piglets are shown in Table 6. In the plasma, the IgA contents at 1 and 21 days of age were also significantly higher in the SeY group than those in the CON group ($p < 0.05$), and an up-regulated trend of the IgM content was observed in the SeY group of piglets at 1 day of age ($p = 0.061$).

Table 6. Effects of SeY supplementation during late gestation and lactation on the immune indicators in the plasma of offspring piglets ($n = 6$).

Item, μg/mL	Piglet		*p*-Value
	CON	SeY	
1 day of age			
IgA	1499.29 ± 67.03	1718.05 ± 61.05	0.036
IgG	2844.21 ± 196.39	2892.61 ± 214.82	0.871
IgM	399.49 ± 57.14	554.61 ± 50.56	0.061
21 days of age			
IgA	210.65 ± 6.90	273.06 ± 17.06	0.007
IgG	1539.68 ± 245.41	1764.78 ± 155.84	0.461
IgM	97.13 ± 10.09	111.01 ± 7.56	0.289

CON, control group, basal diet containing 0.3 mg/kg Se as Na_2SeO_3; SeY, SeY group, basal diet with 0.2 mg/kg Se as Se-enriched yeast; IgA, immunoglobulin A.

3.8. Antioxidative and Oxidative Indicators in the Plasma and Milk of Sows

The oxidative and antioxidative indexes of the plasma and milk of sows are shown in Table 7. In the plasma, the levels of T-AOC and GSH-Px were increased on day 21 of lactation ($p < 0.05$), whereas the MDA concentration was decreased on day 1 of lactation in the SeY group compared with the CON group ($p < 0.05$). In the colostrum and milk, the levels of T-AOC and GSH-Px in the colostrum and T-SOD in milk on day 21 of lactation were increased ($p < 0.05$), while the MDA concentrations in the colostrum and, on day 14 of lactation, the milk were decreased in the SeY group compared with the CON group ($p < 0.05$).

Table 7. Effects of SeY supplementation during late gestation and lactation on the antioxidative and oxidative indicators of the plasma and milk of sows ($n = 6$).

Item	Sow		*p*-Value
	CON	SeY	
Plasma, day 1 of lactation			
T-AOC, mmol/L	0.98 ± 0.03	0.97 ± 0.02	0.845
T-SOD, U/mL	59.52 ± 2.88	64.05 ± 3.64	0.287
GSH-Px, U/mL	494 ± 16.89	556.39 ± 17.75	0.229
GSH, μmol/mL	67.56 ± 1.77	69.18 ± 3.13	0.659
MDA, nmol/mL	4.00 ± 0.31	2.96 ± 0.28 *	0.028
Plasma, day 21 of lactation			
T-AOC, mmol/L	0.92 ± 0.03	1.01 ± 0.03 *	0.047
T-SOD, U/mL	47.34 ± 1.82	53.63 ± 1.29	0.104
GSH-Px, U/mL	847.88 ± 33.36	972.59 ± 22.55 *	0.011
GSH, μmol/mL	54.70 ± 2.44	58.02 ± 3.79	0.472
MDA, nmol/mL	3.17 ± 0.25	2.46 ± 0.34	0.116
Colostrum, day 1 of lactation			
T-AOC, mmol/L	1.10 ± 0.08	1.36 ± 0.07 *	0.035
T-SOD, U/mL	57.12 ± 7.76	71.96 ± 5.65	0.148
GSH-Px, U/mL	93.16 ± 10.38	124.85 ± 8.72 *	0.042
GSH, μmol/mL	11.51 ± 2.07	19.44 ± 3.11	0.055
MDA, nmol/mL	5.67 ± 0.43	3.91 ± 0.61 *	0.039
Milk, day 14 of lactation			
T-AOC, mmol/L	0.96 ± 0.06	1.09 ± 0.07	0.176
T-SOD, U/mL	52.34 ± 7.13	59.72 ± 8.78	0.526
GSH-Px, U/mL	154.9 ± 7.75	178.74 ± 7.12	0.247
GSH, μmol/mL	8.80 ± 1.63	13.1 ± 1.84	0.113
MDA, nmol/mL	4.63 ± 0.44	3.08 ± 0.31 *	0.017
Milk, day 21 of lactation			
T-AOC, mmol/L	0.89 ± 0.04	1.08 ± 0.04	0.154
T-SOD, U/mL	39.40 ± 2.97	55.48 ± 5.52 *	0.050
GSH-Px, U/mL	187.91 ± 9.31	193.41 ± 8.32	0.259
GSH, μmol/mL	8.73 ± 2.14	14.48 ± 3.55	0.190
MDA, nmol/mL	4.34 ± 0.25	3.05 ± 0.30	0.308

CON, control group, basal diet containing 0.3 mg/kg Se as Na_2SeO_3; SeY, SeY group, basal diet with 0.2 mg/kg Se as Se-enriched yeast; T-AOC, total antioxidant capacity; T-SOD, total superoxide dismutase; GSH-Px, glutathione peroxidase; GSH, glutathione; MDA, malondialdehyde; * $p < 0.05$.

3.9. Antioxidative and Oxidative Indicators in the Plasma of Offspring Piglets

The oxidative and antioxidative indexes of the plasma of offspring piglets are shown in Table 8. Compared with the CON group, the content of T-SOD at 1 day of age and the levels of T-AOC and GSH at 21 days of age were significantly raised ($p < 0.05$), while the level of MDA was significantly reduced at 1 and 21 days of age in the SeY group ($p < 0.05$).

3.10. The Small Intestinal Barrier Function of Offspring Piglets

The effect of maternal SeY supplementation during late gestation and lactation on the small intestinal tight junction (TJ) proteins and mucus barrier of weaned offspring piglets are shown in Figures 2 and 3. Compared with the CON group, maternal SeY supplementation tended to up-regulate the relative protein abundances of claudin-1 in the duodenum ($p = 0.085$) and jejunum ($p = 0.066$); ZO-1 ($p < 0.05$) and E-cadherin ($p < 0.05$) in the jejunum; and ZO-1 ($p < 0.05$), E-cadherin ($p < 0.05$), occludin ($p < 0.05$), and claudin-1 ($p < 0.05$) in the ileum of the weaned piglets (Figure 2). Moreover, the abundance and distribution of MUC1,

the major mucus barrier protein, were investigated via immunofluorescence analysis. The localization of MUC1 in the small intestine of offspring piglets was not affected by SeY supplementation (Figure 3); however, maternal SeY supplementation significantly increased the MUC1 expression in the apical intercellular region of the small intestinal epithelium in piglets.

Table 8. Effects of SeY supplementation during late gestation and lactation on the antioxidative and oxidative indicators of the plasma of offspring piglets (n = 6).

Item	Piglet		*p*-Value
	CON	SeY	
1 day of age			
T-AOC, mmol/L	0.81 ± 0.01	0.88 ± 0.08	0.375
T-SOD, U/mL	30.78 ± 1.06	34.09 ± 1.17	0.063
GSH-Px, U/mL	195.57 ± 14.29	243.75 ± 13.89 *	0.036
GSH, μmol/mL	45.98 ± 3.74	55.28 ± 4.86	0.155
MDA, nmol/mL	7.78 ± 0.46	5.70 ± 0.46 *	0.010
21 days of age			
T-AOC, mmol/L	0.79 ± 0.02	0.89 ± 0.03	0.126
T-SOD, U/mL	38.68 ± 1.85	41.89 ± 1.64	0.224
GSH-Px, U/mL	414.63 ± 20.66	460.72 ± 25.71	0.193
GSH, μmol/mL	50.9 ± 6.24	65.13 ± 1.67 *	0.048
MDA, nmol/mL	5.51 ± 0.46	4.54 ± 0.14	0.160

CON, control group, basal diet containing 0.3 mg/kg Se as Na_2SeO_3; SeY, SeY group, basal diet with 0.2 mg/kg Se as Se-enriched yeast; T-AOC, total antioxidant capacity; T-SOD, total superoxide dismutase; GSH-Px, glutathione peroxidase; GSH, glutathione; MDA, malondialdehyde; * $p < 0.05$.

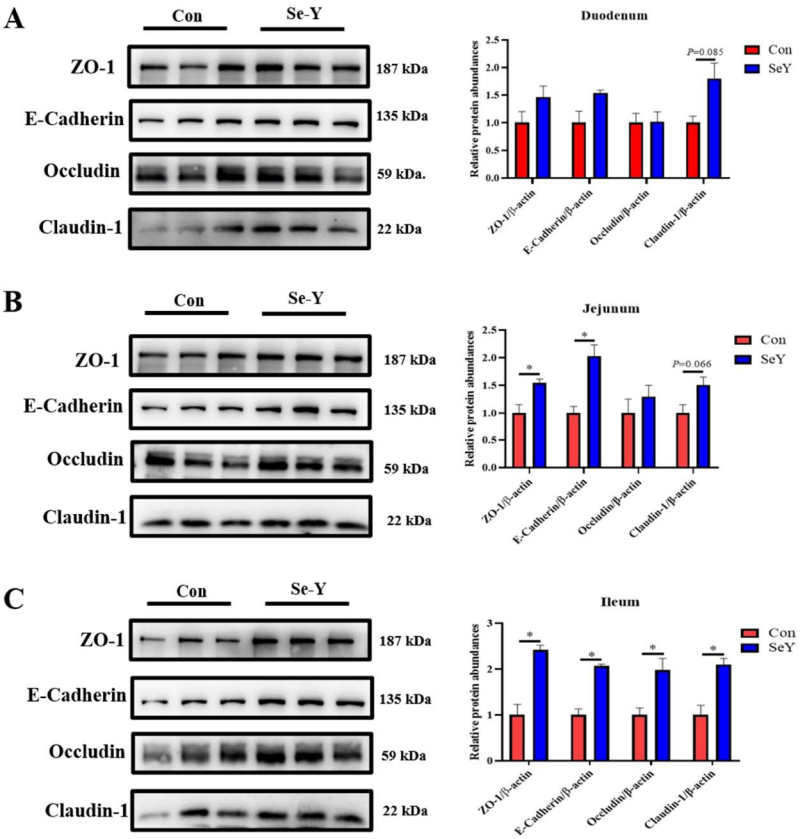

Figure 2. Effects of maternal SeY supplementation during late gestation and lactation on the relative abundances of tight junction (TJ) protein in the duodenum (**A**), jejunum (**B**), and ileum (**C**) of weaned offspring piglets. Data are represented as means ± SEM, n = 6 per group, * $p < 0.05$. CON = control group, sows fed basal diet containing 0.3 mg/kg Se as Na_2SeO_3; SeY = SeY group, sows fed basal diet with 0.2 mg/kg Se as Se-enriched yeast (Se-yeast with 2000 mg/kg Se).

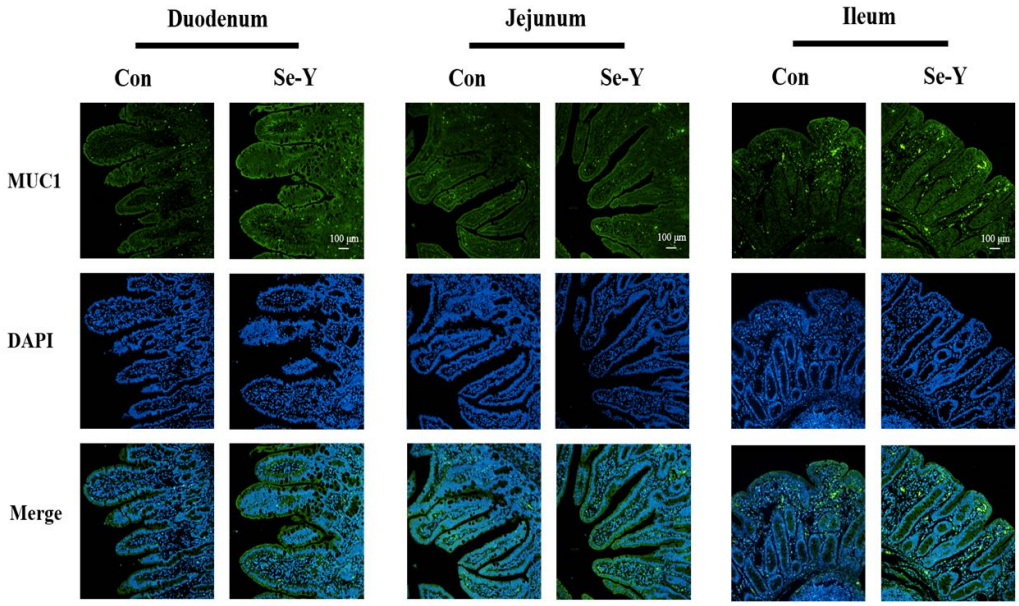

Figure 3. Representative micrographs for mucin (MUC)-1 staining in the small intestine tissues of offspring piglets under a confocal microscope. CON = control group, sows fed basal diet containing 0.3 mg/kg Se as Na_2SeO_3; SeY = SeY group, sows fed basal diet with 0.2 mg/kg Se as Se-enriched yeast (Se-yeast with 2000 mg/kg Se).

3.11. The Expression of Nrf2/Keap1 Pathway-Related Proteins in the Small Intestine of Suckling Offspring Piglets

The effect of maternal SeY supplementation during late gestation and lactation on the small intestinal Nrf2/Keap1 signaling pathway of weaned offspring piglets is shown in Figure 4. Compared with the CON group, maternal SeY supplementation significantly up-regulated the ratio of phosphorylated Nrf2 and total Nrf2 in the small intestine ($p < 0.05$), down-regulated the relative protein abundance of Keap1 in the duodenum ($p < 0.05$), and tended to increase in the jejunum ($p = 0.078$) and ileum ($p < 0.05$) of the weaned piglets.

3.12. Fecal-Associated Microbiota

To understand the effects of dietary SeY supplementation during late gestation and lactation on the composition and structure of the fecal microbiota of sows, 16S rRNA sequencing of fecal samples on day 1 and 21 of lactation was performed. The Pan species curve is flat, indicating that the sample size for this sequencing is sufficient, and almost all bacterial species were captured from feces of sows on day 1 (Figure 5A) and 21 (Figure 6A) of lactation. The Venn diagram showed the OTU network analysis of the fecal microbiota of sows between the two groups on day 1 (Figure 5B) and 21 (Figure 6B) of lactation. A total of 3528 and 3385 microbial OTUs were detected, of which 1121 (31.77%) and 978 (28.89%) individual core microbial OTUs were shared on day 1 of lactation. In addition, a total of 3441 and 3296 microbial OTUs were detected, of which 1121 (31.77%) and 978 (28.89%) individual core microbial OTUs were shared on day 21 of lactation. The microbial alpha diversity indexes of the sow feces, including Ace, Chao1, Simpson, and Shannon indexes, are shown in Figures 5 and 6. The alpha diversity showed no significant differences between the two groups on day 1 (Figure 5C–F) and 21 (Figure 6C–F) of lactation ($p > 0.05$). PCA and PLS-DA analyses were performed to distinguish the differences in the microbial community between the two groups. The microbial beta diversity results showed that there were distinct separations in the microbial OUT levels between the two groups on day 1 (Figure 5G,H) and 21 (Figure 6G,H) of lactation.

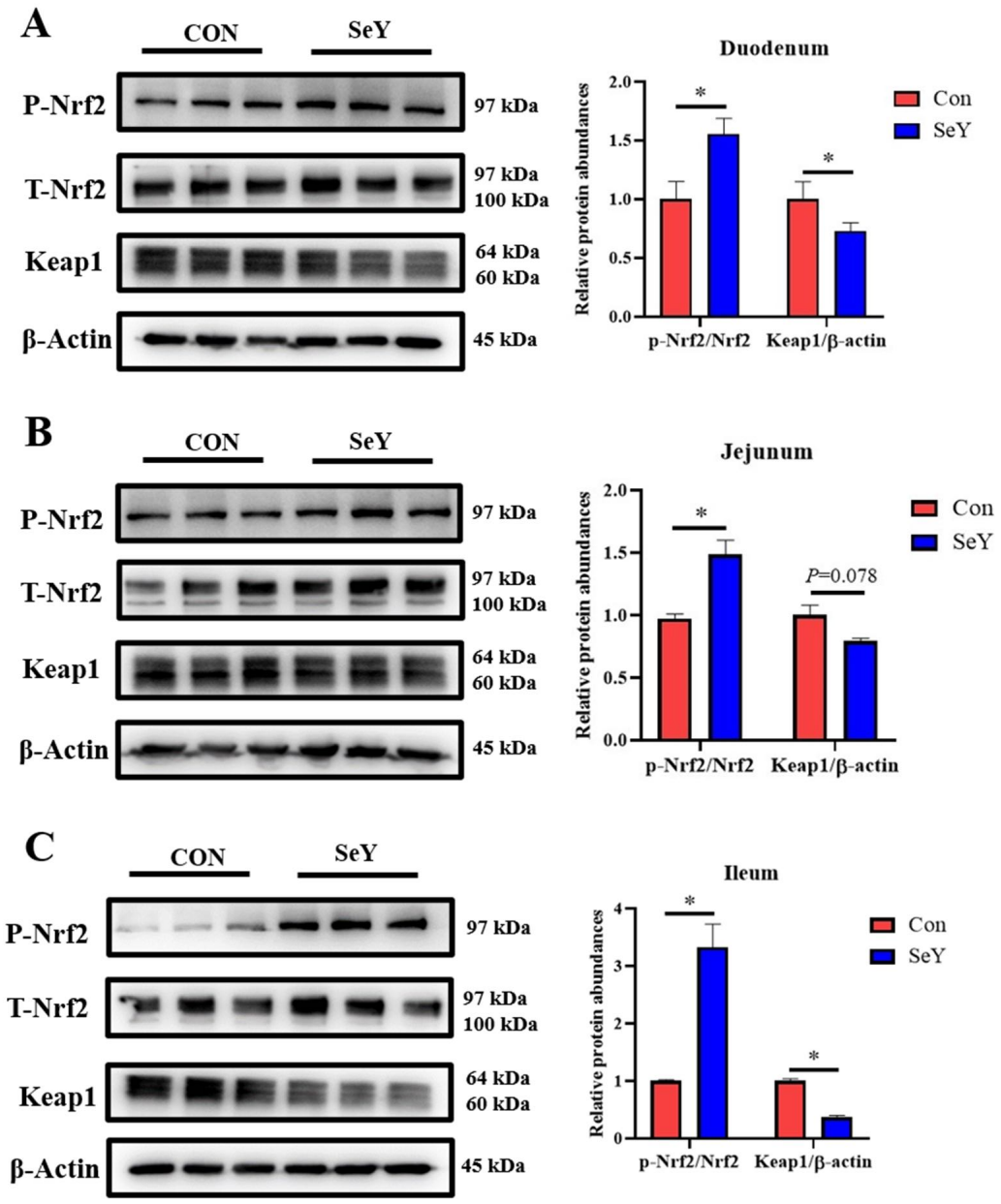

Figure 4. Effects of maternal SeY supplementation on the relative protein abundances involved in the Nrf2/Keap1 signaling pathway in the duodenum (**A**), jejunum (**B**), and ileum (**C**) of suckling offspring piglets. Data are represented as means ± SEM, $n = 6$ per group, * $p < 0.05$. CON = control group, sows fed basal diet containing 0.3 mg/kg Se as Na_2SeO_3; SeY = SeY group, sows fed basal diet with 0.2 mg/kg Se as Se-enriched yeast (Se-yeast with 2000 mg/kg Se).

The fecal microbial communities and differential microbial biomarkers based on the relative abundance of sows on day 1 and 21 of lactation at the phylum and genus levels are displayed in Figures 7 and 8, respectively. At the phylum level, the top four dominant phyla were commonly identified in the two groups of fecal microbiotas of sows on day 1 and 21 of lactation: Firmicutes, Bacteroidota, Proteobacteria, and Spirochactota. In addition, the abundance distribution in the top 20 most abundant fecal microbiota of sows on day 1 (Figure 7B) and 21 (Figure 8B) of lactation at the genus level was plotted as a heatmap. Heatmaps were made using the vegan package in R (v3.3.1). Differences in the relative abundances of the community components of fecal microbiota of sows between the CON and SeY groups were further analyzed using the Mann–Whitney U-test via STAMP software 2.1.3. In addition, the relative abundance differences analyzed via STAMP and LEfSe analyses were

performed together to detect the differential marker bacterium. The linear discriminant analysis (LDA) effect size (LEfSe) (http://huttenhower.sph.harvard.edu/LEfSe (accessed on 5 September 2023)) was calculated to identify differentially abundant bacterium (phylum to genus level) among the different samples (LDA score > 2.0, $p < 0.05$). At the phylum level, the STAMP results and LEfSe analysis together showed no significant difference between the two groups on day 1 and 21 of lactation. At the genus level, compared with the CON group, the SeY group had a significant increase in the relative abundances of *unclassified_f__Lachnospiraceae*, *Prevotellaceae_UCG-001*, *Lachnospiraceae_NK4A136_group*, and *norank_f_ Oscillospirales* on day 1 (Figure 7C,D) of lactation, and there was a significant decrease in the UCG-002 of sows on day 21 (Figure 8C,D) of lactation.

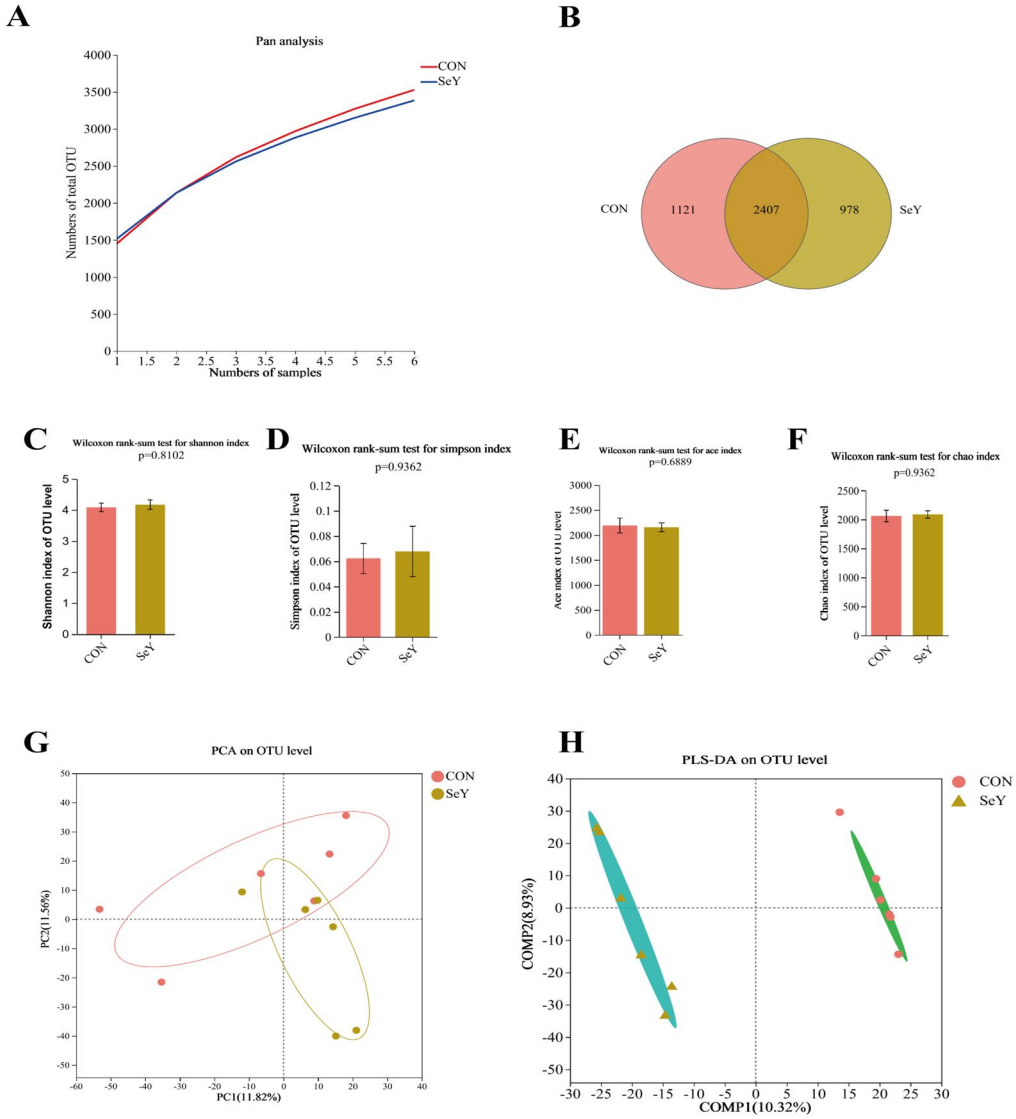

Figure 5. Effects of dietary SeY supplementation on the composition and structure of fecal microbiota in sows on day 1 of lactation. The Pan species curve of the sequencing depth of sows (**A**). The Venn diagram of OTU network analysis (**B**). The fecal microbial alpha diversity of sows, including Shannon (**C**), Simpson (**D**), Ace (**E**), and Chao1 (**F**) indexes. The principal component analysis (PCA) plot (**G**) and partial least squares discrimination analysis (PLS-DA) based on an unweighted UniFrac distance score plot (**H**) of microbial communities. $n = 6$ per group. CON = control group, sows fed basal diet containing 0.3 mg/kg Se as Na_2SeO_3; SeY = SeY group, sows fed basal diet with 0.2 mg/kg Se as Se-enriched yeast (Se-yeast with 2000 mg/kg Se).

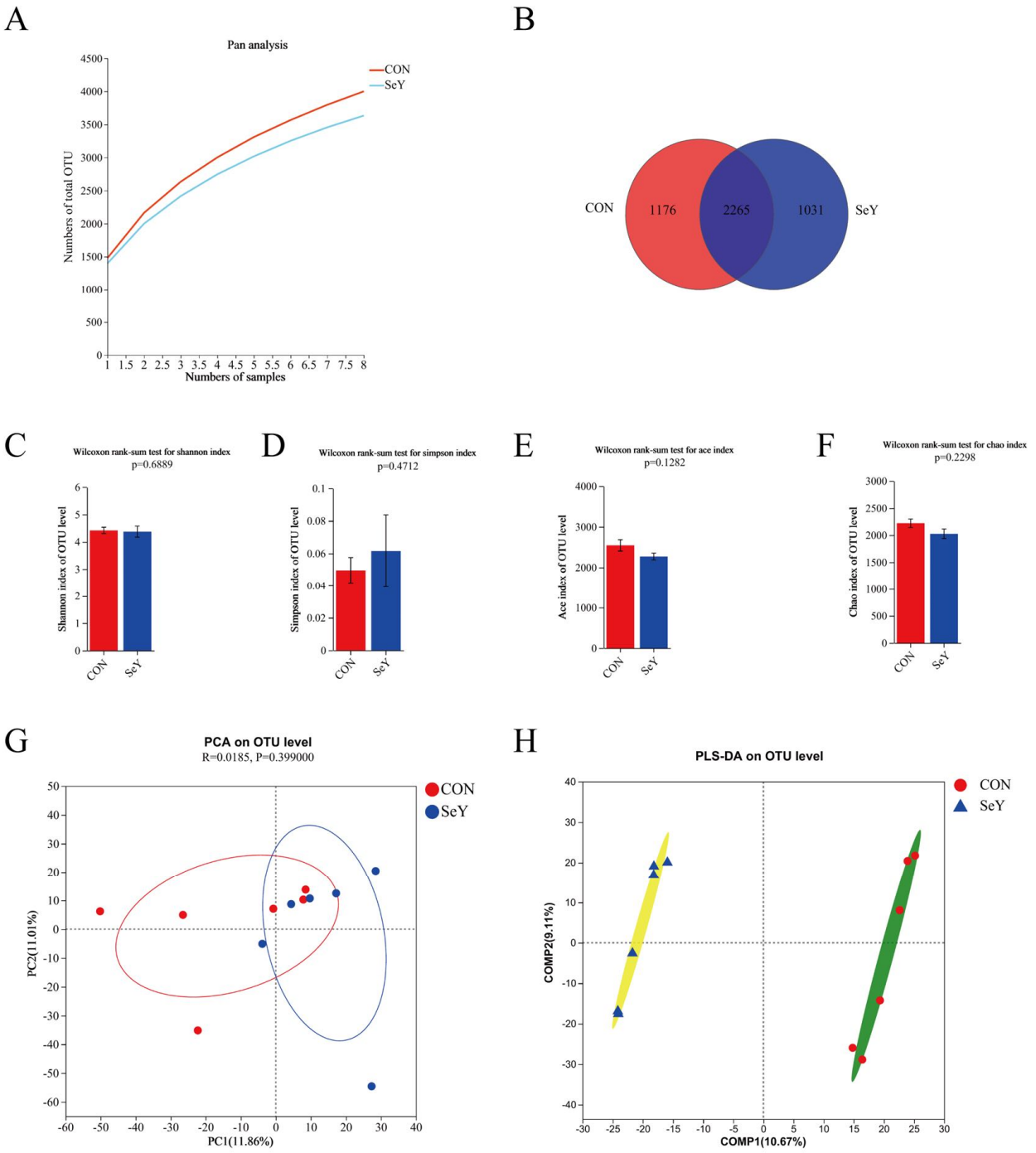

Figure 6. Effects of dietary SeY supplementation on the composition and structure of fecal microbiota in sows on day 21 of lactation. The Pan species curve of sequencing depth of sows (**A**). The Venn diagram of OTU network analysis (**B**). The fecal microbial alpha diversity of sows, including Shannon (**C**), Simpson (**D**), Ace (**E**), and Chao1 (**F**) indexes. The principal component analysis (PCA) plot (**G**) and partial least squares discrimination analysis (PLS-DA) based on an unweighted UniFrac distance score plot (**H**) of microbial communities. n = 6 per group. CON = control group, sows fed basal diet containing 0.3 mg/kg Se as Na_2SeO_3; SeY = SeY group, sows fed basal diet with 0.2 mg/kg Se as Se-enriched yeast (Se-yeast with 2000 mg/kg Se).

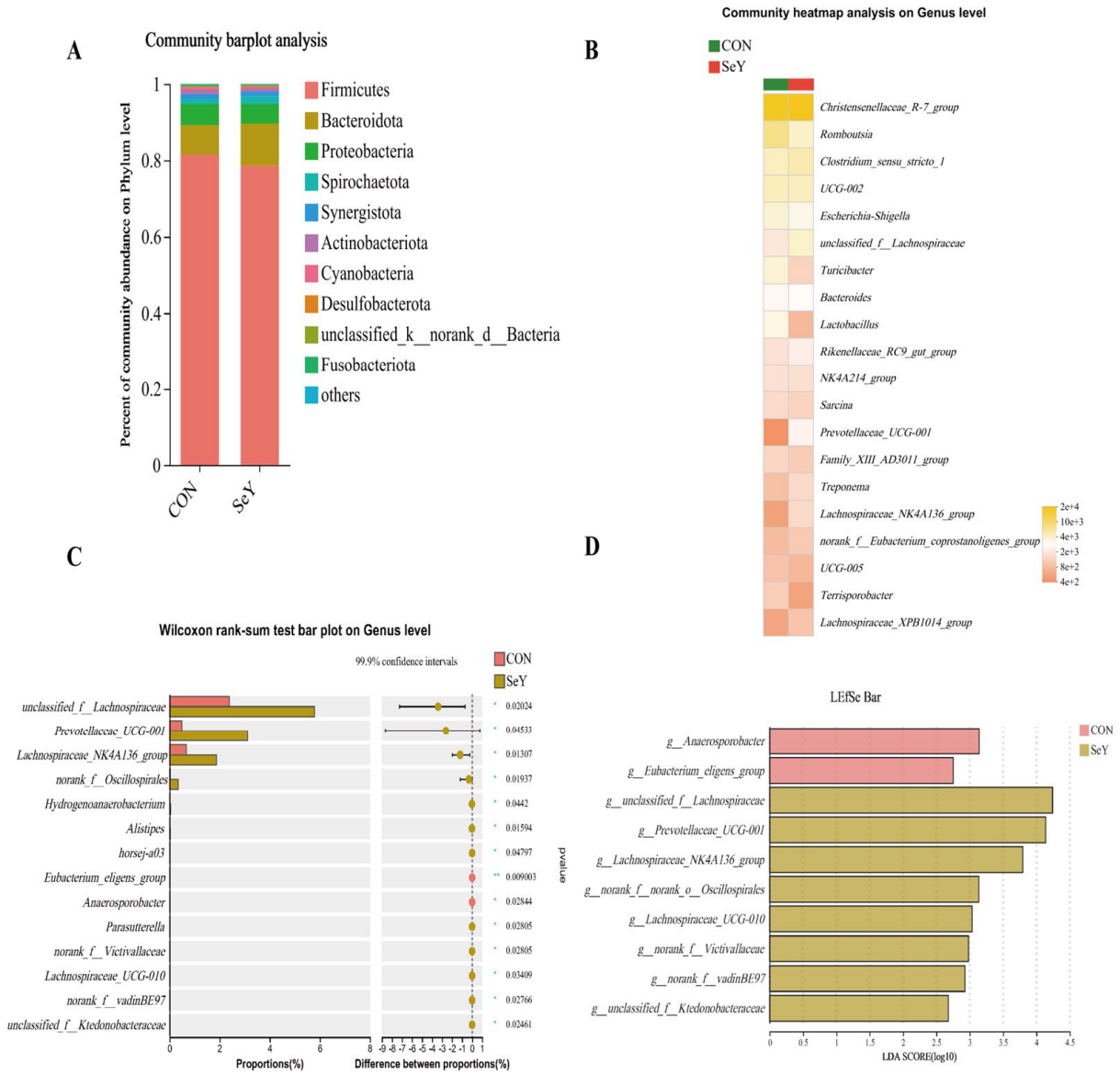

Figure 7. The fecal microbial communities and differential microbial biomarkers based on the relative abundance at phylum and genus levels of sows on day 1 of lactation. The average relative abundances of phylum level greater than 0.1% proportion (**A**) and genus level for the top 20 relative abundance (**B**) were listed. $n = 6$ per group, * $p < 0.05$, ** $p < 0.01$. The relative abundance differences analyzed via STAMP (**C**). Differential microbial biomarkers based on the relative abundance, filtrated via LEfSe analysis (**D**). CON = control group, sows fed basal diet containing 0.3 mg/kg Se as Na_2SeO_3; SeY = SeY group, sows fed basal diet with 0.2 mg/kg Se as Se-enriched yeast (Se-yeast with 2000 mg/kg Se).

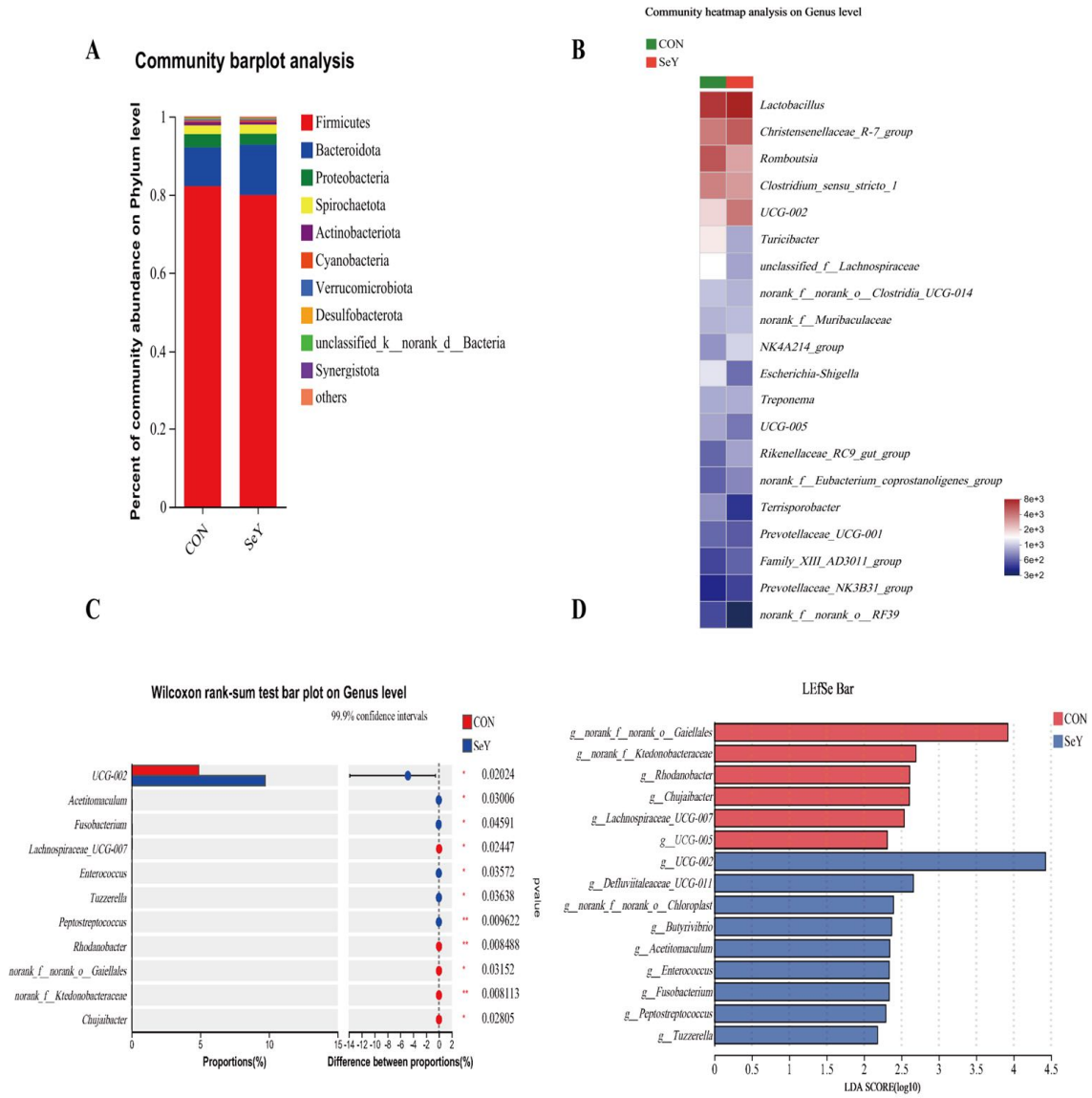

Figure 8. The fecal microbial communities and differential microbial biomarkers based on the relative abundance at phylum and genus levels of sows on day 21 of lactation. The average relative abundances of phylum level greater than 0.1% proportion (**A**) and genus level for the top 20 relative abundance (**B**) were listed. $n = 6$ per group, * $p < 0.05$, ** $p < 0.01$. The relative abundance differences analyzed via STAMP (**C**). Differential microbial biomarkers based on the relative abundance, filtrated via LEfSe analysis (**D**). CON = control group, sows fed basal diet containing 0.3 mg/kg Se as Na_2SeO_3; SeY = SeY group, sows fed basal diet with 0.2 mg/kg Se as Se-enriched yeast (Se-yeast with 2000 mg/kg Se).

4. Discussion

Maternal nutrition is a predominant factor in regulating the redox status, immunity, and gut health of offspring piglets [24]. Thus, this study evaluated the effect of maternal SeY supplementation during late gestation and lactation on sow performance, transfer of Se and antioxidant capacity, and gut microbiota community, as well as on the intestinal health of offspring. Consistent with our experimental hypothesis, the findings in the present study indicated that maternal SeY supplementation during late gestation and lactation improved the lactation performance, redox status, and Se level transfer, and that it altered the gut microbiota in sows. In addition, maternal SeY supplementation enhanced the small intestinal barrier function and microbiota community of the weaned offspring.

The results of this study will offer a reference for the function of SeY in sows' diet and maternal–offspring integration.

As previously mentioned, when supplemented with inorganic selenite (0 to 0.3 ppm), dietary Se ranged from 0.227 to 0.651 ppm [25]. In our study, Se from basal ingredients alone provided 0.34 ppm for gestation diets and 0.37 ppm for lactation diets. These Se concentrations are greater than the NRC (2012) recommendation of 0.15 ppm, and the supplemental level frequently used in the feed industry is 0.30 ppm. In addition, many studies have indicated that maternal Se can transfer to the progeny through the placenta and milk [16,26,27]. Also, Mahan et al. [15] reported that the offspring of sows that were fed organic Se had a higher Se status both at birth and during weaning than those of sows that were fed inorganic Se. This was confirmed in the current study, where the maternal SeY intake increased the levels in the sows' plasma and milk as well as those of the offsprings' plasma. Consistent with a previous study, our results revealed that SeY was delivered from sow to piglet through the colostrum and milk.

Many previous studies have reported that a dietary Se source fed during late gestation and lactation had no effect on the reproductive performance of sows, including the number of pigs per litter, piglet birth weight, or litter gain from birth to weaning [15,16]. Our previous study also reported that increasing the Se supply during late gestation did not affect the reproductive performance of sows [21]. In agreement with the above, the current study indicated that a short-term increase in maternal SeY supply during late gestation did not affect the reproductive performance of sows. Nevertheless, maternal SeY supplementation during late gestation and lactation significantly increased the final average weight during the first week and the mean ADG of offspring piglets during the first week of lactation. Another study also reported that organic Se improved the ADG of suckling piglets at the first week of lactation [13]. Interestingly, we first found that the weaning-to-estrus interval was significantly shortened by SeY supplementation in sow diets. In general, 90–95% of the multiparity sows are predicted to exhibit estrus within a week after weaning. Moreover, the reduced estrus is identified as sufficient nutrient intake and body reserves [28]. Thus, SeY supplementation during late gestation and lactation might promote nutrient absorption in sows, and further nutrient digestibility experiments are needed to verify this finding. Our study indicates that short-term, it did not promote intrauterine fetal growth; however, continuous supplement during lactation increased the lactating performance of sows, including the average weight and mean ADG of piglets during the first week.

ROS overproduction during parturition and lactation may lead to oxidative stress in both the maternal pig and offspring [29]. Moreover, piglets suffer from serious oxidative stress during the newborn and weaned stages [30]. To explore the effect of maternal SeY supplementation on the antioxidant capacity transfer in parturition and lactation, we analyzed the redox status in the plasma of sows and offsprings as well as in milk. Many previous studies have jointly reported that maternal dietary organic Se supplementation during late or entire gestation could both enhance the antioxidant capacities of sows and transfer this to their offspring [13,31,32]. Similar to previous studies, our results showed that maternal SeY supplementation increased the levels of T-AOC, T-SOD, and GSH-Px, while it reduced the MDA content in the plasma and milk of sows and the plasma of their offspring. Our results verified that the maternal enhanced redox status brought about by SeY can transfer to the progeny through milk. In addition, the maternal enhanced redox status transfer was parallel with the increased Se content transfer from sows to offspring.

A previous study suggested that the immune system depends on a sufficient Se status to combat pathogens, viral invasions, and oxidative stress [33]. It has been reported that inorganic Se supplementation in a sow diet obviously increased the blood IgA, IgG, and IgM concentrations of sows and their offspring [34]. Li et al. [35] reported that organic Se addition increased the IgA and IgG in the serum of gilts. In the current study, maternal SeY supplementation during late gestation and lactation also increased the concentrations of IgA in the sow and offspring plasma and the IgG in sow milk. Similarly, other reports have

found that increasing the Se intake in sows and cows during late gestation improved the immunoglobulin transfer through maternal–offspring transmission [36,37]. Our previous study also showed that increasing the organic Se supply in sows' diet led to higher levels of plasma IgA in sows and offspring, plasma IgG in offspring, and IgA as well as IgM in milk [21]. These above results reflect that Se could enhance the passive transfer of antibodies through maternal–offspring transmission.

Furthermore, due to the antioxidant activity of selenoproteins, SeY has long been deemed to improve the intestinal barrier function through alleviating oxidative stress [38]. Proper epithelial cells are held together by the apical junctional complex, which includes transmembrane TJ proteins (claudins and occludin) and the cytosolic scaffold proteins (ZO-1) [39]. In addition, the epithelial monolayer is connected with another adheren junction, the E-cadherin-dependent barrier in the small intestine [40]. The mucus layer, an enormous polymeric network of mucins, is the first line of defense preventing the invasion of potential pathogens, and it maintains homeostasis in the small intestine [38]. Immunofluorescence analysis of MUC1 showed that maternal SeY supplementation increased the MUC1 secretion in the small intestine but did not affect the distribution in the intestinal epithelium of offspring piglets. In addition, the Western blot results showed that maternal SeY supplementation during late gestation and lactation also up-regulated the relative protein abundances of E-cadherin, ZO-1, occludin, and claudin-1 in the small intestine of weaned offspring piglets, suggesting that SeY can promote the development of intestinal barrier function in offspring piglets through mother–child interactions. Consistent with our results, Liu et al. [14] reported that adding SeY effectively improved the distribution and abundance of tight-junction protein in the jejunum. The study suggested that increased SeY addition improved intestinal barrier functions through suppressing epithelial cell apoptosis that is induced by oxidative stress [14]. Our results indicated that maternal SeY supplementation during late gestation and lactation improved the small intestinal chemical barrier and mechanical integrity of the offspring piglets.

It can be seen from previous studies that oxidative stress damages the intestinal barrier functions of the intestinal epithelial cells. Thus, whether the improved TJ protein abundance is related with the increased antioxidant capacity caused by maternal SeY supplementation was further explored. Nrf2 appears to contribute to redox homeostasis in intestinal epithelial cells [41]. Therefore, the Nrf2/Keap1 oxidative pathway was selected in order to detect the above changes in the small intestinal TJ proteins in offspring piglets. Nrf2 is a primary antioxidant transcription factor and participates in regulating the synthesis of antioxidant enzymes (MnSOD and GSH-Px) [42]. Under physiological conditions, Nrf2 is inhibited by Keap1 in the cytoplasm [43]. To combat oxidative stress, the Keap1/Nrf2 dissociation prompts the phosphorylation of Nrf2 to translocate into the nucleus and activate the transcription of an antioxidant gene [44]. Scientific evidence has proven that Nrf2-deficiency-induced oxidative stress and inflammation may indirectly impair the intestinal barrier function [45,46]. It has also been reported that SeY alleviated oxidative-stress-induced small intestinal mucosa disruption through elevating the Nrf2 expression in weaned pigs [14]. Another study found that the SeY supplementation protected chickens against the ochratoxin A-induced oxidative stress through activating in the Nrf2/Keap1 pathway [47]. The cell-cultured experiment also showed that selenomethionine alleviated oxidative stress that was induced by zearalenone via the Nrf2/Keap1 signaling pathway in IPEC-J2 cells [48]. Based on our results, maternal SeY supplementation during late gestation and lactation significantly up-regulated the phosphorylated Nrf2 and down-regulated the Keap1 protein abundances in the small intestine of weaning offspring. The activation of the Nrf2/Keap1 pathway thereby improved the redox status in offspring, which is confirmed by the results of the serum antioxidant capacity indicators.

Nutritional components are also key factors affecting the structure and abundance of the gut community structure [49]. To investigate the effects of SeY supplementation during late gestation and lactation on fecal microbiota proliferation in sows on day 1 and 21 of lactation, the microbiota compositions were analyzed using high-throughput 16S

rRNA sequencing. Microbial diversity is considered to improve the stability of microbiota communities [50]. In the present study, the PCA and PLS-DA results of the beta-diversity analysis showed that the microbial community structure between the CON and SeY groups presented significant separations, suggesting that maternal SeY supplementation altered the sows' fecal microbial community when measured on day 1 and 21 of lactation. The intestine is the harbor of numerous microbial species, belonging predominantly to the phylum Firmicutes or Bacteroidetes [51]. The present study also showed that Firmicutes and Bacteroidetes were the top two dominant phyla between the two groups. To identify the effect of dietary SeY supplement on the fecal differential bacteria in the sows on day 1 and 21 of lactation, the abundance difference and LEfSe analysis were united to analyze the differential bacterium. At the genus level, the STAMP results and LEfSe analysis together showed that dietary SeY addition increased the relative abundances of Lachnospiraceae, Prevotellaceae_UCG-001, Lachnospiraceae_NK4A136_group, and *Oscillospirales* on day 1 of lactation. *Unclassified_f_Lachnospiraceae* had been illustrated to be instrumental in producing butyrate and were associated with the inhibition of intestinal disorders [52]. In addition, to our knowledge, *Lachnospiraceae_NK4A136_group* may improve the intestinal barrier function of rats, comprises one of the main butyrate-producing bacteria, and has potentially beneficial effects on antioxidation [53,54]. *Prevotellaceae_UCG-001* belongs to the Prevotellaceae family, which has been proposed to digest amino acids and enhance calorie extraction from resistant starches, oligosaccharides, and other indigested carbohydrates [55]. In short, *unclassified_f_Lachnospiraceae*, *Prevotellaceae _UCG-001*, and *Lachnospiraceae_NK4A136_group* were all known as SCFAs producing bacteria through indigestible carbohydrates metabolization [56]. Thus, the increased SCFAs-producing bacterium might accelerate the SCFAs synthesis further to promote gut homeostasis of sows in the SeY group. Specifically, SCFAs-activated G-protein coupled receptor (GPCR) signaling has been reported to have essential roles in the colonic barrier function and anti-inflammatory and antioxidative effects in the intestine [57].

5. Conclusions

Taken together, these findings suggest that maternal SeY supplementation during late gestation and lactation could improve the piglets' growth performance during the first week of lactation, Se status, antioxidant capacity, and immunoglobulin transfer, as that it could alter the fecal microbiota composition by increasing antioxidative-related and SCFAs-producing microbiota in sows. Through these mechanisms, these changes contributed to enhancing the small intestinal barrier function and activating the Nrf2/Keap1 pathway in offspring.

Supplementary Materials: The following supporting information can be downloaded at https://www.mdpi.com/article/10.3390/antiox12122064/s1: Table S1: Composition and nutrient levels of basal diets for sows (air-dry basis; %).

Author Contributions: Conceptualization, W.G. and L.X.; methodology, L.X. and S.Z. (Shihai Zhang); software, L.X. and T.L.; investigation, L.X., S.Z. (Shuchang Zhang) and F.C.; data curation, X.L. and X.Y.; writing—original draft preparation, L.X.; writing—review and editing, W.G. and S.Z. (Shihai Zhang); supervision, W.G. All authors have read and agreed to the published version of the manuscript.

Funding: This research was supported by the Project of Swine Innovation Team in Guangdong Modern Agricultural Research System (2022KJ126), National Key Research and Development Program (No. 2021YFD1300401-2), Guangdong Basic and Applied Basic Research Foundation (No. 2021A1515010440).

Institutional Review Board Statement: All animal experiment procedures involving the use and care of animals during this study were approved by the South China Agricultural University Animal Care and Animal Use Committee (no. SYXK2019-0136, Guangzhou, China).

Informed Consent Statement: Not applicable.

Data Availability Statement: Please contact the corresponding author if further information is required.

Acknowledgments: The authors thank Lin Gang of Alltech Inc., Nicholasville, KY, USA for providing the organic SeY source (Sel-Plex2000) and Guangdong Foodstuffs IMP. & EXP. Group Co., Ltd. in Heyuan City, Guangdong Province, China.

Conflicts of Interest: The authors declared no conflict of interest.

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frontiers in Cell and Developmental Biology

REVIEW
published: 17 November 2020
doi: 10.3389/fcell.2020.604896

Regulation of the JAK2-STAT5 Pathway by Signaling Molecules in the Mammary Gland

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Specialty section:
This article was submitted to Signaling, a section of the journal Frontiers in Cell and Developmental Biology

Received: 10 September 2020
Accepted: 29 October 2020
Published: 17 November 2020

Citation:
Tian M, Qi Y, Zhang X, Wu Z, Chen J, Chen F, Guan W and Zhang S (2020) Regulation of the JAK2-STAT5 Pathway by Signaling Molecules in the Mammary Gland. Front. Cell Dev. Biol. 8:604896. doi: 10.3389/fcell.2020.604896

Janus kinase 2 (JAK2) and signal transducers and activators of transcription 5 (STAT5) are involved in the proliferation, differentiation, and survival of mammary gland epithelial cells. Dysregulation of JAK2-STAT5 activity invariably leads to mammary gland developmental defects and/or diseases, including breast cancer. Proper functioning of the JAK2-STAT5 signaling pathway relies on crosstalk with other signaling pathways (synergistically or antagonistically), which leads to normal biological performance. This review highlights recent progress regarding the critical components of the JAK2-STAT5 pathway and its crosstalk with G-protein coupled receptor (GPCR) signaling, PI3K-Akt signaling, growth factors, inflammatory cytokines, hormone receptors, and cell adhesion.

Keywords: Stat5, JAK2, signaling molecules, milk production, mammary gland development

INTRODUCTION

The mammary gland is a critical organ in mammals and is involved in milk production and delivery. The mammary gland is a derivative of the skin, develops early during the embryonic stage and further develops and differentiates into a functional mammary gland during pubertal and adult stages (Gjorevski and Nelson, 2011). The development of the embryonic mammary gland starts with the formation of placodes, which then invaginate the mesenchyme and form mammary gland buds (Robinson, 2008). These buds continue to elongate and bifurcate, developing into a rudimentary gland prior to birth. Subsequently, the rudimentary mammary gland enters a quiescent phase and grows isomorphic with the body. The second stage of mammary gland development is initiated at puberty. During this period, mammary gland development is regulated and sustained by hormones, growth factors and cytokines. The tips of the rudimentary ducts transform into terminal end buds (TEBs) and penetrate into the mammary fat pad (Hinck and Silberstein, 2005). The mature mammary duct is mainly composed of myoepithelial cells (outer layer) and luminal epithelial cells (inner layer). During gestation, these mammary epithelial cells differentiate into milk-producing secretory alveoli, which synthesize the majority of milk fat, protein and lactose (Brisken et al., 1999; Oakes et al., 2008). The mammary gland rapidly undergoes involution after weaning, and approximately 80% of the epithelium is removed via apoptosis (Alexander et al., 2001; Watson, 2006).

Hormones are crucial for ductal morphogenesis in the mammary gland. During puberty, estrogen, growth hormone (GH) and prolactin are required for the development of the mammary gland (McNally and Martin, 2011). Estrogens are mainly secreted by the ovary and sensed by estrogen receptors (ERs), which are nuclear receptors. ERs regulate the transcription of multiple genes with a variety of coregulators [such as steroid receptor coactivator 1 (SRC-1) and Cbp/p300-interacting transactivator 1 (CITED1)] in the mammary gland (Howlin et al., 2006a,b). In contrast to estrogen, GH and prolactin regulate mammary gland function through the phosphorylation of Janus kinase 2 (JAK2) and activation of its downstream regulator signal transducers and activators of transcription 5 (STAT5) (Ihle, 1996). JAK2-STAT5 is proposed to be a critical signaling pathway in the mammary gland. In addition to the abovementioned hormones, JAK2-STAT5 is also regulated by cytokines such as IL-12, INF-γ, IL-4, IL-13, and IL-6. Recent studies have provided additional evidence that other prominent cellular signaling pathways (GPCR, PI3K/Akt and cell adhesion) might also be involved in crosstalk with JAK2-STAT5. The signaling pathways that interact with JAK2-STAT5 are overwhelmingly complex. In this review, we focus on the constitutive and extensive communication between JAK2-STAT5 and other signaling pathways.

STATS AND JAKS IN THE MAMMARY GLAND

In the mammary gland, five STATs (STAT1, 3, 5a,b, and 6) have been identified (Watson and Neoh, 2008). STAT1 and STAT6 have been reported to play minor roles in the mammary gland. Although STAT1 is highly activated in the mammary gland, STAT1 knockout does not significantly affect ductal or alveolar morphogenesis (Klover et al., 2010). STAT6 is downstream of IL-4 and IL-3 and partially regulates the development of the alveoli. However, mice lacking STAT6 are still able to lactate (Khaled et al., 2007). In contrast to STAT1 and STAT6, STAT5, and STAT3 are the key STATs in the mammary gland. STAT5 promotes the proliferation of mammary gland epithelial cells, while STAT3 regulates the process of apoptosis during involution (Chapman et al., 2000). During late pregnancy and lactation, STAT5 is highly activated, as high levels of STAT5 can be detected in the nucleus in epithelial cells of the mammary gland, whereas STAT5 levels are undetectable during the involution period (Bednorz et al., 2011). STAT5 is involved in the side branching and maturation of alveolar cells (Vafaizadeh et al., 2010). Conditional inhibition of STAT5 in the mammary gland at different times further reveals its roles during specific periods of lactation (Reichenstein et al., 2011). Knocking out STAT5 during the first 3 days of lactation affects the expression of ER and connexin 32 (C × 32, a gap junction protein). STAT5 knockout during the first 10 days of lactation decreases neonatal body weight by 30–40% due to changes in mammary gland morphology and a reduction in milk production. Two isoforms of STAT5 (STAT5a and STAT5b) have been identified in the mammary gland (Liu et al., 1996). STAT5a and STAT5b are encoded by two separate genes located on chromosome 11 (mouse) and chromosome 17 (human). These genes are highly homologous (96% conserved at the protein level) and contain different C-terminal regions (Rani and Murphy, 2016). Knocking out STAT5a inhibits the normal development and differentiation of the mammary gland during pregnancy, whereas deletion of STAT5B only impairs body growth (Cui et al., 2004). After weaning, the phosphorylation of STAT5 is significantly decreased with increased phosphorylation of STAT3 (Watson and Neoh, 2008). The switch from the activation of STAT5 to STAT3 indicates the triggering of mammary gland involution. Activated STAT5 and STAT3 can enter the nucleus and regulate related gene expression. STAT5 is thought to regulate genes related to milk protein synthesis (α-casein) and other genes with unclear biological functions (kallikrein-8, prosaposin and Grb10) (Clarkson et al., 2006). In addition, activated STAT5 also regulates ACC1 expression by binding to its promoter and initiating *de novo* synthesis of fatty acids (Mao et al., 2002). Consistently, knocking down STAT5 decreases the expression of ACC1 (Li et al., 2019). It is still not clear whether STAT5 also regulates ACC2. This evidence indicates that STAT5 plays a vital role in milk synthesis. As expected, STAT3 regulates apoptosis by targeting the apoptosis regulator genes CCAAT enhancer binding protein-δ and c-Fos and regulating the PI3K/Akt signaling pathway (Clarkson et al., 2006).

Two isoforms of JAK (JAK1 and JAK2) are expressed in the mammary gland, and these factors are upstream of STAT. The biological functions of these two JAKs are somewhat different. Briefly, prolactin mainly regulates STAT5 activation through JAK2, while JAK1 is primarily regulates STAT3 activation (Xie et al., 2002). It is worth noting that JAK2 not only binds to the prolactin receptor but can also enter the nucleus. The potential mechanisms by which JAK2 regulates nuclear gene expression in the mammary gland are by modulating tyrosine kinase activity and preventing protein degradation. For example, JAK2 interacts with transcription factor nuclear factor 1-C2 (NF1-C) and enhances its stability, which further regulates the expression of genes involved in milk synthesis (Nilsson et al., 2006).

NOVEL FACTORS IN JAK2-STAT5 ACTIVATION

The JAK2-STAT5 signaling pathway was identified long ago in the mammary gland. Recent studies have shown that additional components are required for the activation of JAK2-STAT5 (**Figure 1**). A prolyl isomerase called cyclophilin A (CypA) has been found to be an essential component for JAK2-STAT5 activation. CypA knockout disrupts mammary gland morphogenesis and differentiation by inhibiting the JAK2-STAT5 pathway (Volker et al., 2018). CUB and zona pellucida-like domain-containing protein 1 (CUZD1) is the other component involved in the regulation of mammary gland differentiation. CUZD1 knockout abolishes STAT5 phosphorylation and impairs mammary ductal branching and alveolar development. However, CUZD1 overexpression in mammary epithelial cells increases STAT5 phosphorylation (Mapes et al., 2017). Immunoprecipitation results show that CUZD1 forms a complex with JAK2 and STAT5, and CUZD1

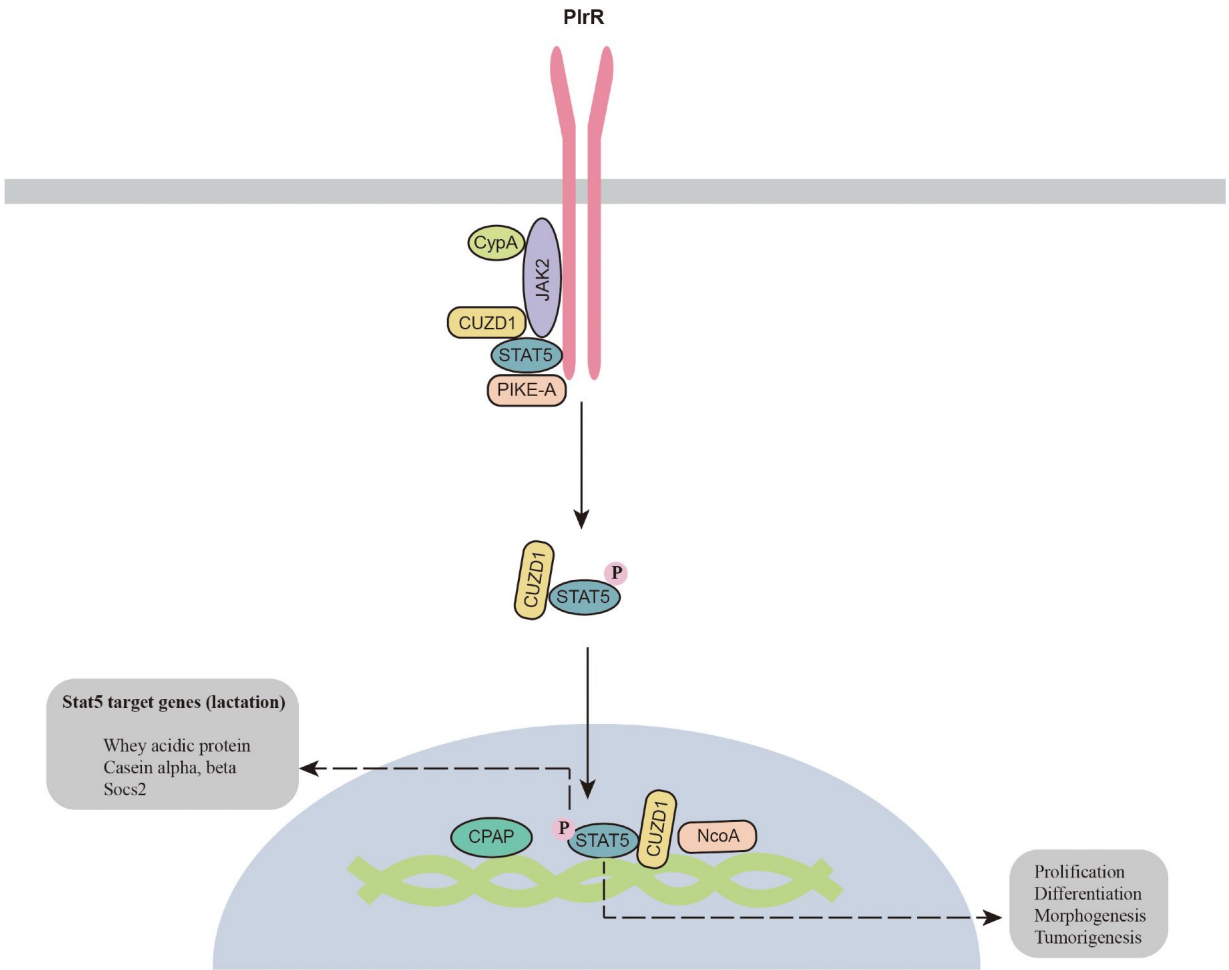

FIGURE 1 | Novel components of the JAK2-STAT5 signaling pathway. CPAP, centrosomal P4.1-associated protein; CypA, cyclophilin A; CUZD1, zona pellucida-like domain-containing protein 1; JAK2, Janus kinase 2; NcoA, nuclear receptor co-activator; PIKE-A, PI 3-kinase enhancer A; PlrR, prolactin receptor; STAT5, signal transducers and activators of transcription 5.

knockout disrupts the connection between JAK2 and STAT5 (Mapes et al., 2017). In addition, PIKE-A has also been reported to participate in complex formation with PRLR and STAT5, which is required for activation of the PRLR and STAT5 signaling pathways (Chan et al., 2010). Knockout of PIKE-A in HC11 mammary gland epithelial cells attenuates cell proliferation by inhibiting STAT5 activation and cyclin D1 expression. The other critical protein for STAT5 activation is zinc finger homeobox 3 (ZFHX3), which is highly expressed during lactation (Zhao et al., 2016). ZFHX3 knockout results in underdevelopment of the mammary gland with decreased PRLR expression and STAT5 phosphorylation. The underlying mechanism by which ZFHX3 regulates the STAT5 signaling pathway is still unknown. The zinc finger transcription factor Miz1, which contains an N-terminal POZ domain and zinc finger motifs, has also been reported to maintain the normal function of the mammary gland. Knockout of Miz1 disrupts the activation of STAT5 signaling through by disrupting intracellular transport and localization of PRLR and ERBB4 (Sanz-Moreno et al., 2014).

In addition, a number of cofactors for p-STAT5 have also been identified in the mammary gland. For example, centrosomal P4.1-associated protein (CPAP) interacts with STAT5 and enhances its activity (Peng et al., 2002). NCoA-1 is another coactivator of STAT5a that regulates the synergistic effects of glucocorticoid receptor and STAT5a on beta-casein expression (Litterst et al., 2003). In summary, JAK2-STAT5 is a complicated signaling pathway, as a number of proteins are required for its activation in the mammary gland. More studies are required to clarify how these components function to regulate the JAK2-STAT5 signaling pathway.

JAK2-STAT5 CROSSTALK WITH THE GPCR SIGNALING PATHWAY

G protein-coupled receptors (GPCRs) are crucial pharmaceutical targets that account for 33% of the targets of Food and Drug Administration (FAD)-approved drugs (Hauser et al., 2017).

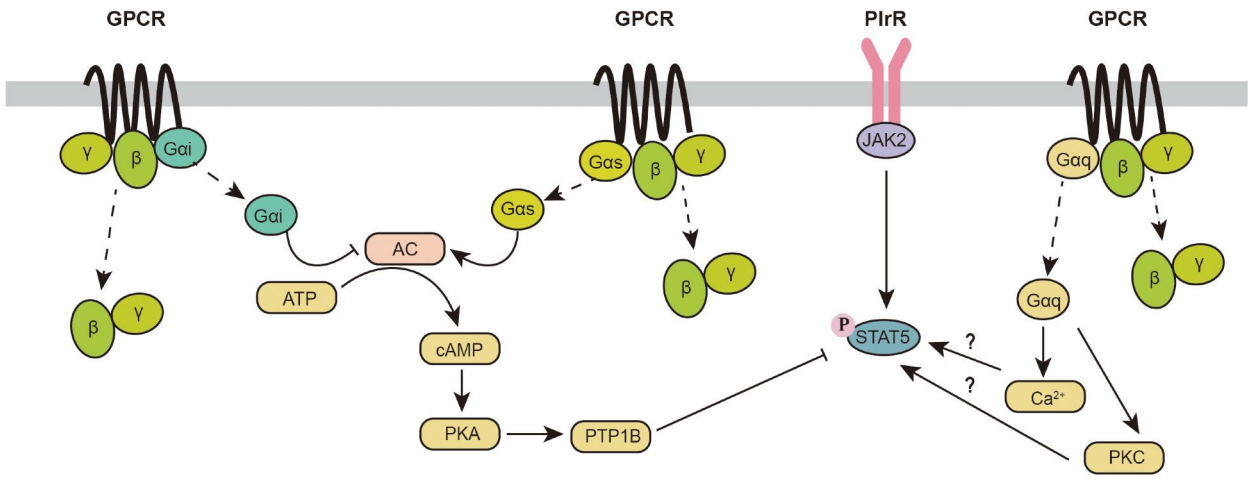

FIGURE 2 | Crosstalk between the GPCR and JAK2-STAT5 signaling pathways. AC, adenylyl cyclase; cAMP, cellular adenosine 3',5'-cyclic monophosphate; GPCR, G protein-coupled receptor; JAK2, Janus kinase 2; PKA, protein kinase A; PKC, protein kinase C; PIrR, prolactin receptor; PTP1B, protein-tyrosine phosphatase 1B; STAT5, signal transducers and activators of transcription 5.

GPCRs are activated by small carboxylic acid metabolites (GPR41, GPR43, GPR81, GPR109A, GPR109B, and GPR84), triglyceride metabolites (GPR40, GPR120, and GPR119), bile acids (GPBAR1), and amino acid and amino acid metabolites (GPR142, CasR, GPR35, TAAR1, and FBR1/2) (Husted et al., 2017). Dietary nutrients and their metabolites can regulate the development and lactation of the mammary gland through the activation of GPCR signaling pathways. In addition, a number of hormones (glucagon, luteinizing hormone, and epinephrine) and neurotransmitters (acetylcholine, dopamine, and serotonin) can also trigger the activation of corresponding GPCRs (Neves et al., 2002). Previously, glucagon, epinephrine and dopamine have been reported to be involved in the regulation of breast cancer (Wang et al., 2002; Ligumsky et al., 2012; Cui et al., 2019), while serotonin controls the development of the mammary gland (Matsuda et al., 2004). These receptors control multiple signaling cascades and regulate various physiological functions. Therefore, understanding the interplay between GPCRs and JAK2-STAT5 is crucial.

GPCRs are 7-transmembrane proteins that are coupled to heterotrimeric G proteins on the intracellular side of the membrane (Thal et al., 2018). The G protein contains G_{α} (binds to GTP/GDP), G_{β} and G_{γ} subunits (Wettschureck and Offermanns, 2005). The unique downstream signal activation of different GPCRs mainly relies on the classification of the G_{α} subunits. To date, four G_{α} subunits ($G_{\alpha s}$, $G_{\alpha i}$, $G_{\alpha q/11}$, and $G_{\alpha\ 12/13}$) have been identified in cells (Syrovatkina et al., 2016). $G_{\alpha i}$ and $G_{\alpha s}$ mainly participate in the regulation of cellular adenosine 3′,5′-cyclic monophosphate (cAMP) through adenylyl cyclase (AC) (Neves et al., 2002). cAMP is a critical second messenger that mainly regulates cellular biological functions through protein kinase A (PKA) and exchange proteins directly activated by PKA and cAMP (EPAC). $G_{\alpha q}$ can increase cellular Ca^{2+} signaling and activate protein kinase C (PKC)-dependent signaling pathways (Neves et al., 2002; Winzell and Ahrén, 2007).

Originally, it was reported that GPCR activation has minor effects on the JAK2-STAT5 signaling pathway. However, recent evidence indicates an intimate relationship between GPCR and JAK2-STAT5 signaling pathways (**Figure 2**). Activation of GPCRs coupled to the $G_{\alpha q}$ subunit has been reported to increase the phosphorylation of STAT5. The oxytocin receptor (OXTR) is a G protein-coupled receptor that binds to $G_{\alpha q}$. Overexpression of OXTR in the mouse mammary gland increases phosphorylation of STAT5 and induces lactation during the early lactation period, whereas OXTR-induced phosphorylation of STAT5 is decreased with attenuated milk production during the peak lactation period (Li et al., 2018). Furthermore, knockout of OXTR can impair milk ejection (Li et al., 2018). This finding indicates that the effect of the $G_{\alpha q}$ signaling pathway in the mammary gland may be dependent on different lactation periods. GPR54 is another GPCR coupled to $G_{\alpha q}$, which can be activated by kisspeptins (Kps). GPR54 is highly expressed during the lactation period. Activation of GPR54 increases β-casein synthesis in the mammary gland with activation of the mTOR, ERK1/2, and STAT5 signaling pathways (Kobayashi et al., 2016). In summary, these data provide some primary evidence for a potential link between the $G_{\alpha q}$ signaling pathway and JAK2-STAT5. However, the underlying mechanism involved in this process is not clear.

In addition to $G_{\alpha q}$, $G_{\alpha s}$- and $G_{\alpha i}$-related signaling pathways also participate in the phosphorylation of STAT5. As a direct downstream target of the $G_{\alpha s/\alpha i}$ signaling pathway, increased cellular cAMP significantly decreases STAT5 phosphorylation and β-casein synthesis through an increase in protein-tyrosine phosphatase 1B (PTP1B) (Chiba et al., 2016). In addition, it has been reported that PKA inhibition inhibits the secretion of newly synthesized caseins (Clegg et al., 1998). PKA inhibits the vesicular structure of the Golgi body and inhibits casein production mainly through exocytosis (Clegg et al., 1998). Furthermore,

PKA is also known to decrease protein synthesis through the inhibition of the mTORC1 signaling pathway (phosphorylation of raptor on Ser792) (Jewell et al., 2019). Because $G_{\alpha s}$ is a positive regulator of cAMP and $G_{\alpha i}$ is a negative regulator of cAMP, activation of the $G_{\alpha s}$ signaling pathway inhibits the stat5 signaling pathway, while triggering $G_{\alpha i}$ signaling can activate the STAT5 signaling pathway.

It is worth noting that many GPCRs are not coupled to a unique G_{α} protein, which makes the situation more complicated. For example, melatonin regulates mammary gland function through the melatonin receptors MT1 and MT2. MT2 is only coupled to a $G_{\alpha i}$ subunit, while MT1 is coupled to both $G_{\alpha i}$ and $G_{\alpha q}$ subunits (Tosini et al., 2014). Although $G_{\alpha i}$ and $G_{\alpha q}$ are thought to individually activate STAT5 in the mammary gland, overexpression of MT1 in the mammary gland surprisingly inhibits mammary gland development and milk synthesis, which is consistent with the decrease in STAT5 phosphorylation and the expression of estrogen and progesterone receptors (Xiang et al., 2012). One possible reason for this contradictory finding might be that the activation of GPCRs activates not only the G_{α} signaling pathway but also $G_{\beta\gamma}$ subunits, regulating many downstream effector targets. At present, the effects of $G_{\beta\gamma}$ on the phosphorylation of STAT5 in the mammary gland are still unclear. In more complicated situations, some GPCRs might be coupled to $G_{\alpha s}$ and $G_{\alpha q}$. Recent studies indicate that the activation of GPCRs could be biased (Qiao et al., 2020; Suomivuori et al., 2020). Thus, it is unrealistic to hypothesize which G_{α} subunits will be dominantly activated. More studies are needed to identify the effects of different GPCRs on the phosphorylation of STAT5.

JAK2-STAT5 CROSSTALK WITH THE PI3K-AKT SIGNALING PATHWAY

During puberty, branching morphogenesis is initiated by GH, estrogen, and IGF1. Intriguingly, GH, estrogen and IGF-1 are all involved in the activation of the PI3K/Akt signaling pathway. Breast cancer is the most common health risk for women. Approximately two-thirds of breast cancers are hormone-dependent (Subramani et al., 2017). Estrogen and GH dysregulation are also closely related to breast cancer. Specifically, genetic ablation of p110α (a catalytic subunit of PI3K) inhibits tumor formation, while knocking out p110β enhances ductal branching and tumorigenesis (Utermark et al., 2012). This evidence suggests that PI3K/Akt signaling is involved in the regulation of normal mammary gland growth and breast cancer development.

Akt is an important regulator of mammary gland development and milk synthesis. During the gestation and lactation periods, total and phosphorylated Akt are significantly increased in the mammary gland and are significantly decreased during the involution period (Schwertfeger et al., 2001; Boxer et al., 2006). Three isoforms of Akt have been identified in the mammary gland. Different Akt subtypes seem to execute different functions. Knockout of Akt1 but not Akt2 or Akt3 interferes with the activation of STAT5, delays differentiation and promotes apoptosis in the mammary gland (Maroulakou et al., 2008). However, Chen et al. (2010) found that knocking out either Akt1 or Akt2 in mice still resulted in normal mammary epithelial differentiation and STAT5 activation. Knockout of one allele of Akt2 in Akt1-deficient mice significantly blocks the phosphorylation of STAT5, which leads to defects in mammary gland differentiation and milk production. This evidence indicates that Akt isoforms might play overlapping regulatory roles and are critical in the mammary gland. Importantly, activation of the PI3K-Akt pathway triggers autocrine-mediated prolactin secretion, which indirectly activates the JAK2-STAT5 signaling pathway. This process is required for the initiation of lactation (Oliver and Watson, 2013). Future studies are needed to verify whether Akt1 and Akt2 are both required for the activation of the JAK2-STAT5 signaling pathway.

In the mammary gland, JAK2 deficiency decreases mammary gland cell proliferation, which can be partially recused by the overexpression of Akt1 (Sakamoto et al., 2007), suggesting a potential link between JAK2 and Akt1. In addition, activation of STAT5 can also directly regulate PI3K-Akt1 signaling through the mechanism described below (**Figure 3**). First, Stat5 directly binds to consensus sites within Akt1 and enhances its transcriptional activation (Creamer et al., 2010; Schmidt et al., 2014). Second, stat5 increases the transcription of two subunits (p85α and p110α) of phosphatidylinositol 3-kinase (PI3K) in the mammary gland (Schmidt et al., 2014). Third, STATs can regulate the activity of PI3K by binding to the p85 regulatory subunit (Rosa Santos et al., 2000; Nyga et al., 2005). This evidence indicates strong crosstalk between the PI3K-Akt and JAK2-STAT5 signaling pathways.

JAK2-STAT5 CROSSTALK WITH GROWTH FACTORS AND INFLAMMATORY CYTOKINES

Growth factors are crucial elements involved in JAK2/STAT5 regulation in the mammary gland (**Figure 4**). Transforming growth factor-β (TGF-β) is considered a crucial factor in the regulation of mammary gland development, as well as mammary tumorigenesis. TGF-β regulates mammary gland epithelial cells through an autocrine mechanism. Three isoforms of TGF-β (TGF-β1, TGF-β2, and TGF-β3) have been identified in mammals. All isoforms negatively regulate the development of the mammary gland (Daniel and Robinson, 1992). TGF-β3 is significantly increased during the involution process in the mouse mammary gland (Faure et al., 2000). A high concentration of TGF-β inhibits the branching process of the mammary gland (Nelson et al., 2006), whereas ductal proliferation and lateral branching are highly increased in TGF-β-mutant mice (Joseph et al., 1999; Crowley et al., 2005). The imbalance between non-canonical and canonical TGF-β signaling leads to mammary tumorigenesis (Parvani et al., 2011). The canonical downstream targets of TGF-β signaling are Smads, which are a group of transcription factors. TGFβ-induced Smad signaling (smad2/3/4 complex) antagonizes prolactin-mediated JAK/STAT signaling by blocking STAT5 transactivation of its target genes

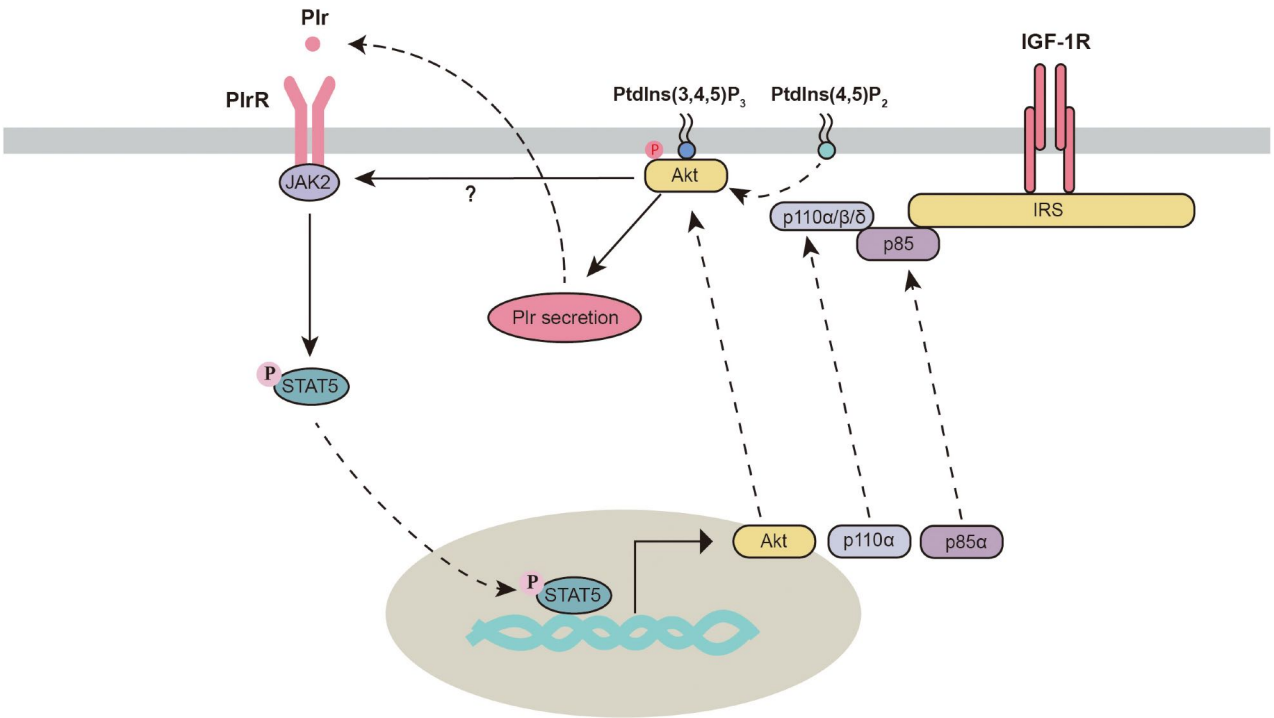

FIGURE 3 | Crosstalk between the PI3K/Akt and JAK2-STAT5 signaling pathways. Akt, protein kinase B; IGF-1R, insulin-like growth factor 1 receptor; IRS, insulin receptor substrate; JAK2, Janus kinase 2; Plr, prolactin; PlrR, prolactin receptor; PtdIns(3,4,5)P_3, phosphatidylinositol 3,4,5-trisphosphate; PtdIns(4,5)P_2, phosphatidylinositol-4,5-bisphosphate; STAT5, signal transducers and activators of transcription 5.

(Cocolakis et al., 2008). Intriguingly, TGFβ is usually highly expressed during the middle of lactation. The antagonistic effect of TGFβ is partially reduced by SnoN, which is an inhibitor of smad proteins (Jahchan et al., 2012). In addition, TGF-β might also regulate the function of the mammary gland via the activation of the non-canonical WNT5A pathway (Roarty and Serra, 2007).

Epidermal growth factor (EGF) is an important factor for mammary gland development (Long et al., 2003). EGF receptors (ERBBs) belong to the tyrosine kinase family, and four ERBBs have been identified (ERBB1, ERBB2, ERBB3, and ERBB4) in the mammary gland. ERBB1 and ERBB4 have been reported to participate in the regulation of mammary gland development. When ERBB1 is knocked out, alveolar development is severely impaired (Fowler et al., 1995). Intriguingly, it has been proposed that ERBB1 is mainly located in the stroma but not in the epithelium (Wiesen et al., 1999; Gallego et al., 2001). These findings suggest an intimate reciprocal stromal-epithelial interaction in the mammary gland. In contrast to ERBB1, the absence of ERBB4 leads to a deficiency in milk secretion (Tidcombe et al., 2003). Specifically, knocking out ERBB4 in mammary epithelial cells significantly impairs the differentiation and proliferation of cells in the mammary gland (Long et al., 2003). ERBB4 tyrosine kinases might also act as scaffold proteins that interact with JAK2 and STAT5 (Muraoka-Cook et al., 2008). When activated, ERBB4 is cleaved at Val-675 and releases a soluble 80-kDa intracellular domain (s80HER4). The kinase activity of s80HER4 is also required for the nuclear translocation of STAT5A (Muraoka-Cook et al., 2006). Thus, similar to PRLR, stimulation of ERBB4 triggers the activation of STAT5. Although some studies indicate cooperative crosstalk between prolactin and EGF (Darcy et al., 1995), other studies showed an antagonistic relationship between these factors (Fenton and Sheffield, 1997; Huang et al., 2006). EGF might inhibit PRL-induced mammary gland functions by modifying STAT5-mediated gene expression. In addition, EGF blocks the STAT5-induced pathway through growth factor receptor-bound protein 2 (Grb2), which is a positive regulator of cell proliferation during morphogenesis (Brummer et al., 2006).

In the context of mammary gland infection by pathogens during lactation, multiple inflammatory cytokines (TNF-α, IL-1β, and IL-6) are released by immune cells in the mammary gland and impair milk production (Shuster et al., 1996; Quesnell et al., 2012). IL-1β and TNF-α have been reported to inhibit milk protein production (β-casein expression) through activation of the NF-κB signaling pathway (Bonizzi and Karin, 2004). NF-κB and AMPK are two critical downstream signals of inflammatory cytokines (TNF-α, IL-1β, and IL-6). To date, JAK2 has been demonstrated to induce the phosphorylation of the inhibitor of NF-κB (Digicaylioglu and Lipton, 2001). However, no evidence indicates that NF-κB can regulate the activation of AMPK, which indicates that this field still needs further research. Interestingly, some experimental clues in non-mammary gland epithelial cells suggest that P38/ERK MAPK signaling pathways might

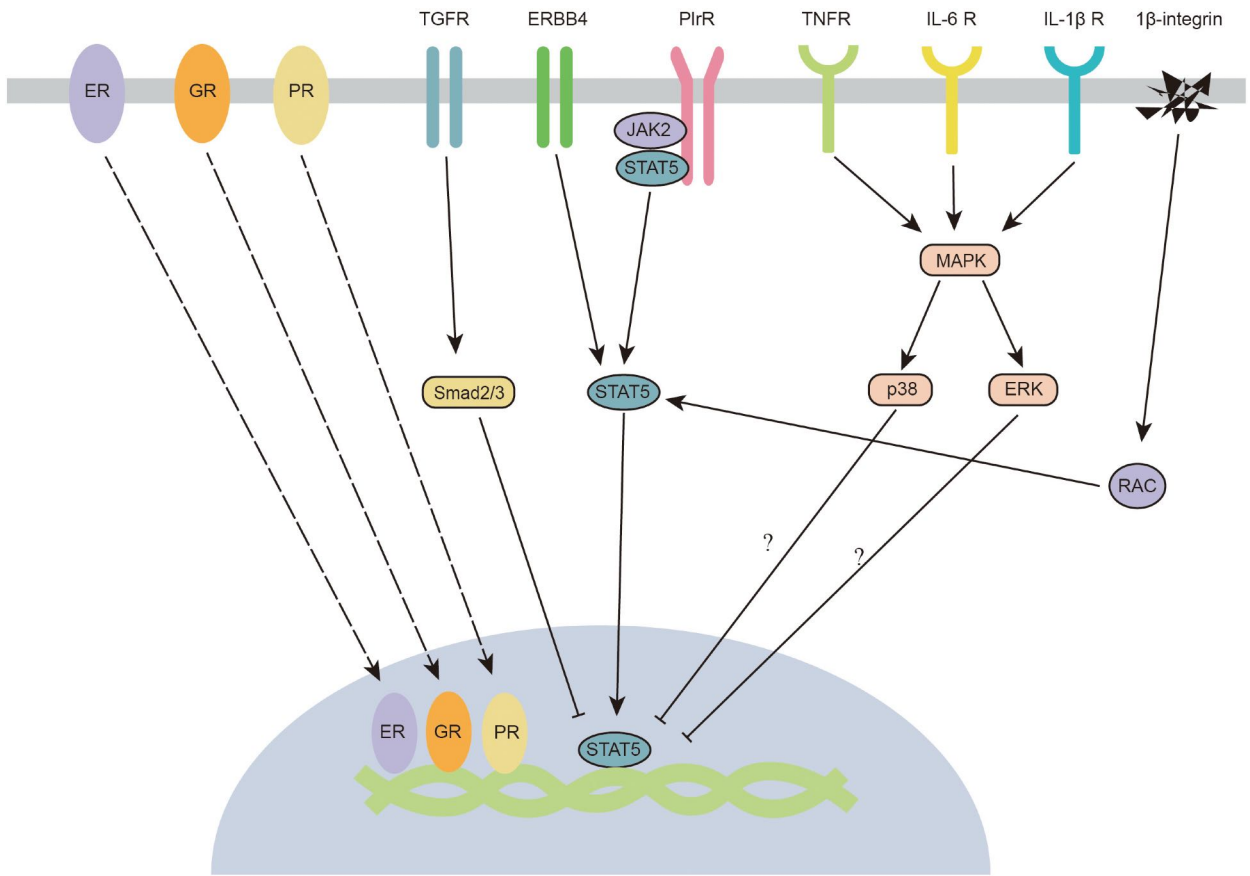

FIGURE 4 | Crosstalk between JAK2-STAT5 and hormone receptors, growth factors, inflammatory cytokines, and integrins. ER, estrogen receptor; ERBB4, epidermal growth factor receptor 4; ERK, extracellular signal-regulated kinase; GR, glucocorticoid receptor; IL-1β R, interleukin-1beta receptor; IL-6 R, interleukin-6 receptor; JAK2, janus kinase 2; MAPK, mitogen-activated protein kinases; PlrR, prolactin receptor; PR, progestinreceptor; Smad2/3, STAT5, signal transducers and activators of transcription 5; TGFR, transforming growth factor receptor; TNFR, tumour necrosis factor receptor.

be involved in the regulation of STAT5 (**Figure 4**). ERK has been reported to inhibit the transcriptional activity of STAT (Krasilnikov et al., 2003). Furthermore, the phosphorylation of p38 is negatively correlated with the phosphorylation of STAT5 (Gaoxia et al., 2018). The potential effects of P38/ERK MAPK of STAT5 in mammary gland epithelial cells still require further study. Recently, TNF-α has been shown to significantly inhibit lactose synthesis by inactivating JAK2 in the mammary gland (Kobayashi et al., 2016). It would be interesting to know if other inflammatory cytokines also affect the activation of the JAK2-STAT5 signaling pathway.

JAK2-STAT5 CROSSTALK WITH HORMONE RECEPTORS

As we previously mentioned in the "JAK2-STAT5 and PI3K-Akt" section, numerous hormones indirectly regulate JAK2-STAT5 through the PI3K/Akt signaling pathway. In this section, we summarize certain hormone receptors that can directly regulate the JAK2-STAT5 signaling pathway. Prolactin is one of the most predominant hormones that regulates mammary gland development (Saunier et al., 2003). The prolactin receptor-dependent signaling pathway is critical for the proliferation and differentiation of mammary alveoli during gestation (Miyoshi et al., 2001). It is widely known that prolactin is mainly responsible for the activation of JAK2-STAT5. In addition to the prolactin receptor, glucocorticoid, estrogen, and progestin receptors have also been reported to directly regulate the JAK2-STAT5 signaling pathway (**Figure 4**). In the mammary gland, glucocorticoid administration can increase milk protein synthesis through glucocorticoid receptors. The glucocorticoid receptor has been reported to interact with STAT5a and enhance STAT5a-mediated gene transcription. Both glucocorticoid receptor and STAT5a recruit the histone acetyltransferase (HAT) p300 coactivator (Pfitzner et al., 1998; Kabotyanski et al., 2006). Intriguingly, a recent study indicated that glucocorticoid receptors regulate beta-casein gene expression by directly interacting with a proximal promoter and a distal enhancer, forming a chromatin loop that connects the promoter and enhancer (Kabotyanski et al., 2009). This chromatin loop is important in regulating milk synthesis gene

expression. Similar to glucocorticoid receptors, estrogen and progestin receptors have also been proposed to regulate STAT5 by interacting with the DNA domain. Estrogen receptor-α and -β enhance prolactin-induced STAT5 activation by directly binding to the STAT5 DNA-binding domain (Björnström et al., 2001; Faulds et al., 2001). The crosstalk between the progestin receptor and the PRLR/STAT5 signaling pathway occurs at the β-casein promoter. Progestin-induced activation of progestin receptor leads to direct binding of progestin receptor to the beta-casein promoter and blocks its activation, which might lead to an inactivated form of STAT5a (Richer et al., 1998).

Recently, some preliminary evidence has indicated that hormones such as insulin, serotonin and leptin also participate in the regulation of the JAK2-STAT5 signaling pathway. Insulin plays an important role in enhancing milk synthesis by phosphorylating STAT5 in the mammary gland (Menzies et al., 2010). The insulin receptor can directly interact with STAT5 and induce its phosphorylation (Chen et al., 1997). In addition, one of the critical downstream signaling pathways of insulin is thought to increase the activity of the PI3K/KAT signaling pathway, which is a critical signaling pathway that crosstalks with JAK2-STAT5. Serotonin inhibits the phosphorylation of STAT5 and decreases β-casein expression (Chiba et al., 2014). Although leptin has not been reported to directly crosstalk with the STAT5 signaling pathway in the mammary gland, it is thought to synergize with prolactin to enhance the expression of beta-casein in the mammary gland through the inactivation of STAT3 (Motta et al., 2007).

INTEGRINS AND JAK2-STAT5

Cell adhesion is a critical factor that determines the fate of epithelial cells (Schmidt et al., 1993). As major receptors associated with cell adhesion, integrins have been reported to regulate cell proliferation, differentiation and migration (Díaz-Coránguez et al., 2019). β1-integrin is thought to maintain the function of the mammary gland via the integrin-containing adhesion complex protein ILK (integrin-linked kinase). ILK regulates STAT5 signaling through Rac1 (Akhtar et al., 2009), which (RAS-related C3 botulinum substrate 1) is a critical downstream factor of integrins (Akhtar and Streuli, 2006; **Figure 4**). Mechanistically, Rac1 recruits STAT5 to kinase complexes and enhances its phosphorylation (Xu et al., 2010). Knocking out β1-integrin decreases the activation of STAT5, impairs the differentiation of secretory epithelial cells, and inhibits the mRNA expression of beta-casein and whey acidic protein (Faraldo et al., 2002).

OTHER SIGNALING PATHWAYS AND THE ACTIVATION OF JAK2-STAT5

In addition to the abovementioned pathways that interact with JAK2-STAT5, other signaling pathways are involved in the regulation of JAK2-STAT5. (1) The Hedgehog signaling pathway negatively regulates mammary gland development. Overexpressing the Hedgehog effector protein GLI1 attenuates the expression of STAT5 through snail and inhibits mammary gland lactation (Fiaschi et al., 2007). (2) Peroxisome proliferator-activated receptor γ (PPARγ) has been shown to regulate STAT5A protein expression (Olsen and Haldosen, 2006). (3) NF-kappa B functions as a negative regulator of the JAK2-STAT5 pathway by interfering with STAT5 tyrosine phosphorylation (Geymayer and Doppler, 2000). However, more evidence is required to support the interplay between JAK2-STAT5 and these signaling pathways.

CONCLUSION AND PERSPECTIVE

Signal transducers and activators of transcription 5 is a crucial transcription factor that directly regulates multiple genes that participate in proliferation, differentiation, and milk secretion in the mammary gland. The current understanding of the crosstalk between JAK2-STAT5 and other signals includes the following: (1) activation of the $G_{\alpha i}$ or $G_{\alpha q}$ GPCR signaling pathway is thought to increase the phosphorylation of STAT5, and the effects of different types of GPCRs could be different due to the bias of agonists; (2) Akt1 activates STAT5 phosphorylation, which can increase the expression of Akt1 and PI3K subunits (p85α and p110α); (3) TGF-β, TNF-α, IL-6, and IL-1β are negative regulators of STAT5 activation, while the effects of EGF on the mammary gland are still controversial; (4) ER, GR and PR are positive regulators of the JAK2-STAT5 signaling pathway by directly interacting with the DNA domain; and (5) cell adhesion is crucial in maintaining the PrlR/STAT5 signaling cascade through β1-integrin.

It is worth noting that in addition to its function in mammary gland epithelial cells, the STAT5 signaling pathway also plays an important role in macrophages in the mammary gland and is required for normal mammary gland development. STAT5 knockout in macrophages leads to decreased ductal elongation but increased epithelial cell proliferation. Mechanistically, STAT5 deletion induces the expression of the proliferative factors Cyp19a1/aromatase and IL-6, which enhance ER signaling in the mammary gland (Brady et al., 2017). It would be interesting to know whether the STAT5 signaling pathway also plays a crucial role in other cell types (fibroblasts, adipocytes, blood vessels, nerves, and various immune cells) in the mammary gland. With the development of single-cell RNA sequencing, it would be possible to identify the potential signals that crosstalk with JAK-STAT5 in individual cells in the mammary gland. More research on the crosstalk among different types of cells in the mammary gland would help us to better understand the signaling networks in the whole mammary gland.

Mammary gland development and lactation are complicated processes that are accompanied by magnificent changes in reproductive hormones. During lactation, mastitis occurs widely and causes inflammatory injury of the mammary gland. Although various signaling interactions have been identified between JAK2-STAT5 and reproductive hormones, growth factors and inflammatory cytokines, the potential challenge in the future is

to precisely predict the biological modifications in the mammary gland mediated by these combinatorial signaling activities.

AUTHOR CONTRIBUTIONS

MT and SZ initiated the idea, the scope, and the outline of this review manuscript. MT, SZ, YQ, XZ, ZW, JC, and FC studied and analyzed all of the publications cited in this manuscript and were involved in the manuscript preparation. SZ and WG conducted the final editing and proofreading. All authors read and approved the final manuscript.

FUNDING

This work was supported by the National Natural Science Foundation of China (nos. 31802067 and 31872364) and the Natural Science Foundation of Guangdong Province (no. 2018A030310201).

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Article

Fat Encapsulation Reduces Diarrhea in Piglets Partially by Repairing the Intestinal Barrier and Improving Fatty Acid Transport

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Simple Summary: Fat is an important energy resource in animal production. Studies of the effect of dietary fat on gut function will facilitate the development and utilization of new fat resources. In our previous study, we found that piglet diarrhea is related to the type of dietary fat ingested. Therefore, we wondered whether dietary fat regulates intestinal function by regulating the expression of key proteins in the piglet intestine. In this study, we confirmed that dietary fat regulates the expression of fatty acid transport, intestinal tight junctions, and β-defensin proteins. Moreover, we have shown for the first time that fat encapsulation reduces the incidence of diarrhea partially by alleviating the damage to intestinal mechanical and immunological barriers induced by fat with a low digestibility.

Abstract: (1) Background: Nutritional strategies to enhance gut function and reduce the piglet diarrhea rate are critical to increase the growth performance of piglets. The purpose of this study was to investigate whether dietary fat types and/or fat microencapsulation techniques are involved in regulating the fatty acid transport system and the mechanical and immunological barriers of the small intestine. (2) Methods: Three hundred twenty-four weaning piglets were randomly divided into three groups fed a soybean oil diet (SBO, control group, 6.0% soybean oil), palm oil diet (PO, 6.0% palm oil), or encapsulated palm oil diet (EPO, 7.5% encapsulated palm oil). (3) Results: A significantly lower mRNA expression of the claudin was observed in the duodenum and jejunum of the PO group than in the SBO group ($p < 0.05$). However, the mRNA expression and protein abundance of claudin and ZO-1 in the jejunum of the EPO group were higher ($p < 0.05$) than in the PO group. Porcine β-defensin (pBD) secretion was not significantly different between the SBO and PO groups ($p > 0.05$), while the pBD-2 levels were significantly different ($p < 0.05$). Compared with the PO group, the EPO group exhibited a significantly increased secretion of pBD-2 and pBD-129 in the small intestine ($p < 0.05$) and pBD-1 in the jejunum and ileum ($p < 0.05$). The protein abundances of apolipoprotein AIV (Apo AIV) and intestinal fatty acid binding protein (I-FABP) were significantly lower in the PO group than in the SBO group ($p < 0.05$). Simultaneously, the protein abundances of fatty acid transport protein 4 (FATP4), fatty acid translocase (CD36), and I-FABP were higher in the EPO group than in the PO group. Furthermore, the low digestibility of palm oil (PO group) might negatively regulate intestinal tight junctions, fatty acid transporters, lipoproteins, and β-defensin through the activation of the AMPK/mTORC1 and AMPK/Sirt1/NF-κB pathways. (4) Conclusions: In summary, microencapsulation techniques might alleviate the negative effects of palm oil and help to improve the intestinal fatty acid transport system and barrier function.

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Citation: Tian, M.; Chen, J.; Wu, Z.; Song, H.; Yang, F.; Cui, C.; Chen, F.; Zhang, S.; Guan, W. Fat Encapsulation Reduces Diarrhea in Piglets Partially by Repairing the Intestinal Barrier and Improving Fatty Acid Transport. *Animals* **2021**, *11*, 28. https://dx.doi.org/10.3390/ani11010028

Received: 20 November 2020
Accepted: 21 December 2020
Published: 26 December 2020

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Keywords: fat; intestinal barrier; fatty acid transport; piglets; AMPK signaling

1. Introduction

Dietary fat is a critical energy source for pigs. The use of an appropriate fat source is expected to increase the performance of pigs. Conversely, the dietary supplementation of fat with a low digestibility might jeopardize the growth of the pig due to an insufficient energy supply. Palm oil is a cheaper alternative oil source to soybean oil in animal husbandry. However, palm oil contains numerous saturated fatty acids and has a lower energy digestibility. Fat encapsulation may change the physical structure of oils and facilitate their digestion and absorption by piglets, which makes palm oil a potential feed ingredient for animals.

The administration of fats with low digestible energy values might induce energy deficiency, retard the growth of pigs, and lead to intestinal dysfunction. The gut is the main site for the digestion and absorption of nutrients. In addition, the gut also forms the boundary between mammalian internal and external environments. The intestinal barrier is composed of a physical barrier, a chemical barrier, an immune barrier, and microorganisms, and these barriers jointly resist the invasion of foreign substances [1]. The maintenance of the integrity of intestinal barrier function is a key prerequisite to ensure the health of piglets [2–4]. As the predominant energy sensor in cells, adenosine 5′-monophosphate (AMP)-activated protein kinase (AMPK) is proposed to detect changes in cellular energy. Energy deprivation activates AMPK to regulate multiple biological functions upon the activation of numerous downstream signaling pathways. Nuclear factor kappa-B (NF-κB) is reported to be involved in the regulation of β-defensin expression [5,6]. Activation of AMPK leads to an increase in the sirtuin 1 (Sirt1) activity, which suppresses NF-κB-driven immune responses [7,8]. In addition, AMPK inhibits the activity of mechanistic target of rapamycin complex 1 (mTORC1) through the phosphorylation of the raptor on Ser792 [9]. Since mTORC1 is a master regulator of protein synthesis and cell proliferation [10], AMPK might regulate functional protein expression (tight junctions proteins, fatty acid transporters, etc.) in the intestine through the mTORC1 pathway.

This study was designed to elucidate the effects of the dietary administration of soybean oil, palm oil, and encapsulated palm oil on intestinal function. As mentioned above, palm oil has a lower digestible energy which is expected to trigger the activation of AMPK and regulate gut function. In this study, palm oil impaired the expression of intestinal tight junction proteins, fatty acid transporters, and lipoproteins through the AMPK/mTORC1 pathway. At the same time, palm oil administration inhibited the β-defensin expression and activated AMPK/Sirt1/NF-κB signaling. Importantly, no significant difference in gut function was observed between soybean oil and encapsulated palm oil treatments, indicating that microencapsulation techniques could increase the digestibility of palm oil. These observations provide novel molecular biological evidence that encapsulated palm oil is an alternative energy source to soybean oil.

2. Materials and Methods

All the animal care and handling procedures conducted during this study were approved by the South China Agricultural University Animal Care and Use Committee (No. 20110107-1, Guangzhou, China).

2.1. Diets

Three experimental diets based on corn and soybean meal are shown in Table S1: soybean oil diet (SBO, control group, 6.0% soybean oil), palm oil diet (PO, 6.0% palm oil), and encapsulated palm oil diet (EPO, 7.5% encapsulated palm oil). All the diets meet the nutrient requirements of 7 to 11 kg piglets [11]. Soybean oil was obtained from Cargill Grain and Oil Co., Ltd. (Dongguan, China). Palm oil was purchased from Yihai Kerry Grain

& Oil Ltd. (Guangzhou, China). The same source of palm oil was encapsulated using 8.5% dried casein and 11.5% whey powder. The three ingredients were stirred at 60 °C for 30 min to uniformly mixed them in liquid form, pasteurized at 65 °C for 30 min, and homogenized at a pressure of 250 kg/cm^2 before spray drying (EYELA Spray Drier SD-1000, Tokyo Rikakikai Co., Ltd., Tokyo, Japan). The inlet temperature of the drier was 180 °C and the outlet temperature was 80 °C. The size of the dried fat powder particles ranged from 30 to 50 µm. The fatty acid composition of the fat sources used in the experiment was analyzed using gas chromatography (GC-2010 Pro, SHIMADZU Corporation, Kyoto, Japan) and is shown in Table S2.

2.2. Animals and Experimental Design

After a 7-day adaptation period, 324 weaning piglets (8.23 ± 0.22 kg, Duroc × Landrace × Large White) were randomly classified into the three treatment groups with six replicates (pens) per treatment and eighteen piglets per replicate based on their initial body weight and sex, and the experiment lasted for 28 days. All the piglets were provided with *ad libitum* access to feed and fresh water during the experiment. One heathy piglet of each replicate was sacrificed by electrocution on day 28. The piglets were selected for slaughter according to body weight (close to average weight) and sex (half of each). The duodenum, jejunum, and ileum were collected, snap-frozen in liquid nitrogen, and then stored at −80 °C until further analysis.

2.3. Data Collection

The initial body weight and final body weight were measured on days 0 and 28, respectively, with the pen as the replicate. During the trial, the number of the piglets with diarrhea symptoms was recorded by the attending veterinarian. Diarrhea symptoms were scored using the fecal scoring system (0—normal, hard form; 1—soft feces, soft form; 2—mild diarrhea, no form, loose, puddles; 3—severe diarrhea, watery) reported by Ren, et al. (2020) [12]. The diarrhea incidence is the ratio of the total number of diarrhea events to the product of the total piglet number and the number of test days. At the end of the experiment, the total feed intake of each group was measured.

2.4. Chemical Analyses

The gross energy (GE) of the feed sample was analyzed using an adiabatic oxygen bomb calorimeter (IKA C200, IKA Company, Staufen, Germany). The crude protein (CP) content was measured according to the method of the Association of Official Analytical Chemists [13]. The dietary ether extract was determined using the method described by Thiex, et al. (2003) [14]. Moreover, the dietary contents AAs was analyzed using ion-exchange chromatography (Hitachi L-8800 amino acid analyzer, Tokyo, Japan) [13]. The tissues (20–50 mg) of duodenum, jejunum, and ileum were rinsed with 1× PBS and homogenized in 1 mL of 1× lysis buffer. Then, the homogenate was broken with ultrasound until it was clear and centrifuged for 5 min at 10,000× *g* and 4 °C. The supernatant was obtained and assayed immediately. The concentrations of pBD-1, pBD-2, pBD-3, pBD-114, and pBD-129 were measured using commercially available ELISA kits from USCN Life Sciences (Wuhan, China), Shanghai Renjie Biotechnology (Shanghai, China) and Research Biological Technology (Shanghai, China), respectively. These kits are sandwich enzyme immunoassays for in vitro quantitative analysis. The absorbance of the colored product was determined using a Multiskan GO Microplate Spectrophotometer (Thermo Fisher Scientific, Rockford, IL, USA). The pBD concentrations of the samples were calculated with reference to the standard curve obtained from the standards of known concentrations.

2.5. RNA Extraction and Relative Qualification of mRNA

Total RNA was extracted from intestinal tissues using the RNAiso Plus reagent (Takara, Dalian, China). The concentration and purity of the RNA samples were assessed with a NanoDrop spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA), and only

RNA samples with an absorbance ratio (260/280) greater than 1.8 were used. Subsequently, RNA was reverse-transcribed into cDNA using the PrimeScript First Strand cDNA Synthesis Kit (Takara, Dalian, China) according to the manufacturer's protocol. Real-time quantitative PCR (RT-qPCR) was performed using an ABI Prism 7500 sequence detection system (Applied Biosystems, Carlsbad, CA, USA) in a total 20 μL volume composed of 10 μL of RT-qPCR Master Mix (SYBR Green, Takara, Dalian, China), 2 μL of cDNAs, 0.8 μL of each PCR primer (10 μM), 0.4 of μL ROX (10 μ/μL, Takara), and 6 μL of dd water. The PCR program was set as follows: denaturation at 94 °C for 5 min followed by 40 repeated cycles of 94 °C for 30 s, 60 °C for 30 s, and 72 °C for 30 s. The relative gene expression was analyzed using the relative quantification ($2^{-\Delta\Delta Ct}$) method. The primers used for RT-qPCR are shown in Table 1.

Table 1. Real-time PCR primer sequence.

Genes	Accession	Direction	Sequences (5′–3′)	Size
Apo AI	NM_214398.1	Forward Reverse	CGTGTATGTGGATGCGATCAAA CCCAGTTGTCCAGGAGTTTCAG	104
Apo AIV	AJ222966.1	Forward Reverse	TCAACACCCAGGTTCAGCAG AACTCATCCGCATAGGGTGC	121
Apo B48	AH001207.2	Forward Reverse	GAGGTAACTGTGCCTGCATA CTGCACTAAGGTCACGATGT	436
GPR120	HQ662564.1	Forward Reverse	CTCTTCCTGCTCATGATCTCCTTC GTGACATGTTGTAGAGAATGGGGTT	178
FATP4	XM_003353676.1	Forward Reverse	AGCCGCATCCTGTCCTTT GACATCCTTGGCGATCTTTT	213
CD36	DQ192230.1	Forward Reverse	GGACTCAT TGCTGGTGCTGT GTCTGTAAACTTCCGTGCCTGT	169
I-FABP	NM_001031780.1	Forward Reverse	GAGCTTGGTGTCACTTTTAACTAC CCTCTTGGCTTCTACTCCTTCATA	192
IL-FABP	NM_214215.2	Forward Reverse	GGAGGTCTCCACTGTCGG CTGTTACACCAGGTTTATTTG	115
L-FABP	NM_001004046.2	Forward Reverse	GAAGGGGAAGGACATCA CAGTCAGGGTCTCCATCTCACA	138
Claudin	NM_001244539.1	Forward Reverse	TCTTTCTTATTTCAGGTCTGGCTATCT CCACTGGAAGGCGAAGGTTTT	247
Occludin	NM_001163647.2	Forward Reverse	ACTGGCGGCGAGTCCTGCGACGAGC TATTGTATTCATCAGCAGCAGCCAT	244
ZO-1	XM_021098856.1	Forward Reverse	AAAGCCCTAAGTTCAATCACAATCT TCCTCATCTTCATCATCTTCTACAG	253
pBD-1	NM_213838.1	Forward Reverse	TGCCACAGGTGCCGATCT CTGTTAGCTGCTTAAGGAATAAAGGC	81
pBD-2	NM_214442.2	Forward Reverse	CCAGAGAGGTCCGACACTACA GGTCCCTTCAATCCTTGTAGGTGAA	168
pBD-3	XM_021074698.1	Forward Reverse	ACCAAGCACGCCTTCCTATC GCATTTTCGGCCACTCACAG	236
pBD-114	NM_001129973.1	Forward Reverse	TGTACCTTGGTGGATCCTGAACGA CGCCCTCTGAATGCAGCATATCTT	240

Table 1. *Cont.*

Genes	Accession	Direction	Sequences (5′–3′)	Size
pBD-129	NM_001129975.1	Forward Reverse	CAAAGACCACTGTGCCGTGAATGA TTGATGCTGGCGAAAGGGTTGGTA	131
β-actin	397563	Forward Reverse	TGCGGGACATCAAGGAGAAG AGTTGAAGGTGGTCTCGTGG	176

Apo AI, apolipoprotein AI; Apo AIV, apolipoprotein AIV; Apo B48, apolipoprotein B48; FATP4, fatty acid transport protein 4; CD36, fatty acid translocase; GPR120, G protein coupled receptors 120; I-FABP, intestinal fatty acid binding protein; IL-FABP, ileum fatty acid binding protein; L-FABP, liver fatty acid binding protein; pBD-1, porcine β-defensin 1; pBD-2, porcine β-defensin 2; pBD-3, porcine β-defensin 3; pBD-114, porcine β-defensin 114; pBD-129, porcine β-defensin 129.

2.6. Western Blotting Analysis

Total protein was extracted from intestine tissues using the RIPA lysis buffer (Beyotime, Shanghai, China) supplemented with 1% of the protease inhibitor PMSF (100 mM) and 1% phosphatases inhibitor (100×). A BCA protein assay kit (Beyotime, Shanghai, China) was used to determine the total protein concentrations. Then, equal amounts of samples (20 μg of total protein) were resolved on 10% a polyacrylamide gel and transferred onto polyvinylidene difluoride (PVDF) membranes (Millipore, Bedford, MA, USA). After blocking with 5% skim milk for 2 h at room temperature, each membrane was washed five times with TBST buffer for 5 min each. Subsequently, the membranes were incubated at 4 °C overnight in TBST containing 5% BSA with the primary antibody. Antibodies against target proteins were obtained from commercial companies (Table 2). Subsequently, the membranes were incubated with the corresponding secondary antibodies (511203, ZenBio, Chengdu, China) at room temperature for 1.5 h. After five rinses, band signals were detected using a chemiluminescent ECL Western blot detection system (ProteinSimple, Santa Clara, CA, USA). Finally, the protein bands were quantified by densitometry using ImageJ software, and the relative protein abundance was normalized to total β-actin or total target protein levels (Image-Pro Plus 6.0) (Rockville, MD, USA).

Table 2. Antibody information used in this experiment.

Antibodies	Code No.	Company	kDa
Apo AI	ab64308	Abcam	27
Apo AIV	ab239480	Abcam	43
FATP4	ab200353	Abcam	72
CD36	D161529	Sangon	53
I-FABP	ab60272	Abcam	15
Claudin	ab15098	Abcam	23
Occludin	ab31721	Abcam	59
ZO-1	ab96587	Abcam	187
AMPK	2532S	Cell Signaling Technology	62
p-AMPK	2535S	Cell Signaling Technology	62
NF-κB	10745-1-AP	Proteintech Group	65
p-NF-κB	3033S	Cell Signaling Technology	65
mTOR	2983S	Cell Signaling Technology	289
p-mTOR	5536S	Cell Signaling Technology	289
p70 S6K	9202S	Cell Signaling Technology	70, 85
p-p70 S6K	9234S	Cell Signaling Technology	70, 85
4EBP1	ab2606	Abcam	20–21
p-4EBP1	9451S	Cell Signaling Technology	15–20
β-actin	bs-0061R	Bioss	42

2.7. Statistical Analysis

Data with values that exceeded 3 standard deviations from the mean were considered outliers and excluded from the analysis. All the data were tested for normality using the

Shapiro–Wilk test. Statistical significance was analyzed with a one-way ANOVA using the MIXED procedure of the statistical software SAS version 9.4 (SAS Institute, Cary, NC, USA) with the pen as the experimental unit. Diet, sex, and their interaction were defined as the main effects. Means were generated and separated using the LSMEANS and PDIFF options. Statistical significance was considered if $p \leq 0.05$ and trends were indicated at $0.05 < p \leq 0.10$. The results are presented as the means ± standard errors of the means.

3. Results

3.1. mRNA Expression and Protein Abundance of Tight Junction Proteins in the Intestine

The mRNA expression of tight junction proteins (claudin, occludin, and ZO-1) in the duodenum, jejunum, and ileum is shown in Figure 1A–C. A significantly lower mRNA expression of claudin was detected in the duodenum of the PO group ($p < 0.05$) than in the SBO group. A significantly higher mRNA expression of claudin was observed in the duodenum of the EPO group ($p < 0.05$) than in the PO group. In the jejunum, the mRNA expression of claudin in the PO group was significantly lower ($p < 0.05$) than that in the SBO group. Simultaneously, the mRNA expression of claudin and ZO-1 was significantly ($p < 0.05$) higher than in the PO group. Furthermore, the mRNA expression of tight junction proteins in the ileum was not significantly different among the three groups in the ileum.

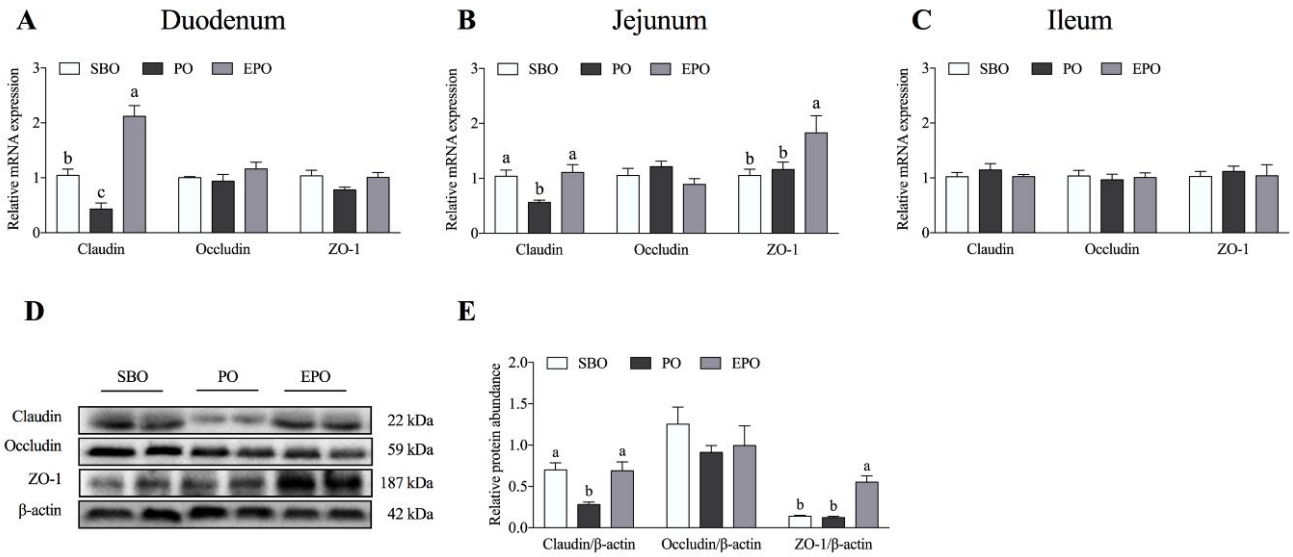

Figure 1. Expression and abundance of intestinal tight junction mRNAs and proteins. (**A**–**C**) The mRNA expression of tight junction proteins in the duodenum (**A**), jejunum (**B**), and ileum (**C**). (**D**,**E**) The abundance of tight junction proteins in the jejunum. SBO, soybean oil group; PO, palm oil group; EPO, encapsulated palm oil group. Different letters (a, b, and c) indicate a significant difference among different treatments ($p < 0.05$).

Based on the mRNA results, we detected the protein abundance of tight junction proteins in the jejunum (Figure 1D,E). The protein abundance results were similar to those for the mRNA expression. A significantly lower protein abundance of claudin was detected ($p < 0.05$) in the PO group than in the SBO group. The protein abundances of claudin and ZO-1 were significantly higher in the EPO group ($p < 0.05$) than in the PO group. Additionally, the protein abundance of occludin was not significantly different among the three groups.

3.2. Secretion of β-defensin in the Small Intestine of Piglets.

The differences in the intestinal porcine β-defensin expression (mRNA and tissue concentrations) among different treatments groups are shown in Figure 2. In the duodenum (Figure 2A,D), a lower mRNA expression of pBD-1, pBD-2, and pBD-129 was detected in the PO group than in the SBO group ($p < 0.05$). When animals were fed encapsulated palm oil,

the mRNA expression of pBD-1 and pBD-2 was significantly higher in the EPO group than that in the PO group ($p < 0.05$) and showed no significant difference compared with the SBO group. Simultaneously, a significantly higher mRNA expression of pBD-129 was observed than in the other two groups ($p < 0.05$). Furthermore, the concentrations of pBD-2 and pBD-129 in the duodenal tissues were significantly higher than in the other two groups ($p < 0.05$).

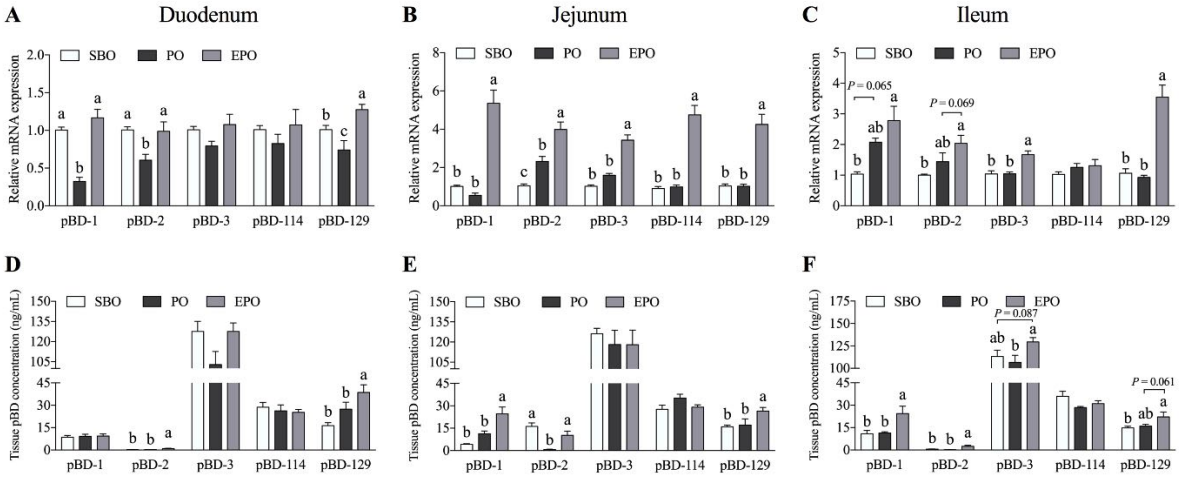

Figure 2. Secretion of β-defensins in the porcine small intestine. The mRNA expression and concentrations of porcine β-defensins in the duodenum (**A**,**D**), jejunum (**B**,**E**), and ileum (**C**,**F**). SBO, soybean oil group; PO, palm oil group; EPO, encapsulated palm oil group. Different letters (a, b, and c) indicate a significant difference among different groups ($p < 0.05$).

In the jejunum (Figure 2B,E), the mRNA expression of β-defensins in the EPO group was significantly higher than that in the other two groups ($p < 0.05$). Moreover, the concentrations of pBD-1 and pBD-129 in the jejunum were significantly higher than in the other two groups ($p < 0.05$). Interestingly, the defensin mRNA expression in the PO group and the EPO group was significantly higher than in the SBO group ($p < 0.05$). However, the pBD-2 concentration in the PO group was significantly lower than that in the other two groups ($p < 0.05$). No significant differences were observed between the SBO group and the EPO group.

In the ileum (Figure 2C,F), the mRNA expression and secretion of five β-defensins were not significantly different between the SBO group and the PO group. However, the pBD-3 and pBD-129 mRNAs were expressed at significantly higher levels in the EPO group than in the other two groups. The expression of the pBD-2 mRNA in the EPO group showed a trend toward higher expression ($p = 0.069$) compared with the PO group. At the same time, the tissue concentrations of pBD-1, pBD-2, and pBD-3 in the EPO group were significantly higher ($p < 0.05$) than the PO group. The tissue concentration of pBD-129 in the EPO group showed a higher secretion trend ($p = 0.061$) than in the PO group.

3.3. Fatty Acid Transport System in the Jejunum Tissues

The mRNA expression and protein abundance of fatty acid transporters and lipoproteins are shown in Figure 3. Compared with the SBO group, the mRNA expression of fatty acid transport protein 4 (FATP4), apolipoprotein A-I (Apo AI), and apolipoprotein A-IV (Apo AIV) was significantly lower in the PO group ($p < 0.05$). The mRNA of Apo AI, apolipoprotein B48 (Apo B48), and fatty acid translocase (CD36) was expressed at a significantly higher level in the EPO group than in the PO group ($p < 0.05$); the free fatty acid receptor 4 (GPR120) ($p = 0.058$) and intestinal fatty acid binding protein (I-FABP) ($p = 0.064$) mRNAs showed a trend toward higher expression in the EPO group than in the PO group. Simultaneously, the relative abundances of the Apo AI and I-FABP proteins were significantly lower in the PO group than in the SBO group ($p < 0.05$). However, no significant difference in the FATP4 and CD36 levels was observed between the SBO and PO

groups. Compared with the PO group, the relative abundances of the FATP4 ($p = 0.031$), CD36 ($p < 0.05$), and I-FABP ($p = 0.065$) proteins were higher in the EPO group.

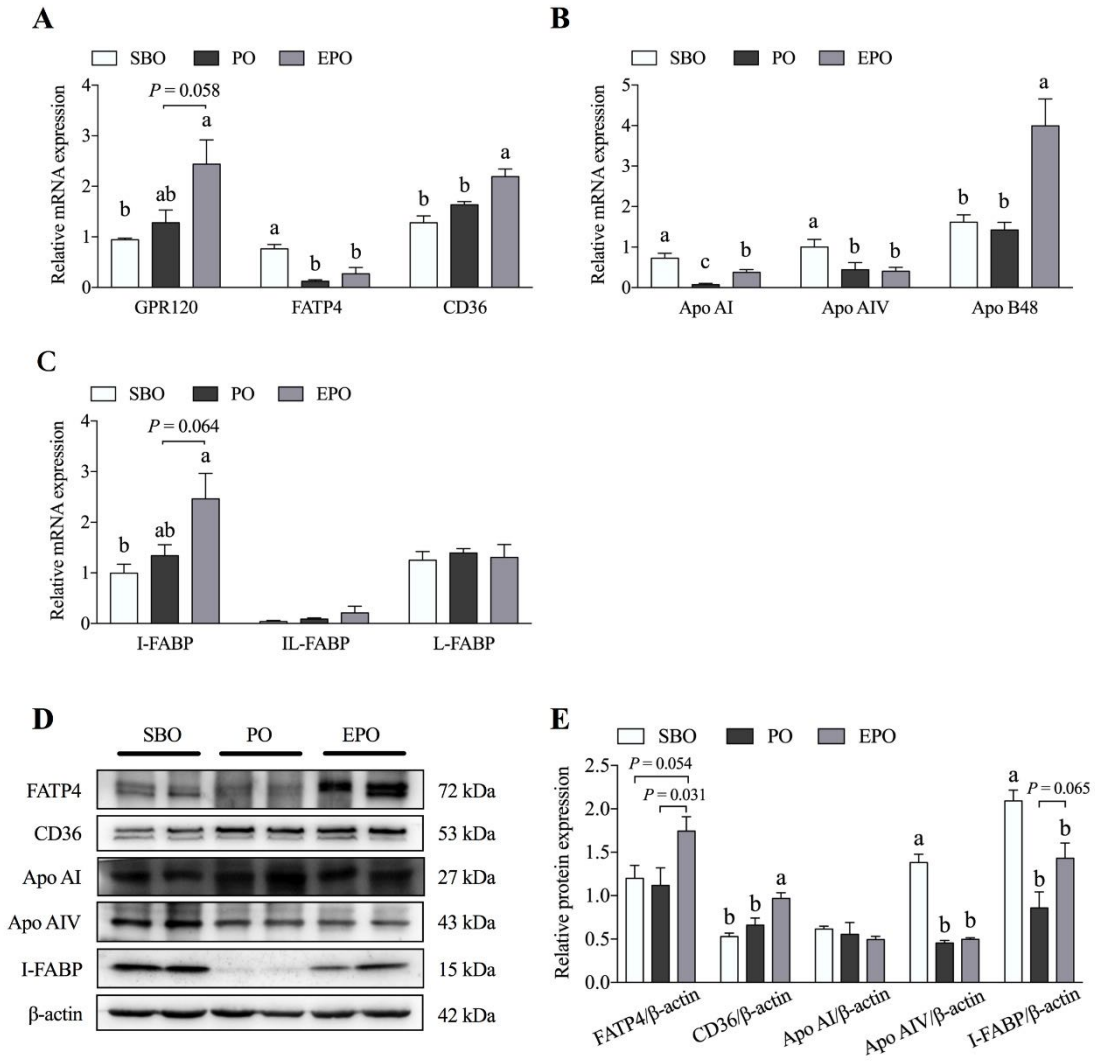

Figure 3. Expression of fatty acid transport system-related proteins in the porcine jejunum. The mRNA expression (**A**–**C**) and protein abundance (**D**,**E**) of proteins involved in fatty acid transport in the porcine jejunum. SBO, soybean oil group; PO, palm oil group; EPO, encapsulated palm oil group. Different letters (a, b, and c) indicate a significant difference among different groups ($p < 0.05$).

3.4. AMPK/mTORC1 and AMPK/Sirt1/NF-κB Pathways in the Jejunum

We examined the activation of the AMPK/mTORC1 and AMPK/Sirt1/NF-κB pathways in porcine jejunum to elucidate the underlying mechanisms of the effect of the dietary administration of soybean oil, palm oil, and encapsulated palm oil on intestinal function (Figure 4A,B). Our results showed a higher phosphorylation of AMPK and NF-κB in the PO group than in the SBO group ($p < 0.05$). Interestingly, when the animals were treated with encapsulated palm oil, the phosphorylation of AMPK and NF-κB was significantly decreased compared with the PO group ($p < 0.05$). Compared with the SBO group, the phosphorylation of mTOR ($p < 0.05$) and p70 S6K ($p = 0.059$) was lower in the PO group. However, the phosphorylation of mTOR, p70 S6K, and 4EBP1 was significantly increased in the EPO group compared with the PO group ($p < 0.05$).

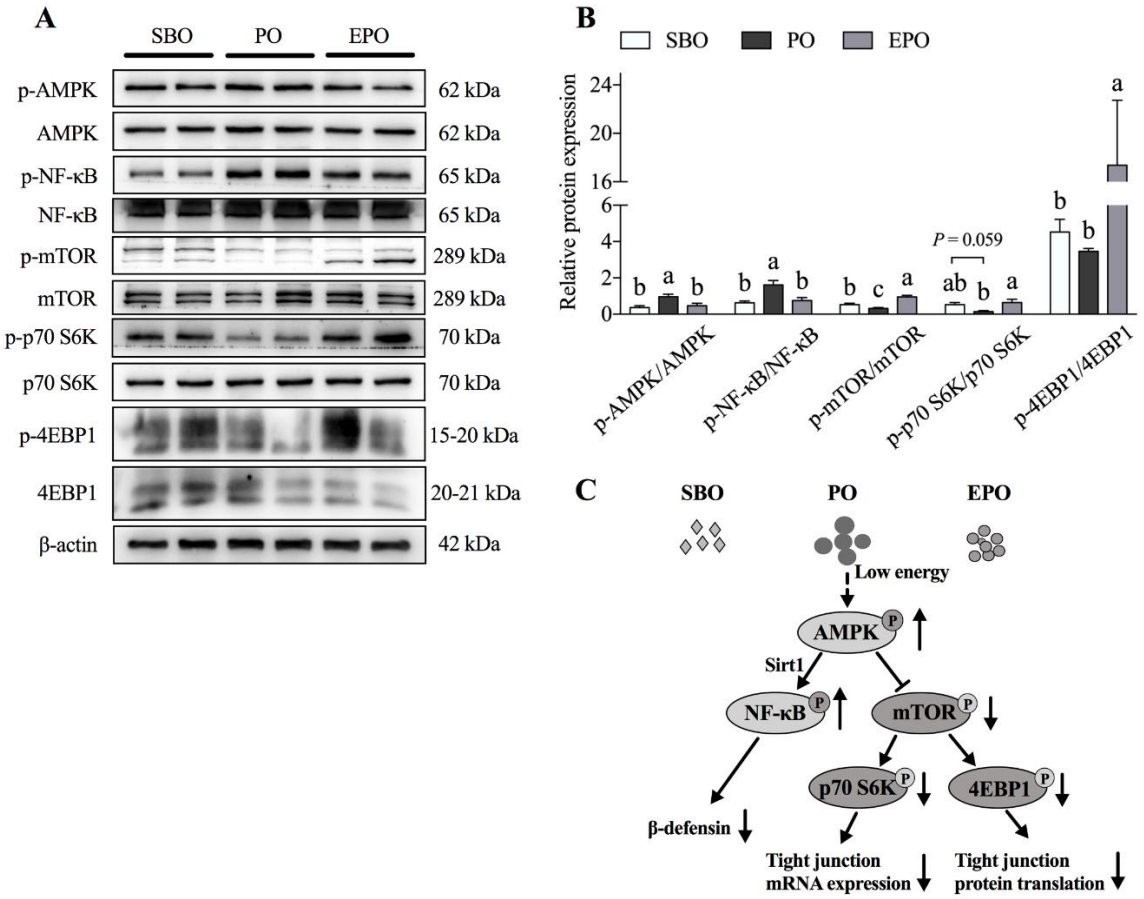

Figure 4. AMPK/mTORC1 and AMPK/Sirt1/NF-κB pathways in the jejunum. (**A**,**B**) The levels of phosphorylated proteins in the AMPK/mTORC1 and the AMPK/Sirt1/NF-κB pathways in the jejunum. (**C**) The energy produced by lipid metabolism regulates the pig intestinal barrier through the AMPK/mTORC1 and AMPK/Sirt1/NF-κB pathways. SBO, soybean oil group; PO, palm oil group; EPO, encapsulated palm oil group. Different letters (a, b, and c) indicate a significant difference among different groups ($p < 0.05$).

4. Discussion

The intestinal epithelium is a selective permeability barrier that effectively absorbs nutrients and protects the internal environment from the penetration of harmful molecules (such as pathogens, toxins, and antigens) [15]. Intestinal barrier disruption will increase the intestinal cell permeability and the penetration of proinflammatory factors in the intestinal cavity, thereby inducing the activation of the intestinal mucosal immune system and leading to intestinal inflammation and tissue damage [4,16,17]. After weaning, drastic changes in the diet structure and incomplete intestinal development might disrupt the intestinal flora balance, induce intestinal inflammation, and even result in diarrhea in piglets. In addition, early weaning may increase intestinal permeability and reduce the expression of tight junction proteins in piglets [18]. Thus, nutritional strategies designed to enhance the intestinal barrier function are crucial during the weaning period.

Dietary fat is generally added to diets to provide energy. Fatty acids from dietary fat participate in various metabolic reactions [19–21]. However, the effects of fatty acids with different chain lengths differ. Short-chain fatty acids (SCFAs) maintain intestinal biological functions such as energy recovery, cell proliferation, and intestinal barriers [22–25]. The addition of sodium butyrate to the diet of weaned piglets reduces the incidence of diarrhea because the butyrate treatment increases the mRNA expression of the occludin and ZO-1 in the intestinal mucosa [26]. Similarly, Peng et al. [27] reported that butyric acid increases tight junction integrity and promotes Ca^{2+}-induced tight junction assembly in

Caco-2 cells. Medium-chain fatty acids (MCFAs) are used directly by intestinal cells to generate energy, which helps support the integrity of piglet intestines [28]. Long-chain fatty acids (LCFAs) ameliorate intestinal inflammation by inhibiting the excessive release of proinflammatory factors [19,20,29], particularly n-3 polyunsaturated fatty acids (PUFAs), arachidonic acid (ARA), and conjugated linoleum acid (CLA) [30–32]. The n-3 and n-6 polyunsaturated fatty acids (PUFAs), such as DHA, reduce inflammatory damage to the occludin and ZO-1 [33]. In addition, compared with corn oil, the addition of 5% fish oil to the diet improves the intestinal morphology and increases the expression of the tight junction proteins occludin and claudin-1 [34]. As shown in our previous study, when 6% palm oil is added to the diet, the diarrhea rate of piglets in the palm oil group is significantly higher than that in the soybean oil group (Table S3) [35], which may be related to the substantial difference in fatty acid composition (saturated and unsaturated fatty acids) between soybean oil and palm oil. AMPK is significantly activated in the small intestine of piglets fed palm oil, potentially due to the energy deficiency caused by the low digestibility of PO. AMPK is upstream of mTORC1 and functions as a critical regulator of protein synthesis, including tight junction proteins. In this experiment, claudin expression and mTORC1 signaling pathway activity were inhibited upon the administration of palm oil. Furthermore, the microencapsulation of palm oil activated the mTORC1 signaling pathway and increased the expression of claudin and ZO-1 compared with palm oil in the present study. This evidence suggests that the regulation of intestinal tight junctions by different types of oil may be partially related to their digestibility in the intestine.

Fatty acids are absorbed by the transportation system in intestinal epithelial cells and then transported into the bloodstream via the lymphatic circulation through the interaction with lipoproteins. Intriguingly, proteins involved in fatty acid absorption and transportation are also regulated by palm oil administration due to energy deficiency. Multiple proteins in the intestinal fatty acid transport system have been identified. For example, FAT/CD36, FATP4, and I-FABP are membrane transporters involved in fatty acid transport. Apo AIV is a glycoprotein that is involved with fat absorption. It is secreted by the intestine and interacts with chylomicron. In this experiment, the microencapsulation of palm oil also increased the expression of CD36, FATP4, I-FABP, and Apo AIV compared with the palm oil.

In addition to regulating intestinal mucosal permeability and fatty acid transport, fatty acids also affect intestinal defensin secretion [29]. A clear correlation between the fatty acid chain length and the efficiency of induced defensin expression has been identified. When the carbon number of the fatty acid is four or less, the defensin-inducing activity gradually increases as the carbon number increases [36]. However, when the carbon number of a fatty acid exceeds four, its ability to induce defensin activity decreases as the carbon number increases [36]. Caprylic acid and nonanoic acid enhance the immune barrier function of the intestinal epithelium by increasing the expression of the pBD-1 and pBD-2 genes [37]. Octanoic acid and nonanoic acid regulate β-defensin expression by inhibiting the histone deacetylase (HDAC) activity [37]. These results collectively imply that fatty acid-induced β-defensin expression depends on the fatty acid type. Thus, a study of the effects of fat sources and/or types on β-defensin expression is interesting. In the present study, the encapsulation of palm oil significantly increased the duodenal, jejunal, and ileal pBD-2 secretion compared with the palm oil treatment, which may be due to the modification of oil digestion and absorption efficiency in the intestine. Previously, pigs fed encapsulated palm oil were reported to display increased average daily gain (ADG) and G:F levels compared with pigs fed palm oil without encapsulation [35]. In addition, increased fat digestibility is also accompanied by an increased utilization of dry matter (DM) and GE. Thus, fat encapsulation might regulate nutrient digestibility and the activity of AMPK, which modulate the expression of pBD-2 through SIRT1/NF-κB signaling. Consistent with these findings, NF-κB signaling was inhibited by palm oil treatment compared with encapsulated palm oil treatment in this trial. The enhanced physical barrier and immune barrier functions might partially explain how lipid encapsulation alleviates diarrhea occurrence.

5. Conclusions

Fat is an important energy source in animal production. Studies of the effect of oil on gut function will facilitate the development and utilization of new fat sources. In summary, our experiments confirmed that dietary fat regulates the expression of fatty acid transport proteins, intestinal tight junction proteins, and β-defensins. Moreover, we have shown for the first time that fat encapsulation reduces the incidence of diarrhea partially by alleviating the damage to the intestinal mechanical and immunological barriers induced by fat with a low digestibility. At the same time, we hypothesize that the improvement in gut function mediated by microencapsulated fat may be related to the increased efficiency of fatty acid utilization through the AMPK/mTORC1 and AMPK/Sirt1/NF-κB pathways.

Supplementary Materials: The following materials are available online at https://www.mdpi.com/2076-2615/11/1/28/s1: Table S1: Composition and nutrient content of experimental diets (as-fed basis) [35], Table S2: Analyzed fatty acid compositions of the fat sources used in the experiment [35], and Table S3: Diarrhea incidence in piglets [35].

Author Contributions: M.T., F.C., S.Z., and W.G. conceived and designed the experiments. M.T., J.C., Z.W., H.S., C.C., and F.Y. performed the experiments. M.T. and J.C. analyzed the data and wrote the paper. S.Z. and W.G. conducted the final editing and proofreading. All authors have read and agreed to the published version of the manuscript.

Funding: This study was supported by National Natural Science Foundation of the China (Nos. 31802067 and 31872364), National Key R&D Program of China (No. 2017YFD0500500), and Natural Science Foundation of Guangdong Province (No. 2018A030310201).

Institutional Review Board Statement: The study was conducted according to the guidelines of the South China Agricultural University Animal Care and Use Committee (No. 20110107-1, Guangzhou, China).

Informed Consent Statement: Not applicable.

Data Availability Statement: All data used in the current study are available from the corresponding author on reasonable request.

Conflicts of Interest: The authors declare no conflict of interest.

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ORIGINAL RESEARCH
published: 25 March 2021
doi: 10.3389/fmicb.2021.647242

Anethole Attenuates Enterotoxigenic *Escherichia coli*-Induced Intestinal Barrier Disruption and Intestinal Inflammation via Modification of TLR Signaling and Intestinal Microbiota

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Edited by:
Takahiro Masuda,
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Reviewed by:
Shiyan Qiao,
China Agricultural University, China
Xin Wu,
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Specialty section:
This article was submitted to Microbial Immunology, a section of the journal Frontiers in Microbiology

Received: 29 December 2020
Accepted: 25 February 2021
Published: 25 March 2021

Citation:
Yi Q, Liu J, Zhang Y, Qiao H, Chen F, Zhang S and Guan W (2021) Anethole Attenuates Enterotoxigenic Escherichia coli-Induced Intestinal Barrier Disruption and Intestinal Inflammation via Modification of TLR Signaling and Intestinal Microbiota. Front. Microbiol. 12:647242. doi: 10.3389/fmicb.2021.647242

This study aimed to investigate the effects of dietary anethole supplementation on the growth performance, intestinal barrier function, inflammatory response, and intestinal microbiota of piglets challenged with enterotoxigenic *Escherichia coli* K88. Thirty-six weaned piglets (24 ± 1 days old) were randomly allocated into four treatment groups: (1) sham challenge (CON); (2) *Escherichia coli* K88 challenge (ETEC); (3) *Escherichia coli* K88 challenge + antibiotics (ATB); and (4) *Escherichia coli* K88 challenge + anethole (AN). On day 12, the piglets in the ETEC, ATB, and AN group were challenged with 10 mL *E. coli* K88 (5×10^9 CFU/mL), whereas the piglets in the CON group were orally injected with 10 mL nutrient broth. On day 19, all the piglets were euthanized for sample collection. The results showed that the feed conversion ratio (FCR) was increased in the *Escherichia coli* K88-challenged piglets, which was reversed by the administration of antibiotics or anethole ($P < 0.05$). The duodenum and jejunum of the piglets in ETEC group exhibited greater villous atrophy and intestinal morphology disruption than those of the piglets in CON, ATB, and AN groups ($P < 0.05$). Administration of anethole protected intestinal barrier function and upregulated mucosal layer (mRNA expression of mucin-1 in the jejunum) and tight junction proteins (protein abundance of ZO-1 and Claudin-1 in the ileum) of the piglets challenged with *Escherichia coli* K88 ($P < 0.05$). In addition, administration of antibiotics or anethole numerically reduced the plasma concentrations of IL-1β and TNF-α ($P < 0.1$) and decreased the mRNA expression of TLR5, TLR9, MyD88, IL-1β, TNF-α, IL-6, and IL-10 in the jejunum of the piglets after challenge with *Escherichia coli* K88 ($P < 0.05$). Dietary anethole supplementation enriched the abundance of beneficial flora in the intestines of the piglets. In summary, anethole can improve the growth performance of weaned piglets infected by ETEC through attenuating intestinal barrier disruption and intestinal inflammation.

Keywords: anethole, piglet, *Escherichia coli* K88, antibiotics, microbiota

INTRODUCTION

Enterotoxigenic *Escherichia coli* (ETEC) is considered one of the main causes of diarrhea in weaning piglets (Fairbrother et al., 2005). Generally, a poor breeding environment causes an increase in intestinal ETEC (Yokoyama et al., 1992), disrupts the balance of intestinal flora (Li et al., 2012) and affects the digestion and absorption of nutrients (Gao et al., 2013). The enterotoxins secreted by ETEC can destroy the intestinal mucosa layer and tight junction structure, which leads to increased permeability of the intestine (Fleckenstein et al., 2010; Dubreuil, 2012). Bacteria or antigens that pass through the intestinal mucosa are captured and recognized by immune cells, which further activate the immune response and inflammatory process (Moretó and Pérez-Bosque, 2009; Turner, 2009). Damage to the intestine could reduce growth performance, cause severe diarrhea, and even lead to piglet death (Fleckenstein et al., 2010).

A large number of antibiotics are used in animal production worldwide each year, of which most are antibiotic growth promoters (AGPs). Based on a survey of antibiotic usage in China for 2013, total antibiotic production usage was approximately 162,000 tons, of which 52% was used in animals (Ying et al., 2017). According to the requirements of Chinese government, feed manufacturers is not allowed to produce commercial feeds containing growth-promoting drug feed additives since July 1, 2020.

"Medicine and food are homologous" comes from a view of traditional Chinese medicine, meaning that some of food can have a certain therapeutic effect. Anethole (AN) was originally extracted from fennel and has long been proven to have anti-inflammatory effects, and that it's also has been used in animal production (Windisch et al., 2008). Previous studies have reported that AN can improve the growth performance of animals at an appropriate dosage (Kim et al., 2013; Zeng et al., 2015; Charal et al., 2016). However, to the best of our knowledge, there is no comprehensive report on the effects of AN on ETEC-infected piglets. Thus, the primary aim of this study was to determine the effects of AN on the growth performance, intestinal barrier function, inflammatory response and intestinal microbiota of piglets challenged with ETEC.

MATERIALS AND METHODS

Ethics Approval

All the experimental protocols in this study were satisfy the needs of animal welfare and conducted in strict accordance with the Guidelines for the Protection and Use of Laboratory Animals issued by the South China Agricultural University Animal Care and Use Committee (No. 20110107-1, Guangzhou, China).

Animals, Housing, and Experimental Design

This trial is conducted in an experimental house with a controlled temperature at 30 ± 2°C and humidity below 80%. Piglets were individually fed in metabolic cages (1.2 m × 0.4 m × 0.5 m) with a three-day adaptation period to adapt to the new environment and feed. All piglets had free access to feed and water. During the adaptation period, piglets did not show any symptoms of diarrhea, skin lesions and obvious inflammation, which indicated that piglets were healthy and suitable for this experiment. After adaption, 36 male piglets (Duroc × (Landrace × Yorkshire), initial weight 7.5 ± 1 kg) were assigned to one of four treatments according to the principle of similar weight (n = 9). This experiment last for 19 days. Four treatments are listed as follows: (1) sham challenge (CON); (2) *Escherichia coli* K88 challenge (ETEC); (3) *Escherichia coli* K88 challenge + antibiotics (ATB); and (4) *Escherichia coli* K88 challenge + anethole (AN). CON and ETEC groups receives the control diet, ATB group receives the control diet supplemented with antibiotics (50 mg/kg quinocetone, 75 mg/kg chlortetracycline, 50 mg/kg kitasamycin), and AN group receives the control diet supplemented with AN (300 mg/kg, pure AN ≧ 7.5%, coating with corn starch, Pancosma, Switzerland). The feed formula was prepared according to NRC (2012). The ingredient composition and nutrient levels of control diet are presented in **Supplementary Table 1**.

Enterotoxigenic *Escherichia coli* K88 Challenge

Escherichia coli K88 (CVCC225) was purchased from the Chinese Veterinary Medicine Collection Center, and it was confirmed to have heat labile enterotoxin (LT), heat stable enterotoxin (ST), and F4 fimbriae in our laboratory (Ren et al., 2019). On day 12, piglets from the ETEC, ATB, and AN group orally challenged with 10 mL nutrient broth (NB) containing 5 × 10^9 CFU/mL ETEC K88 via a syringe, CON group orally injected with 10 mL of sterilized NB. CON group was kept in isolation in order to avoid cross-contamination.

Sample Collection

Blood and feces samples were collected on day 19. Five milliliter of blood samples were collected into tubes containing EDTA via the anterior vena cava puncture and quickly centrifuged (1000 × g, 4°C, 10 min) for plasma samples in 30 min, then stored at −80°C. At the same time, over 5 g fresh feces samples were collected into centrifuge tubes and stored at −80°C. After blood and feces samples collection, piglets were immediately euthanized. About 2 cm length of duodenum (about 10 cm near the pylorus) and jejunum (about 60 cm near the pylorus) were collected, then stored into 4% paraformaldehyde solution for histological analyses. Jejunal and ileal segments (10 cm length) were opened longitudinally and the contents were flushed in cold

Abbreviations: ADFI, average daily feed intake; ADG, average daily gain; AN, anethole; ATB, antibiotics; BW, body weight; CON, control; ETEC, enterotoxigenic *Escherichia coli*; F/G, feed conversion ratio; IL-1β, interleukin-1β; IL-6, interleukin-6; IL-10, interleukin-10; LPS, lipopolysaccharide; MLN, mesenteric lymph nodes; MyD88, myeloid differentiation factor 88; NF-κB, nuclear factor kappa B; NMDS, Non-metric multi-dimensional scaling; OTU, operational taxonomic unit; PCoA, principal coordinate analysis; SEM, standard error of mean; SIGIRR, single immunoglobulin and toll-interleukin 1 receptor domain; TLR, toll like receptor; TNF-α, tumor necrosis factor-α; TOLLIP, toll interacting protein; TRAF6, tumor necrosis factor receptor-associated factor 6; VCR, villi height and crypt depth ratio; ZO-1, Zonula occludens protein-1.

normal saline (NS) solution for twice. Mucosa was collected by scraping using a sterile glass microscope slide at 4°C, rapidly frozen in liquid N_2 and stored at −80°C for the analysis of mRNA and protein expression. Similarly, mesenteric lymph node (MLN) was collected and rapidly frozen in liquid N_2 for the analysis of mRNA expression. The time from anesthesia to complete sampling was controlled at about 30 min per piglet.

Measurements

Growth Performance

Feed intake of each piglets was daily recorded. Body weight of each piglets were recorded on day 0, day 12, and day 19 to calculate average daily gain (ADG), average daily feed intake (ADFI) and F/G (Feed conversion ratio) respectively.

Immunological Parameters

Plasma IL-1β, TNF-α, IL-6, and IL-10 were analyzed by using commercially available porcine ELISA kits (Huamei, Wuhan), according to the manufacturer's instructions. All assays were run in duplicate and diluted if necessary.

Intestinal Morphology

The samples of duodenum and jejunum were embedded in paraffin. Each sample was used to prepare one slide with two sections (4 μm thickness), which were stained with Hematoxylin-Eosin. Three views of each section and three well-oriented villi and crypts of each view were selected for intestinal morphology measurement. Villi height and crypt depth ratio (VCR) was calculated after measure.

Quantitative PCR for Gene Expressions

Total RNA was extracted from the frozen jejunum, ileum, and MLN tissues by using total RNA extraction kit (LS040, Promega, Shanghai, China) according to the manufacturer's instruction. The quality, purity and concentrations of RNA samples were assessed by electrophoresis on 1.5% agarose gel (130 V, 18 min) and NanoDrop Spectrophotometer (A260/A280). Then, the RNA was adjusted to a uniform concentration by using RNase-free ddH_2O. Subsequently, reverse transcription of the RNA to complementary cDNA was performed using a cDNA reverse transcription kit (RR047A, Takara, Tianjin, China). Quantitative PCR by using the SYBR green system (RR820A, Takara) was performed on QuantStudioTM 6 Flex (Applied Biosystems, CA, United States). The reaction mixture (10 μL) contained 5 μL of SYBR Green PCR Master Mix, 1 μL of cDNA, 0.4 μL of forward and reverse primer (10 μM/L), 0.2 μL of ROX Reference Dye II (50×), and 3 μL of RNase-free ddH_2O. The PCR reaction was repeated three times for each gene and carried out as following: one cycle at 50°C for 120 s and 95°C for 600 s, forty cycles at 95°C for 15 s and 60°C for 60 s and one cycle at 95°C for 15 s, 60°C for 60 s, and 95°C for 15 s. Target gene expression was calculated based on the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001) and normalized to GAPDH. The primer sequences were designed by using Primer 3.0 (**Supplementary Table 2**).

Western Blot Analysis for Protein Expressions

The total protein in the frozen jejunal and ileal tissue samples was lysed in RIPA (P0013B, Biyuntian, Shanghai, China). The protein concentration of each sample was measured using BCA protein assays (P0010, Biyuntian, Shanghai, China). Equal amounts of denatured protein (25 μg) from each sample were separated on 10% SDS-PAGE and then electroblotted onto PVDF membranes. Membranes were blocked for 2 h with 5% skimmed milk in TBST at room temperature. Subsequently, the membranes were incubated with specific antibody [ZO-1 (ab96b87, Abcam, United States), Occludin (ab31721, Abcam, United States), Claudin-1 (ab15098, Abcam, United States), and β-actin (bs-0061R, Bioss, China)] for 12 h at 4°C, and then incubate with secondary antibody for 1 h at room temperature. Finally, the proteins were detected using ECL chemiluminescence reagents (P1020, ApplyGen, Beijing, China) and FluorChem M Fluorescent Imaging System (ProteinSimple, CA, United States). Protein expression were analyzed by using image J software.

16S rDNA Sequencing

Gut microbiome of feces was analyzed by High-throughput 16S rDNA sequencing technology. Sequencing (PE250, NovaSeq6000, Illumina Inc., CA, United States) was performed by Novogene Co., Ltd. (Beijing, China), the V3–V4 region of the 16S rDNA was amplified using primers 341F (5′-CCTAYGGGRBGCASCAG) and 806R (5′-GGACTACNNGGGTATCTAAT). For 16S rDNA sequencing data, statistical analyses were performed with NovoMagic (Novogene Co., Ltd.) online tools.

Statistical Analysis

All data of this experiment were analyzed by using one-way ANOVA according to the general linear models (GLM) procedure of SPSS 22.0 (IBM Inc., United States). Data were expressed as means ± SEM. Comparisons between the values of mean treatment were made by LSD using Duncan's multiple test. $P < 0.05$ was considered as statistically significant differences and as tendency when $0.05 < P < 0.10$.

TABLE 1 | Effects of anethole on growth performance of piglets challenged with enterotoxigenic *Escherichia coli* K88.

Items	Treatments				SEM	*P*-value
	CON	ETEC	ATB	AN		
Initial BW (kg)	7.17	7.13	7.32	7.25	0.16	0.977
Final BW (kg)	13.78	12.69	13.60	13.51	0.41	0.795
Pre-challenge (0–12 days)						
ADFI (g)	357	358	355	371	18	0.990
ADG (g)	275	272	280	285	14	0.989
F/G	1.33	1.30	1.31	1.33	0.04	0.995
Post-challenge (13–19 days)						
ADFI (g)	623	559	623	608	27	0.832
ADG (g)	445	307	406	394	22	0.136
F/G	1.47^b	1.87^a	1.52^b	1.55^b	0.06	0.050

n = 9. Different letters mean statistically significant difference among the groups (P < 0.05).

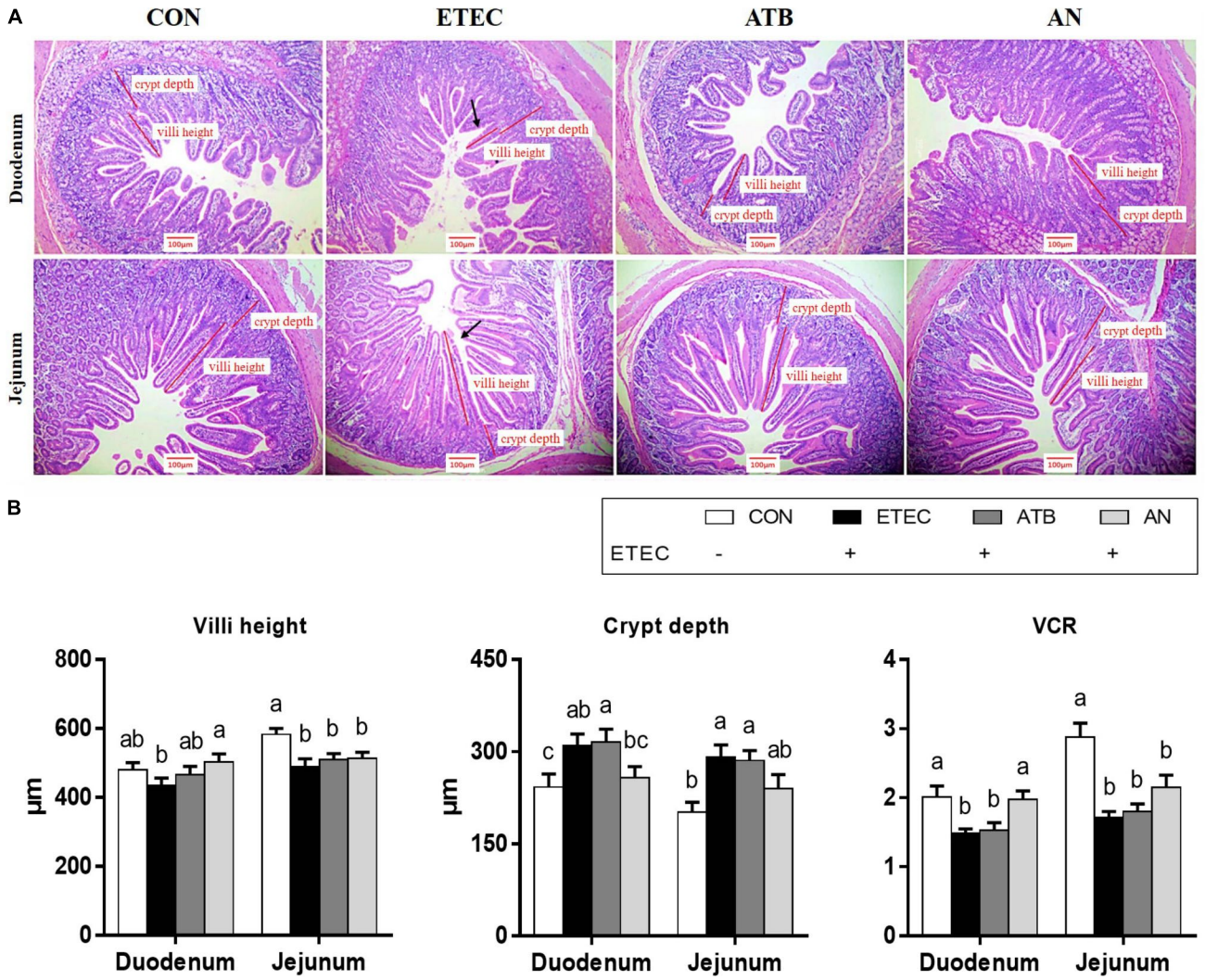

FIGURE 1 | Effects of anethole on intestinal morphology of piglets challenged with enterotoxigenic *Escherichia coli* K88. **(A)** HE staining of intestine **(B)** Villi height, crypt depth and VCR of intestine. The data in each group is expressed as the mean ± SE (n = 9). Different letters mean statistically significant difference among the groups (P < 0.05).

RESULTS

Performance

After ETEC challenge, the piglets fed diets containing antibiotics or AN had lower (P < 0.05) F/G than the piglets challenged with ETEC, and this F/G was similar to that of the piglets given the CON treatment (**Table 1**).

Intestinal Morphology

The duodenums of the piglets in the AN group exhibited higher villus heights (P < 0.05) and VCRs (P < 0.05) than those of the piglets in the ETEC group. The piglets in the ATB group had greater duodenal crypt depths (P < 0.05) than those in the CON and AN groups. The jejunums of the piglets in the CON group had higher villus heights (P < 0.05) and VCRs (**Figure 1**, P < 0.05) than those of the piglets in the other groups. In addition, the piglets in the CON group had lower crypt depths (**Figure 1**, P < 0.05) than those in the ETEC and ATB groups.

Barrier Function

The relative mRNA expression of mucin-1, mucin-2, ZO-1, Occludin, and Claudin-1 in the jejunum and ileum of the piglets was significantly downregulated (P < 0.05, **Figure 2**) in response to ETEC challenge ($P_{mucin-2}$ = 0.063). However, this downregulation was partially mitigated by dietary supplementation with antibiotics or AN. In addition, the ZO-1, Occludin, and Claudin-1 protein levels were also markedly decreased (P < 0.05, **Figure 2**) after ETEC challenge, which can be attenuated by dietary supplementation with antibiotics or AN.

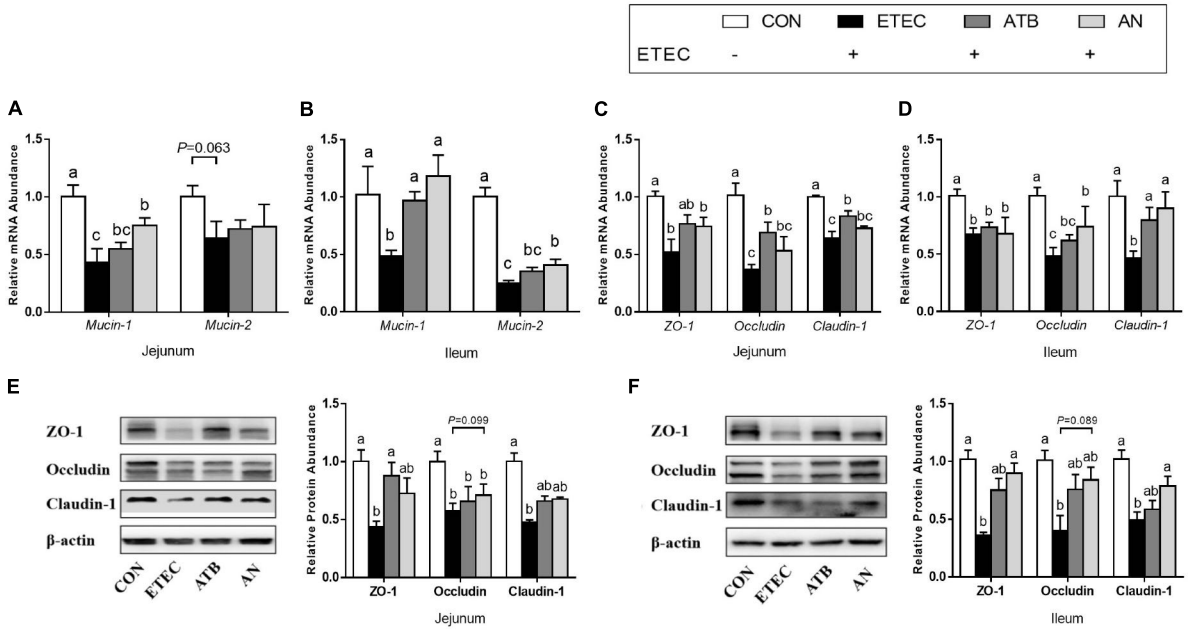

FIGURE 2 | Effects of anethole on **(A–B)** intestinal secretory and **(C–F)** barrier function of piglets challenged with enterotoxigenic *Escherichia coli* K88. For PCR assays, $n = 8$, *GAPDH* as reference gene; For western blot, $n = 3$, β-actin as reference protein; The data in each group is expressed as the mean ± SE. Different letters mean statistically significant difference among the groups ($P < 0.05$).

Inflammatory and Immunological Responses

The plasma levels of IL-1β and TNF-α in the piglets tended to increase after ETEC challenge ($P < 0.1$), whereas this tendency was not observed in the ETEC-challenged piglets in the ATB and AN groups. Compared with those of the piglets in the CON group, the relative mRNA expression levels of certain genes were upregulated ($P < 0.05$) in the jejunum (TLR9, MyD88 and IL-10), ileum (TLR5), and MLN (NF-κB) of the piglets in the ETEC group, which could be attenuated by supplementation with antibiotics and AN. Compared with those of the piglets in the ATB group, the relative mRNA expression levels of certain genes was significantly decreased ($P < 0.05$) in the jejunum (TLR5 and TLR9) and significantly increased ($P < 0.05$) in the MLN (TRAF6) of the piglets in the AN group. In addition, compared with those of the piglets in the CON or ETEC group, the relative mRNA expression levels of genes related to the MyD88/NF-κB signaling pathway were upregulated ($P < 0.05$) in the jejunum (SIGIRR) and ileum (SIGIRR and IL-10) of the piglets in the ETEC group and the ileum (SIGIRR) and MLN (SIGIRR) of the piglets in the AN group (**Figure 3**).

Gut Microbiome

A total of 1,775,153 high-quality sequences were generated from 20 fecal samples (four treatments, $n = 5$), with an average of 88,758 sequences per sample, and 64,854 ± 2,566 effective tags were obtained for subsequent analysis after the noise sequences were discarded. Finally, all the effective tags were clustered to operational taxonomic units (OTUs) at 97% sequence similarity and then allotted to 23 phyla, 39 classes, 81 orders, 145 families, 322 genera, and 1,738 OTUs. For alpha-diversity, the bacterial richness ACE and Chao1 index of the AN group were markedly higher than those of the CON and ETEC groups ($P < 0.05$), the richness Observed_species index of the ETEC and AN group had a tendency of significant difference ($0.05 < P < 0.10$), the diversity Shannon and the Simpson index had no significant difference among four treatment groups ($P > 0.05$). For beta diversity, the PCoA (PC1 32.33% vs PC2 20.26%) and NMDS (stress = 0.133) analyses based on Weighted UniFrac distances showed that the microbiota from the piglets in the ETEC group obviously tended to separate from that of the piglets in both the ATB and AN group (**Figure 4**).

At the phylum level, five major bacteria in the feces of piglets were Firmicutes (51.74–9.85%), Bacteroidetes (6.97–34.30%), Spirochaetes (0.35–13.06%), Actinobacteria (0.84–7.26%), and Euryarchaeota (0.01–5.69%). At the genus level, unidentified_*Clostridiales* (1.59–35.52%), *Catenibacterium* (0.30–16.21%), *Blautia* (0.81–15.16%), *Lactobacillus* (0.51–13.55%), *Terrisporobacter* (0.37–12.64%), and *Catenisphaera* (0.20–10.97%) were the most predominant genera in all the samples, and three genera (*Lactobacillus*, unidentified_*Ruminococcaceae*, and *Selenomonas*) were significantly different among the different groups on top 10 (**Figure 5** and **Supplementary Table 4**). *Lactobacillus* abundance in the ATB group was significantly higher ($P < 0.05$) than that in the ETEC group. unidentified_*Ruminococcaceae* abundance in the ATB and AN groups was significantly lower ($P < 0.05$) than that in the ETEC group. *Selenomonas* abundance in the AN group was significantly higher ($P < 0.05$) than that in the ETEC group.

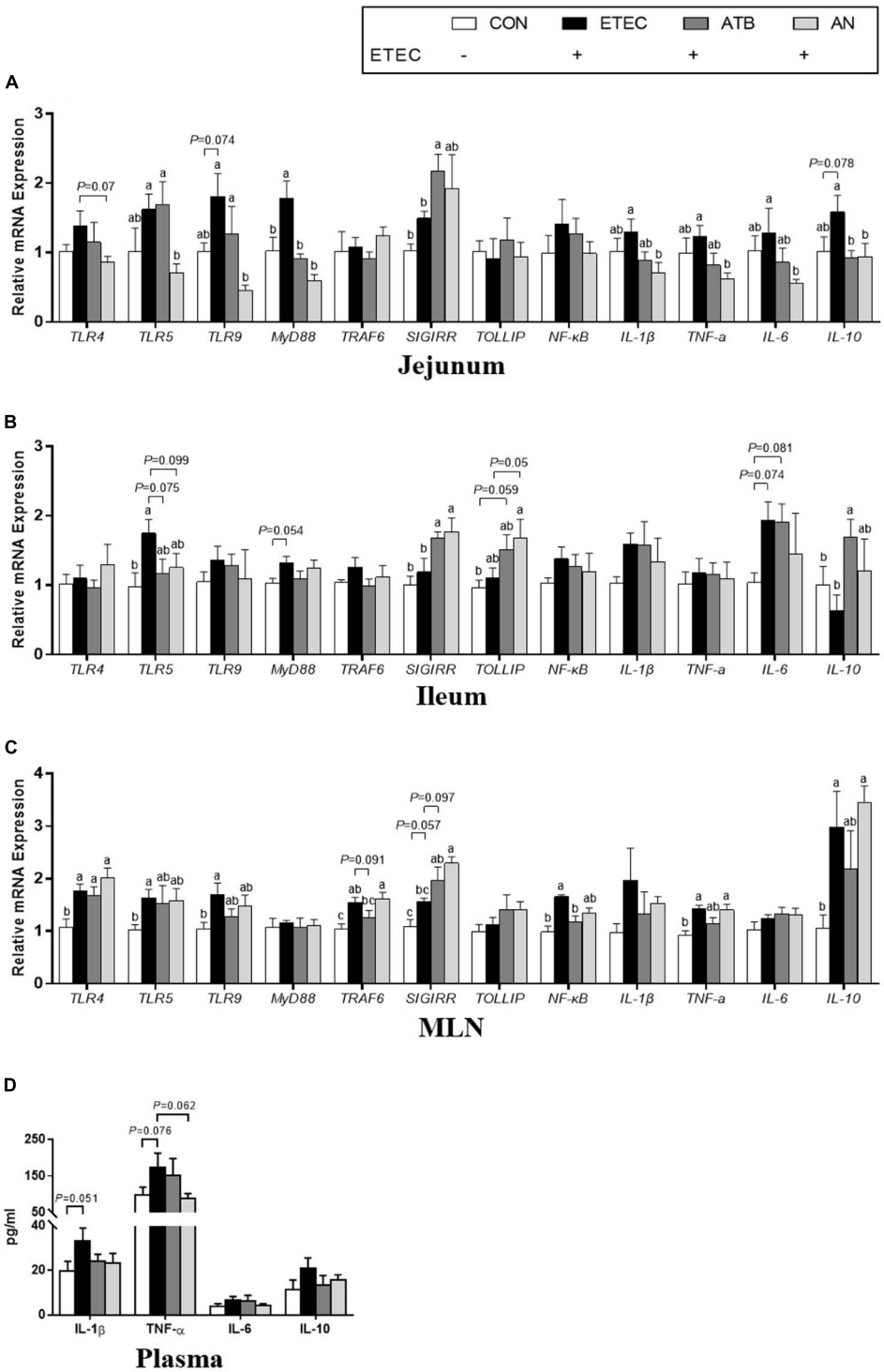

FIGURE 3 | Effects of anethole on concentration of relative mRNA expression of genes related to inflammation **(A)** jejunum, **(B)** ileum, **(C)** MLN, and **(D)** plasma cytokine and of piglets challenged with enterotoxigenic *Escherichia coli* K88. ELISA for plasma, $n = 9$; For PCR assays, $n = 8$, *GAPDH* as reference gene; The data in each group is expressed as the mean ± SE. Different letters mean statistically significant difference among the groups ($P < 0.05$).

DISCUSSION

Enterotoxigenic *Escherichia coli* regulates the secretion of enterotoxins and induces diarrhea and intestinal impairment in weaned piglets (Che et al., 2017). In the present study, the FCR of piglets was significantly increased after ETEC challenge. Correspondingly, after statistical analysis of the internal morphology of piglets, we can find that after ETEC

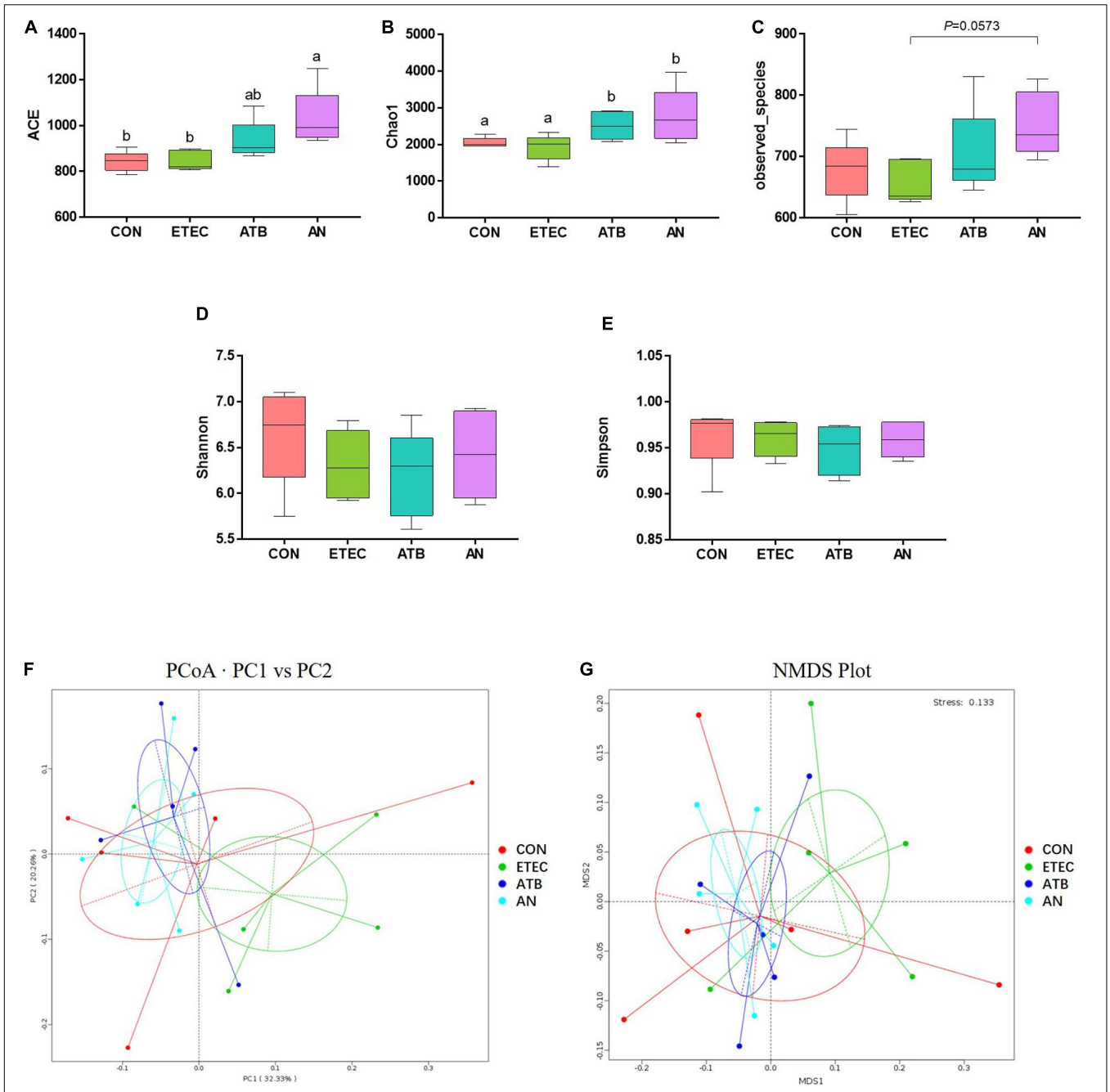

FIGURE 4 | Effects of anethole on alpha diversity and beta diversity of fecal microbiota of piglets challenged with enterotoxigenic *Escherichia coli* K88 (n = 5). **(A–E)** Alpha Diversity index: ACE, Chao1, Observed_species, Shannon, Simpson. **(F)** Principal Coordinates Analysis based weighted Unifrac. **(G)** NMDS Plot Analysis based weighted Unifrac.

challenge, the VCR values of duodenum and jejunum of piglets in ETEC group were significantly reduced ($P < 0.05$), while the VCR value of duodenum of piglets in AN group did not decrease significantly, which is the most direct evidence that AN can help piglets resist ETEC infection. Maruzzella and Freundlich found that AN has the strong bacteriostatic effect (Maruzzella and Freundlich, 2010). Meanwhile, some other studies have found that AN inhibits the secretion of acetylcholinesterase (AchE) and increases the concentration of acetylcholine (Ach; Bhadra et al., 2011), high Ach levels trigger intestinal smooth muscle contraction and enhance gastrointestinal motility, which may decrease the opportunities for ETEC colonization of the intestine.

Toll-like receptors (TLRs) play an important role in the regulation of innate immunity in animals. Numerous pathogenic molecules have been reported to be recognized by TLRs. For

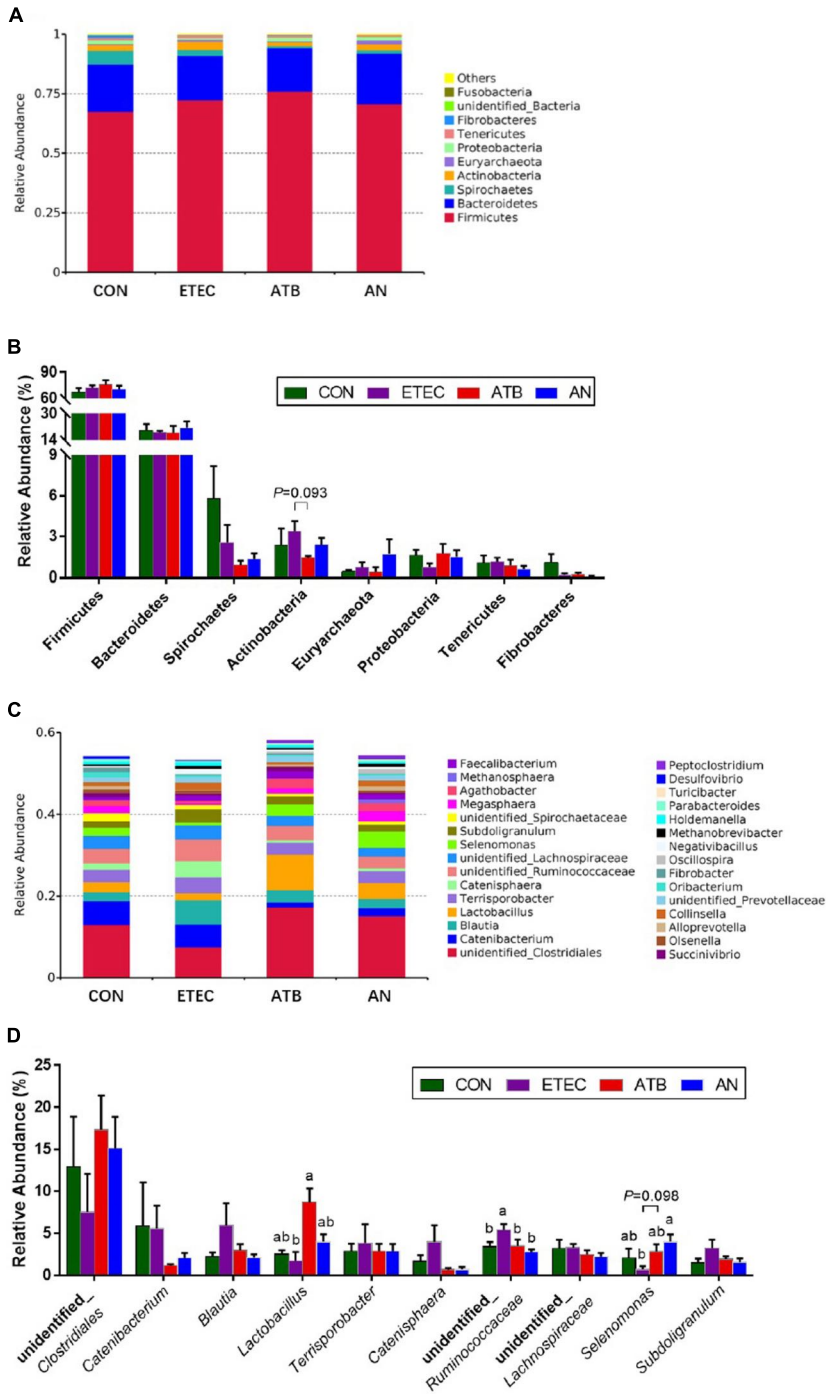

FIGURE 5 | Effect of anethole on microbial composition of fecal microbiota of piglets challenged with enterotoxigenic *Escherichia coli* K88 (n = 5). **(A)** Relative abundance in phyla. **(B)** Top eight in phyla. **(C)** Relative abundance in genus. **(D)** Top 10 in genus.

example, TLR4 proactively identifies the lipopolysaccharide (LPS) of ETEC, while TLR5 and TLR9 recognize flagellin and CpG-DNA, respectively (Cario, 2005; Lu et al., 2008; Kim et al., 2015). The activation of TLRs leads to the secretion of a large number of proinflammatory cytokines via the myeloid differentiation factor 88 (MyD88)/nuclear factor-kappa B (NF-κB) signaling pathway (Kawai and Akira, 2007). The present study showed that seven days after ETEC challenge, the relative mRNA expression of MyD88 in the jejunum was significantly upregulated in the piglets challenged with ETEC. Similarly, TLR5 in the ileum and TLR4, TLR5, TLR9, TRAF6, and NF-κB in the MLN were significantly upregulated. However,

most of these genes (except TLR4 and TRAF6 in MLN) were not altered in the piglets given dietary supplementation of ATB or AN. To maintain the stability of the immune system, the MyD88/NF-κB signaling pathway is negatively regulated by TOLLIP and SIGIRR (Burns et al., 2000; Wald et al., 2003). In this study, the relative mRNA expression of SIGIRR in the jejunum, ileum, and MLN and the relative mRNA expression of TOLLIP in the ileum were upregulated to varying degrees with the administration of AN and ATB. Additionally, no significant difference in the MLN and TOLLIP mRNA expression was identified between the AN and ATB group. These results indicated that the AN supplements had functions similar to those antibiotics, which can inhibit the MyD88/NF-κB signaling pathway by activating its negative regulators. This has shown direct evidence that AN alleviates the inflammation induced by ETEC challenge in piglets.

Activated NF-κB regulates the expression of proinflammatory cytokines (Kawai and Akira, 2007). In the present study, we found that the mRNA expression of IL-1β and TNF-α in the jejunum, ileum and MLN was increased to varying degrees after ETEC challenge. The concentrations of IL-1β and TNF-α in the plasma also tended to increase. Elevated concentrations of IL-1β and TNF-α generate heat and lead to elevated rectal temperature (Yi et al., 2005; Tesch et al., 2018). This observation might partially explain the rapid increase in the intestinal temperature of piglets in response to intestinal infection in our study. Tight junctions (TJs) are the most important connections between cells, TJs only allow soluble, small molecule substances to pass through them, which hinders the passage of macromolecular substances and microorganisms (Lee, 2015), Excessive production of the proinflammatory cytokines IL-1β and TNF-α also break tight junction and increase cell bypass permeability in the gut (McKay and Baird, 1999; Ma et al., 2004; Al-Sadi et al., 2010). In addition, mucin protects the biological function of epithelial cells and participates in the processes of epithelial cell renewal and cell signaling activation, studies have found that downregulated mucin-1 can increase TNF-α expression (Guang et al., 2010). In our study, we observed the disruption of tight junctions and mucin secretion in response to ETEC infection. It is worth noting that AN is not the only essential oil that can regulate the inflammation of the intestine. In previous studies, thymol and oregano were also found to significantly alleviate the increase in IL-1β and TNF-α in the piglet jejunum mucosa caused by challenge with ETEC (Pu et al., 2018).

As we known, intestinal inflammation and gut microorganisms have relations, to investigate the effects of AN on the proliferation of intestinal microbiome in piglets, the microbes in the feces were analyzed by high-throughput 16S rDNA sequencing. In this study, the alpha diversity of the fecal microbiota in the AN group was significantly higher than that in the CON and ETEC group. Beta diversity showed that the microbiota from the ETEC group obviously tended to separate from that of both the ATB and AN group. Thus, the AN group had more similar microbial structures than the ATB group, which is different from the ETEC group. This evidence indicates that AN supplements have functions similar to those of antibiotics in modifying the structure of the intestinal flora. Specifically, we found the ATB group exhibited a significantly increased abundance of *Lactobacillus*, while the AN group exhibited a significantly increased abundance of *Selenomonas*, and both the ATB and AN group exhibited a significantly reduced abundance of unidentified_Ruminococcaceae. Under the normal condition, *Lactobacillus* can inhibit the TLR4 inflammatory signaling triggered by ETEC, which is conducive to the maturation of the intestinal mucosal immune system and triggers local immunomodulatory activity (Zhang et al., 2011; Finamore et al., 2014). *Selenomonas* can produce SCFAs which inhibit inflammation and enhance barrier function (Bladen et al., 1961; Rajilić-Stojanović et al., 2015). Recently study was found that Ruminococcaceae is a biomarker of microbes in oxidative damage and is highly abundant in many intestinal injury models (Zhou et al., 2018). Moreover, several studies have shown that Ruminococcaceae could be involved in recovery after ETEC infection (Salonen et al., 2010; Rajilić-Stojanović et al., 2015). These signs indicated that AN has a positive regulatory effect on intestinal microbiota of piglets infected with ETEC, but its mechanism may be different from the antibiotics. Overall, dietary supplementation with AN enriches the abundance of beneficial flora in the intestines of piglets, which enhances the intestinal functions of piglets and reduces the occurrence of inflammation.

CONCLUSION

In summary, AN can attenuate enterotoxigenic *E. coli*-induced intestinal barrier disruption and intestinal inflammation via modification of TLR signaling and intestinal microbiota, then improving the growth performance of weaned piglets infected by ETEC. Meanwhile, AN is a promising alternative to antibiotics in animal husbandry.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The name of the repository and accession number can be found below: National Center for Biotechnology Information (NCBI) Sequence Read Archive (SRA), https://www.ncbi.nlm.nih.gov/sra, SRR13728343.

ETHICS STATEMENT

The animal study was reviewed and approved by South China Agricultural University Animal Care and Use Committee.

AUTHOR CONTRIBUTIONS

QY was the principal investigator that designed the study, wrote the manuscript, carried out the animal trials, sample analysis, data collection, and statistical analysis. JL, YZ, and HQ carried out the animal trials and sample analysis. FC supervised the study. SZ revised the manuscript. WG designed and supervised the study

and revised the manuscript. All authors read and approved the final manuscript.

FUNDING

This work was supported by National Natural Science Foundation of China (No. 31872364).

ACKNOWLEDGMENTS

We thank Dr. Alexandra Blanchard for technical assistance.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2021.647242/full#supplementary-material

Supplementary Figure 1 | The experimental procedure.

Supplementary Table 1 | Composition and nutrient levels of basal diets (as fed-basis).

Supplementary Table 2 | Primer sequences of target and reference genes.

Supplementary Table 3 | The relative abundances of 8 phyla.

Supplementary Table 4 | The relative abundances of 30 genera.

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Artemisinin Protects Porcine Mammary Epithelial Cells against Lipopolysaccharide-Induced Inflammatory Injury by Regulating the NF-κB and MAPK Signaling Pathways

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Simple Summary: Sow mastitis is a serious breast disease that can cause severe inflammation, agalaxia and even lead to death of piglets. Porcine mammary epithelial cells (pMECs) are the main cell types that affect sow milk secretion, therefore, when swine mastitis occurs, the inflammatory response of pMECs directly affects the mammary gland health and sow's lactation ability. Promoting the health of mammary gland epithelial cells is an important method for treating mastitis. Thus, in the current study, we investigated the effects of artemisinin on the inflammatory response of pMECs induced by lipopolysaccharide (LPS), and proposed a potential anti-inflammatory mechanism. We confirmed that artemisinin can reduce the inflammatory damage of pMECs induced by LPS by inhibiting MAPK and NF-κB signaling pathways. Pretreatment of pMECs with artemisinin showed enhanced anti-inflammatory activity against LPS-induced inflammation. Artemisinin could be a useful, safe and natural anti-inflammatory feed additive to prevent sow mastitis.

Abstract: Artemisinin performs a variety of biological functions, such as anti-cancer, anti-inflammatory, anti-viral, and anti-oxidant effects. However, the effects of artemisinin on sow mastitis have not been studied. The results of the current study showed that mRNA expression abundance and content of the inflammatory factors interleukin-1β (IL-1β), tumor necrosis factor α (TNF-α), and interleukin-6 (IL-6) were significantly increased when using 50 μg/mL LPS to stimulate pMECs for 24 h ($p < 0.05$). Pretreatment with 20 μM artemisinin weakened LPS-induced inflammatory damage in pMECs and decreased mRNA expression abundance and the content of inflammatory factors (IL-1β, IL-6, and TNF-α) in pMECs ($p < 0.05$). Mechanistically, artemisinin inhibited LPS-induced activation of the mitogen-activated protein kinase (MAPK) and nuclear factor-κB (NF-κB) signaling pathways. In summary, the pretreatment of pMECs with artemisinin showed enhanced anti-inflammatory activity against LPS-induced inflammation.

Keywords: porcine mammary epithelial cells; lipopolysaccharide; anti-inflammation; artemisinin

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Citation: Zhang, W.; Xiong, L.; Chen, J.; Tian, Z.; Liu, J.; Chen, F.; Ren, M.; Guan, W.; Zhang, S. Artemisinin Protects Porcine Mammary Epithelial Cells against Lipopolysaccharide-Induced Inflammatory Injury by Regulating the NF-κB and MAPK Signaling Pathways. *Animals* **2021**, *11*, 1528. https://doi.org/10.3390/ani11061528

Received: 15 April 2021
Accepted: 21 May 2021
Published: 24 May 2021

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1. Introduction

Mammalian mastitis is one of the common diseases worldwide, which leads to increased veterinary expenses, and significant economic losses to the breeding industry every year [1,2]. The clinical signs of mammalian mastitis are agalaxia (decreases in milk production and quality) and dolor, which are accompanied with increased inflammatory

mediators in milk. During mammalian mastitis, piglets can develop hypoglycemia and hypothermia, which might finally lead to death [3,4].

Breast tissue contains large numbers of mammary epithelial cells for milk synthesis [5,6], which can also react with pathogens. This response is important for determining the outcome of mammary gland infection [7]. A large number of studies have shown that the inflammatory response of mammary epithelial cells induced by lipopolysaccharide (LPS) can not only reduce milk production and the levels of fat and protein in milk, but also destroy the milk–blood barrier [8–10]. Therefore, it is important to control and reduce the inflammation of mammary epithelial cells during mastitis. Although the use of antibiotics is still an effective way to treat animal mastitis, the use of antibiotics is severely restricted due to the emergence of more and more serious bacterial resistance and food safety [11]. It is important to seek novel alternatives to antibiotics for the effective and safe treatments of mastitis in veterinary research. Chinese herbal medicine with anti-inflammatory activity is a potentially effective option for the treatment of mastitis [12].

Artemisinin (chemical formul, $C_{15}H_{22}O_5$; molecular mass, 282.34 g/moL) is a sesquiterpene lactone compound with a peroxy bridge structure [13]. This specific structure is an important reason for artemisinin and its derivatives being believed to have antimalarial and antibacterial activities [14]. This chemical is isolated from the Chinese plant *Artemisia annua* L. and is effective for the treatment of severe and multidrug-resistant malaria [15–17]. Previous studies have shown that artemisinin has a variety of biological functions, such as antibacterial [18], anti-inflammatory [19], anti-viral [20], antioxidant [21–23], and immunomodulatory activities [24,25]. Although many studies have described the anti-inflammatory functions of artemisinin, the effects of artemisinin on LPS-induced inflammatory damage in porcine mammary epithelial cells (pMECs) remain to be clarified. We hypothesized that artemisinin might have a protective effect on the LPS-induced inflammatory response of pMECs.

2. Materials and Methods

2.1. PMECs Isolation, Cell Culture, and Treatments

pMECs were isolated from the mammary gland of a lactating sow as previously described [26] and keep refrigerated in liquid nitrogen. pMECs were cultured in Dulbecco's modified Eagle's medium/F12 nutrient mixture (DMEM/F12) supplemented with 5 μg/mL hydrocortisone, 10% fetal bovine serum (FBS), 10 ng/mL insulin-like growth factors-1 (IGF-1), 5 μg/mL insulin–transferrin–selenium, 10 ng/mL epidermal growth factor, and 100 U/mL antibiotics (streptomycin and penicillin) at 37 °C in a humidified atmosphere with 5% CO_2. Medium was changed every 48 h, and cells were subcultured by trypsin digestions at a subcultivation ratio of 1:2.

LPS (lipopolysaccharide, Escherichia coli serotype O55:B5, Sigma, Waltham, MA, USA) was diluted in DMEM/F12. Artemisinin (purity > 99%, The National Institutes for Food and Drug Control, Beijing, China) was dissolved in DMSO (Sigma, Waltham, MA, USA) and diluted in DMEM/F12.

2.2. Cell Viability Assay and Flow Cytometric Analysis

To test the cell viability, equal amounts of pMECs were seeded in 96-well plates at a density of 2×10^4 cells/mL and cultured in DMEM/F12 containing 10% FBS. After cultured for 48 h, cells are washed three times with PBS, and then treated with different concentrations of artemisinin or LPS for different times. The cell counting kit - 8 (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) was used to analyze cell viability according to the instruction. An automatic microplate reader (Bio-Rad Laboratories Inc., Foster City, CA) was used to measure the absorbance of all wells at a wavelength of 450 nm. Cell viability is the quantification of No. live cells and is expressed as a percentage of the control.

To test cell apoptosis, pMECs were pretreated with 0 or 20 μM of artemisinin for 12 h before being stimulated with 50 μg/mL LPS for 24 h. After treatment, all cells were

trypsinized, washed 2~3 times with PBS, stained with Annexin V-FITC/PI (Invitrogen Inc., Carlsbad, CA, USA), and analyzed by flow cytometry, according to the instructions.

2.3. Real-Time PCR

According to the experimental requirements, pMECs were treated with LPS or artemisinin, and then the mRNA expression of inflammatory cytokines and apoptosis-related genes were measured by real-time PCR as described previously [27]. Real-time PCR was performed using the ABI StepOnePlus™ real-time PCR system (Applied Biosystems, Grand Island, NE, USA). The amplification of real-time PCR was performed with a reaction volume of 20.0 μL. Samples were analyzed in duplicate.

Real-time PCR reaction protocol: heating at 94 °C for 5 minutes for initial enzyme activation, then performing 40 cycles at 94 °C for 30 s, and then annealing at 60 °C for 30 s, last extension at 72 °C for 20 s. In this study, β-actin was stability expressed among different treatments, which was selected as a reference gene by geNorm 3.5 (http://medgen.ugent.be/~jvdesomp/genorm. accessed on 10 December 2009) in pMEC [28]. The expression level of each gene was calculated according to the $2^{-\Delta\Delta Ct}$ method [29]. Table 1 shows the primer sequences of target genes.

Table 1. Primer sequences for the mRNA.

Genes	Accession	Sequence Primers (5′-3′)	Size (bp)
TNF-α	NM_214022.1	F-ATGGGCTGTACCTCATCTACTC R- GGCTCTTGATGGCAGAGAGG	141
IL-1β	XM_021081828.1	F-CCGAAGAGGGACATGGAGAA R-AGTTGGGGTACAGGGCAGAC	88
IL-6	NM_214399.1	F-TGGCTACTGCCTTCCCTACC R-CAGAGATTTTGCCGAGGATG	132
IL-8	NM_213867.1	F-AGGACCAGAGCCAGGAAGAGAC R-CACAGAGAGCTGCAGAAAGCAG	108
β-actin	XM_021086047.1	F-TGCGGGACATCAAGGAGAAG R-AGTTGAAGGTGGTCTCGTGG	176

Abbreviations: TNF-α, tumor necrosis factor-α; IL-1β, interleukin-1β; IL-6, interleukin-6; IL-8, interleukin-8.

2.4. Measurement of Inflammatory Factor Levels

Cell culture and treatments were performed as described previously [30]. The culture medium in the 6-well plate was aspirated and discarded, the cells were washed with precooled PBS 3 times, and 200 μL of RIPA lysis buffer was added to every well, which was pipetted several times to fully lyse the cells. The lysate was transferred to a 2.0 mL centrifuge tube and centrifuged at 10,000 rpm for 10 min, and the cell supernatant was used to analyze the content of inflammatory factors. Cell supernatants were subsequently used for inflammatory factor analysis with a pig ELISA kit (TNF-α, #CSB-E16980p; IL-1β, #CSB-E06782p; IL-6, #CSB-E06786p), Cusabio Biotech Company, Wuhan, China) according to the manufacturer's instructions.

2.5. Western Blot Analysis

Western blotting was performed as previously described [31]. Proteins were extracted from pMECs using the radio immunoprecipitation assay (RIPA) lysis buffer (#P0013B, Beyotime, Shanghai, China) and quantified using a BCA protein assay kit (#P0009, Beyotime, Shanghai, China). Proteins (10–20 μg/sample) were separated by SDS-PAGE (Invitrogen Inc.), transferred to nitrocellulose membranes (Millipore, Bedford, MA, USA.), and then hybridized with specific antibodies.

Primary antibodies for TLR-4 (1:500, ab13556), p65 (1:1000, ab16502), phospho-p65 (1:1000, ab183559)), β-actin (1:1000, ab5694), p38 (1:1000, ab182453), phospho-p38 (1:1000, ab207483), ERK (1:1000, ab32537), phospho-ERK (1:1000, ab207470), JNK (1:1000, ab126424), and phospho-JNK (1:1000, ab279842) were from the Abcam Company Ltd. (Cambridge, MA, USA). Primary antibodies (dilution, cat. no. follow in parentheses) for IκBα (1:1000,

#9242), and phospho-IκBα (1:1000, #2859) were from the Cell Signaling Technology (Woburn, MA, USA).

2.6. Statistical Analysis

All data are derived from at least 3 independent experiments performed in triplicate, and the results are expressed as mean ± SEM. One-way ANOVA was used for statistical analysis, and then Duncan's test was used for multiple comparisons. $p < 0.05$ was used as the criterion for judging the significance of the difference, and $p < 0.01$ was the criterion for judging the extremely significant difference.

3. Results

3.1. Inflammatory Injury in LPS-Induced PMECs

In order to determine the effect of inflammatory damage on LPS-induced pMEC, cell viability was measured by the CCK-8 assay after the pMECs were treated with various concentrations (5, 10, 25, 50, 100, and 200 μg/mL) of LPS for 12, 24, and 48 h. As shown in Figure 1A, cell viability was significantly inhibited ($p < 0.05$) after stimulation with 200 μg/mL LPS for 12 h. Cell viability was also significantly inhibited ($p < 0.05$) after stimulation with 50, 100 or 200 μg/mL LPS for 24 h. In addition, the cell viability was significantly decreased ($p < 0.05$) after stimulation with all concentrations of LPS for 48 h. As shown in Figure 1B, after pMECs were treated with 50 μg/mL LPS for 24 h, cell apoptosis was significantly increased ($p < 0.05$), and mRNA expression abundance and the content of inflammatory factors (IL-1β, TNF-α, and IL-6) were also significantly increased ($p < 0.05$; Figure 1C,D). Thus, after comprehensive consideration of cell viability, apoptosis and inflammatory factors, 50 μg/mL LPS for 24 h was selected to establish inflammatory injury in pMECs for subsequent experiments.

3.2. Effects of Artemisinin on the Viability of PMECs

In order to investigate the effect of artemisinin on pMECs viability, cells were treated with various concentrations (0.1, 1, 10, 20, 40, and 80 μM) of artemisinin for 6, 12, 24, and 48 h and analyzed by CCK-8 assay. As shown in Figure 2, low concentrations of artemisinin promoted cell growth and induced cell death in a concentration-dependent manner at higher concentrations. Maximum cell viability significantly increased ($p < 0.05$) when pMECs were treated with 20 μM artemisinin for 12 h. According to these results, 20 μM artemisinin (treated with 12 h) was chosen to examine the protective effects of artemisinin against inflammatory injury in LPS-induced pMECs.

3.3. Effects of Artemisinin on Viability and Apoptosis in LPS-Induced PMECs

In order to determine the protective effect of artemisinin on inflammatory injury in LPS-induced pMECs, cell viability and apoptosis were measured after treatment with artemisinin or LPS. As shown in Figure 3, the LPS-induced decrease in cell viability was significantly inhibited ($p < 0.05$) by 20 μM artemisinin (Figure 3A), and LPS-induced pMECs apoptosis was significantly reduced ($p < 0.05$) by artemisinin (Figure 3B,C).

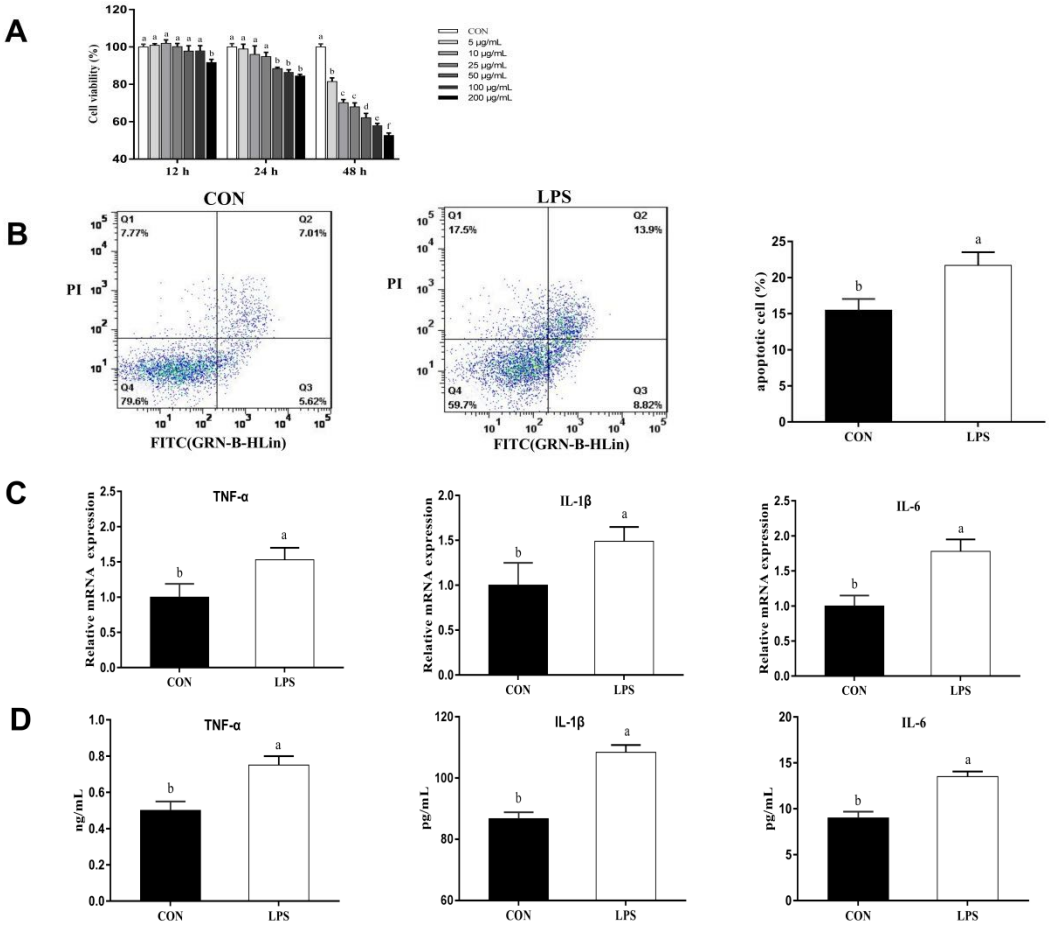

Figure 1. Inflammatory injury in LPS-induced porcine mammary epithelial cells (pMECs). (**A**) Cell viability was measured by CCK-8 following treatment with different concentrations (5, 10, 25, 50, 100, and 200 μg/mL) of lipopolysaccharide (LPS) for 12, 24, and 48 h. The data are the means ± SEM (n = 6 to 12). Different superscript letters (a,b,c) indicate that there are significant differences between the groups ($p < 0.05$). CON = control cells without any treatment. (**B**) After pMECs were treated with 50 μg/mL LPS for 24 h, the percentage of cells undergoing apoptosis was determined by flow cytometry and annexin V/propidium iodide (PI) staining. (**C**) Effects on the mRNA expression of inflammatory factors (IL-1β, TNF-α, and IL-6) after pMECs were treated with 50 μg/mL LPS for 24 h. (**D**) Effects on the levels of inflammatory factors after pMECs were treated with 50 μg/mL LPS for 24 h.

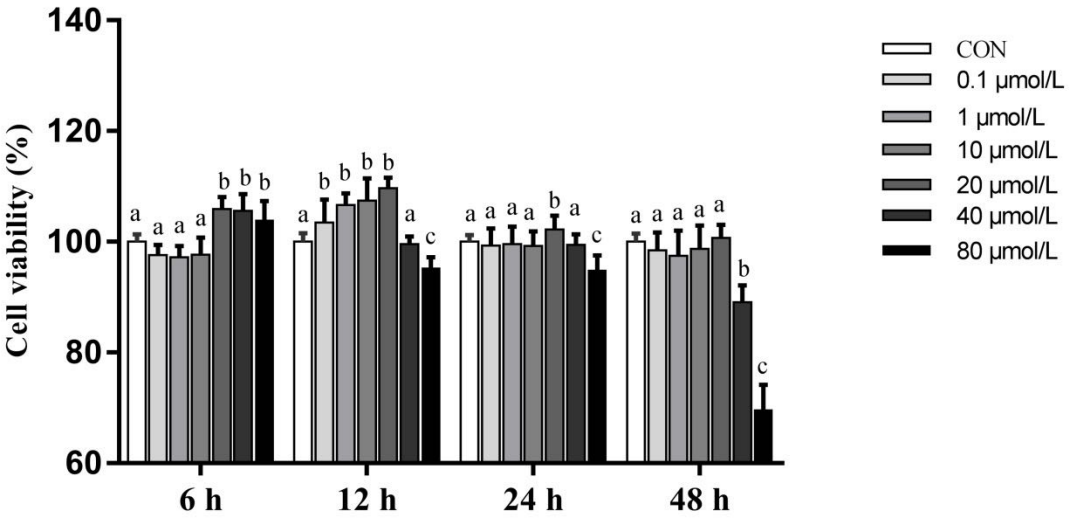

Figure 2. The effects of artemisinin on the viability of pMECs. Cells were treated with different concentrations (0.1, 1, 10, 20, 40, and 80 μM) of artemisinin for 6, 12, 24, and 48 h. Cell viability was determined by the CCK-8 assay. Different superscript letters (a,b,c) indicate that there are significant differences between the groups ($p < 0.05$).

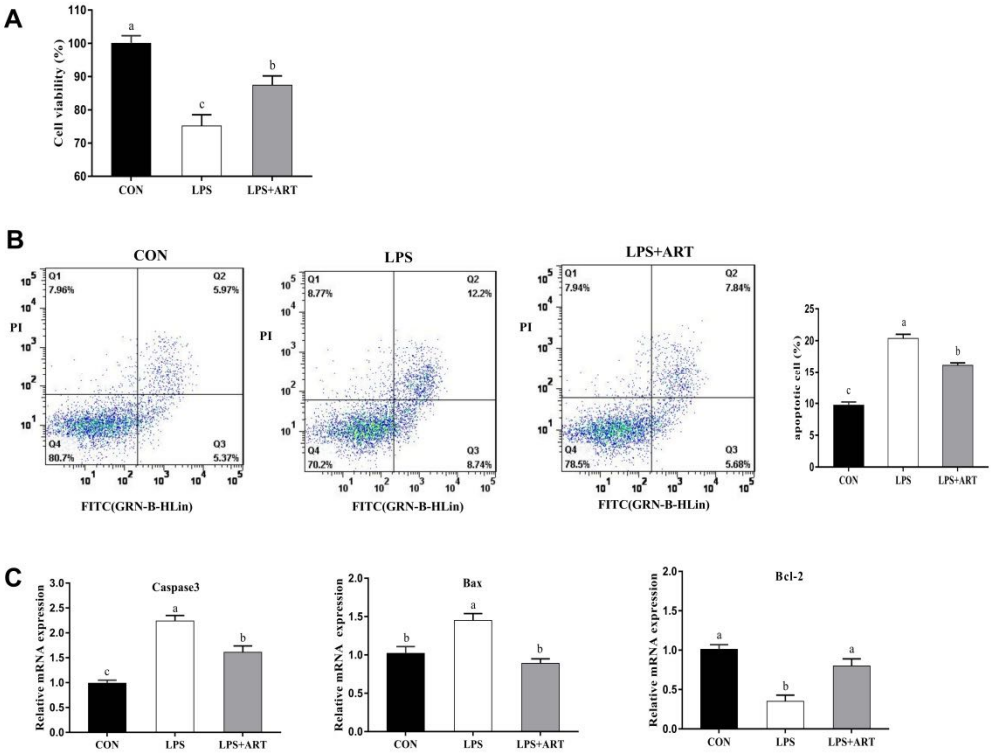

Figure 3. The effects of artemisinin on cell viability (**A**) and apoptosis (**B**,**C**) in LPS-induced pMECs. Cells were pretreated with the indicated concentrations (0 and 20 μM) of artemisinin for 12 h before being stimulated with 50 μg/mL LPS for 24 h. Cell viability was measured by the CCK-8 assay. The percentage of cells undergoing apoptosis was determined by flow cytometry and annexin V/propidium iodide (PI) staining. The mRNA expression levels of apoptotic genes (Caspase-3, Bax, and Bcl-2) were quantified using reverse-transcription PCR. Different superscript letters (a,b,c) indicate that there are significant differences between the groups ($p < 0.05$). CON = control cells without any treatment; LPS = cells treated with only 50 μg/mL LPS; LPS + ART = LPS (50 μg/mL) + artemisinin (20 μM).

3.4. Effects of Artemisinin on LPS-Induced Inflammatory Factors

To analyze the anti-inflammatory effects of artemisinin, pMECs were treated with 20 μM artemisinin for 12 h before being exposed to 50 μg/mL LPS for 24 h. As shown in Figure 4A,B, the mRNA expression abundance and content of inflammatory factors (IL-1β, TNF-α, and IL-6) induced by LPS were significantly downregulated ($p < 0.05$) by artemisinin; however, artemisinin supplementation did not affect ($p > 0.10$) LPS-induced IL-8 mRNA expression abundance. Thus, the anti-inflammatory effect of artemisinin was confirmed in LPS-induced pMECs.

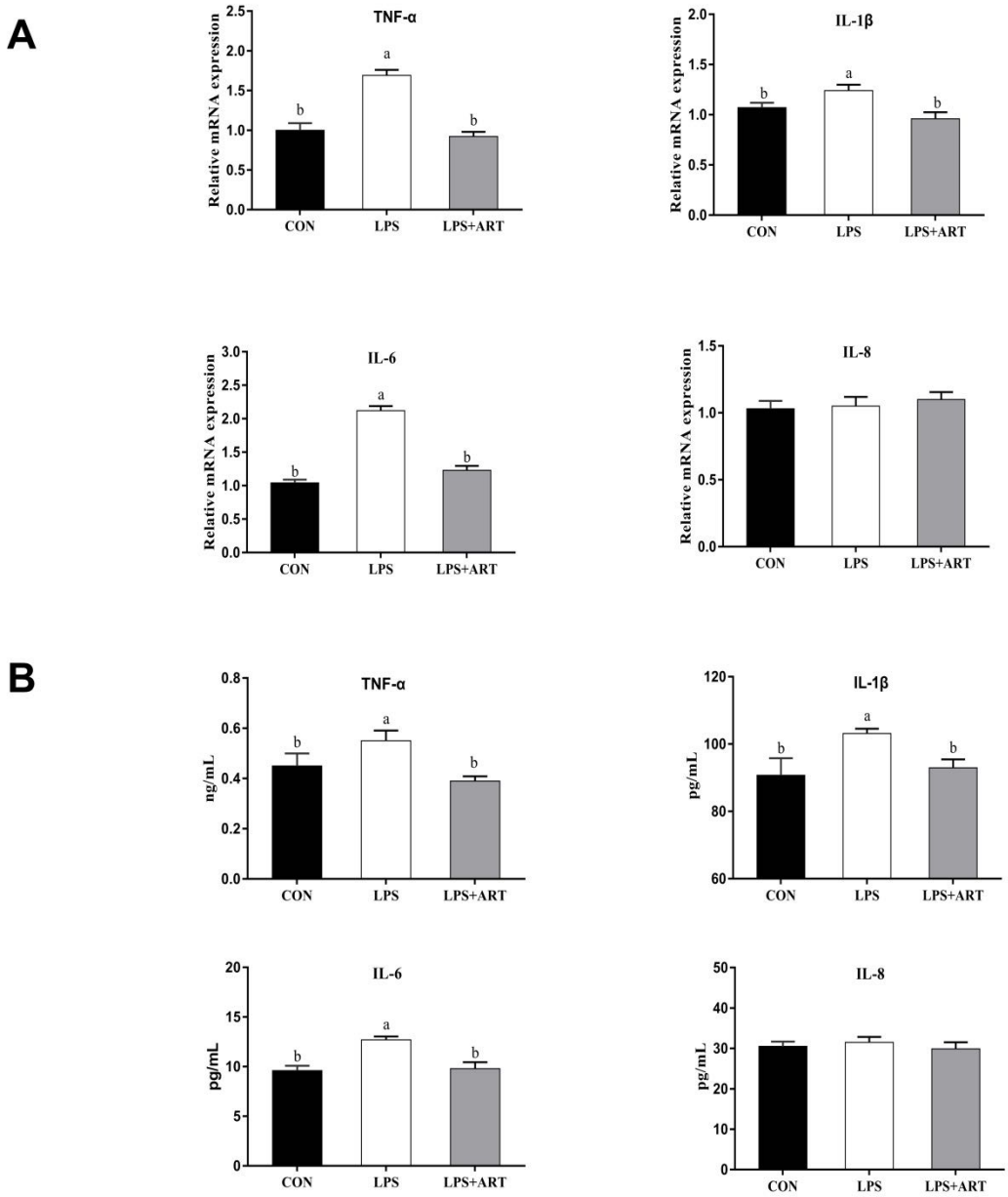

Figure 4. The effects of artemisinin on LPS-induced inflammatory factor (IL-1β, TNF-α, IL-6, and IL-8) gene expression (**A**) and protein expression (**B**). Cells were pretreated with the indicated concentrations (0 and 20 μM) of artemisinin for 12 h before being stimulated with 50 μg/mL LPS for 24 h. The mRNA expression of inflammatory factors (IL-1β, TNF-α, IL-6, and IL-8) was quantified using reverse-transcription PCR, and the protein levels were measured by ELISA. Different superscript letters (a,b,c) indicate that there are significant differences between the groups ($p < 0.05$). CON = control cells without any treatment; LPS = cells treated with only 50 μg/mL LPS; LPS + ART = LPS (50 μg/mL) + artemisinin (20 μM).

3.5. Effects of Artemisinin on the NF-κB and MAPK Signaling Pathways in LPS-Induced PMECs

The nuclear factor κB (NF-κB) and mitogen-activated protein kinase (MAPK) signaling pathways are the most classic inflammatory signaling pathways. To determine the effect of artemisinin on the NF-κB and MAPK signaling pathways, we investigated the critical proteins of these signaling pathway by Western blot analysis. LPS activated cellular NF-κB and MAPK signaling pathways, causing inflammation. The phosphorylation levels of the NF-κB signaling pathway proteins p65 and IκBα were significantly increased ($p < 0.05$) (Figure 5), and the phosphorylation levels of the MAPK signaling pathway proteins p38, ERK and JNK were significantly increased ($p < 0.05$) (Figure 6) after the cells were treated with 50 μg/mL LPS for 24 h. When the cells were pretreated with 20 μM artemisinin for 12 h before being stimulated with 50 μg/mL LPS for 24 h, the phosphorylation levels of critical NF-κB and MAPK signaling pathway proteins were significantly decreased ($p < 0.05$; Figures 5 and 6). These data showed that the activities of NF-κB and MAPK in LPS-induced pMECs were significantly inhibited by artemisinin.

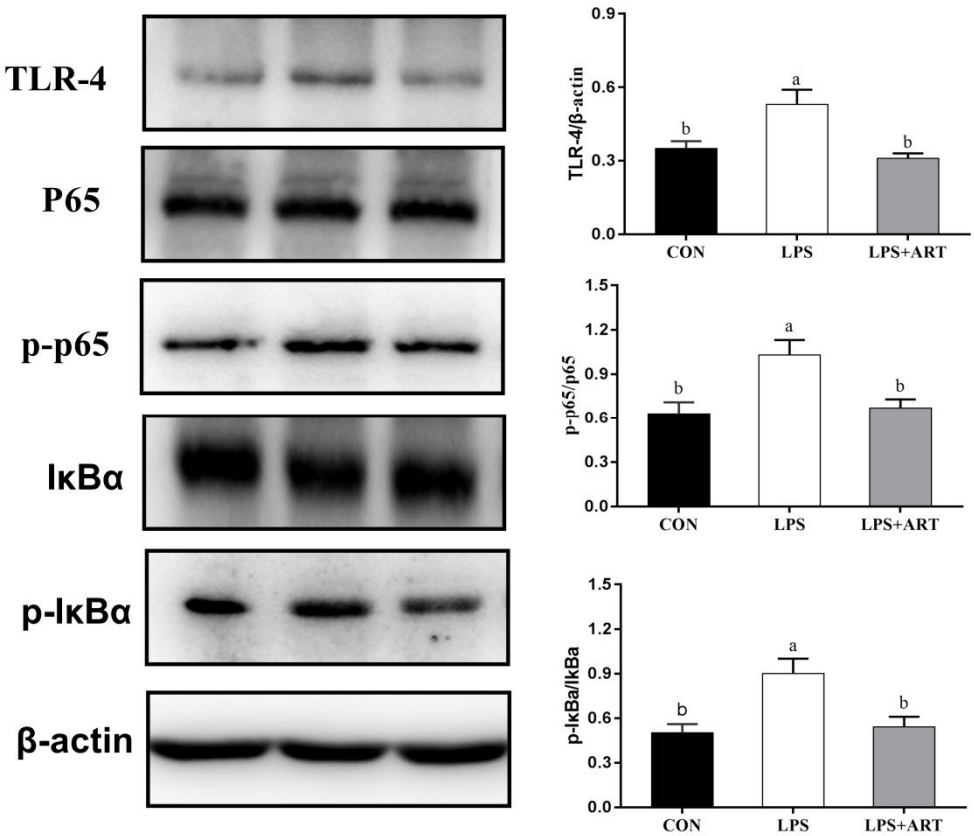

Figure 5. The effects of artemisinin on the expression of NF-κB signaling pathway-related proteins in LPS-induced pMECs. The data are the means ± SEM ($n = 3$). Different superscript letters (a,b) indicate that there are significant differences between the groups ($p < 0.05$). CON = control cells without any treatment; LPS = cells treated with only 50 μg/mL LPS; LPS + ART = LPS (50 μg/mL) + artemisinin (20 μM). Original western blot figures in Figure S1.

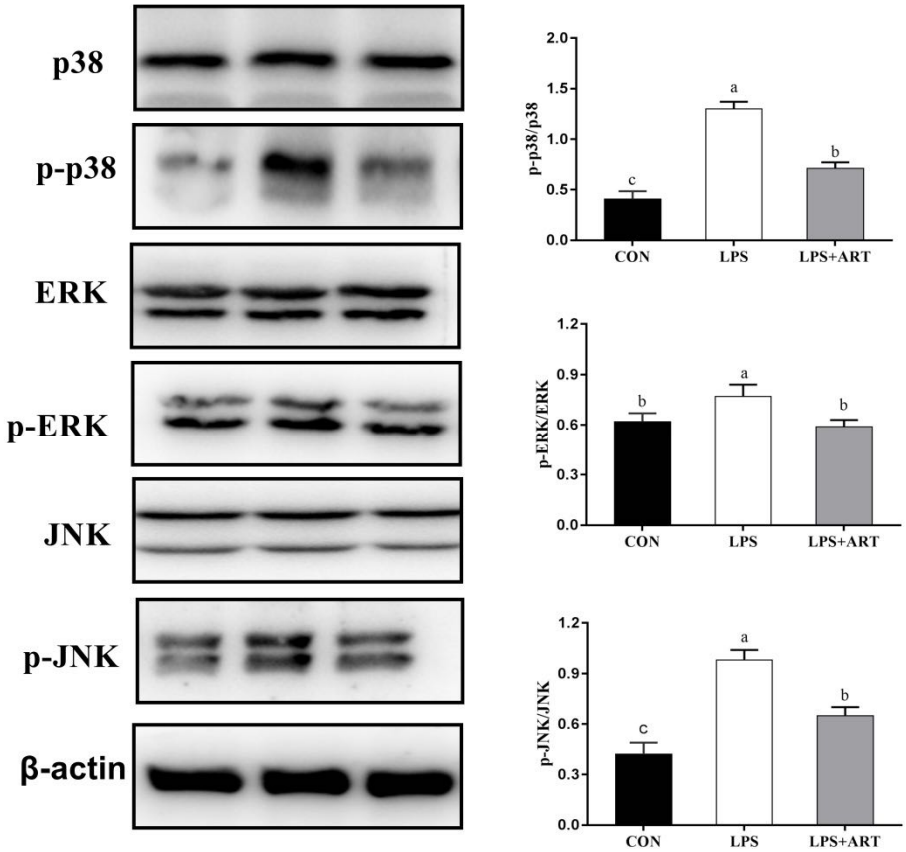

Figure 6. The effects of artemisinin on the expression of MAPK signaling pathway related proteins in LPS-induced pMECs. The data are the means ± SEM (n = 3). Different superscript letters (a,b,c) indicate that there are significant differences between the groups ($p < 0.05$). CON = control cells without any treatment; LPS = cells treated with only 50 μg/mL LPS; LPS + ART = LPS (50 μg/mL) + artemisinin (20 μM). Original western blot figures in Figure S2.

4. Discussion

Escherichia coli (*E. coli*) is one of the main pathogens causing clinical mastitis in animals [32]. LPS is the main component of the outer membrane of *E. coli*, which is an effective initiator of inflammation and endotoxic shock. In this study, LPS was used to stimulate inflammatory response in porcine mammary epithelial cells in vitro to study the effects of artemisinin on sow mastitis. The results showed that, after pMECs were stimulated with 50 μg/mL LPS for 24 h, cell viability was significantly decreased, inflammatory factor mRNA and protein expression and apoptosis were significantly increased (Figure 1), suggesting that the cells exhibited an inflammatory response. This finding indicates that the pMECs inflammation model was successfully established.

Artemisinin has the potential anti-inflammatory effects [19], but the underlying mechanism of artemisinin in regulating inflammation has not been fully explained. Therefore, we studied the anti-inflammatory effect of artemisinin against LPS-induced inflammatory injury in pMECs and examined its mechanism. Previously, it has been confirmed that LPS stimulates mammary epithelial cells to produce a rapid inflammatory response, which is characterized by the release of a large number of pro-inflammatory cytokines (IL-1β, TNF-α, and IL-6) [33]. This response is good for attracting circulating immune effector cells (such as neutrophils) to fight infection, but excessive inflammation can damage cells and tissues [34]. Therefore, the expression of pro-inflammatory cytokines needs to be strictly regulated in the inflammatory response [35]. Studies have shown that artemisinin has a variety of biological functions, such as anti-inflammatory effects. Wang et al. [36] reported that artemisinin pretreatment potently inhibited IL-6 and TNF-αrelease induced

by LPS in RAW264.7 cells, and artemisinin synergized with antibiotics to protect against LPS challenge by decreasing pro-inflammatory cytokine release. Yuan et al. [37] reported that proinflammatory factor levels (IL-1β, IL-6, TNF-α) and TLR-2 were significantly ameliorated by artemisinin treatment in LL37-induced rosacea-like mice, and artemisinin significantly decreased the LL37-induced expression of inflammatory biomarkers via the NF-κB signaling pathway in HaCaT cells. Li et al. [38] also reported that dihydroarteannuin can strongly reduce TNF-α levels in the culture supernatant of peritoneal macrophages and the serum of BXSB mice in vitro and in vivo, and the inhibitory effects on TNF-α production resulted from blockade of the NF-κB signaling pathway upstream of IκB degradation. Similarly, in this research, we examined the protective effect of artemisinin against LPS-induced inflammatory injury in pMECs and observed that the LPS-induced expression of IL-1β, TNF-α, and IL-6 was significantly down-regulated by artemisinin (Figure 4). It is worth noting that, in this experiment, we did not analyze the effects of artemisinin on inflammatory response without LPS challenge in pMECs, which is required to be addressed in the future.

In the present study, LPS-induced inflammatory damage to pMECs were weakened by artemisinin without cytotoxicity (Figures 3 and 4). In addition, we identified artemisinin as an inhibitor of the NF-κB and MAPK signaling pathways. LPS leads to the rapid and coordinated activation of various intracellular signaling pathways, including NF-κB and MAPK signaling pathways [39]. NF-κB regulates the expression of chemokines, cytokines, anti-apoptotic factors and cell growth factors, and can also trans-activate various physiological and pathological processes, such as innate and adaptive immune responses, cell proliferation, cell death, inflammation and, in some cases, tumorigenesis [40,41]. MAPK contains at least three MAPK families (ERK, p38 and JNK), which could be activated and phosphorylated by LPS, thereby activating transcription factors during inflammation [42,43]. Thus, inhibiting NF-κB and MAPK activation can be used as a potential therapeutic strategy for treating inflammatory events [44,45]. Previous studies have shown that pretreatment of Hep3B cells with artemisinin significantly inhibited TNF-α-induced expression of the NF-κB reporter gene. Artemisinin can also inhibit TNF-α-induced degradation of IκBα and p65 nuclear translocation [46]. In addition, artemisinin could decrease the expression of the inflammatory proteins COX-2 and iNOS and effectively inhibit vascular smooth muscle cells inflammation induced by TNF-α through the NF-κB signaling pathway [47]. Zhu et al. [48] reported that artemisinin attenuates LPS-induced proinflammatory responses by inhibiting the NF-κB pathway in microglial cells. Aldieri et al. [43] also showed that artemisinin inhibited NF-κB activation in cytokine-stimulated human astrocytoma T67 cells. Mechanistically, artemisinin suppressed LPS-induced NF-κB activation by blocking IκBα degradation and p65 phosphorylation and inhibited MAPK activation by blocking ERK, JNK, and P38 phosphorylation (Figure 7). It would be interesting to investigate whether artemisinin could be used as a drug to treat LPS-induced inflammatory. In addition, future studies are needed to study the anti-inflammatory activity of artemisinin in an *in vivo* model of sow mastitis.

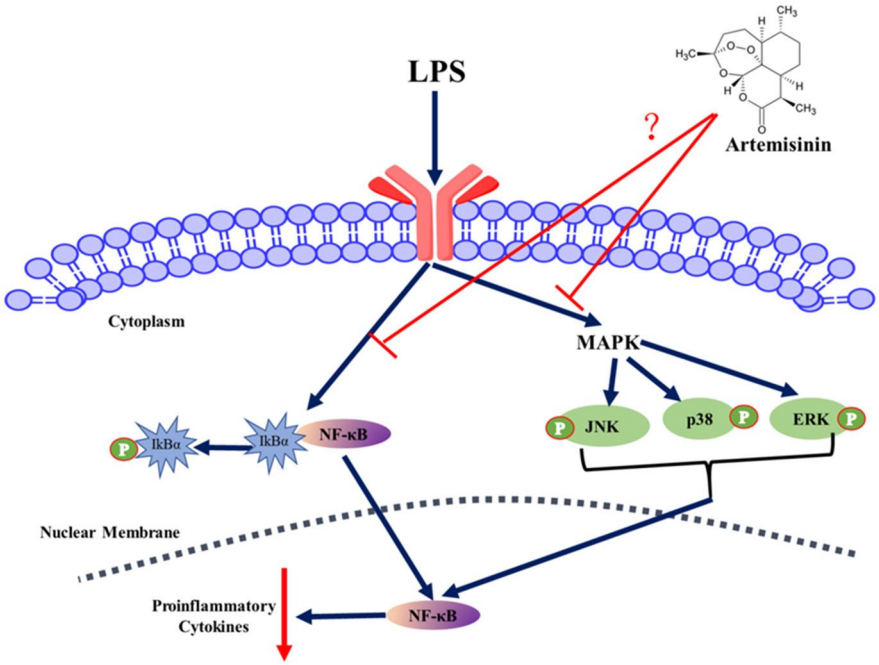

Figure 7. Possible mechanisms underlying the effects of artemisinin and LPS on pMECs. TLR4-LPS interaction leads to the rapid and coordinated activation of NF-κB and MAPK signaling pathways. Artemisinin reduces LPS-induced inflammatory damage to pMECs by inhibiting the MAPK and NF-κB signaling pathways.

5. Conclusions

In summary, the pretreatment of pMECs with artemisinin showed enhanced anti-inflammatory activity against LPS-induced inflammation. Artemisinin reduces the inflammatory damage of pMECs partially through the inhibition of NF-κB and MAPK signaling pathways.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/ani11061528/s1, Figure S1: Full original Western blot (WB) images for Figure 5, Figure S2: Full original Western blot (WB) images for Figure 6.

Author Contributions: W.Z., F.C., S.Z., and W.G. designed the experiments. W.Z., L.X., J.C., J.L., and Z.T. performed the experiments. W.Z. analyzed the data and wrote the manuscript. M.R., W.G., and S.Z. conducted the final proofreading. All authors have read and agreed to the published version of the manuscript.

Funding: This study was financially supported by the National Natural Science Foundation of the P.R. of China (No. 31872364 and No. 31802067), Guangdong Basic and Applied Basic Research Foundation (No. 2021A1515010440) and Major Science and Technology Projects in Anhui Province (201903a06020002).

Institutional Review Board Statement: The study was conducted according to the guidelines of the Declaration of Helsinki, and approved by the Ethics Committee of South China Agricultural University (No. 2019g003, 2/8/2019).

Data Availability Statement: All data used in this study can be obtained from the corresponding author according to the author's reasonable request.

Conflicts of Interest: All authors declare that there is no conflict of interest.

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An update to this article is included at the end

Journal of Thermal Biology 94 (2020) 102751

Contents lists available at ScienceDirect

Journal of Thermal Biology

journal homepage: http://www.elsevier.com/locate/jtherbio

Short Communication

Dietary enzymatically-treated *Artemisia annua* L. supplementation could alleviate oxidative injury and improve reproductive performance of sows reared under high ambient temperature

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ARTICLE INFO

Keywords:
Artemisia annua L
Heat stress
Reproductive performance
Oxidative injury
Gene expression

ABSTRACT

The medicinal plant *Artemisia annua* L. is well known for its antimalarial compound artemisinin and the antioxidant capacity of its active ingredients. However, low bioavailability of *Artemisia annua* L. limits its therapeutic potential, fermentation of *Artemisia annua* L. can improve its bioavailability. This study was aimed to investigate the effects of dietary supplementation of enzymatically-treated *Artemisia annua* L. (EA) on reproductive performance, antioxidant status, milk composition of heat-stressed sows and intestinal barrier integrity of their preweaning offspring. 135 multiparous sows of average parity 4.65 (Landrace × large white) at day 85 of pregnancy were randomly distributed into 3 treatments. Sows in the control group were housed at control rooms (temperature: 27.12 ± 0.18 °C, temperature-humidity index (THI): 70.90 ± 0.80) and fed the basal diet. Sows in the HS, HS + EA groups were fed the basal diet supplemented with 0 or 1.0 g/kg EA respectively, and reared at heat stress rooms (temperature: 30.11 ± 0.16 °C, THI: 72.70 ± 0.60). Heat stress increased the malondialdehyde (MDA) content, reduced the activities of total antioxidant capacity (T-AOC) and total superoxide dismutase (T-SOD) of sows and piglets, and seriously compromised the antioxidant capacity of the sows and the intestinal integrity of their offspring. However, dietary supplementation of 1.0 g/kg EA reduced the MDA content, increased the activities of T-SOD and T-AOC in serum, colostrum, and milk of heat-stressed sows, and increased colostrum yield and 14-d milk fat content. EA supplementation also increased piglet weaning weight and the activities of T-SOD and T-AOC in serum. In addition, the abundances of intestinal tight junction proteins claudin-1 and occludin were up-regulated in piglets in EA-supplemented group. In conclusion, dietary EA supplementation at 1.0 g/kg can alleviate the oxidative stress in heat-stressed sows, improve the antioxidant capacity in both sows and their offspring, and promote the intestinal barrier integrity in their offspring. EA may be a potent dietary supplement that ameliorates oxidative stress in livestock production by improving the antioxidant capacity.

1. Introduction

Heat stress is one of the important factors that challenge the production of livestock and seriously affects the production performance (Niu et al., 2009; Zhong et al., 2018), immunity (Quinteirofilho et al., 2010; Jafari et al., 2020; Nawab et al., 2020), antioxidant status (Yi et al., 2016; Hosseinivashan et al., 2016), intestinal barrier integrity and function (Pearce et al., 2013), meat quality (Lu et al., 2017) and even death (Nardone et al., 2010), causing tremendous economic losses to the global livestock and poultry industry (Stpierre et al., 2003; Cheng et al., 2018). Due to thicker skin and higher subcutaneous fat deposition, sows are especially vulnerable to heat stress. It has become one of the main

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https://doi.org/10.1016/j.jtherbio.2020.102751
Received 8 June 2020; Received in revised form 31 August 2020; Accepted 4 October 2020
Available online 8 October 2020
0306-4565/© 2020 Published by Elsevier Ltd.

Table 1
Ingredient composition and nutritional levels of the basic diet (air-dried basis).

Ingredient	Content (%)	Nutritional level[b]	Content (%)
Corn	52.20	Digestible energy, MJ/kg	14.13
Soybean meal	24.00	Crude protein	18.13
Wheat bran	6.00	Ash	5.76
Fish meal	2.50	Crude fiber	3.31
Wheat standard powder	7.50	Dry matter	87.10
Soybean oil	4.00	Crude fat	6.81
Dicalcium phosphate	1.20	Calcium	0.77
Llimestone	0.90	Total phosphorus	0.66
Salt	0.30	Available phosphorus	0.41
Vitamin and mineral premix[a]	0.30	Digestible Lys	0.86
Choline chloride (50%)	0.20	Digestible Met + Cys	0.51
Sodium bicarbonate	0.20	Digestible Thr	0.60
Mold removal agent	0.05	Digestible Trp	0.18
Mold inhibitor	0.20		
Zeolite (carrier)	0.45		
Total	100.00		

[a] Per kg of diet: 50.0 mg zinc ($ZnSO_4 \cdot H_2O$), 80.0 mg iron ($FeSO_4 \cdot H_2O$), 20.0 mg manganese ($MnSO_4 \cdot H_2O$), 5.00 mg copper ($CuSO_4 \cdot 5H_2O$), 0.14 mg I (CaI_2O_6), 0.3 mg selenium (Na_2SeO_3), 13,000 IU vitamin A, 90 IU vitamin E, 4000 IU vitamin D_3, 4 mg vitamin B_1, 4 mg vitamin K_3, 10.0 mg vitamin B_2, 4.8 mg vitamin B_6, 0.034 mg vitamin B_{12}, 20.0 mg D-pantothenate, 40.0 mg niacin, 2 .0 mg folic acid, 0.16 mg D-biotin.
[b] Calculated values according to the tables of feed composition and nutritive values in Chinese Feeding Standard of Swine (2004).

problems in the pig industry in tropical and subtropical regions around the world, especially in the summer (Gourdine et al., 2006).

Heat stress induces oxidative stress, which is an imbalance between anti-inflammatory system and reactive oxygen species (ROS). During late gestation and lactation, sows are especially sensitive to heat stress-induced oxidative stress, which leads to serious harm to the sows (Fuquay, 1981).

Supplementation of plant extracts in feed has been found to reduce heat stress in animals (Zheng and Wang, 2001; Cai et al., 2004; Song et al., 2017; Kim et al., 2003) through reducing or scavenging free radical formation (Wright et al., 2001; Pietta, 2000). The medicinal plant *Artemisia annua* L. (A. annua) is an annual herb belonging to *Artemisia* species of *Asteraceae*, which is diffusely used in traditional Chinese medicine and now distributed in many countries worldwide (Tu, 2011). Previous studies have demonstrated that the extracts of A. annua can increase antioxidant status and immunity in rats (Ryu et al., 1998), and enhance growth performance (De Almeida et al., 2012), antioxidant capacity (Brisibe et al., 2009) and immune function in broilers (Bhakuni et al., 2000; Hwang et al., 2013).

Table 3
Effects of dietary supplementation with EA on reproductive performance of heat-stressed sows.

Item	Control	HS	HS + EA	SEM[a]	*P*-value[b]
Colostrum yield, kg/sow	4.41[b]	4.23[c]	4.58[a]	0.05	0.026
Parturition					
Litter birth weight, kg	15.11	15.34	15.67	0.25	0.444
Individual piglet weight, kg	1.50	1.48	1.51	0.01	0.263
Weaning, 21 days					
Litter weight, kg	50.25[b]	48.68[c]	52.52[a]	0.79	0.023
No. of pigs weaned/litter	8.81	8.56	8.82	0.13	0.353
Individual piglet weight, kg	5.77[b]	5.70[c]	5.93[a]	0.05	0.040

[a] SEM means standard error of the mean. No. of sows used in this trial (n = 45).
[b] $P < 0.05$ indicates a significant difference.

Plant cell walls limit the release and utilization of active ingredients within the cytoplasm. Enzymatic hydrolysis technology can be used to degrade or destroy cell walls, thereby enabling better release and more extraction of biologically active compounds from *Artemisia annua* L. (Puri et al., 2012; Wan et al., 2016a,b). Furthermore, no information is available regarding the effects of enzymatically-treated *Artemisia annua* L. (EA) or A. annua on ameliorating the adverse effects caused by heat stress in pigs. Therefore, this study was to investigate the effects of dietary EA supplementation on reproductive performance, antioxidant capacity, milk composition of heat-stressed multiparous sows and intestinal barrier integrity of their preweaning offspring.

2. Materials and methods

2.1. Preparation of enzymatically treated artemisia annua L. (EA)

EA was obtained from China, which was harvested in the stage of flourish. The EA was produced by Kehu Biotechnology Research Center, Guangzhou, P. R. China. Pectinase and cellulases were used to digest the entire herb of *Artemisia annua* L. and then filtered and dried. Its main active ingredients were tested according to AOAC (Horwitz, 1975), the main active ingredients of EA include 4.77 ± 0.22 mg gallic acid equivalents (GAE)/g phenols, 1.68 ± 0.13 mg rutin equivalents (RE)/g flavonoids, 156.2 ± 0.20 mg/kg arteannuinic acid, 62.0 ± 0.08 mg/kg deoxyartemisinin, and 26.0 ± 0.06 mg/kg artemisinin.

Table 2
Primer sequences for the mRNA.

Gene	Accession Number	Sequences (5'→3')	Product size (bp)
Occludin	NM_001163647.2	F- ACTGGCGGCGAGTCCTGCGACGAGC R- TATTGTATTCATCAGCAGCAGCCAT	244
ZO-1	XM_021098856.1	F- AAAGCCCTAAGTTCAATCACAATCT R- TCCTCATCTTCATCATCTTCTACAG	253
ZO-2	XM_021070931.1	F- TTATTCGGACCCATAGCAGACA R- GTCCTGTTCAATAATTTGCCTCAC	156
ZO-3	XM_005661351.3	F- TGTGCTGGTGCGGAGGAGAG R- AGCCAAGCCTGAATCTGTGATGTG	91
Claudin-1	NM_001244539.1	F- TCTTTCTTATTTCAGGTCTGGCTATCT R- CCACTGGAAGGCGAAGGTTTT	247
Claudin-2	NM_001161638.1	F- TGACGGCAGTCGGCTTCTCC R- CACGGTGATGATACAGGCCAACG	176
Claudin-3	NM_001160075.1	F- CTTCATCGGCAGCAGCATTATCACAGC R- GGATCTTGGCTTTGGCCGTGTCG	251
Claudin-4	NM_001161637.1	F- TCATCGGCAGCAACATCGTCAC R- CAGCAGCGAGTCGTACACCTTG	110
Claudin-5	NM_001161636.1	F- CGCCTTCCTGGACCACAACATC R- CAGCACCGAGTCGTACACCTTG	115
β-actin	XM_003357928	F-TGCGGGACATCAAGGAGAAG R-AGTTGAAGGTGGTCTCGTGG	176

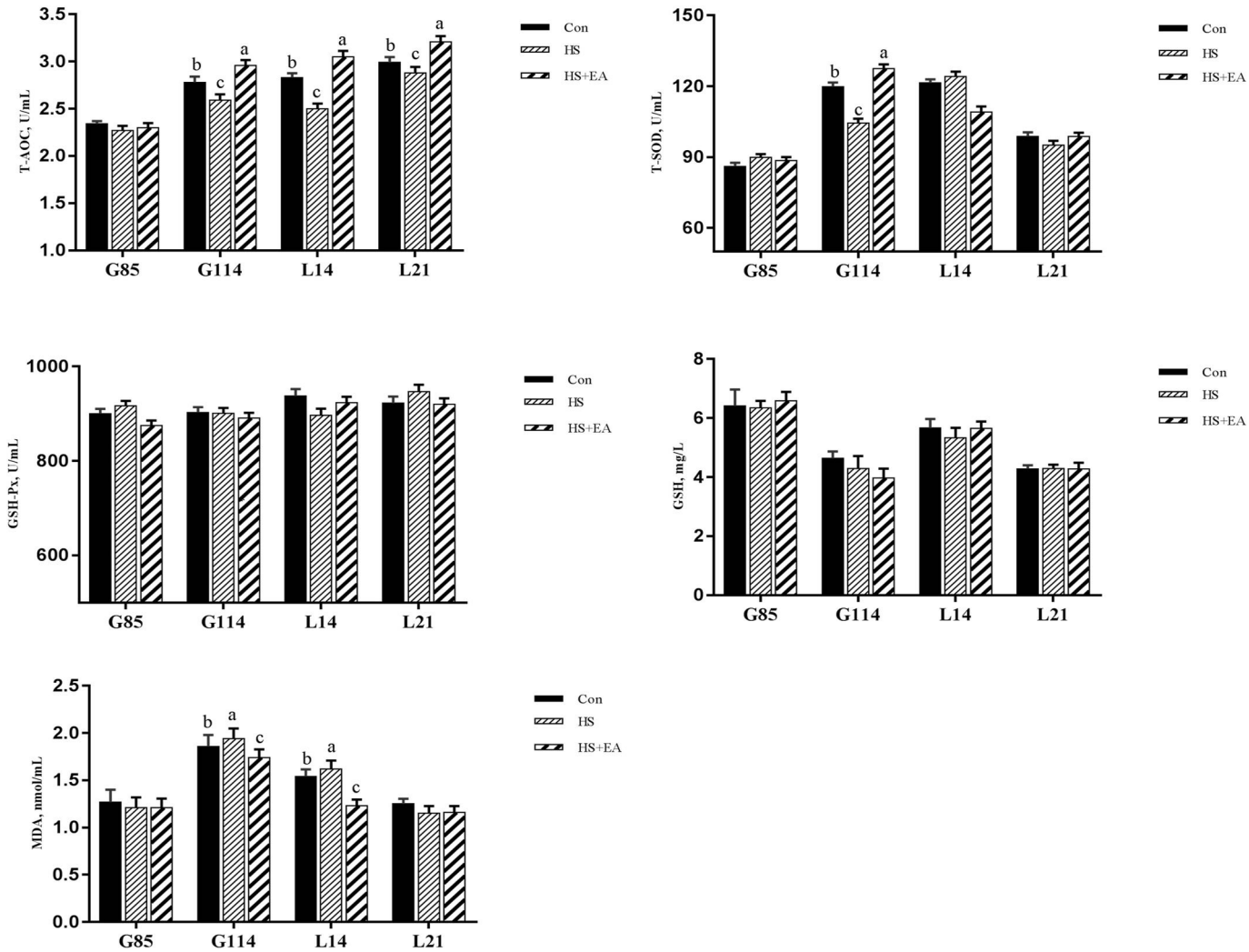

Fig. 1. Effects of dietary supplementation of EA on the antioxidant status in the serum of sows. Data are shown as mean ± SEM (n = 10). Different letters indicate a significant difference among different treatments ($P < 0.05$). Abbreviations: G85, day 85 of gestation; G114, day 114 of gestation; L14, day 14 of lactation; L21, day 21 of lactation (weaning); T-SOD, total superoxide dismutase; T-AOC, total antioxidant capacity; GSH-Px, glutathione peroxidase; GSH, glutathione; MDA, malondialdehyde.

2.2. Animals and experimental design

The experiment was conducted in a subtropical city (Jiang men) in Guangdong Province in South China (subtropical climate) from July to October 2018, and the sow farm is located at latitude and longitude of 21° 85′ N and 112° 48′ E, respectively. Heat stress rooms (HS) were conventional rearing rooms, which were a semi-opened rooms; Control rooms (Control) contained pad curtained with circulating water and large fans.

135 crossbred sows (Landrace × large white, average parity = 4.65 ± 0.25) of similar parity and weight (average weight = 260 kg) on day 85 of gestation were randomly distributed into 3 treatments (45 sows per treatment). Sows in the control group were housed at control rearing rooms (temperature: 27.12 ± 0.18 °C (25.26–29.18 °C), temperature-humidity index (THI): 70.90 ± 0.80 (68.90–72.01)) and fed the basal diet. Sows in the HS, HS + EA groups were fed basal diet supplemented with 0 or 1.0 g/kg EA respectively, and reared at heat stress rooms (temperature: 30.11 ± 0.16 °C (26.08–32.86 °C), THI: 72.70 ± 0.60 (69.34–74.27)).

2.3. Diets and management

The feed composition and nutrient levels of the basal diet are shown in Table 1. During experiment, the ambient real-time relative humidity (RH) and temperature (T) were recorded every hour using an automatic humidity and temperature recorder (W-series, Henan, China). THI was calculated according to the formula (Wegner et al., 2014).

Sows were individually housed in stalls (2.10 m × 1.10 m). Each sow was fed three times per day (approximately 3.00 kg in total). On day 107 of gestation, sows were transferred into respective farrowing rooms, where all experimental settings were held constant as during the gestational period. During this time, sows had free access to feed.

2.4. Data and sample collection

Within the first 8 h after birth, the number of piglets, individual birth weight, and litter birth weight were collected. 24 h after birth, every piglet was weighed individually again to estimate the colostrum yield of sows according to the methods published before (Theil et al., 2014). The piglets were cross-fostered only within each treatment group by 48-h postpartum, and the litter size was standardized to 10 ± 1 pigs.

Blood was collected by ear venipuncture from a random subset of sows (n = 10 per treatment) on the day 85 of gestation (G85), day 114 of gestation (G114), day 14 and day 21 of lactation (L14 and L21). Blood was collected from the same subsets of sows in every period. Sows were fed at 06:30 a.m., the blood samples were taken 3 h after morning meal

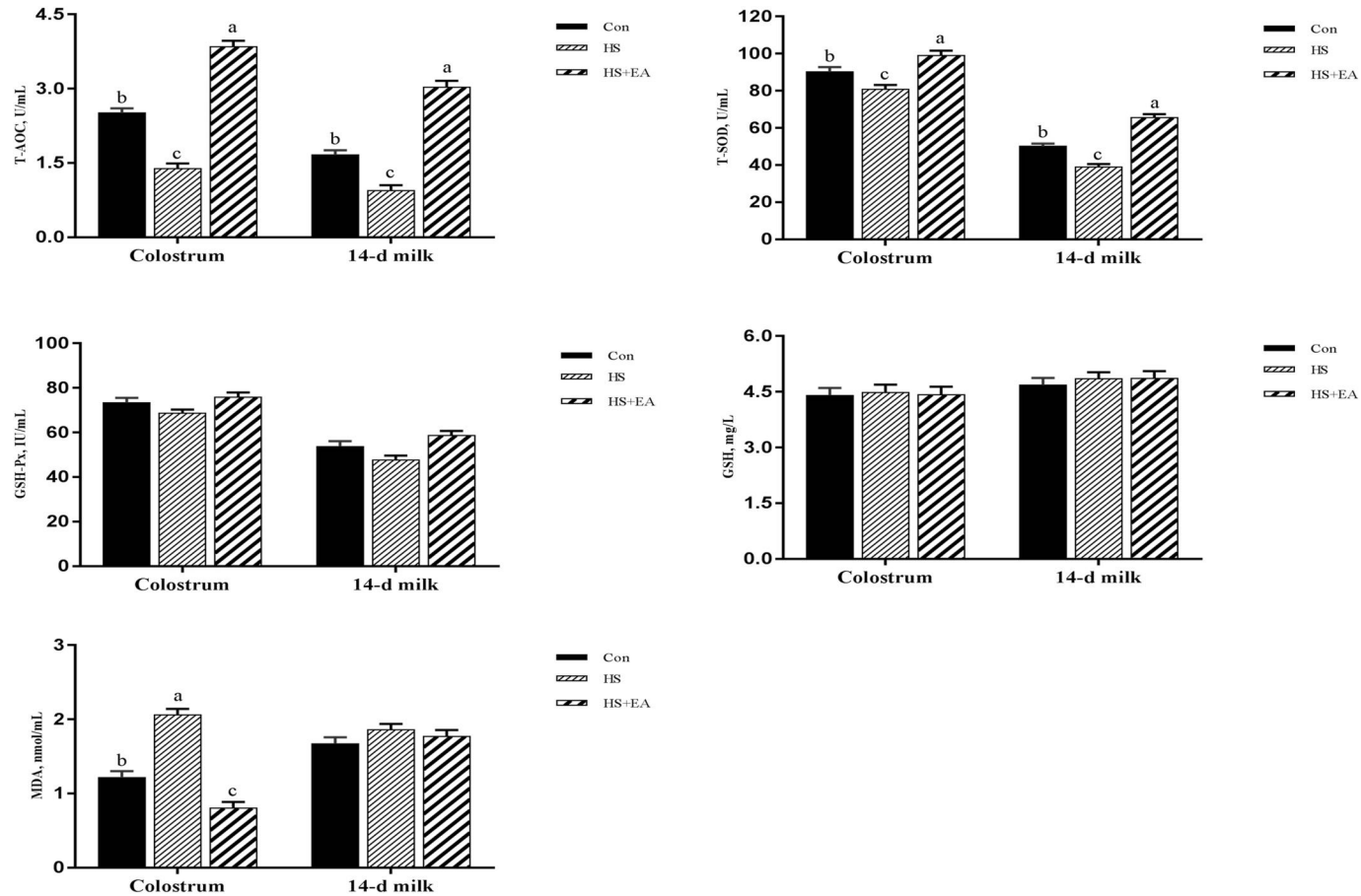

Fig. 2. Effects of dietary supplementation of EA on the antioxidant status in the colostrum and 14-d milk of sows. Data are shown as mean ± SEM (n = 10). Different letters indicate a significant difference among different treatments ($P < 0.05$).

Table 4
Effects of dietary supplementation with EA on the nutrient composition of colostrum and 14-d milk of heat-stressed sows.

Item	Control	HS	HS + EA	SEM[a]	*P*-value[b]
Colostrum, %					
Fat	4.03	3.99	3.88	0.16	0.834
Lactose	11.38	11.44	11.40	0.19	0.618
Protein	7.94	7.97	7.94	0.16	0.378
Solids-not-fat	21.04	21.12	21.04	0.36	0.880
14-d milk, %					
Fat	6.15[b]	6.21[b]	6.95[a]	0.16	0.048
Lactose	5.84	5.98	5.95	0.05	0.890
Protein	3.84	3.96	3.94	0.38	0.982
Solids-not-fat	10.47	10.73	10.72	0.10	0.929

[a] SEM means standard error of the mean. No. of sows used in this trial (n = 10).
[b] $P < 0.05$ indicates a significant difference.

(around 9:30 a.m.). They were collected in 10 mL sterile vacutainer tubes, and centrifuged at 4 °C, 3500×*g* for 10 min and frozen immediately at −80 °C for later analysis.

Colostrum was collected from every teat within 24 h of postpartum (n = 10 per treatment), 14-d milk was collected after injection of 30 IU oxytocin postpartum at the day 14 of lactation. Approximately 20 mL were collected and stored at −80 °C until analysis.

On the day of birth (L0) and weaning (L21), 6 piglets (3 males and 3 females, one pig per litter) were randomly selected from each treatment, weighted and blood samples were collected (10 mL) through the anterior vena cava of piglets. The whole gastrointestinal tracts were rapidly removed after piglets were sacrificed by exsanguination. A 4.0 cm intestinal segment was collected at mid-duodenum, mid-jejunum and mid-ileum. Each collected segment was opened longitudinally and rinsed with saline and then snap frozen in liquid nitrogen and stored at −80 °C for the analysis of tight junction proteins.

2.5. Laboratory analysis

The antioxidant status, such as total superoxide dismutase (T-SOD), glutathione (GSH), total antioxidant capacity (T-AOC), glutathione peroxidase (GSH-Px) and malonaldehyde (MDA) levels, were measured in serum, colostrum and 14-d milk according to the method published before (Chen et al., 2016) by commercial kits (Cayman, Ann Arbor, MI, USA). Colostrum and 14-d milk samples were analyzed by a fully automated milk composition analyser (Chen et al., 2019) (ULTRA-MILER-UL40AC, China).

2.6. Real-time PCR analysis

The mRNA expression of tight junction proteins was measured by real-time PCR as described before (Heng et al., 2019). Total RNA from intestinal tissues was isolated, and the RNA concentration and purity were measured. 2.0 μg of total RNA was reverse transcribed to cDNA in a 20.0 μL reaction mixture using the PrimeScript™ RT reagent kit (TaKaRa) according to the manufacturer's protocols.

Real-time PCR was carried out using ABI StepOnePlus™ Real-Time PCR systems (Applied Biosystems, Grand Island, NY). The Real-time PCR amplification was performed in a 20.0 μL reaction volume. Each sample was tested in duplicate. The thermal profile was used for qRT-

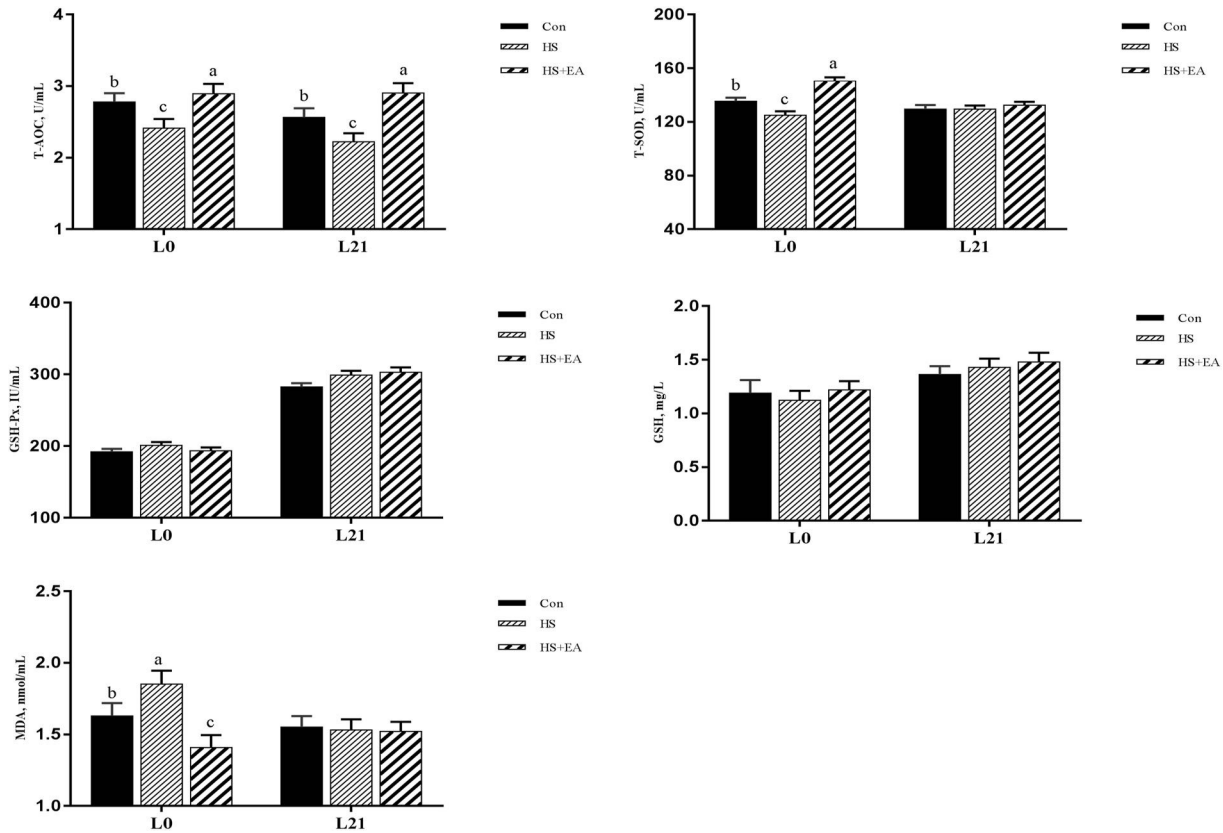

Fig. 3. Effects of dietary supplementation of EA on the antioxidant status in the serum of piglets. Data are shown as mean ± SEM (n = 6). Different letters indicate a significant difference among different treatments ($P < 0.05$). Abbreviations: L0, day 0 of birth; L21, day 21 of lactation (weaning).

PCR: heating at 95 °C for 6 min for initial enzyme activation, followed by 42 cycles of 95 °C for 25 s, and annealing at 60 °C for 30 s. The relative gene expression levels were calculated according to the $2^{-\Delta\Delta Ct}$ method and β-actin was used as reference gene. Primer sequences of the target genes are listed in Table 2.

2.7. Statistical analysis

The statistical analyses were conducted using one-way ANOVA procedure of SPSS 21.0 software (SPSS, INC., Chicago, IL, USA), Differences among groups were evaluated by Duncan's test. The individual sow and its litter were used as the experimental unit. For all data analyses, significance was defined as $P < 0.05$.

3. Results

3.1. Reproductive performance

Compared with the control and HS groups, the HS + EA group had more colostrum yield ($P < 0.05$). Greater litter weight and individual weaning piglet weight were observed in piglets from HS + EA compared with HS and control groups ($P < 0.05$; Table 3).

3.2. Antioxidant status in serum, colostrum and 14-d milk of sows

Sows in the HS group had lower activities of T-AOC (G114, L14, L21) and T-SOD (G114) and higher concentrations of MDA (G114, L14) in the serum ($P < 0.05$). On the contrary, dietary EA supplementation resulted in higher activities of serum T-AOC (G114, L14, L21) and T-SOD (G114) and lower concentrations of MDA (G114, L14) compared with the control and HS groups ($P < 0.05$; Fig. 1). Furthermore, the HS + EA group had higher activities of T-AOC, T-SOD in colostrum and 14-d milk and lower concentrations of MDA in colostrum ($P < 0.05$; Fig. 2).

3.3. The nutrient composition of colostrum and 14-d milk of sows

There were no differences among groups for the levels of fat, solids-not-fat, lactose and protein in colostrum ($P > 0.10$), but sows fed EA had higher levels of fat in 14-d milk compared with the control group ($P < 0.05$; Table 4).

3.4. Antioxidant status in serum of piglets

Compared with the control and HS groups, piglets in the HS + EA group had higher activities of T-AOC (L0, L21) and T-SOD (L0), and lower concentration of MDA (L0) ($P < 0.05$; Fig. 3).

3.5. mRNA expression level

Compared with the control group, HS down-regulated duodenal claudin-1 and ileal occludin mRNA expression in piglets at birth and weaning ($P < 0.05$). However, the dietary supplementation of EA significantly up-regulated the mRNA expression of tight junction protein in duodenum and ileum of piglets compared with the control and HS groups ($P < 0.05$; Figs. 4 and 5).

4. Discussion

Heat stress is induced when the ambient temperature exceeds the sows' tolerance (Quiniou and Noblet, 1999). It can cause a series of detrimental effects to animal health. Due to the thicker skin and higher fat deposition, sows are more susceptible to heat stress (Pearce et al., 2013; Stpierre et al., 2003). In order to decrease metabolic heat production, one common consequence induced by heat stress is the

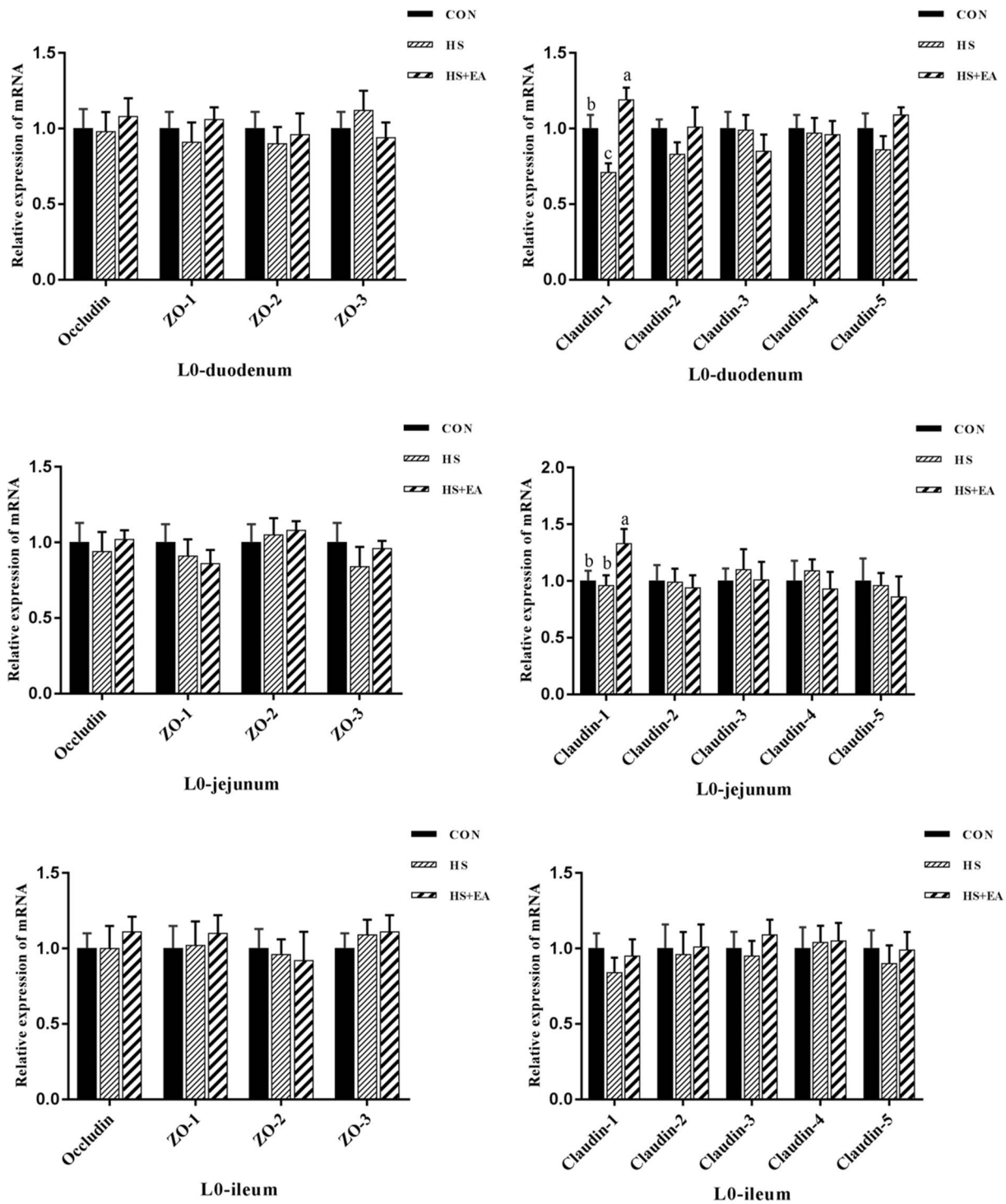

Fig. 4. Effects of dietary supplementation of EA on relative mRNA expression of intestinal tight junction proteins of nursing piglets at birth. Data are shown as means ± SEM (n = 6). The mRNA level of each gene for the CON group is assigned a value of 1 and normalized against β-actin. Different letters (a, b, c) indicate significant differences ($P < 0.05$). Abbreviations: L0, day 0 of birth.

reduction of feed intake (Mack et al., 2013). Studies have shown that decreased feed intake in sows under heat stress significantly reduces the reproductive performance, lactation performance, milk production and feed conversion rate (Renaudeau et al., 2011, 2012). Thus, it is especially important to find ways to alleviate heat stress in sows. Previous studies have demonstrated that the extracts of *Artemisia annua* L. increase antioxidant capacity in rats (Ryu et al., 1998), enhance growth performance and improve antioxidant capacity and immune function (Brisibe et al., 2009), compensate for necrotic enteritis or coccidiosis associated growth depression in chicks (Hwang et al., 2013). However, there is a lack of research of the effects of EA in heat-stressed sows. Therefore, the aim of this study was to explore the effects of EA supplementation on reproductive performance, antioxidant status of heat-stressed multiparous sows and their offspring.

In the current study, sows fed EA diet had more colostrum yield, greater litter weight and individual piglet weaning weight. These results were in agreement with above-mentioned studies that EA can effectively mitigate the detrimental effects of heat stress and improve the growth

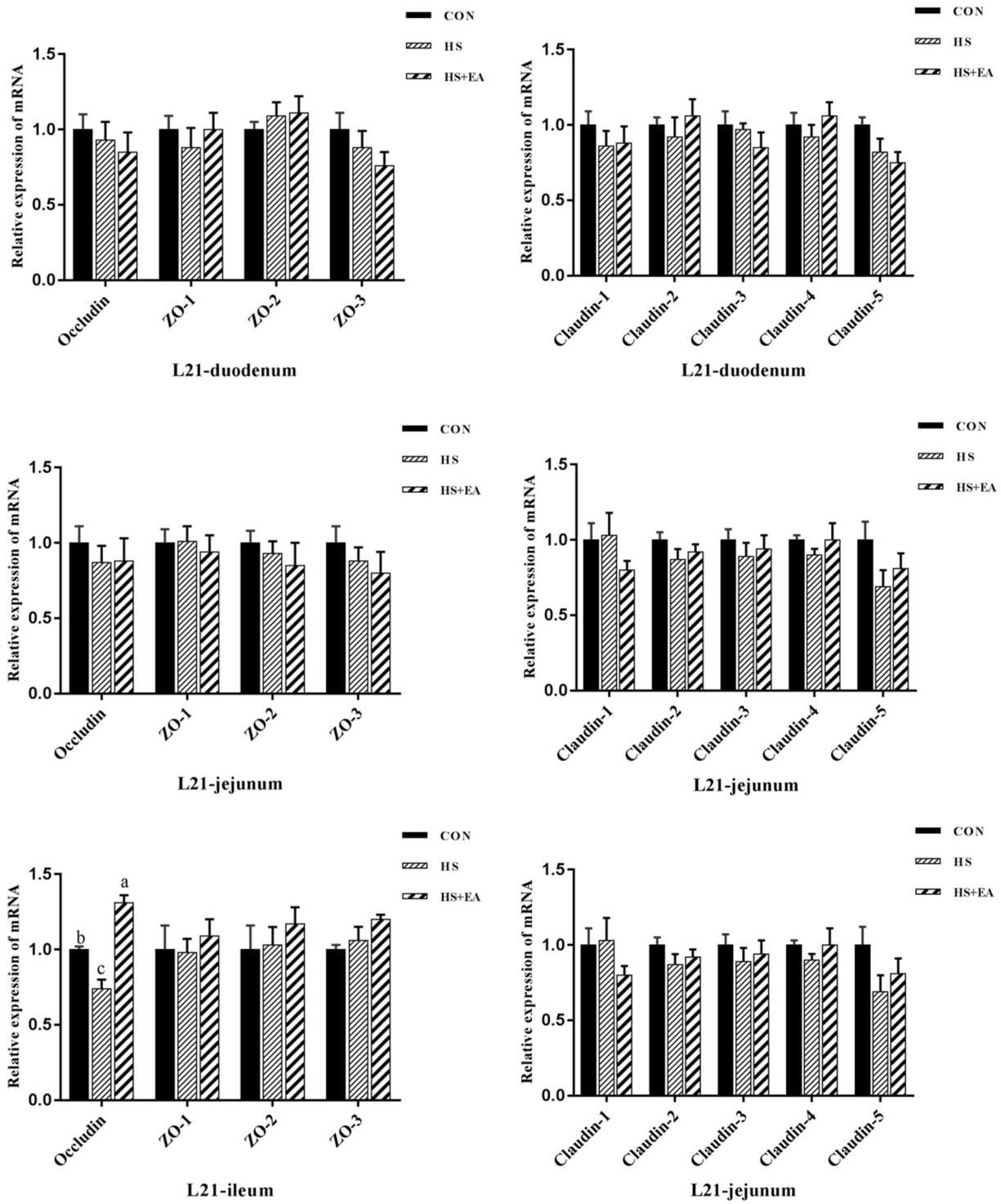

Fig. 5. Effects of dietary supplementation of EA on the relative mRNA expression of intestinal tight junctions proteins of weaning piglets. Data are shown as means ± SEM (n = 6). Different letters (a, b, c) indicate significant differences ($P < 0.05$). Abbreviations: L21, day 21 of birth (weaning).

performance of heat-stressed broilers. The beneficial effects of EA supplementation may be because it is rich in phenols and flavonoids, which can promote the release of prolactin, enhance the antioxidant capacity of the sow and reduce metabolic disorders, thereby increasing the milk production and ultimately promoting the growth of their offspring (Ferreira et al., 2010).

Oxidative stress is the caused by the imbalanced relationship between antioxidation system and reactive oxygen species. Sows increase oxidative stress when they are under heat stress during late gestation and lactation (Zhao et al., 2011). The level of MDA reflects the oxidative state of the body. Oxidative stress decreases activities of GSH-Px, CAT, and T-SOD, disturbs the steady state levels of free radicals, results in cellular oxidative damage, and enhances ROS production, which collectively leads to cytotoxicity (Bernabucci et al., 2002; Belhadj et al., 2016). The antioxidant defense system can protect the organelles and cellular components against ROS-induced damage (Li et al., 2015; Zhang et al., 2015). Previous studies have shown that dietary EA supplementation reduces MDA concentrations and lipid oxidation products in

muscle and serum (Wan et al., 2016a,b; Cherian et al., 2013), improves the CAT, T-SOD and GSH activities in broilers (Song et al., 2017; Wan et al., 2016). In addition, several studies have also proven that rats fed diets supplemented with *Artemisia* extracts improve the CAT, SOD, and GSH-Px activities and reduce MDA concentration in the liver (Kim et al., 2003; Ryu et al., 1998). Similarly, in the current study, the increased MDA concentrations in serum and colostrum indicated that heat exposure induced oxidative damage to sows. The higher activities of T-SOD, T-AOC in serum of sow and piglets, and lower concentrations of MDA in colostrum were observed when sows fed 1.0 g/kg EA compared with the control and HS groups, which were in agreement with previous studies (Song et al., 2017; Kim et al., 2003; Wan et al., 2016a,b; Cherian et al., 2013).

The healthy growth of piglets during lactation is influenced by their living environment and maternal transmission (such as colostrum and milk quality and yield). Colostrum and milk are important substances for piglets to get nutrients (include fat, lactose, proteins, and non-fat solids), immune agents, and antioxidant protection (Albera and Kankofer, 2009). High quality and yield of colostrum and milk are the guarantee for healthy growth of piglets during lactation (Quesnel et al., 2012). Previous studies have shown that heat stress reduces feed intake in sows, which further decreases its milk production and quality (Silva et al., 2009; Renaudeau et al., 2003). In dairy cows and goats, *Artemisia annua* L. supplementation improves milk fat (Cabiddu et al., 2006; Khiaosaard et al., 2009), due to the increase of butyric acid vibrio (Jenkins et al., 2008; Wallace et al., 2007). In our study, we also observed increased milk fat of sows fed EA supplemented diet, which may share similar mechanism as mentioned earlier and needs further exploration.

The development of the intestine in piglets plays a crucial role in their healthy growth. Impaired intestinal barrier is one of the important causes of intestinal inflammation and diarrhea in piglets, which could lead to serious economic losses in the pig industry (Clayburgh et al., 2004; Shih and Targan, 2007). Heat stress causes serious damage to the structural integrity and barrier function of piglets (Pearce et al., 2012). Tight junctions are the primary means of attachment among intestinal epithelial cells and play an important role in maintaining the mechanical barrier and permeability in pigs (Tsukita et al., 2001). Tight junctions mainly consist of the transmembrane protein complexes (claudins and occludins) and the cytosolic proteins zonula occludens (ZO-1, ZO-2, and ZO-3) (Kim et al., 2012).

To better clarify the molecular mechanism of the effect of EA on the barrier function in piglet, we quantified the changes in tight junction proteins for the first time. Previous studies have shown that dietary *Artemisia annua* L. supplementation at 1.0 g/kg improves intestinal development and alleviates the intestinal inflammatory response in broilers (Song et al., 2017). The current study indicated that heat stress reduced the protein levels of occludin and claudin-1, which is consistent with the previous study (Song et al., 2014). Importantly, EA supplementation significantly increased intestinal claudin-1 and occludin levels in the current study, which indicated that EA could alleviate the intestinal damage caused by heat stress and improve intestinal barrier function. These results also suggest that EA may improve the growth performance of piglets by promoting the tight junction protein levels in the intestines.

5. Conclusions

In conclusion, dietary supplementation of enzymatically-treated *Artemisia annua* L. at 1.0 g/kg can alleviate the oxidative stress in heat-stressed sows, improve the antioxidant capacity, and promote the intestinal barrier integrity in their offspring. Improving the antioxidant capacity may be an effective approach to attenuate the detrimental effects of heat stress on reproductive performance and health in sows. The results of this study provide a novel perspective for improving the antioxidant capacity of heat-stressed sows, and offer a good nutritional management strategy to extenuate heat stress in livestock production.

Funding information

This research work was financially supported by the National Key Research and Development Program of China (2018YFD0501002).

Compliance with ethical standards

All animal protocols used in this study were conducted under the protocol (SCAU-AEC-2010-0416) approved by the South China Agricultural University Institutional Animal Care and Use Committee.

Conflicts of interest

The authors declare no conflicts of interest.

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Update

Journal of Thermal Biology
Volume 100, Issue , August 2021, Page

DOI: https://doi.org/10.1016/j.jtherbio.2021.103035

Journal of Thermal Biology 100 (2021) 103035

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Corrigendum to "Dietary enzymatically-treated *Artemisia annua* L. supplementation could alleviate oxidative injury and improve reproductive performance of sows reared under high ambient temperature" [J. Therm. Biol. 94 (2020) 102751.1–9]

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The authors regret that Fig. 5 is incorrect. The L21-jejunum figure appears twice in Fig. 5. The authors have made changes and corrections. The authors would like to apologise for any inconvenience caused.

Please see the attachment for details.
The wrong Fig. 5:

DOI of original article: https://doi.org/10.1016/j.jtherbio.2020.102751.

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https://doi.org/10.1016/j.jtherbio.2021.103035

Available online 26 June 2021
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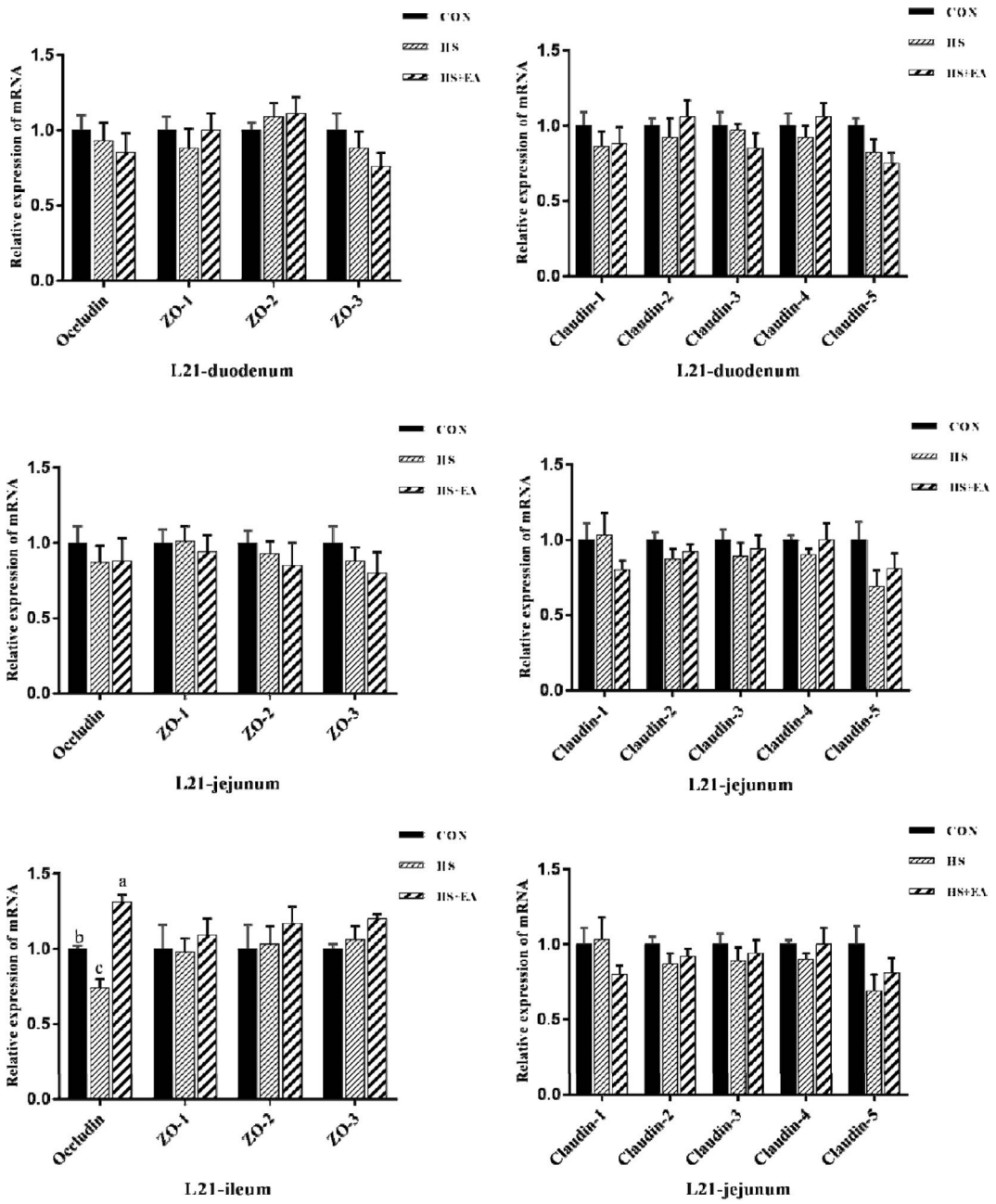
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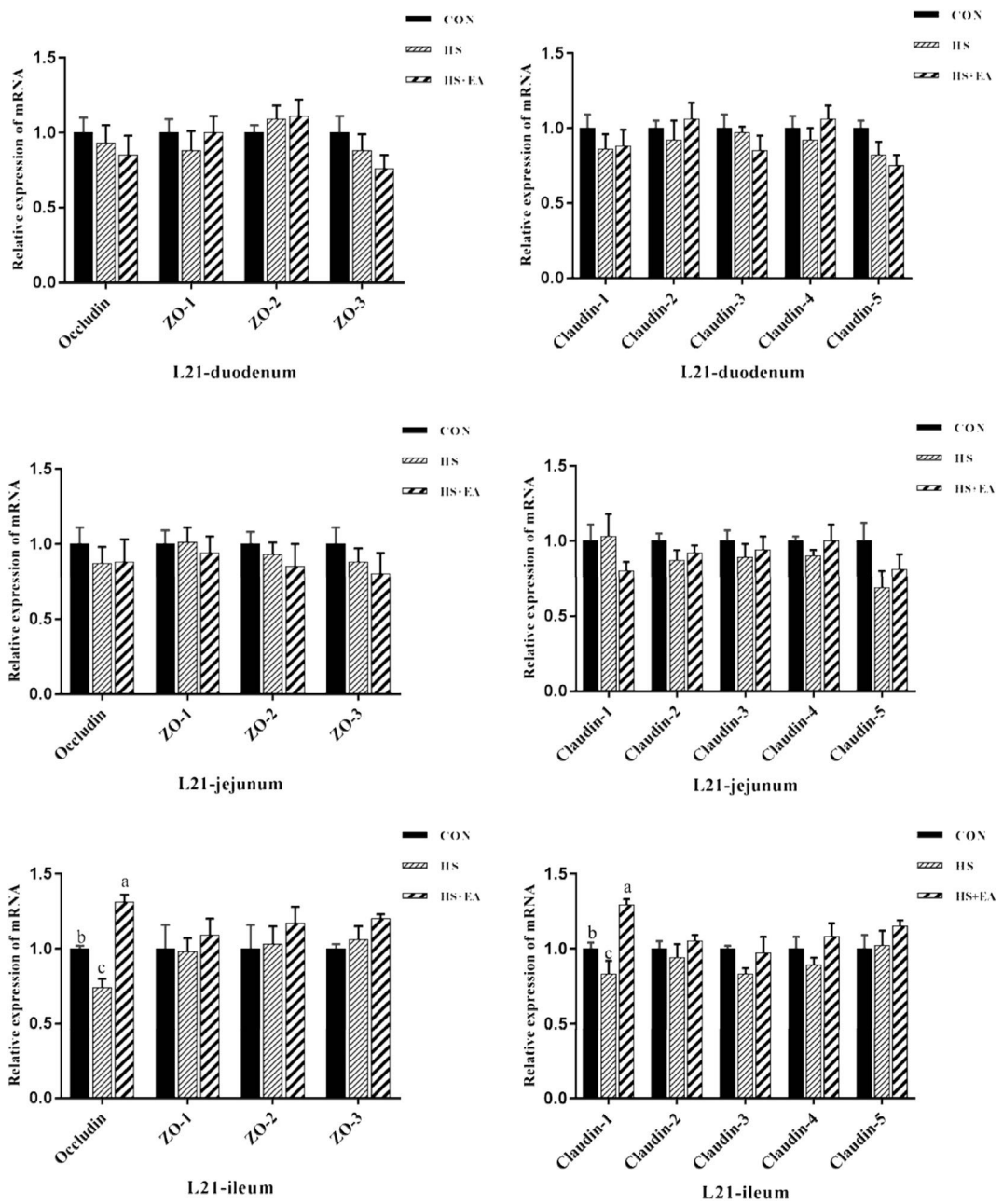

Nutrition Research Reviews, page 1 of 13 doi:10.1017/S0954422421000226
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Ferroptosis Regulation by Nutrient Signalling

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Abstract

Tremendous progress has been made in the field of ferroptosis since this regulated cell death process was first named in 2012. Ferroptosis is initiated upon redox imbalance and driven by excessive phospholipid peroxidation. Levels of multiple intracellular nutrients (iron, selenium, vitamin E and coenzyme Q_{10}) are intimately related to the cellular antioxidant system and participate in the regulation of ferroptosis. Dietary intake of monounsaturated fatty acids (MUFA) and polyunsaturated fatty acids (PUFA) regulates ferroptosis by directly modifying the fatty acid composition in cell membranes. In addition, amino acids and glucose (energy stress) manipulate the ferroptosis pathway through the nutrient-sensitive kinases mechanistic target of rapamycin complex 1 (mTORC1) and AMP-activated protein kinase (AMPK). Understanding the molecular interaction between nutrient signals and ferroptosis sensors might help in the identification of the roles of ferroptosis in normal physiology and in the development of novel pharmacological targets for the treatment of ferroptosis-related diseases.

Key words: **Nutrient sensing: Se: Fe: AMPK: mTORC1: Ferroptosis**

(Received 17 March 2021; revised 19 June 2021; accepted 30 June 2021)

1. Introduction

Cell death is a predominant process in normal physiological development and the maintenance of homeostasis, and the dysregulation of cell death leads to various diseases, including cancer. Programmed cell death is characterised by necroptosis, apoptosis, autophagy and ferroptosis. The process of necroptosis is identified by swollen organelles and ruptured cell membranes. The main characteristics of apoptosis is cell shrinkage with chromatin condensation and DNA fragmentation. Intriguingly, the hallmarks of ferroptosis are condensed mitochondria, increased bilayer membrane density and a reduction in the number of mitochondrial cristae[1,2]. Ferroptosis, a form of iron-dependent cell death, was first named in 2012. It is caused by the depletion of glutathione (GSH) and the inactivation of the phospholipid peroxidase glutathione peroxidase 4 (GPX4)[1,3]. GPX4 is the only major antioxidant enzyme known to convert toxic lipid hydroperoxides (L-OOH) into non-toxic lipid alcohols (L-OH)[3]. Thus, the predominant characteristic of ferroptosis is the accumulation of oxidative phospholipids in the cell membrane, which could trigger cell death through unclear mechanism[4]. Ferroptosis has attracted considerable interest from basic and clinical researchers since it has been implicated in the occurrence and development of multiple diseases.

Cellular biological functions are largely regulated by extracellular and intracellular nutrients and their metabolite concentrations. A rapid and efficient response to nutrient levels is crucial for maintaining cellular homeostasis. Recently, substantial progress has been made in the field of ferroptosis and nutrient sensing. In this review, we mainly focus on the potential cross talk between ferroptosis and nutrient signalling factors.

2. The general pathway of ferroptosis

As the hallmark of ferroptosis, lipid peroxidation is triggered by non-enzymatic and enzymatic processes[5]. The non-enzymatic process is mainly catalysed by cellular free iron and oxygen and is known as the Fenton reaction (Figs. 1 and 2). Bis-allylic

Abbreviations: AA, arachidonic acid; ACS, acyl-CoA synthetase; ACSL 3/4, acyl-CoA synthetase long-chain 3 and 4; ACSL, acyl-CoA synthetase long-chain family member; AdA, adrenic acid; AMPK, AMP-activated protein kinase; FA, fatty acid; FSP1, ferroptosis suppressor protein 1; GPX4, glutathione peroxidase 4; GSH, glutathione; IRP1 and IRP2, iron-regulatory proteins 1 and 2; ISC, increases iron-sulphur cluster; L-OH, lipid alcohols; L-OOH, lipid hydroperoxides; LOX, lipoxygenase; MDA, malondialdehyde; mTORC1, mechanistic target of rapamycin complex 1; MUFA, monounsaturated fatty acid; PL•, phospholipid radicals; PLO•, pLOOH reacts with cellular ferrous iron to generate additional alkoxyl radicals; PLOO•, phospholipid peroxyl radical; PLOOH, PLOO• reacts with a PLH molecule to yield a molecule of hydroperoxide; PUFA, polyunsaturated fatty acid; ROS, reactive oxygen species; SCD1, stearoyl-coenzyme A desaturase-1; SFA, saturated fatty acid; SREBP1, sterol regulatory element-binding protein 1; SREBP1c, sterol regulatory element-binding protein 1c; TF, transferrin; TfR1, transferrin receptor 1; THN, tetrahydronaphthyridinol; TSC, tuberous sclerosis complex.

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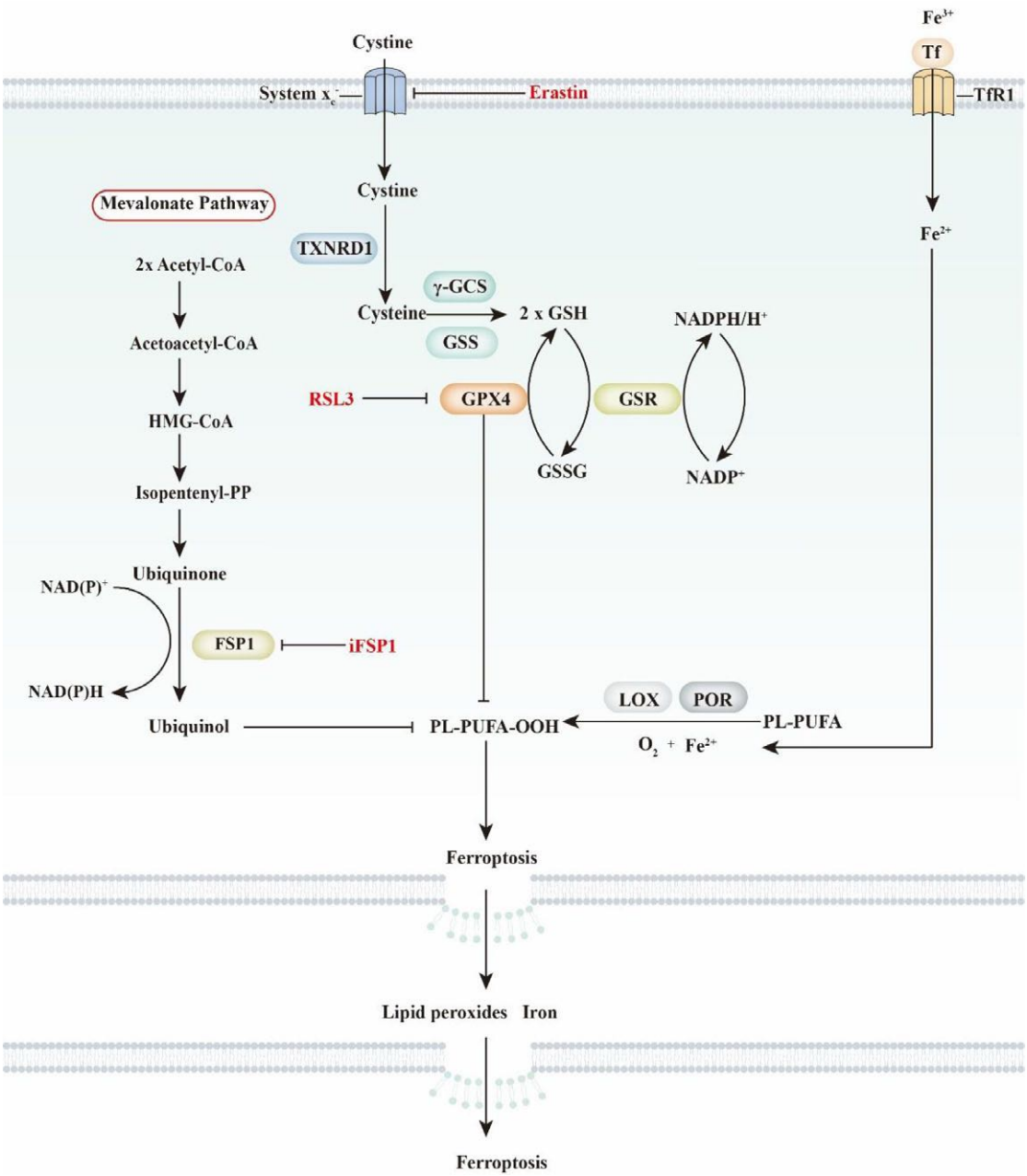

Fig. 1. General view of ferroptosis. Ferroptosis is characterised by iron-dependent phospholipid peroxidation. It is mainly regulated by selenium-dependent and CoQ_{10}-dependent pathways. The selenium-dependent pathway is primarily dependent on the enzymatic activity of glutathione peroxidase 4 (GPX4), which is a unique selenoprotein that functions as a phospholipid peroxidase. Cellular cystine transportation has an intimate relationship with the selenium-dependent pathway. Briefly, cystine is transported by amino acid transporter system x_c^- and further oxidised into cysteine by thioredoxin reductase 1 (TXNRD1). Then, cysteine, glutamate and glycine are used to synthesise glutathione (GSH) by γ-glutamylcysteine synthetase (γ-GCS) and glutathione synthetase (GSS). As a cofactor of GPX4, GSH functions in conjunction with GPX4 to reduce L-OOH into L-OH. In the CoQ_{10}-dependent pathway, the suppressor protein 1 (FSP1)–ubiquinone system is critical for lipid peroxidation. FSP1 reduces ubiquinone to ubiquinol, which further decreases lipid radicals to prevent lipid oxidation. Ubiquinone is derived from dietary CoQ_{10} intake or synthesis through the mevalonate pathway. FSP1, ferroptosis sensitising protein 1; GSH, glutathione; GSR, glutathione reductase; GSSG, glutathione disulfide; PL-PUFA, polyunsaturated fatty acid- containing phospholipid; Tf, transferrin; TfR1, transferrin receptor 1.

hydrogen atoms (the weakest C-H bond) in polyunsaturated fatty acyl moieties are vulnerable to oxidation by oxygen species and preliminarily form phospholipid radicals ($PL^{\bullet}$). After the initiation of lipid oxidation, $PL^{\bullet}$ is converted into a phospholipid peroxyl radical ($PLOO^{\bullet}$), releasing one molecule of oxygen. Subsequently, $PLOO^{\bullet}$ reacts with a PLH molecule to yield a molecule of hydroperoxide (PLOOH) and $PL^{\bullet}$[4]. As a form of reactive oxygen species (ROS), PLOOH reacts with cellular ferrous iron to generate additional alkoxyl radicals ($PLO^{\bullet}$) and $PLOO^{\bullet}$. The reactions of lipid peroxidation lead to the accumulation of lipid-based oxidative secondary products such as 4-hydroxynonenal and malondialdehyde (MDA). This propagation process of lipid peroxidation is terminated when PLOOH is cleared in a timely manner by GPX4. The enzymatic process is

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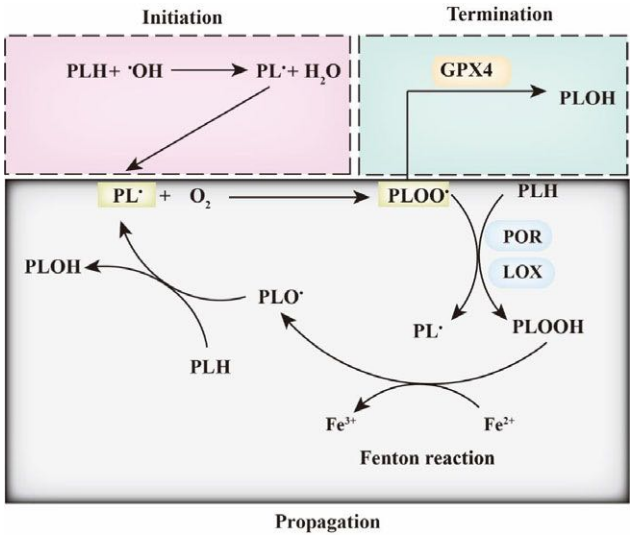

Fig. 2. Ferroptosis and phospholipid peroxidation. Polyunsaturated fatty acids contain bis-allylic hydrogen atoms, which are prone to removal, leading to the formation of phospholipid radicals (PL•). PL• reacts with O_2 to form PLOO• and is subsequently converted into PLOOH by lipoxygenase (LOX) and/or cytochrome P450 oxidoreductase (POR). Then, the iron-dependent Fenton reaction yields PLO• and PL•, which leads to the induction of PLOOH production. This lipid propagation is terminated when PLOO• is reduced by glutathione peroxidase 4 (GPX4).

also required to trigger ferroptosis (Fig. 2). Lipoxygenases (LOX) target PUFA located in lipid bilayers and regulate lipid peroxidation[6]. Inhibition of LOX makes cells more resistant to ferroptosis[7]. Cytochrome P450 oxidoreductase (POR) is the other critical regulator of ferroptosis; it induces lipid peroxidation by directly transferring hydrogen from PUFA or by indirectly converting Fe^{3+} into Fe^{2+} [8,9].

Cells have a protection program to safeguard them from ferroptosis. The canonical pathway to prevent ferroptosis in cells is mainly dependent on system x_c^- and GPX4 (Fig. 2). Mechanistically, system x_c^- regulates the cellular concentration of cystine, which could be converted into cysteine and further act as a critical substrate for GSH synthesis. GSH is a cofactor of GPX4, which reduces toxic PLOOH to corresponding non-toxic PLOHs. Inhibition of either system x_c^- or GPX4 induces ferroptosis. The system x_c^-–GSH–GPX4 axis is regarded as the most classical and is the best-studied pathway in the regulation of ferroptosis. Recently, studies identified a novel ubiquinone-dependent pathway in which FSP1 functions as an oxidoreductase to reduce ubiquinone to ubiquinol, which prevents lipid peroxidation[10,11].

3. Ferroptosis and iron

Iron has been known as a critical component for haemoglobin synthesis since 1932[12]. The role of iron in haemoglobin formation and oxygen transport has attracted the attention of nutritionists for many years. Insufficient iron intake has a direct connection to anaemia in many developing countries[13]. Recently, the accumulation of iron has been found to be closely related to ferroptosis. Iron is proposed to enhance lipid peroxidation through the Fenton reaction or activation of enzymes such as LOX[14]. Mechanistically, iron reacts with PLOOH to generate the free radicals PLO• and PLOO•, which propagate peroxidation chain reactions[4].

Feeding animals an iron-enriched diet increases cellular iron loading (Fig. 3)[15]. Iron is absorbed in the upper intestine in the form of ferrous iron (Fe^{2+}), which is then quickly oxidised into a ferric ion (Fe^{3+}). Once exported from enterocytes, Fe^{3+} binds with transferrin (TF) and circulates in the blood circulatory system. This complex is recognised and imported by the transferrin receptor (TFRC) located in the cell membrane. Once taken up, the iron bound to TF is reduced from Fe^{3+} to Fe^{2+} by the STEAP3 metalloreductase in the endosome and is then transferred to the cytosol via solute carrier family 11 member 2 (SLC11A2). The enrichment of iron further sensitises cells to ferroptosis.

Iron storage and export are both critical processes that regulate intracellular iron levels (Fig. 3). Cellular free iron is stored by ferritins, which are composed of ferritin light chain (FTL) and ferritin heavy chain 1 (FTH1). Knocking down *Ftl* and *Fth1* increases the concentration of free iron, sensitising cells to ferroptosis triggered by GPX4 inhibition[16]. Similarly, overexpression of mitochondrial ferritin inhibits erastin-induced ferroptosis[17]. In addition to ferritin, multiple mitochondrial proteins (such as NFS1 cysteine desulphurase, CDGSH iron sulphur domain 1 and CDGSH iron sulphur domain 2) also regulate cellular iron concentrations by manipulating iron–sulphur cluster biogenesis[18–21]. Iron chelators (such as deferoxamine, deferasirox, deferiprone, ciclopirox and dexrazoxane) mimic the effects of ferritin and are widely used to treat iron overload diseases[22]. The other way to relieve iron overloading is through the regulation of iron export. Solute carrier family 40 member 1 (SLC40A1), which is also known as ferroportin, is the only known transporter of iron exportation. Siramesine is a sigma-2 receptor ligand, which is a lysosomotropic agent that can be used to treat depression[23], while lapatinib is originally identified as a dual tyrosine kinase inhibitor of ErbB1 and ErbB2 tyrosine kinase receptors[24]. The combination of siramesine and lapatinib has been reported to synergically regulate ferroptosis through iron regulatory proteins[25]. Overexpression of *SLC40A1* inhibits siramesine- and lapatinib-induced ferroptosis, whereas knockdown of *SLC40A1* promotes ferroptosis[25]. Consistently, prominin 2 (PROM2), a member of the prominin family of pentaspan membrane glycoproteins, regulates exosome-dependent iron exportation and desensitises cells to ferroptosis[16]. Some regulators participate in the modulation of cellular iron homeostasis. Iron-regulatory proteins 1 and 2 (IRP1 and IRP2) regulate the genes involved in iron uptake, export, storage and utilisation[26,27].

4. Ferroptosis and selenium

Selenium was discovered approximately 200 years ago by Swedish scientists. This element was originally regarded as a toxin in animals because livestock fed selenium-enriched plants suffered hair loss and a necrotic hoof malady[28]. With the gradual understanding of selenium, scientists have found that

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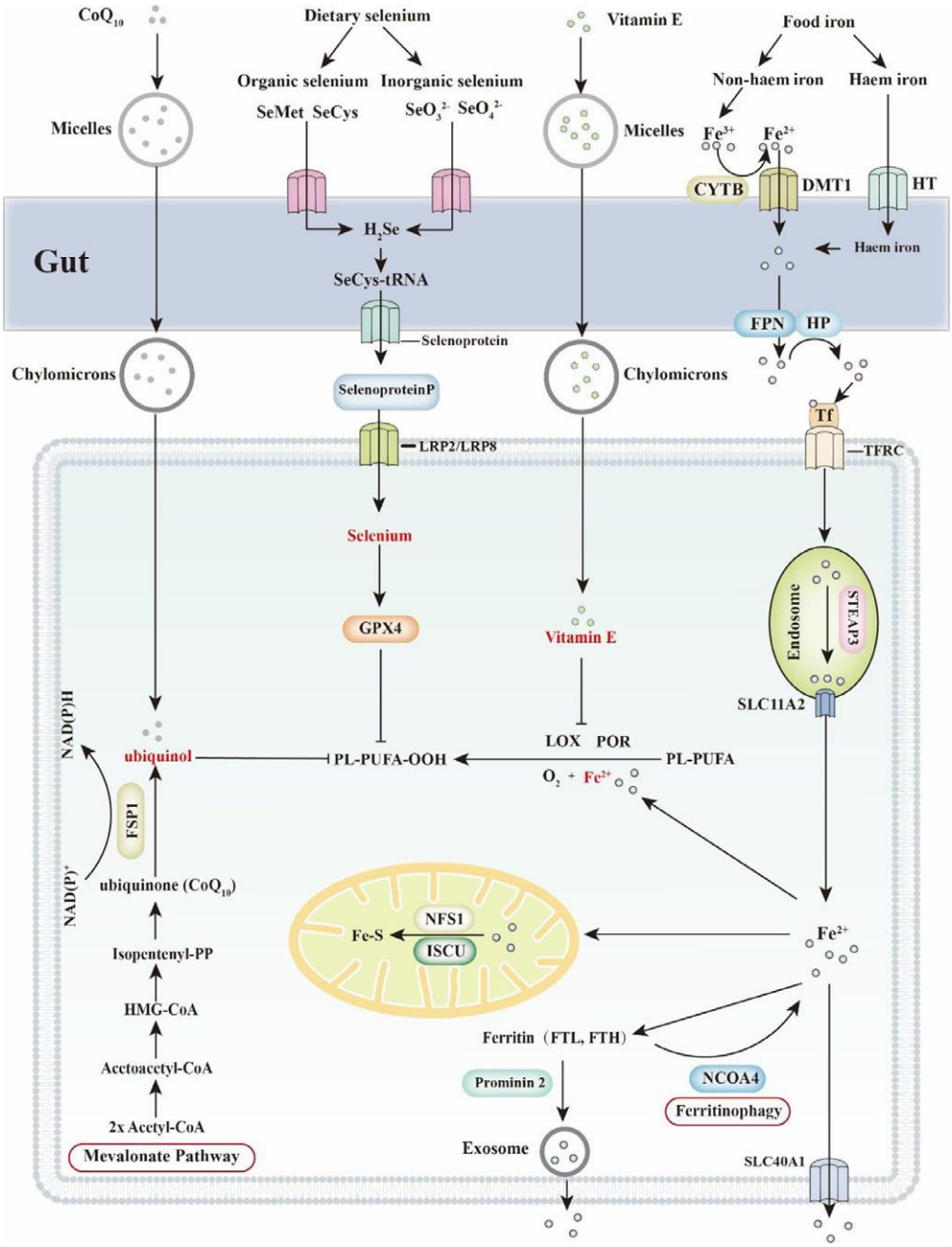

Fig. 3. Ferroptosis and dietary nutrients (CoQ10, selenium, vitamin E and iron). Ferroptosis is an iron-dependent form of cell death. Thus, regulation of cellular iron concentration is critical to ferroptosis. Dietary iron includes haem iron and non-haem iron. Haem iron is directly transported into enterocytes through haem transporters (HT). Dietary ferric iron (Fe^{3+}) is reduced into ferritin iron (Fe^{2+}) by the ferric reductase enzyme cytochrome b (CYTB) and then transported into cells via divalent metal transporter 1 (DMT1). Subsequently, Fe^{2+} is exported by FPN1 and oxidised into Fe^{3+} by hephaestin (HP). Finally, Fe^{3+} is combined with transferrin (TF) and transported to targeted organs, which are recognised by the transferrin receptor (TFRC) in the cell membrane. Selenium is classified into organic selenium and inorganic selenium, both of which are absorbed in the small intestine. SeO_3^{2-} is passively absorbed by the gut, while SeO_4^{2-} seems to be transported by a sodium-mediated carrier. Organic forms of selenium are not recognised and are transported as minerals. For instance, selenomethionine (SeMet) and selenocysteine (SeCys) are transported in the form of amino acids. More than 50 % of selenium is converted into selenoprotein P (SELENOP) and transported to the targeted organs through blood. Low-density lipoprotein receptor-related protein 2 and 8 (LRP2/LRP8) are critical for receptor-dependent selenium uptake in different tissues. Cellular selenium is subsequently synthesised into GPX4 and protects cells from ferroptosis. Vitamin E and CoQ_{10} are both regarded as fat-soluble vitamins. Dietary vitamin E and CoQ_{10} are incorporated into micelles and absorbed by the small intestine. In enterocytes, vitamin E and CoQ_{10} are attached to chylomicrons and then transported to target organs through the lymph and blood circulation systems. CoQ_{10} (ubiquinone) is largely transformed into ubiquinol in lymph. In blood vessels, CoQ_{10} is attached to low-density lipoprotein (LDL) and very-low-density lipoprotein (VLDL), while vitamin E is attached to high-density lipoprotein (HDL). Mechanistically, ubiquinol directly prevents lipid peroxidation, whereas vitamin E regulates ferroptosis mainly by competing with lipoxygenases (LOX) for PUFA substrate-binding sites. FSP1, ferroptosis sensitising protein 1; FTH, ferritin heavy chain; FTL, ferritin light chain; FPN, fragipain; GPX4, glutathione peroxidase 4; HMG-CoA, β-Hydroxy β-methylglutaryl-CoA; ISCU, iron–sulphur cluster assembly enzyme; NCOA4, nuclear receptor coactivator 4; NFS1, nitrogen fixation 1 homologue; PL-PUFA, polyunsaturated fatty acid- containing phospholipid; POR, cytochrome P450 oxidoreductase; SLC40A1, solute carrier family 40 member 1; SLC11A2, solute carrier family 11, member 2.

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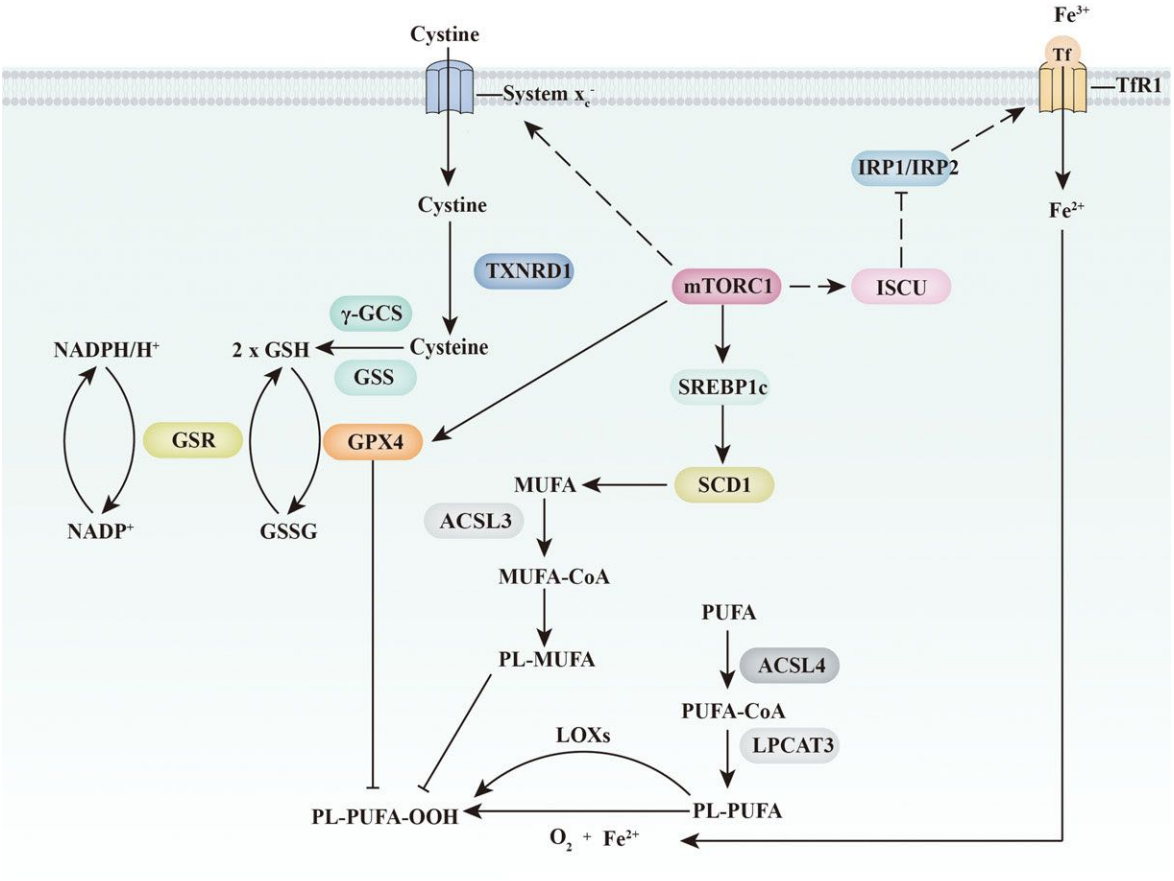

Fig. 4. Ferroptosis and the mTORC1 signalling pathway. mTORC1 inhibits ferroptosis through three independent pathways. On the one hand, mTORC1 increases the expression of GPX4 and the abundance of GSH (through the up-regulation of amino acid transport system x_c^-). On the other hand, mTORC1 increases MUFA abundance in the plasma membrane through the SREBP1c/SCD1 pathway. Furthermore, mTORC1 might directly inhibit an increase the intracellular iron concentration through the ISCU/IRP/TfR1 signalling pathway. ACSL 3/4, acyl-CoA synthetase long-chain 3 and 4; γ-GCS, γ-glutamylcysteine synthetase; GPX4, glutathione peroxidase 4; GSH, glutathione; GSR, glutathione reductase; GSS, glutathione synthetase; GSSG, glutathione disulfide; IRP1/IRP2, iron regulatory protein 1 and 2; ISCU, iron–sulphur cluster assembly enzyme; LOX, lipoxygenases; LPCAT3, lysophospholipid *O*-acyltransferase 3; mTORC1, mechanistic target of rapamycin complex 1; PL-MUFA, monounsaturated fatty acids-containing phospholipid; PL-PUFA, polyunsaturated fatty acid-containing phospholipid; SCD1, stearoyl-CoA desaturase-1; SREBP1c, sterol regulatory element binding protein-1c; Tf, transferrin; TfR1, transferrin receptor 1; TXNRD1, thioredoxin reductase 1.

selenium is toxic at high levels but essential at low levels[29]. Insufficient selenium induces various diseases, such as cancer, inflammation, immune disorders and nervous system diseases.

Selenium is a critical component of selenoproteins. Briefly, selenide (HSe^-) is converted into selenophosphate ($H_2SePO_3^-$), which is incorporated into P-Ser-tRNA$^{[Ser]Sec}$ and used for selenoprotein synthesis[30]. Knockout of the Sec-specific tRNA gene *Trsp* (*nuclear encoded tRNA selenocysteine 2*) leads to lethality in mice during embryonic development[31]. To date, approximately twenty-five selenoproteins have been identified in humans[32,33]. Among these selenoproteins, GPX4 is the only antioxidant enzyme protecting cells from lipid peroxidation and ferroptosis. Similar to *Trsp* deficiency, knocking out *GPX4* in mice is lethal during the early embryonic period. Importantly, selenium utilisation by GPX4 is critical to inhibit ferroptosis[34]. The replacement of the catalytically active selenium to a cysteine in GPX4 makes cells highly sensitive to peroxide-induced ferroptosis[34]. The evidence obtained from this mutation indicates that selenolate-based catalysis of GPX4 is critical to prevent ferroptosis in cells.

Increasing selenium intake is an efficient way to increase the cell selenium concentration and protect cells from ferroptosis (Fig. 3). Dietary selenium is usually taken up as selenoproteins. Selenomethionine (SeMet, organic form) is absorbed most rapidly by all the intestinal segment, while selenate and selenite (inorganic form) can only be efficiently absorbed by the ileum[35]. Selenium salts are required to be converted into selenoamino acids before being absorbed by the intestine[36]. Thus, for dietary sources of selenoproteins, organic forms of selenium are absorbed more efficiently than inorganic forms. Selenoproteins can then be transported into the liver for selenoprotein P (SELENOP) synthesis[32]. Subsequently, SELENOP is delivered to target organs, which are recognised by low-density lipoprotein receptor-related protein 2 and 8 (LRP2 and LRP8)[37,38].

Ingestion of selenocysteine-containing peptides activates homeostatic transcription to inhibit ferroptosis and alleviate haemorrhagic or ischaemic stroke[39]. Notably, ingested selenium not only acts as a component in GPX4 synthesis but also drives the transcription of GPX4 through coordinated

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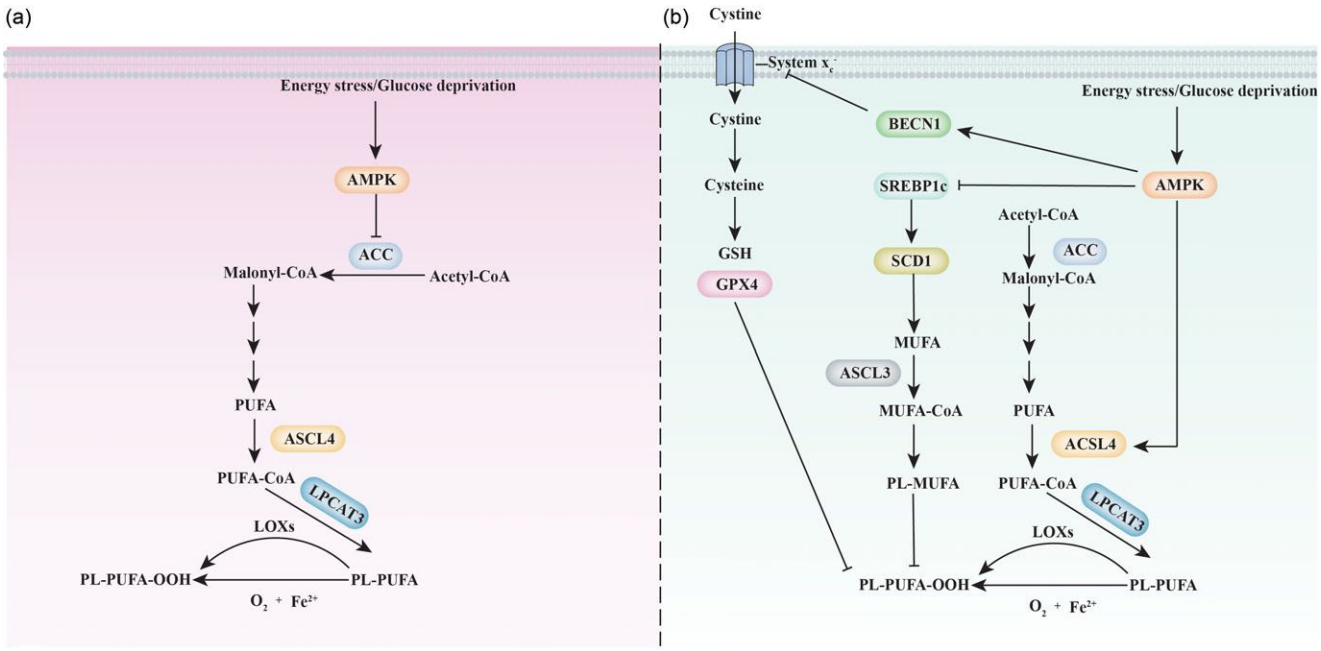

Fig. 5. Ferroptosis and the AMPK signalling pathway. Inconsistent and contradictory results have been obtained in studies of AMPK and ferroptosis. (a) A group of scientists found that AMPK prevents ferroptosis by decreasing PUFA levels and increasing MUFA levels through the inhibition of ACC function. (b) Another group of scientists promotes an alternative view, suggesting that AMPK promotes ferroptosis with an increase in PUFA levels and a decrease in MUFA levels through activation of **ACSL4** and inactivation of the SREBP1c/SCD1 pathway. In addition, AMPK also inhibits the activity of amino acid transport system x_c^- via BECN1, which further triggers the initiation of ferroptosis. ACC, Acetyl-CoA carboxylase; ACSL 3/4, acyl-CoA synthetase long-chain 3 and 4; AMPK, 5'AMP- activated protein kinase; BECN1, beclin-1; GPX4, glutathione peroxidase 4; GSH, glutathione; LOXs, lipoxygenases; LPCAT3, lysophospholipid *O*-acyltransferase 3; PL-MUFA, monounsaturated fatty acids-containing phospholipid; PL-PUFA, polyunsaturated fatty acid-containing phospholipid; SCD1, stearoyl-CoA desaturase-1; SREBP1c, sterol regulatory element binding protein-1c.

activation of the transcription factors TFAP2c and Sp1 in neurons[39]. Interestingly, selenium deficiency drives adaptive transcription to attenuate ferroptotic insults, but this adaptation appears to be insufficient to protect cells from undergoing ferroptosis[39]. When dietary selenium is limited, selenium is preferentially enriched in the brain and testes, which indicates the predominant function of selenium-dependent enzymes in these organs[40]. This selective selenium distribution might partially explain why Parkinson's disease, Alzheimer's disease, dementia, seizures, coordination and cognitive decline are commonly identified in selenium-deficient individuals[41]. Together, nutritional supplementation with selenium is an efficient way to protect the cell from ferroptosis and its related metabolic diseases through the regulation of GPX4 in selenium-deficient individuals.

5. Ferroptosis and CoQ_{10}

Coenzyme Q_{10} (CoQ_{10}, also known as ubiquinone), a vitamin-like substance, is a lipid-soluble antioxidant used for the treatment of cardiovascular disease. CoQ_{10} maintains the mitochondrial membrane potential, promotes ATP synthesis and inhibits ROS generation. CoQ_{10} protects neuronal cells from oxidative stress and prevents related neurodegenerative diseases, including Parkinson's and Alzheimer's diseases[42,43].

Recently, CoQ_{10} has been reported to prevent the production of lipid peroxides. In addition to the canonical GSH-based GPX4 pathway, the non-mitochondrial CoQ_{10} antioxidant system acts in parallel to protect the cell from ferroptosis[11]. Ferroptosis suppressor protein 1 (FSP1) has been identified by two individual groups as a crucial ferroptosis-resistance factor[10,11]. In the plasma membrane, FSP1 functions as an oxidoreductase to reduce ubiquinone to ubiquinol and prevents lipid peroxidation. In addition, CoQ_{10} increases the phosphorylation of AMPK[44–46], which might be the other pathway that prevents ferroptosis. However, since the biological effects of AMPK signalling on ferroptosis are still unclear (additional details are discussed in the AMPK and ferroptosis sections), more research is warranted to determine whether CoQ_{10} is connected to ferroptosis through AMPK.

To date, no direct *in vivo* or *in vitro* evidence has indicated that administration of CoQ_{10} can rescue cells from ferroptosis. However, over many decades, the pharmaceutical use of CoQ_{10} has been demonstrated to restore respiratory chain activities and prevent oxidative stress. Dietary supplementation with CoQ_{10} prevents the initiation of lipid peroxidation[47,48], which is consistent with the results observed *in vitro*[49]. Similar to CoQ_{10}, the CoQ_{10} analogue idebenone also halts cellular lipid peroxidation[50]. In addition to dietary supplementation, CoQ_{10} is also synthesised through the mevalonate pathway. It has been reported that an inhibitor of HMG-CoA reductase (cerivastatin) or a scavenger of CoQ_{10} (compound FIN56) sensitises cells to ferroptosis[50]. Similarly, 4-chlorobenzoate inhibits CoQ_{10} production and triggers ferroptosis[11]. Thus, dietary

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supplementation with CoQ_{10} or an increase in CoQ_{10} *de novo* synthesis might efficiently protect cells from ferroptosis.

6. Vitamin E and ferroptosis

Discovered approximately 100 years ago, vitamin E is one of most predominant inhibitors of ferroptosis[51]. Dietary vitamin E comprises eight homologues: α-, β-, γ- and δ-tocopherols and α-, β-, γ- and δ-tocotrienols. All of these isoforms are found to break the autoxidation chain reaction, especially α-tocopherol, which has the highest antioxidant activity[52,53]. Thus, clinial symptoms of vitamin E deficiency are similar to those of selenium deficiency. LOX-mediated lipid peroxidation has been implicated as a central event in ferroptosis. Tocotrienols and tocopherols regulate ferroptosis mainly through their competition with LOX (through a corking mechanism) for PUFA-substrate binding sites[54]. Tocotrienols are more effective than tocopherols in protecting cells from ferroptosis, as they are more effective LOX inhibitors[54]. Mechanistically, vitamin E inhibits LOX by reducing the non-haem iron in the enzyme (converts Fe^{3+} to Fe^{2+}).

Dietary supplementation with the lipophilic antioxidant vitamin E is an effective way to prevent ferroptosis and its related diseases. In an *in vivo* study, *Gpx4*-knockout mice fed a vitamin-E-deficient diet exhibited increased lipid peroxidation and hippocampal neurodegeneration[55]. Similarly, mice with endothelial cell knockout of *Gpx4* appear normal when fed a vitamin-E-sufficient diet but abnormal when fed a vitamin-E-insufficient diet[56]. Dietary supplementation with vitamin E restores the dysfunction of T cells induced by *Gpx4* deficiency[57]. In addition, severe embryo lipid peroxidation is observed when vitamin E is absent. Dietary supplementation with vitamin E during gestation prevents disruption in mouse embryonic fibroblasts, cortical neurons and even fetal lethality caused by GPX4 knockout[58,59]. Recently, α-tocopherol hydroquinone, an endogenous metabolite of vitamin E, was implicated as a more powerful inhibitor of ferroptosis than vitamin E[60].

To improve the biological function of vitamin E as ferroptosis inhibitors, scientists have attempted to develop better analogues of vitamin E. Tetrahydronaphthyridinols (THN) constitute a specific group of aza-phenols, which are considered the most promising synthetic compounds to inhibit ferroptosis[61]. The speed at which THN react with peroxyl radicals is almost 30-fold faster than that of α-tocopherol[62]. THN with alkyl chains ranging from twelve to fifteen carbons are more effective in protecting cells from ferroptosis than the classical ferroptosis inhibitors Fer-1 and Lip-1[63]. This evidence indicates that THN can efficiently inhibit ferroptosis.

7. Fatty acids and ferroptosis

Fatty acids (FA) are lipids that not only act as an energy sources and constitute portions of cellular membranes but also play multiple physiological roles. FA can be classified into saturated fatty acids (SFA), MUFA and PUFA based on the degree of hydrocarbon chain saturation. PUFA are predominant components in cellular, mitochondrial and retinal membranes. Cells (such as neurons) with higher levels of PUFA are more sensitive to ferroptosis[64].

By using quantitative redox lipidomics, a study made clear that ferroptosis occurs in cells with high levels of plasma membrane phosphatidylethanolamines (PE), which are a class of specific phospholipids with two fatty acid chains – arachidonic acid (AA) and adrenic acid (AdA)[54]. Fatty acid elongation by very-long-chain fatty acid protein 5 (ELOVL5) and fatty acid desaturase 1 (FADS1) is required to maintain the intracellular levels of AA and AdA[65]. Blockage of AA or AdA esterification into PE efficiently prevents ferroptosis in cells. FA must be activated by acyl-CoA synthetase (ACS) before they can be incorporated into triacylglycerol (TAG)[66]. Among the five isoforms of acyl-CoA synthetase long-chain family members (ACSL), ACSL4 is specific for the activation of AA and AdA[67]. Intriguingly, ACSL4 seems to serve as a double-edged sword in terms of cell survival. On the one hand, ACSL4 decreases fatty acid-induced lipotoxicity[68]. On the other hand, ACSL4 drives esterification of AA and AdA into PE, which triggers ferroptosis[54]. Excess AA increases Fenton reaction-induced oxidative stress with the up-regulation of anti-inflammatory lipoxinA4 (LXA4) and down-regulation of prostaglandin E2 (PGE2) (LXA4 and PGE2 are proposed to promote and inhibit cell growth, respectively), which further induce cell death[68]. Thus, the effects of PUFA (AA and AdA) to promote cell survival or induce ferroptosis may be dose dependent[66].

Administration of cells with PUFA is an efficient way to promote ferroptosis. PUFA, especially AA, can induce inflammatory bowel disease (IBD) by triggering ferroptosis in *Gpx4*-deficient mice[69]. Dietary ingestion of the PUFA dihomo-γ-linolenic acid (DGLA; 20:3 n-6) induces ferroptosis in the nematode *Caenorhabditis elegans* and human cells[70]. These experiments propose that the enrichment of PUFA in the plasma membrane makes cells more susceptible to ferroptosis.

In contrast to PUFA, the administration of MUFA causes cells to enter a ferroptosis-insensitive state. Exogenous MUFA decrease lipid ROS accumulation and inhibit ferroptosis in an acyl-CoA synthetase long-chain family member 3 (ACSL3)-dependent manner[71]. Mechanistically, MUFA are converted into MUFA-PL by ACSL3, and they displace PUFA in the plasma membrane and hinder lipid ROS accumulation[71]. Clinically, increased serum MUFA prevent ferroptosis induced by neurodegeneration or kidney failure[72]. Overloading intracellular SFA triggers a specified apoptosis process called lipotoxicity[73]. Intriguingly, MUFA also protect cells from apoptotic lipotoxicity caused by SFA. Thus, modification of dietary MUFA levels can be an efficient way to regulate ferroptosis and lipotoxicity.

In addition to replacing PUFA with MUFA in the plasma membrane, increasing ether lipid abundance is an alternative way to protect cells from ferroptosis. Endogenous ether lipids are regarded as oxidative 'sinks' that protect PUFA chains from oxidation[74,75]. Infants with inherited ether lipid synthesis enzyme deficiency present with severe growth and mental disorders[76]. The synthesis of ether lipids protects *C. elegans* and human cancer cells from undergoing ferroptosis[70]. Future

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studies need to clarify the potential methods to manipulate the synthesis of ether lipids.

8. mTOR signalling and ferroptosis

mTORC1 is a master regulator of protein synthesis, which is triggered by multiple stimuli, such as growth factors, energy levels and amino acids[77–79]. Amino acids regulate mTORC1 lysosomal localisation and subsequently activate mTORC1 through Rag GTPase-dependent and Rag GTPase-independent pathways[80–82]. The small G-protein Ras homologue enriched in the brain (Rheb) contains a CAAX (C = Cys, A = aliphatic, X = terminal amino acid) box, which facilitates its localisation to lysosomes[83,84]. Rheb directly interacts with mTORC1 and triggers conformational changes in mTOR, triggering its activation[85]. In contrast to amino acids, growth factors activate mTORC1 by stimulating the dissociation of the tuberous sclerosis complex (TSC) from the lysosome and Rheb[83]. TSC is a negative regulator of mTORC1, and its mutation constitutively activates mTORC1[86–88]. mTORC1 is composed of three components: the catalytic component mTOR, the regulatory-associated protein of mTOR (Raptor) and mammalian lethal with Sec13 protein 8 (mLST8, also known as $G_{\beta L}$). Raptor recognises mTORC1 substrates, and mLST8 acts as a positive regulator of mTOR activity. Activation of mTORC1 regulates mRNA translation and protein transcription through the phosphorylation of eukaryotic initiation factor 4E-binding protein 1 (4E-BP1) and S6 kinase (S6K1), which are the two key downstream substrates of the mTORC1 complex.

Recently, activation of mTORC1 was reported to prevent lipid peroxide accumulation and suppress ferroptosis[89]. This effect was partially due to mTORC1 signalling participating in the regulation of lipid metabolism. As a transcriptional target of sterol regulatory element-binding protein 1 (SREBP1), stearoyl-coenzyme A desaturase-1 (SCD1) is critical for the conversion of SFA into MUFA. Synthesised MUFA displace PUFA in the plasma membrane and inhibit the accumulation of lipid ROS[71]. Knocking out either *SREBP1* or *SCD1* impaired the effects of mTORC1 on ferroptosis[89]. Exogenous MUFA palmitoleic acid (16:1, PO) or oleate acid (18:1, OA) supplementation also restores ferroptosis resistance when mTORC1 signalling is depressed. This evidence indicates that mTORC1 signalling protects cells from ferroptosis through the SREBP1/SCD1-mediated synthesis of MUFA (Fig. 4).

Another connection between mTORC1 signalling and ferroptosis is GPX4 (Fig. 4). Han *et al.*[90] also observed that inhibition of mTORC1 was accompanied by a decrease in GPX4 level. In addition, some preliminary evidence suggests that mTOR signalling might be required to maintain the expression of SLC7A11[91]. SLC7A11 is critical for cystine transportation, which is required for GSH synthesis and GPX4 activity.

In addition, mTORC1 is proposed to regulate ferroptosis directly through the modulation of the iron transport system (Fig. 4). Some evidence indicates that the activation of mTORC1 decreases the cellular iron concentration. mTORC1 phosphorylates iron–sulphur cluster assembly enzyme (ISCU) at serine 14 and increases iron–sulphur cluster (ISC) assembly[92,93]. Stabilisation of ISCU further inhibits IRP1/IRP2 function, promoting ferroportin 1 (Fpn1, a protein that mainly mediates iron export) and decreasing cellular iron concentration[94]. Notably, however, the effect of mTORC1 on cellular iron concentration is inconsistent. Bayeva *et al.*[94] reported that activation of mTORC1 negatively regulates the expression of tristetraprolin, which binds to Transferrin receptor 1 (TfR1) mRNA to enhance its degradation. TfR1 is a protein that mainly mediates iron import. Thus, activation of mTORC1 is proposed to increase cellular iron concentration. Future experiments are needed to clarify the effect of mTOR on iron transport.

In clinical research, mTORC1 plays a vital role in iron metabolism in cardiomyocytes. Approximately 80 % of mTOR-treated orthotopic heart transplant patients have functional iron deficiency[95]. In cardiomyocytes, activation of mTOR protects against ferroptosis partially by decreasing ROS production[96]. An in-depth understanding of the cross talk between mTOR signalling and ferroptosis will lead to a novel therapy for patients with myocardial infarction.

9. Energy sensing and ferroptosis

Energy homeostasis is important to sustain the biological function of cells. AMPK acts as the predominant energy sensor in eukaryotic cells[97]. The adenylation system (ATP + ADP + AMP) has been measured to estimate cellular energy levels since 1976[98,99]. Increased ADP and AMP indicate a decrease in cellular energy status. AMPK is a heterotrimer that contains an α catalytic subunit, β regulatory subunit and γ regulatory subunit[100]. AMPK subunits include multiple isoforms, such as α1, α2, β1, β2, γ1, γ2 and γ3[101]. The classical pathway to AMPK activation (phosphorylation at Thr172) is dependent on increased levels of ADP and AMP[102].

Recently, AMPK has been shown to be a critical regulator of ferroptosis (Fig. 5). Cells with AMPK activation are more resistant to ferroptosis, while those with AMPK inactivation are more sensitive to ferroptosis[103–105]. This AMPK effect is due to the energy stress-impaired biosynthesis of PUFA, especially arachidonic acid (C20:4) and adrenic acid (C22:4), which are required for ferroptosis[54]. Mechanistically, AMPK regulates ferroptosis partially through phosphorylation of ACC, which inhibits PUFA-containing lipid biosynthesis and makes cells more resistant to ferroptosis[103,105]. Inhibition (treatment with 5-(tetradecyloxy)-2-furoic acid) or site mutation (mutation of ACC1 Ser79 to ACC1 Ala79; mutation of ACC2 Ser212 to ACC Ala212) of ACC mimics the protective role of AMPK activation in ferroptosis[103]. This evidence proposes a protective mechanism that protects cells from organ injury induced by energy failure. For instance, energy stress was found to protect against renal ischaemia–reperfusion injury[103].

Notably, there is a fierce debate regarding the role of AMPK in the regulation of ferroptosis (Fig. 5). Cells under energy stress commonly show increased ROS production, which indicates the promotion of ferroptosis[106]. Song *et al.*[107] observed that activation of AMPK is required for BECN1-induced ferroptosis, a finding in opposition to the results shown by Lee *et al.*[103]. BECN1 is a crucial regulator of macroautophagy/autophagy[108].

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Mechanistically, activation of AMPK phosphorylates BECN1 at Ser90/93/96 and promotes BECN1–SLC7A11 complex formation; this complex blocks system x_c^--regulated homeostasis in GSH and induces ferroptosis[107,109]. In addition, Zhao *et al.*[110] found that inactivation of AMPK promotes SREBP1/SCD1 expression, which inhibits ferroptosis with the production of MUFAs. However, it is worth noting that activation of SREBP1, a critical transcriptional regulator of lipogenesis, is proposed to promote the production of both MUFA and PUFA, but not specific MUFA. Thus, more studies are required to confirm this finding. Notably, AMPK is the upstream and negative regulator of mTORC1[111]. As activation of mTORC1 protects the cell from ferroptosis, it is reasonable to hypothesise that the activation of AMPK promotes ferroptosis.

Mitochondria are predominant organelles that participate in energy production and the regulation of ferroptosis. It is worth noting that the potential mechanism of mitochondria-triggered ferroptosis is also controversial. Some evidence indicates that the mitochondrial TCA cycle and electron transfer chain increase lipid peroxide accumulation and promote ferroptosis induced by cysteine deprivation[112,113]. However, one study reported that functional mitochondrial electron transfer chain is not required in erastin-induced cell death[1]. In addition, mitochondria have many other functions independent of the electron transfer chain that could contribute to ferroptosis, such as tumor suppressor protein p53[114] and enzymes involved in mitochondrial respiration[114,115]. Interestingly, mitochondria were involved only in ferroptosis caused by cysteine deprivation but not by GPX4 inhibition (induced by RSL3)[112]. This result might be due to inhibited GPX4 quickly enhancing the accumulation of lipid ROS in other pathways that remain active, a supposition that requires further study. Clinically, MitoTEMPO (a superoxide scavenger targeted to the mitochondria) decreases lipid peroxidation and protects the heart from ischemia/reperfusion (IR)-induced damage[116]. Understanding the physiological functions of mitochondria and AMPK in the regulation of ferroptosis will help in identifying novel pharmacological targets for ferroptosis-related diseases.

10. Conclusions and perspectives

Tremendous progress has been made in the field of ferroptosis in recent years. Multiple nutrients have been reported to regulate redox homeostasis and lipid peroxidation. To date, nutrients participating in ferroptosis regulation are classified into three categories: (1) selenium, iron, vitamin E and CoQ_{10}, which mediate the production of PLOOH generated in cells; (2) exogenous fatty acids that manipulate the fatty acid profiles of cellular membranes (mainly in monogastric animals); and (3) amino acids and energy stress, which trigger the activation of mTORC1 and AMPK signalling pathways related to ferroptosis. However, our understanding of ferroptosis and the cross talk of its factors with nutrient signalling factors is still limited but is expected to explode in the coming years. Critical issues in this field that must be addressed are listed below.

First, ferroptosis is not only involved in diverse diseases but might also be critical to regulate normal physiology. Under physiological conditions, iron and ROS are involved in the regulation of cellular biological functions. Once a threshold is crossed, iron overload and oxidative damage can cause ferroptotic cell death. MDA, a critical marker of lipid peroxidation, is generally increased in animals under heat and oxidative stress[117,118]. In contrast to classical ferroptosis, the accumulation of MDA under heat and oxidative stress is usually insufficient to induce a notable increase in the cell death rate. Thus, the possibility that sublethal ferroptosis can reprogram cell metabolism and biological functions in different cells and/or organs needs to be studied in the future. In addition, the thresholds for inducing sublethal ferroptosis-related damage are unknown. Nutritional strategies may be an efficient way to manipulate sublethal ferroptosis-related disorders.

Second, current findings on the cross talk between ferroptosis and nutrient sensing factors are not consistent, especially results related to ferroptosis and AMPK. To date, an intense debate rages regarding the biological function of AMPK in cancer cells[119,120]. The signalling model of AMPK and ferroptosis might be more complicated than the current model. For instance, AMPK regulates not only lipid metabolism through the phosphorylation of ACC1 and ACC2 but also sterol regulatory element-binding protein 1c (SREBP1c) and PPAR γ coactivator 1α (PGC1α)[121,122]. The expression and effects of SREBP1c and PGC1α vary by cell type. Therefore, the effects of AMPK-regulated ferroptosis could be cell type- or tissue-dependent.

Third, iron is not the only ion to trigger the Fenton reaction, which raises the question, why is ferroptosis more sensitive to liable iron concentrations? Recently, some studies have indicated that other ions also participate in the regulation of ferroptosis. For instance, under specific conditions, copper regulates the initiation of ferroptosis[123]. Endoplasmic reticulum zinc homeostasis is closely related to ferroptosis[124]. Thus, future studies are required to determine whether current findings are missing critical information regarding other ions with respect to ferroptosis.

In addition to the unclear connections between nutrient sensing and ferroptosis, precise biological markers for ferroptosis need to be clarified in the future. Although the expression of some genes (for instance, prostaglandin-endoperoxide synthase 2 (PTGS2/COX2), ChaC glutathione-specific γ-glutamylcyclotransferase 1 (CHAC1/BOTCH), nuclear factor erythroid 2-like 2 (NFE2L2/NRF2)) is related to the process of ferroptosis, these genes are not uniquely involved in ferroptosis[125]. Furthermore, potential connections among ferroptosis, apoptosis and autophagy need to be explored. It is difficult to separate ferroptosis from other types of cell death. Nutrient signalling regulates not only ferroptosis but also apoptosis[126,127] and autophagy[128,129]. Future studies need to answer the following questions: What is the precise relationship among different types of cell death? Does nutrient signalling individually regulate different types of cell death, or does it regulate cell death processes in combination?

In summary, an in-depth understanding of ferroptosis and nutrient signalling might lead to a new approach for the therapeutic intervention of ferroptosis-related diseases, such as cancer, neurodegeneration and inflammation. Further study should focus on ferroptosis in both non-pathological and the normal physiology contexts.

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Financial Support

This work was supported by National Natural Science Foundation of the P.R. of China (no. 31802067 and 31872364) and the Natural Science Foundation of Guangdong Province (No. 2018A030310201).

Authors' contributions

Yingao Qi and Shihai Zhang initiated the idea, the scope and the outline of this review paper. Yingao Qi, Xiaoli zhang, Min Tian, Shihai Zhang, Zhihui Wu, Jinghui Heng and Fang Chen studied and analysed all of the publications cited in this paper and were involved in the manuscript preparation. Shihai Zhang and Wutai Guan conducted the final editing and proofreading. All authors read and approved the final manuscript.

Conflicts of interest

The authors declare that they have no competing interests.

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frontiers in Cell and Developmental Biology

REVIEW
published: 26 June 2020
doi: 10.3389/fcell.2020.00530

Current Evidences and Future Perspectives for AMPK in the Regulation of Milk Production and Mammary Gland Biology

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Specialty section:
This article was submitted to Signaling, a section of the journal Frontiers in Cell and Developmental Biology

Received: 25 March 2020
Accepted: 05 June 2020
Published: 26 June 2020

Citation:
Wu Z, Tian M, Heng J, Chen J, Chen F, Guan W and Zhang S (2020) Current Evidences and Future Perspectives for AMPK in the Regulation of Milk Production and Mammary Gland Biology. Front. Cell Dev. Biol. 8:530. doi: 10.3389/fcell.2020.00530

Activated protein kinase (AMP)-activated protein kinase (AMPK) senses the cellular energy status and coordinates catabolic and anabolic processes. Extensive studies have proposed that AMPK regulates energy homeostasis, cell growth, autophagy, mitochondrial biology and inflammation. The biological functions of AMPK vary in different tissues or organs. As a unique organ that produces milk, the mammary gland has recently attracted substantial research attention. This review discusses how AMPK in the mammary gland is activated by energy deprivation and heat stress via the activation of canonical and non-canonical pathways. In addition, the important downstream targets of AMPK and their functions in the mammary gland, especially during milk synthesis, are summarized in the review.

Keywords: mammary epithelial cells, AMPK, milk fat, milk protein, lactose

INTRODUCTION

Energy homeostasis plays a critical role in maintaining the survival and function of cells. In 1967, Atkinson proposed using the adenylate system [ATP (adenosine 5′ triphosphate) + ADP (adenosine diphosphate) + AMP (adenosine monophosphate)] to estimate cellular energy levels (Atkinson and Fall, 1967; Atkinson and Walton, 1967). ATP acts as a high-energy bond donor, and ATP can be broken down into ADP and AMP, which is accompanied by the release of energy.

Abbreviations: 4EBP1, eukaryotic initiation factor 4E binding protein 1; ACC, acetyl-coA carboxylase; ADP, adenosine diphosphate; AMP, adenosine monophosphate; AMPK, AMP-activated protein kinase; ATGL, adipose triglyceride lipase; ATM, ataxia telangiectasia mutated; ATP, adenosine 5' triphosphate; CaMKKβ, Ca^{2+}/calmodulin-activated protein kinase kinase-β; CPT1, carnitine palmitoyltransferase 1; CSN1S1, Casein Alpha S1; DMI, dry matter intake; eEF2K, eukaryotic translation elongation factor 2 kinase; FA, fatty acid; FABP3, fatty acid-binding protein 3; FAS, fatty acid synthase; GLUT4, glucose transporter 4; Jak, Janus kinase; LKB1, liver kinase B1; LXRα, liver X recepter α; mTORC1, mammalian target of rapamycin complex 1; PIKK, phosphoinositide 3 kinase-like kinase; PPARγ, peroxisome proliferators-activated receptors γ; ROS, reactive oxygen species; S6K1, ribosomal protein S6 kinase 1; SCD, stearoyl-CoA desaturase; SREBP1c, sterol regulatory element-binding protein 1c; Stat, signal transducer and activator of transcription; TAG, triacylglycerol; TBC1D1, TBC1 domain family member 1; TSC2, Tuberin 2; TXNIP, thioredoxin-interactingprotein.

Increased levels of cellular ADP and/or AMP are indicators of decreased energy status. AMP-activated protein kinase (AMPK) is the dominant energy sensor in eukaryotic cells (Hardie, 2007). As a master regulator of metabolism, AMPK is proposed to regulate energy homeostasis (Hardie et al., 2012), cell growth (Mihaylova and Shaw, 2011), autophagy (Mihaylova and Shaw, 2011), and mitochondrial biology (Herzig and Shaw, 2018). Recent advances in research have proposed that the biological effects of AMPK can be tissue- and/or cell type-specific. Hypothalamic AMPK modulates the energy balance of the whole body via the regulation of food intake (López et al., 2016). In T cells, AMPK regulates cell migration and determines CD8+ T cell fate (differentiation into effector or memory CD8+ T cells) (Finlay and Cantrell, 2011). However, the functions of AMPK in the mammary gland are still unclear.

The mammary gland is a unique organ for the secretion of milk. The mammary gland contains not only the epithelial cells but also the stromal compartment (also known as connective tissue or fat pad) (Hennighausen and Robinson, 2005). Mammary epithelium includes luminal and secretory cells that are involved in milk production during lactation period. The primary components of the mammary stroma are adipocytes, but it also contains fibroblasts, blood vessels and neurons. Milk directly regulates the early growth and health of neonates. The main components of milk are carbohydrates, lipids, and proteins with small proportions of minerals and vitamins. Colostrum is the milk secreted shortly after fallowing and consists a higher level of immunoglobulins (IgG, IgM, and IgA). These immunoglobulins are involved in the establishment of passive immunity for neonates. The energy requirements and mobilization in the mammary gland are high and very complicated. During lactation, energy is not only consumed by the mammary gland to maintain its basic metabolism but also largely used for milk production (Sampson and Jansen, 1984). In this review, we summarized recent advances in the understanding of the mechanism by which AMPK regulates milk production and mammary gland biology. Understanding the functions of AMPK and its downstream targets in the mammary gland will contribute to the development of nutritional or drug-related strategies for improving mammary gland health and promoting milk production.

AMP-ACTIVATED PROTEIN KINASE

AMP-activated protein kinase exists as a heterotrimer, which consists of one catalytic subunit (α) and two regulatory subunits (β and γ) (Xiao et al., 2011). There are multiple isoforms of these AMPK subunits, including α1, α2, β1, β2, γ1, γ2, and γ3 (Hardie et al., 2006). AMPKα1 is dominantly expressed in the mammary gland (Quentin et al., 2011), while AMPK α2 and γ3 are undetectable (McFadden and Corl, 2009). This evidence indicates that the expression of AMPK subunits could be tissue dependent.

The canonical mechanism by which AMPK is activated (phosphorylation at Thr172) relies on increased levels of ADP, AMP or Ca^{2+}. LKB1 (liver kinase B1) and CaMKKβ (Ca^{2+}/calmodulin-activated protein kinase kinase-β) are the primary upstream kinases of AMPK (Hawley et al., 2003, 2005). During conditions of energy deprivation, ADP can directly bind to the γ regulatory subunit of AMPK (Hardie et al., 2012). In even more severe situations, ADP is converted to AMP, which leads to a conformational change in AMPK and increases its activity by approximately 10-fold (Hardie et al., 2012). This conformational change also protects AMPK from being dephosphorylated and ensures its activation. In addition, increased levels of Ca^{2+} can directly regulate AMPK activity through CaMKKβ and in manner that is independent of the level of ADP or AMP (Hawley et al., 2005). In the human mammary gland, activated LKB1 and CaMKKβ are also reported to increase the activity of AMPK (Høyer-Hansen et al., 2007; Chung et al., 2017).

In addition to ADP, AMP and Ca^{2+}, AMPK can also be activated by non-canonical signals, such as low oxygen (Marsin et al., 2000), reactive oxygen species (ROS) (Alexander et al., 2010; Ditch and Paull, 2012) and DNA damage (Ji et al., 2010; Sanli et al., 2010). When oxygen is limited, nutrients are not completely oxidized, which limits the production of ATP (Depré et al., 1998). ROS are proposed to regulate AMPK through two different pathways: (1) oxidation of the two conserved Cys residues in the AMPK α subunit (Alexander et al., 2010) and (2) activation of ataxia telangiectasia mutated (ATM), which is a phosphoinositide 3 kinase-like kinase (PIKK) that triggers the activation of AMPK (Ditch and Paull, 2012). DNA damage-induced activation of AMPK is also ATM-dependent (Storozhuk et al., 2013; Tripathi et al., 2013).

ACTIVATION OF AMPK IN THE MAMMARY GLAND

Negative energy balance (NEB) is widely observed in swine and cow production, especially during lactation. Elevated ADP or AMP is often associated with a NEB (**Figure 1**). The feed intake of primiparous sows usually fails to meet the nutritional requirements for fetal growth and milk production, leading to the mobilization of protein and fat reserves in the body. Similarly, the peak lactation period of cows generally occurs 4–6 weeks after delivery, but the dry matter intake (DMI) does not reach its maximum until 8–10 weeks after delivery (Bertics et al., 1992). Thus, during this period, the energy intake in dairy cows is not able to meet the demand for milk production (especially in high-yielding cows). AMPK signaling in the mammary gland is highly active during this period, which results in a 15–20% decrease in milk production in sows and cows (Eastham et al., 1988; Wang et al., 2018). *In vitro* experiments also demonstrated that AMPK is significantly activated in mammary epithelial cells during energy deprivation (cells cultured without glucose or amino acids) (Zhang M. et al., 2018).

In addition to NEB, heat stress is reported to negatively regulate mammary gland development and milk production in cows (Tao et al., 2011) and sows (Renaudeau and Noblet, 2001). Intriguingly, recent findings indicate that heat stress triggers the activation of AMPK in the mammary gland. In

the murine mammary gland, the AMPK signaling pathway is significantly upregulated by heat stress (Han et al., 2019). In a transcriptomic study of the bovine mammary gland, AMPK signaling was the most highly activated pathway in response to heat stress (Gao et al., 2019). The non-canonical pathway could be a potential link between heat stress and AMPK (**Figure 2**). First, under heat stress, ROS are increased and accumulate in the bovine mammary gland (Li et al., 2019). Activation of AMPK decreases the production of ROS (El-Sisi et al., 2019) and enhances the antioxidant capacity (Guo et al., 2020) of the mammary gland. Second, oxygen uptake is significantly decreased in sows during heat stress (Black et al., 1993). Third, heat stress induces DNA damage in the mammary gland (Nair et al., 2010). In addition, heat stress also decreases feed intake in mammals, which indirectly triggers a decrease in energy intake and subsequently increases the levels of ADP and AMP. Therefore, heat stress can coordinately regulate AMPK through canonical and non-canonical pathways in the mammary gland.

AMPK REGULATES MILK SYNTHESIS

Milk Fat

The process of milk fat synthesis in different species has been previously well summarized (Bionaz and Loor, 2008; Osorio et al., 2016; Zhang S. et al., 2018). Briefly, the process includes *de novo* fatty acid (FA) synthesis, FA uptake, FA activation, FA intracellular transport, FA elongation, FA desaturation, triacylglycerol (TAG) synthesis and lipid droplet formation. The FAs used for milk fat synthesis are either derived from blood circulation or are originally synthesized in the mammary gland. AMPK is a critical sensor that regulates fat metabolism in the mammary gland (**Figure 3**). It has been reported

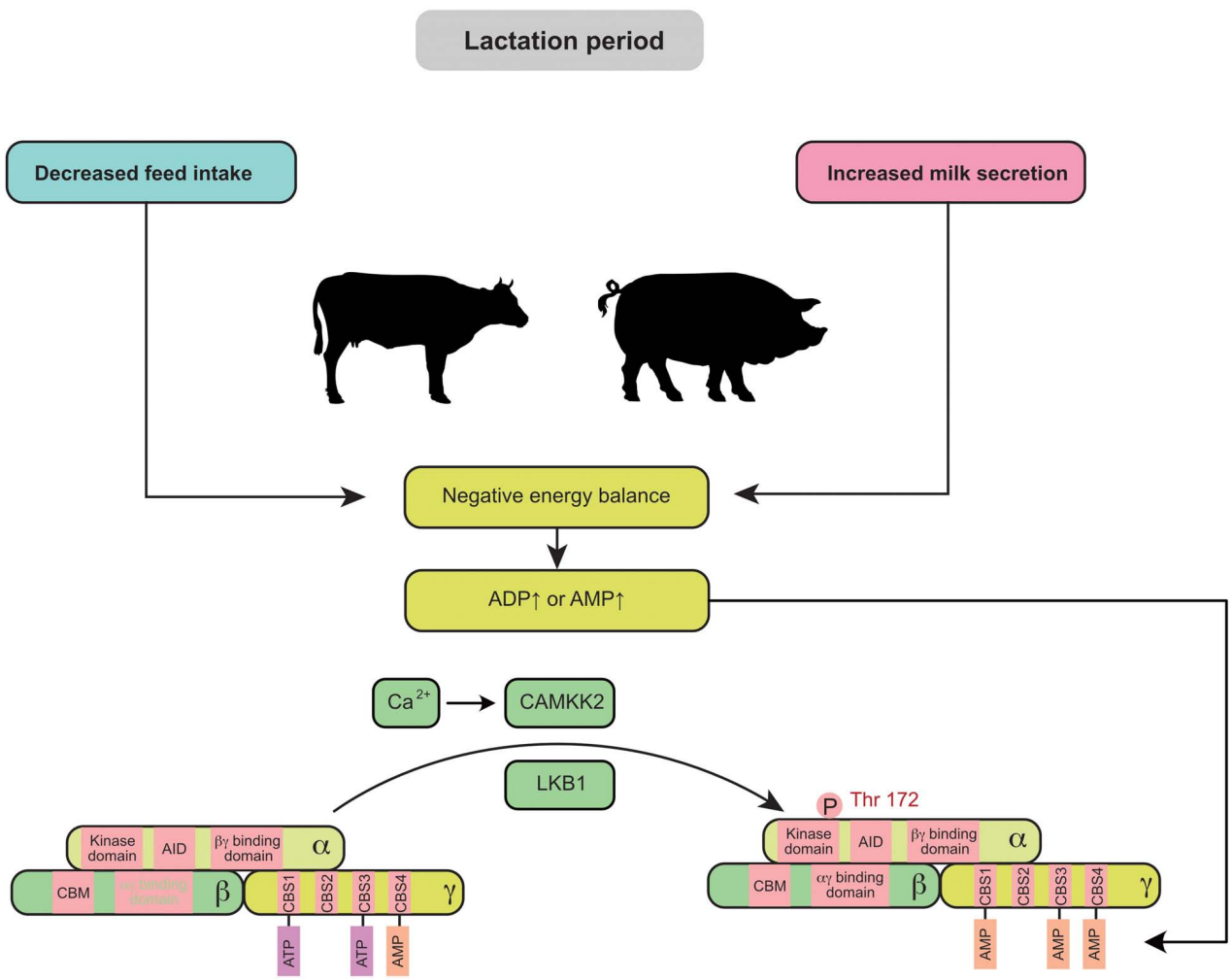

FIGURE 1 | Negative energy balance induces the activation of mammary AMPK through the canonical pathway: during lactation, decreased maternal feed intake usually fails to meet the requirement for milk secretion and leads to a negative energy balance. Elevated ADP or AMP in the mammary gland is associated with a negative energy balance and promotes AMPK activity. AID, auto-inhibitory domain; CAMKK2, calmodulin dependent protein kinase kinase 2; CBM, carbohydrate-binding module; CBS, cystathionine-beta-synthase; LKB1, liver kinase B1.

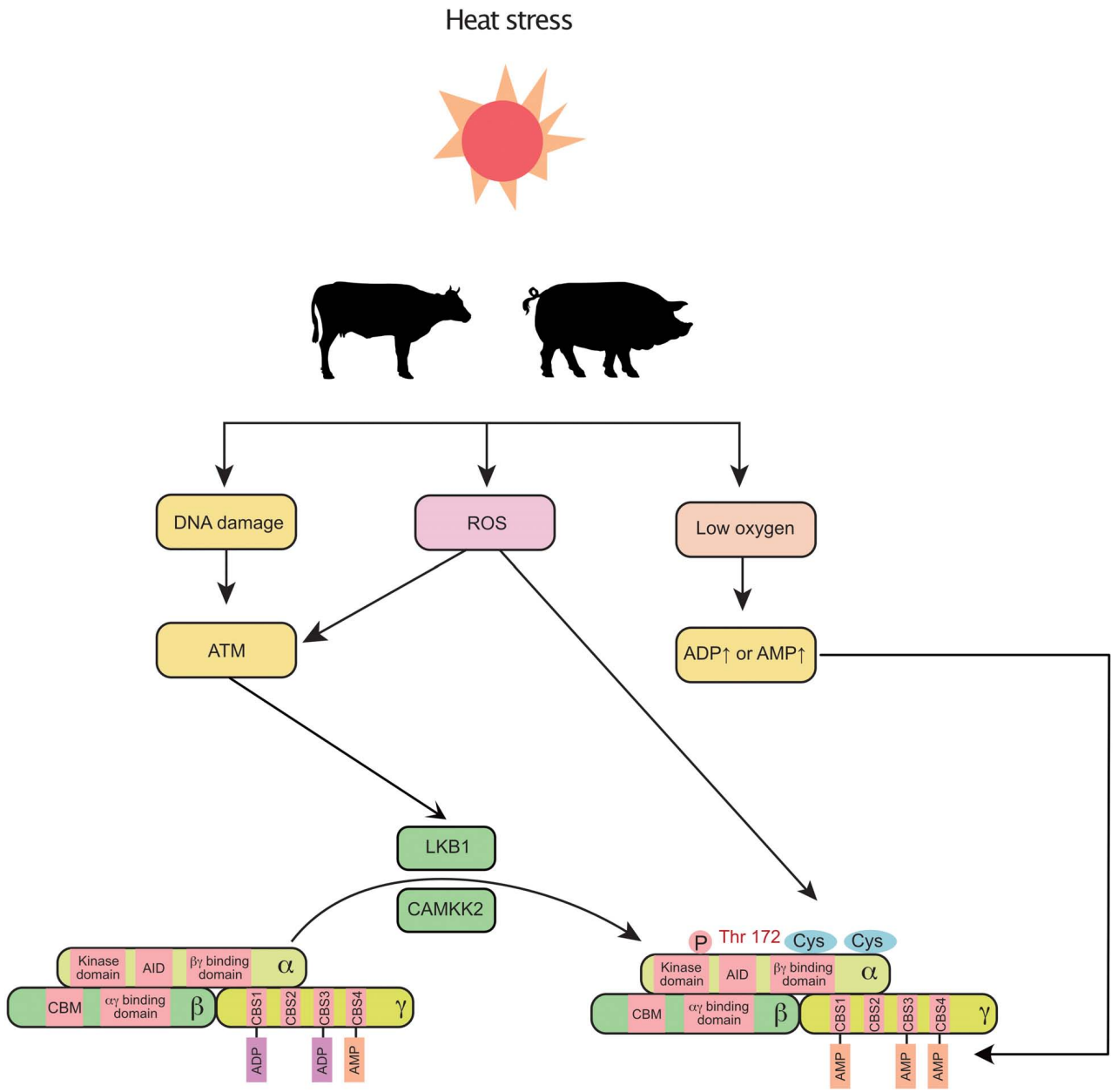

FIGURE 2 | Heat stress induces the activation of mammary AMPK through canonical and non-canonical pathways: heat stress increases ROS, decreases blood oxygen, and alters DNA integrity, which further activates AMPK (non-canonical pathway). Additionally, the decreased feed intake (increased ADP and AMP) caused by heat stress also activates AMPK. ROS, reactive oxygen species; AID, auto-inhibitory domain; ATM, ataxia telangiectasia-mutated gene; CAMKK2, calmodulin dependent protein kinase kinase 2; CBM, carbohydrate-binding module; CBS, cystathionine-beta-synthase; LKB1, liver kinase B1.

that AMPK activators 5-aminoimidazole-4-carboxamide 1-β-D-ribofuranoside (AICAR) and A-769662 (A76) are reported to inhibit fat synthesis in the bovine mammary gland (McFadden and Corl, 2009; Huang et al., 2020).

AMPK phosphorylates its downstream proteins at Ser/Thr residues (recognition motifs of AMPK: Φ(X,β)XXS/TXXXΦ) (Φ, hydrophobic; β, basic) (Dale et al., 1995). Acetyl-coA carboxylase (ACC) is a rate-limiting enzyme in fatty acid metabolism. It is one of the primary downstream pathways of AMPK. AICAR activates AMPK and inhibits ACC1 in goat mammary epithelial cells (Zhang et al., 2011). The coding and amino acid sequences of ACC were first identified in the murine mammary gland in 1988 (López-Casillas et al., 1988). Two isoforms of ACC have been identified in animals, namely, ACC1 (ACACA) and ACC2 (ACACB). ACC1 is a cytosolic protein that catalyzes the conversion of acetyl-CoA to malonyl-CoA during *de novo* FA synthesis (Abu-Elheiga et al., 2000). ACC2 is associated with the outer membrane of mitochondria. The malonyl-CoA

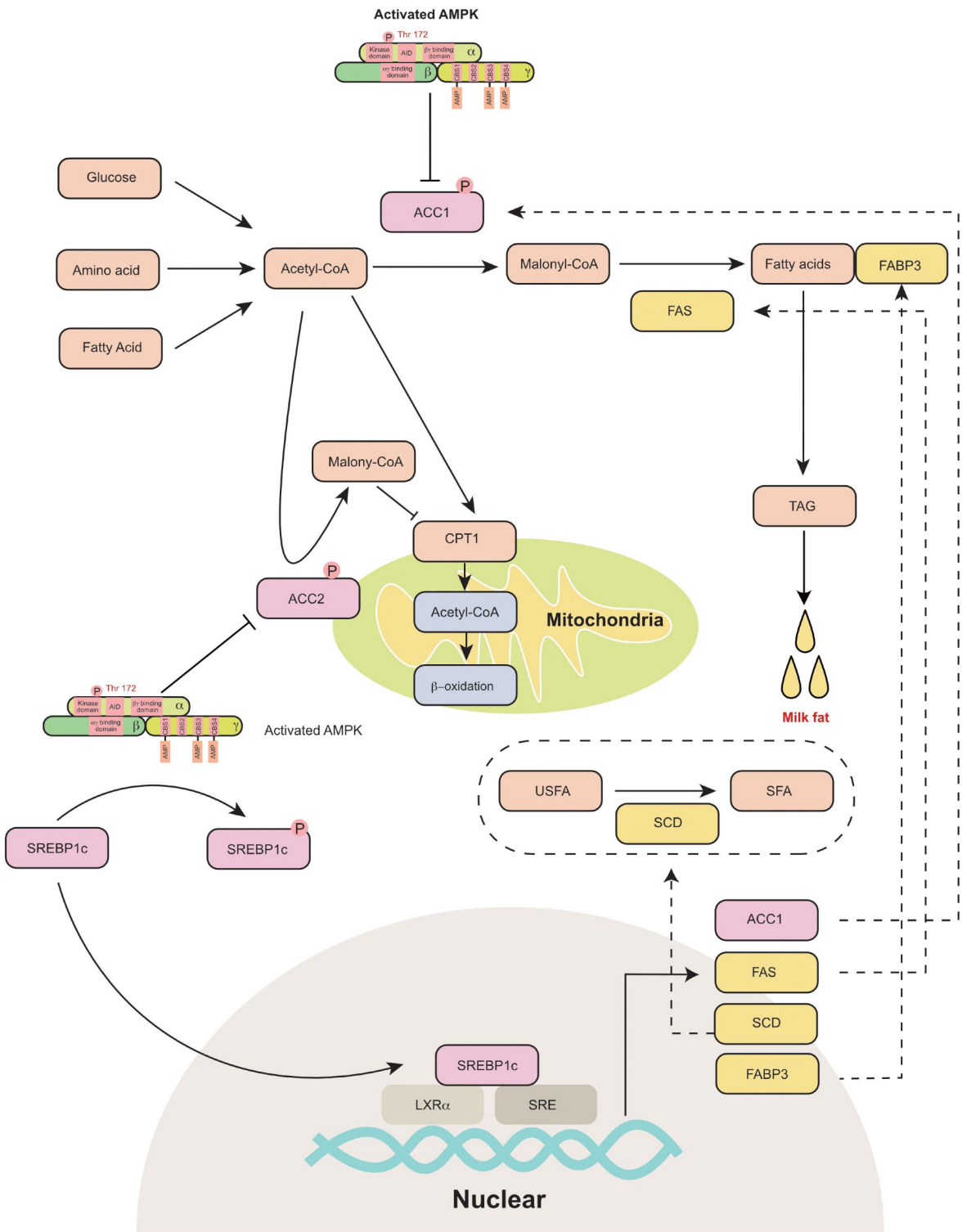

FIGURE 3 | AMPK regulates mammary milk fat synthesis: AMPK phosphorylates and inactivates ACC1 and ACC2. ACC1 is a cytosolic protein that converts acetyl-CoA to malonyl-CoA during *de novo* fatty acid synthesis. ACC2 is associated with mitochondria and regulates mitochondrial fatty acid oxidation through the inhibition of CPT1 by malonyl-CoA. AMPK inhibits the transcriptional activity of SREBP-1c through the phosphorylation of SERBP1c at Ser372, which further decreases the expression of ACC1, FAS, SCD and FABP3, which participate in fatty acid synthesis. ACC1, acetyl-coA carboxylase 1; ACC2, acetyl-coA carboxylase 2; AID, auto-inhibitory domain; CBS, cystathionine-beta-synthase; CPT1, carnitine palmitoyl transferases 1; FABP3, fatty acid binding protein 3; FAS, fatty acid synthase; LXRα, liver X receptor α; SCD, stearoyl-CoA desaturase; SFA, saturated fatty acid; SRE, sterol-regulatory element; SREBP1c, sterol-regulatory element binding protein 1c; TAG, triacylglyceride; USFA, unsaturated fatty acid.

produced by ACC2 regulates mitochondrial fatty acid oxidation through the inhibition of carnitine palmitoyltransferase 1 (CPT1) (Abu-Elheiga et al., 2000). ACC is highly expressed in the mammary gland during the lactation period (López-Casillas et al., 1991). The dominant isoform of ACC in the mammary gland varies among different animals. In the rat mammary gland, the main type of ACC is ACC2, while ACC1 is mainly expressed in white adipose tissue (López-Casillas et al., 1991; Ponce-Castañeda et al., 1991). However, both ACC1 and ACC2 are highly expressed in the murine mammary gland (Takebe et al., 2009). Similarly, ACC1 (Lv et al., 2015) and ACC2 (Palombo et al., 2018) are also detected in the porcine mammary gland and are upregulated during lactation. In dairy cows, ACC1 is considered a critical regulator of fatty acid synthesis in the mammary gland (Matsumoto et al., 2012; Tao et al., 2015). Short-term energy deprivation induces ACC phosphorylation and inhibits fatty acid synthesis in bovine mammary gland epithelial cells (McFadden and Corl, 2009). Thus, AMPK is proposed to control the switch between lipogenesis and lipid oxidation through the phosphorylation or dephosphorylation of ACCs.

The other critical downstream target of AMPK is sterol regulatory element-binding protein 1c (SREBP1c), which is a lipogenic transcription factor. Previously, AMPK activation (triggered by AICAR or A-769662) was reported to decrease the mRNA expression of lipogenic genes, including FAS and FABP3 in the bovine mammary gland (Zhang et al., 2011; Huang et al., 2020). AMPK inhibits the transcriptional activity of sterol regulatory element binding protein-1c (SREBP-1c) through the phosphorylation of SERBP1c at Ser372 (Li et al., 2011). SREBP1c was initially identified as a regulator of lipogenesis in the liver (Horton et al., 2002). Subsequently, SREBP1c has been proposed to regulate milk fat synthesis in cows (Li et al., 2014), goats (Xu et al., 2016b), and swine (Che et al., 2019). SREBP-1c binds to the promoter of lipogenic genes and increases their mRNA expression (Horton et al., 1998). The core promoter region of SREBP1c in the goat mammary gland is located from −395 to +1 bp upstream of the transcriptional start site and includes liver X receptor α (LXRα) binding elements and sterol regulatory elements (Xu et al., 2016a). In bovine mammary epithelial cells, the overexpression of SREBP1c significantly increases the expression of ACC1, FAS (fatty acid synthase), SCD (stearoyl-CoA desaturase) and FABP3 (fatty acid-binding protein) (Li et al., 2014). Consistently, the knockdown of SREBP1c decreases the expression of lipogenic genes (Li et al., 2014). In goat mammary epithelial cells, SREBP1c overexpression upregulates numerous genes that are responsible for *de novo* fatty acid synthesis and fatty acid transportation (Xu et al., 2016b). In the murine mammary gland, the knockdown of SREBP1c decreases the expression of FAS and SCD2 (Rudolph et al., 2010). Thus, AMPK can indirectly control transcription of lipogenic genes in the mammary gland through the phosphorylation of SREBP1c.

In addition to ACC and SREBP-1c, AMPK also regulates milk fat synthesis through adipose triglyceride lipase (ATGL) and TBC1 domain family member 1 (TBC1D1). ATGL mainly regulates the first step of the lipid lipolysis process. ATGL is a downstream target of peroxisome proliferator-activated receptor γ (PPARγ) (Shi et al., 2013) but not of SREBP1c (Xu et al., 2016b). Recently, AMPK was reported to activate ATGL through the phosphorylation of Ser406 (Ahmadian et al., 2011; Kim et al., 2016). In the goat mammary gland, ATGL is considered an important enzyme that regulates milk fat synthesis (59). In contrast to ATGL, TBC1D1 has been shown to regulate the translocation of glucose transporter 4 (GLUT4), which is an important insulin-sensitive transporter of glucose (Sakamoto and Holman, 2008). AMPK regulates the activity of TBC1D1 through the phosphorylation of Ser237 and Thr596 (Pehmøller et al., 2009). Interestingly, a whole genome resequencing study in cows showed that TBC1D1 expression is correlated with milk fat percentage (Jiang et al., 2016, 2019).

It is worth noting that some potential targets of AMPK might also regulate milk fat synthesis in the mammary gland. For example, AMPK increases the activity of Coactivator 1 alpha (PGC-1α) through the phosphorylation of Thr177 and Ser538 (Jäger et al., 2007). PGC-1α is a transcriptional coactivator of transcription factor PPARγ (Handschin and Spiegelman, 2006). In the liver, PGC-1α is proposed to regulate fatty acid β-oxidation through PPARγ (Inagaki et al., 2007). The other potential target protein is Liver X receptors (LXRs), which regulates the expression of SREBP1c and the process of lipogenesis. In the liver, AMPK is found to inhibit the ligand-induced LXR activity on Srebp-1c promoter (Yap et al., 2011). Intriguingly, recent studies reported that the activation of LXRs also increases the fatty acid synthesis in the mammary gland (McFadden and Corl, 2010; Oppi-Williams et al., 2013). To date, PGC-1α and LXR have been identified as downstream targets of AMPK in the liver. It would be interesting to know whether these mechanisms could be validated in the mammary gland as well.

Milk Protein

Milk proteins, such as caseins and whey proteins, are important sources of nutrition for neonates. Colostrum contains immunoglobulins, such as IgG, IgM, and IgA, which are important to establish the early immunity of neonates. The amino acids used for milk protein synthesis are transported via a complicated transportation system (Zhang Y. et al., 2018). The process of milk protein synthesis requires a significant amount of energy (Moe, 1981). The master regulator of protein synthesis is mammalian target of rapamycin complex 1 (mTORC1). Dietary energy deprivation activates AMPK and inhibits mTORC1 in the rat mammary gland (Jiang et al., 2008). Similarly, in bovine mammary epithelial cells, energy deprivation increases the phosphorylation of AMPK by inhibiting the mTORC1 signaling pathway (Burgos et al., 2013). Glucose deprivation in bovine mammary epithelial cells leads to the downregulation of the mRNA expression of Casein Alpha S1 (CSN1S1), CSN2 and CSN3, which encode a_{s1}-casein, β-casein and κ-casein, respectively (Zhang M. et al., 2018). However, glucose promotes αS1-casein and β-casein protein synthesis in the bovine mammary gland (Wang et al., 2019).

mTORC1 is a master regulator of anabolic and catabolic metabolism in cells (Howell and Manning, 2011). More specifically, mTORC1 participates in the regulation of gene transcription (Mayer and Grummt, 2006), protein translation (Mamane et al., 2006), and apoptosis (Thedieck et al., 2013).

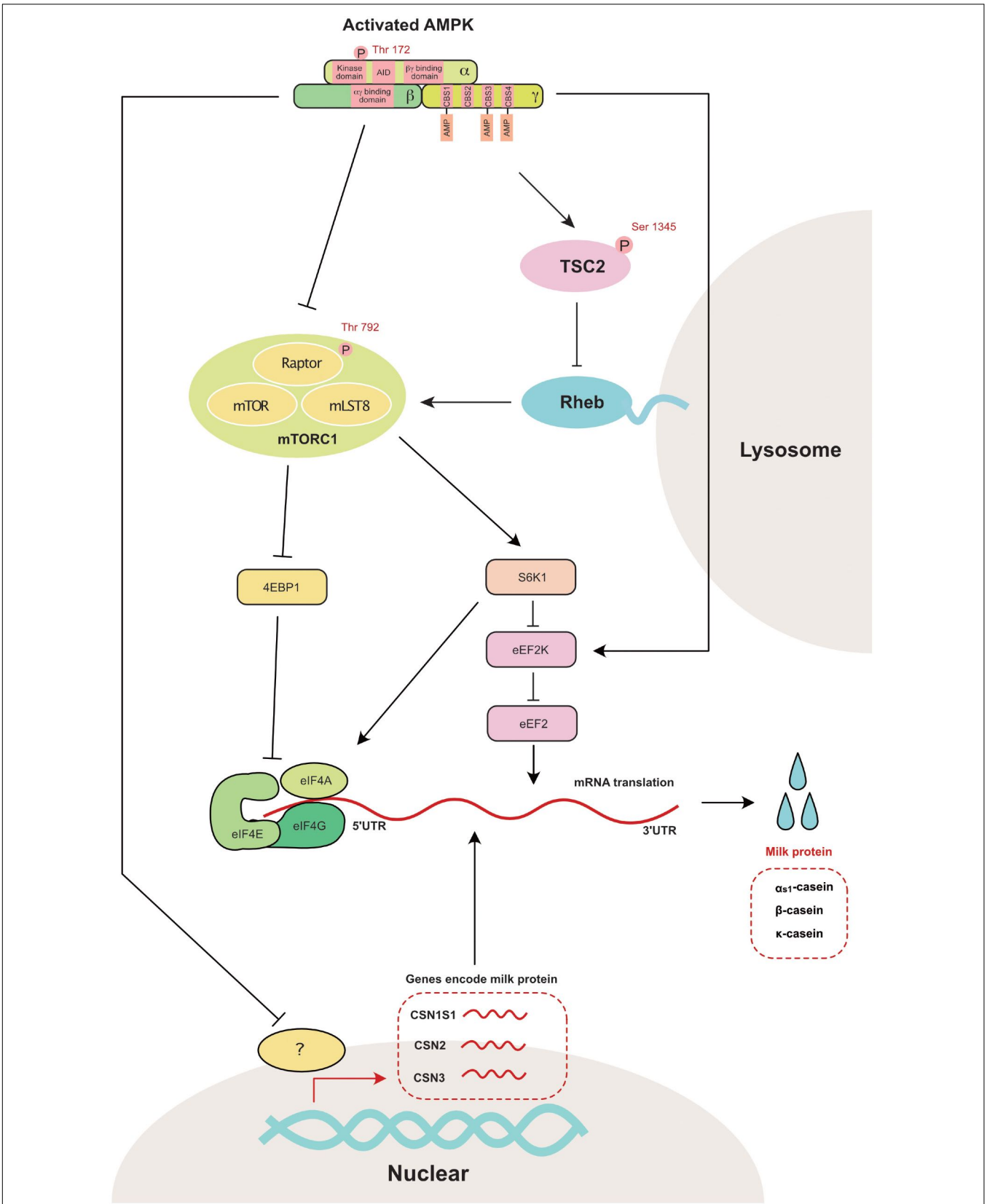

FIGURE 4 | Continued

FIGURE 4 | AMPK regulates mammary milk protein synthesis: mTORC1 is the master regulator that controls milk protein synthesis in the mammary gland through 4EBP1 and S6K1. AMPK directly inhibits mTORC1 through the phosphorylation of raptor at Ser 792. AMPK also decreases mTOR activity through the phosphorylation of TSC2 (a negative mTORC1 regulator) at Ser1345. The genes encoding a_{S1}-casein (CSN1S1), β-casein (CSN2) and κ-casein (CSN3) are also decreased with the activation of AMPK. 4EBP1, eukaryotic initiation factor 4E binding protein 1; AID, auto-inhibitory domain; CBS, cystathionine-beta-synthase; eEF2, eukaryotic elongation factor 2; eEF2K, eukaryotic elongation factor 2 kinase; eIF4A, eukaryotic initiation factor 4A; eIF4E, eukaryotic initiation factor 4E; eIF4G, eukaryotic initiation factor 4G; Raptor, regulatory associated protein of TOR; Rheb, ras homolog enrichedin brain; S6K1, s6 kinase 1; TSC2, tuberin 2; mTOR, mammalian target of rapamycin; mTORC1, mammalian target of rapamycin complex 1; mLST8, mammalian ortholog of LST8.

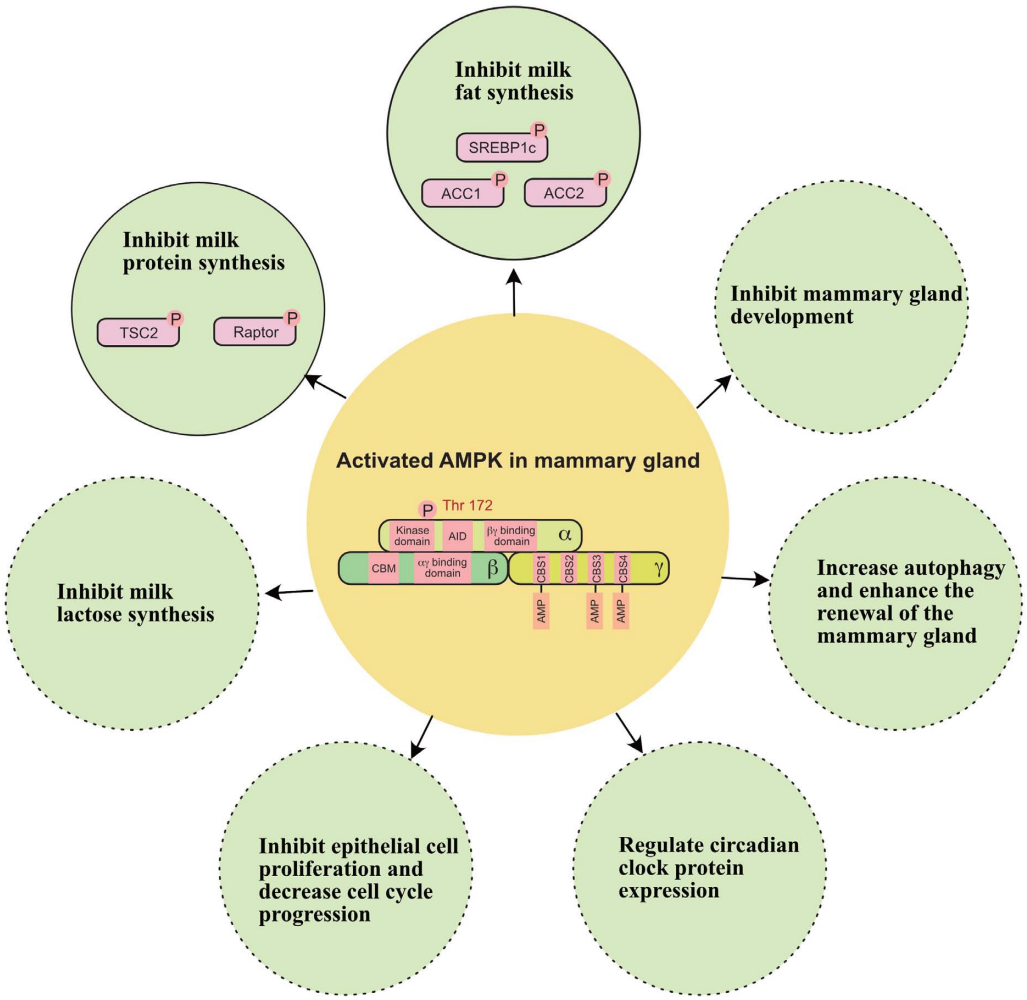

FIGURE 5 | A working model of how AMPK regulates milk secretion and mammary gland function. ACC1, acetyl-coA carboxylase 1; ACC2, acetyl-coA carboxylase 2; SREBP1c, sterol regulatory element binding protein-1c; TSC2, tuberin 2. Solid circle indicates this function has been widely verified in the mammary gland. Dashed circle indicates this function still needs to be verified in the mammary gland.

Eukaryotic initiation factor 4E binding protein (4EBP1) and ribosomal protein S6 kinase 1 (S6K1), which mainly regulate protein translation, are two independent downstream regulators of mTORC1 (Kim and Guan, 2019). Two independent pathways have been previously reported to inhibit mTOR activity through AMPK activation (**Figure 4**). First, AMPK inhibits TSC2 (Tuberin 2, which is a negative regulator of mTORC1) through the phosphorylation of Ser1345 (Inoki et al., 2006). Second, AMPK inhibits Raptor (a component of mTORC1) through the phosphorylation of Ser792 (Carling et al., 1987). In the mammary gland, AMPK is also reported to regulate the phosphorylation of TSC2 (Inoki et al., 2006) and Raptor (Burgos et al., 2013).

The other potential downstream target of AMPK in the regulation of protein synthesis is eukaryotic translation elongation factor 2 kinase (eEF2K), which is a negative regulator of protein elongation (Leprivier et al., 2013). As a downstream target of eEF2K, eEF2 is significantly increased during the lactation period in the bovine mammary gland (Bionaz and Loor, 2011). This observation suggests that eEF2K is a crucial regulator of milk synthesis during lactation.

Milk Lactose

In the mammary gland, 55–70% of glucose is used for lactose synthesis (Guinard-Flament et al., 2006). Glucose acts as a substrate and provides energy for lactose synthesis. Compared with studies regarding AMPK signaling during milk protein and fat synthesis, studies regarding AMPK signaling during lactose production are limited. This gap in the literature might be because the mammary gland is the only model in which lactose synthesis can be studied. In addition, technical challenges make it difficult to induce high levels of lactose synthesis in the mammary epithelial model *in vitro*. The mammary lactose synthesis pathway has been previously summarized (Zhang S. et al., 2018). Briefly, glucose and UDP-galactose (derived from glucose) are synthesized into lactose in the Golgi bodies. Some evidence indicates that lactose synthesis is inhibited by AMPK activation. AMPK activation (stimulated by A-769662) is found to decrease the rate of lactose synthesis in the bovine mammary gland (Huang et al., 2020). Interestingly, activated AMPK increases GLUT1 mRNA expression and glucose uptake in the goat mammary gland (Zhang et al., 2011). AMPK-induced increased GLUT1 expression occurs through the phosphorylation of thioredoxin-interacting protein (TXNIP) at Ser308 (Wu et al., 2013). Although glucose is a critical source for lactose synthesis, the increase in GLUT1 does not indicate increased mammary lactose synthesis. Under energy deprivation conditions, increased cellular glucose is directly used to generate energy to maintain basic cellular function rather than to synthesize lactose. The detailed mechanism by which AMPK regulates the enzymes involved in lactose synthesis is still unclear and requires further research.

AMPK REGULATES MAMMARY GLAND DEVELOPMENT

The Janus kinase (Jak)-signal transducer and activator of transcription (Stat) signaling pathway is critical for mammary gland development (Hennighausen and Robinson, 2001; Wagner et al., 2004). Either glucose or amino acid deprivation activates AMPK and simultaneously decreases the Jak2/STAT5 signaling pathway in bovine mammary epithelial cells (Zhang M. et al., 2018). Initially, it was proposed that Jak2/STAT5 is involved in alveologenesis and mammary epithelial cell maintenance (Wagner et al., 2004; Caffarel et al., 2012). Recently, Jak2/STAT5 was also found to regulate milk protein synthesis through the regulation of casein translation (Yang et al., 2015). However, the evidence supporting a direct connection between AMPK and Jak2/STAT5 is still unclear and requires more study in the future.

AMPK FUNCTIONS BEYOND MILK SYNTHESIS AND MAMMARY GLAND DEVELOPMENT

In addition to regulating milk production, recent evidence indicates that AMPK is involved in the regulation of other biological functions of mammary glands (**Figure 5**); these other biological functions include: (1) inhibiting epithelial cell proliferation and decreasing cell cycle progression in the mammary gland (Wang et al., 2019); (2) regulating circadian clock protein expression in the mammary gland (Hu et al., 2020); (3) relieving oxidative stress in the mammary gland (Guo et al., 2020); and (4) increasing autophagy and enhancing the renewal of the mammary gland (Li et al., 2020).

CONCLUSION AND OUTLOOK

To date, AMPK appears as a critical sensor in the mammary gland that detects nutrient fluctuations and environmental changes. Energy deprivation and heat stress are two major elements involved in the activation of AMPK in the mammary gland. AMPK regulates milk production (fat, protein, and lactose) and mammary gland biology (development and proliferation) through the regulation of transcription and post-translational modifications.

To the best of our knowledge, the existing evidence regarding the effects of the AMPK pathway on the mammary gland was mainly obtained under conditions of energy deprivation (amino acid or glucose deprivation). It is important to understand the function of AMPK and its related signaling pathways in the mammary gland using tissue-specific knockout mice. Future studies using AMPK-specific agonists and antagonists may also extend our understanding of AMPK signaling in the mammary gland. To date, most research studying normal mammary gland were performed in cows, mice and sows, rather than humans. Thus, it is of great importance to investigate the functions of AMPK in humans.

The most direct way to inactivate AMPK is to increase energy intake. However, according to production experience, it is very challenging to increase energy intake during the lactation period in mammals due to the limited gut volume. Thus, it would be interesting to determine whether an AMPK antagonist could rescue the decrease in milk production caused by energy deprivation and/or heat stress. The identification of novel nutritional and pharmacological inhibitors of AMPK, which can be widely and safely used as food or feed additives in mammalian diets, remains a major challenge.

AUTHOR CONTRIBUTIONS

SZ initiated the idea, the scope, and the outline of this review. ZW, MT, JC, JH, and FC studied and analyzed all of the publications cited in this manuscript and were involved in the manuscript preparation. WG conducted the final editing and proofreading. All authors read and approved the final manuscript.

FUNDING

This work was supported by the National Natural Science Foundation of China (Nos. 31802067 and 31872364) and the Natural Science Foundation of Guangdong Province (No. 2018A030310201).

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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动物营养学报 2020,32(12):5587-5595
Chinese Journal of Animal Nutrition

doi:10.3969/j.issn.1006-267x.2020.12.012

氧化应激对母畜乳腺功能的影响及其营养调控策略

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摘　要: 高温环境导致的热应激常引起机体的有害自由基增加,致使母畜乳腺组织发生氧化损伤。由氧化应激引起的母畜乳腺功能下降,是影响仔猪早期养分摄入的重要限制因素之一。本文整理了母畜氧化应激产生的机理及其对母畜乳腺组织的影响,并阐述了实际生产中减少母畜氧化应激的营养调控策略,以期为改善畜牧生产中母畜氧化应激、提高母畜乳腺功能提供理论支撑。

关键词: 热应激;氧化应激;乳腺;泌乳;营养调控

中图分类号: S811.2　　**文献标识码:** A　　**文章编号:** 1006-267X(2020)12-5587-09

泌乳是哺乳动物的重要生理机能,乳腺组织发育良好是母体正常泌乳的结构基础[1]。乳汁成分对新生儿的生存、正常发育和动态生长至关重要。乳糖、乳蛋白和乳脂为母畜乳汁中三大主要成分,是新生儿个体正常发育的重要物质基础[2-3]。动物幼仔出生后,免疫系统处于脆弱或尚未发育完全的状态,需尽快从初乳中摄取能量物质和免疫球蛋白[4]。另外,随着人们对生活品质要求的提高,乳制品作为人类生活的必需品也牵动着畜牧业的经济发展[5]。乳腺是一种特化的皮肤腺,当其受到某些外部条件刺激时,会产生大量有害自由基,诱导乳腺氧化损伤,进而会导致乳产量和乳汁品质下降,最终会影响哺乳母畜的健康生长[6-7]。因此,采取有效的措施提高母畜乳腺发育和乳汁质量就显得格外重要。

在我国华南地区,夏季炎热多雨,高温(温度35 ℃以上)高湿(相对湿度85%以上)的环境极易引起泌乳母畜热应激(heat stress,HS),导致其生产及生理机能异常[8-10]。热应激条件下,动物体内代谢酶的活性会随机体热负荷的增加而受到影响,最终机体由于过量自由基无法清除而导致抗氧化能力下降[11]。有研究发现,夏季高温高湿环境造成的机体热应激使哺乳期母猪的泌乳量减少、乳蛋白含量下降[12-13]和激素分泌紊乱[14]。在奶牛上,权素玉等[15]研究证实,高温刺激能使其乳腺上皮细胞的成活率明显下降,并造成其氧化损伤。另外,高温环境(热应激)也可导致泌乳中期奶牛的产奶量减少30%~40%[16-17];并且暴露于高温环境下的原代牛乳腺上皮细胞(bovine mammary epithelial cells,BMECs)表现出较高的死亡率[18-20]。也有报道,长期热应激条件也可抑制小鼠乳腺上皮细胞中的超氧化物歧化酶(superoxide dismutase,SOD)活性,这从而显著削弱细胞本身的抗氧化能力[21]。综上所述,由热应激引起的氧化应激会诱发母畜乳腺组织发病率提高,乳产量和乳品质下降。因此,采取有效的营养调控措施来减缓母畜乳腺的氧化应激、保持乳腺细胞内氧化-抗氧化系统的平衡,对维持哺乳动物乳腺的结构和功能的完整性及后代的生长发育具有重要意义。

收稿日期: 2020-05-25
基金项目: 国家自然科学基金(31802067,31872364);广东省自然科学基金(2018A030310201)
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1 热应激诱导氧化应激的机理

热应激是指机体产生热量无法快速有效地排出,从而引起体内热负荷快速上升,最终导致热应激反应的过程[22]。氧化应激(oxidative stress)即机体产生的过多自由基不能及时清除,进而致使机体组织细胞、蛋白质和核酸等一系列生物大分子损伤的过程[23]。研究表明,在畜禽生产中,高温引起的热应激是导致机体氧化应激的重要环境因素之一[24-26],其主要表现在:1)机体在遭受热应激刺激时,体内高活性分子如活性氧自由基(reactive oxygen species,ROS)和活性氮自由基(reactive nitrogen species,RNS)产生过多过的氧化硝基和氧化性很强的过氧亚硝基($ONOO^-$)[27];2)热应激会破坏机体的抗氧化酶[SOD、过氧化氢酶(catalase,CAT)和谷胱甘肽过氧化物酶(glutathione peroxidase,GPx)]系统。由于环境温度升高或者自身热代谢出现问题,体内积热增加,代谢明显增加,体温明显上升,最终导致机体内氧化-抗氧化系统紊乱[28]。有学者指出,慢性热应激和急性热应激不同程度地影响着体内抗氧化酶的活性,较长时间的慢性热应激会致使机体抗氧化酶活性下降,进而使得过多有害自由基不能及时排出,最终导致氧化损伤;而急性热应激对抗氧化酶活性效果却反之,其最终会缓解氧化应激[11]。对于影响哺乳动物抗氧化能力的因素,目前的研究大都是在表观上说明部分酶活性发生了变化,而其他具体的影响机制还鲜有报道。

2 氧化应激对母畜乳腺功能的影响

在畜牧生产中,热应激是导致母畜乳腺炎症反应、乳脂率和乳蛋白率下降以及乳腺细胞凋亡的重要因素之一[29-30]。有文献报道,热应激刺激能致使哺乳动物乳房温度增高,最终可能影响乳房发生乳腺炎症反应;并且在严重热应激条件下乳腺使用负调控反馈机制,以减少奶产量[17]。梁学武等[31]和张凡健等[32]研究指出,在高温条件下牛奶中的乳蛋白率、乳脂率均有不同程度地降低。大量体外试验也表明,环境温度达到 42 ℃时,显著抑制了 BMECs 中 αs1-酪蛋白和 β-酪蛋白的基因表达水平,这表明热应激不仅制约着牛奶的产量和品质,还对乳腺上皮细胞的合成能力有着直接的影响[33]。另外,高温条件或氧化应激还会影响泌乳高峰期的奶牛 BMECs 热休克蛋白的 mRNA 表达,进而影响细胞的成活和凋亡情况,严重制约了乳腺正常的发育和功能[34-35]。近年来,氧化应激是制约高遗传哺乳动物乳腺发育的重要因素之一[36],因此在实际生产中采取必要的措施来缓解母畜乳腺氧化应激显得至关重要。

3 缓解母畜氧化应激的营养调控措施

3.1 酶类抗氧化物

抗氧化酶是机体抵御氧化应激的重要物质,其主要包括 SOD、CAT、乳过氧化物酶(lactoperoxidase,LPX)、GPx 等[37-39]。SOD 主要存在于细胞的过氧化物酶体中,其可通过歧化反应来消除动物机体内过量的 ROS[40]。CAT 功能与 GPx 作用类似,都能将过氧化氢分解为水和氧,具备及时消除机体产生的大量自由基、分解过氧化物的功效[41]。但是,酶类抗氧化物由于具有分子量大、难以纯化、造价昂贵、难以维持酶活性等特点,在实际应用中较少。在生产中,我们可以通过在动物饲粮中添加可调控抗氧化酶表达的物质对其进行间接调控。

3.2 非酶类抗氧化物

非酶类抗氧化物与酶类抗氧化物相比较而言,具有分子质量小、稳定性高等特点,其在实际应用中更为广泛。其中较为常见的有以下几类。

3.2.1 维生素类

维生素 A(又名视黄醇)是维持动物体正常生长和发育的一种微量营养物质。同时,维生素 A 也是改善免疫、抗氧化应激的重要因子[42-44]。大鼠饲粮中添加适量的维生素 A 能增强机体抗氧化能力[45];并且,维生素 A 含量每增加 100 ng/mL,其临床性乳腺炎症风险可降低 60%左右[46]。在泌乳期奶牛饲粮中添加维生素 A(220 IU/kg)可显著提高奶牛泌乳性能、抗氧化机能和免疫功能[47]。有体外试验显示,维生素 A 可缓解由过氧化氢(H_2O_2)、一氧化氮(NO)和脂多糖(lipopolysaccharides,LPS)诱导引起的 BMECs 氧化应激损伤[48-49];并且,维生素 A 也可通过硫氧还蛋白氧化还原酶(thioredoxin reductase,TrxR)和丝裂原活化蛋白激酶(mitogen-activated protein kinase,MAPK)途径减少花生四烯酸(arachidonic acid,ARA)的过量生成来缓解奶牛 BMECs 的氧化损伤[50]。虽然维生素 A 对动物体的抗氧化效果已

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被大量报道,但是,关于维生素 A 调控动物体氧化应激的具体分子调控机理还有待深度挖掘。

除维生素 A 外,维生素 C 在动物饲粮中也广泛添加。维生素 C 也叫做抗坏血酸,其能很好地抵御自由基氧化损害并且能够预防热应激[51]。郭文晋等[52]研究指出,在奶牛复合添加剂中添加维生素 C(40 mg/kg)可增加热应激奶牛产奶量、乳脂率及乳蛋白率,这暗示了维生素 C 对泌乳期奶牛热应激引起的氧化应激具有改善效果。

此外,维生素 E 也是最主要的生物自由基清除剂之一,并且足量的维生素 E 具有提高畜禽抗应激和机体免疫力的能力[46]。有研究报道,维生素 E 通过清除自由基可增加 SOD 产量、减轻乳房炎症状和缩短乳房炎的持续时间[53-54];并且在生产试验中,添加含有 66 IU/kg 维生素 E 的母猪饲粮可以显著减少乳房炎-子宫炎-无乳综合征的发病率[55]。

3.2.2　微量元素类

微量元素硒(Se)在改善动物体抗氧化功能方面具有较强的生物学功能。研究发现,硒可以通过促进奶牛 BMECs 内抗氧化硒蛋白、CAT 和 SOD 活性,降低丙二醛含量等来提高抗氧化功能相关指标[56-57]。有研究报道,利用含 0.6 mg/kg 硒培养基培养奶牛 BMECs 能显著增强其抗氧化水平[58];并且,饲喂添加含硒的有机螯合物的饲粮,能显著改善奶牛乳成分的比例,但产奶量不变[59]。此外,Chen 等[60]研究也证实,饲粮中添加硒(1.2 mg/kg)可降低母猪初乳和 21 d 乳中丙二醛的含量,这说明饲粮中添加硒可以改善乳汁的抗氧化状态。在细胞培养过程中,硒元素是以亚硒酸钠的形式存在的,其可通过减少自由基和脂质过氧化物来的生成来保护细胞免受氧化损伤[61-63]。奶山羊的体外研究表明,亚硒酸钠(0.5 μg/mL)可通过提高细胞活力、减少细胞内 ROS 含量和细胞凋亡来保护 H_2O_2 所致的乳腺细胞的氧化应激[64]。

锌(Zn)是超过 10%的真核蛋白质组所需的关键催化、结构和调节辅助因子,并且在细胞凋亡、细胞信号传导、增殖、分化、运动性和抗氧化防御作用等多方面发挥着重要作用[65]。机体内锌的过量积累或消耗都对细胞具有毒性,严重时会导致体内线粒体功能障碍、ROS 升高、炎症发生和细胞凋亡[66-67]。啮齿类动物围产期缺锌会严重损害乳腺功能,同时改变哺乳期间乳成分[68-69],这可能是因为缺锌导致胶原蛋白沉积和乳腺发育受损的结果。已有研究表明,有机锌不仅增强母畜对乳腺炎的抵抗力,同时也能提高产奶量;并且补充氧化锌也可使奶中炎症细胞减少 30%[70]。

3.3　植物提取物

植物提取物作为一类饲料抗氧化剂,具备了安全性高、无残留以及无毒害等优点;依据其活性成分基本可以分为植物多酚类、生物碱类、挥发油类、黄酮类、植物多糖类、有机酸类、皂苷类等[71],其中酚类和黄酮类是当今研究的热点[72]。

3.3.1　植物多酚类

植物多酚在抗氧化方面已取得良好的效果,其主要在茶叶、水果、蔬菜等产品中以茶多酚、葡多酚、苹果多酚、橄榄多酚、石榴多酚等形式存在[73]。茶多酚(tea polyphenols,TP)又叫茶单宁或茶靴质,属于多酚类物质,是茶叶中的一种主要化学成分[74]。有文献报道,20~100 μg/mL TP 处理 H_2O_2 诱导损伤的 BMECs 后,均能显著降低细胞凋亡和提高部分抗氧化酶的活性,并以100 μg/mL 效果最好;这说明一定范围浓度的 TP 可以缓解 H_2O_2 氧化应激所致的 BMECs 损伤并抑制其凋亡[75]。原花青素(proantho cyanidins,PC)是目前国际上公认的一种最高效的植物性天然多酚类抗氧化剂,有研究结果显示,奶牛饲粮中添加 20 mg/kg PC,明显增加了血浆总抗氧化能力(total antioxidant capacity,TAC)和 GPx、SOD 活性,改善了奶牛泌乳早期抗氧化水平[76]。白藜芦醇(又称芪三酚)也是一种多酚类化合物。有研究显示,白黎芦醇对细胞的抗氧化应激作用主要是通过激活核因子 E2 相关因 2(nuclear factor erythroid-2 related factor 2,Nrf2)-抗氧化反应元件(antioxidant response element,ARE)信号通路实现的[77-79]。另外,也有学者指出,在母鼠哺乳期间,饲喂添加 0.2%的白黎芦醇饲粮能提高乳腺抗氧化酶链中关键基因的表达,显著减少了母鼠由哺乳应激引起的乳腺氧化损伤[37]。

3.3.2　黄酮类

忍冬藤、忍冬叶、黄芩茎叶这类植物中含有酚酸、黄酮及生物碱类成分,其中黄芩茎叶中黄酮类成分最为丰富,具有较好的抑菌、抗炎作用[80]。有研究表明,黄芩茎叶、忍冬藤配伍使用可显著改善金黄色葡萄球菌所致小鼠乳腺炎症损伤,降低炎

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症因子水平;并且二者配伍使用效果优于两者单独使用[81]。飞蓟素是一种类黄酮提取物;有研究发现,每天给妊娠第90~100天母猪口服8 g水飞蓟素在一定程度上可缓解母猪妊娠后期的氧化应激反应[82]。另外,黄酮类化合物为紫花苜蓿的主要代谢产物,已有文献表明,在培养基中添加75 μg/mL苜蓿黄酮会促进LPS诱导的奶牛BMECs中乳蛋白和乳糖的合成[83]。并且,苜蓿黄酮还有保护LPS诱导的奶牛乳腺细胞的氧化损伤作用,其机制主要是通过降低细胞ROS含量和炎症因子水平,抑制细胞凋亡来实现的[84]。

3.4 生物碱类、挥发油类、皂苷类等

生物碱具有抗氧化、抗炎、抗菌等多种生物学活性,其中提高畜禽抗氧化能力的效果已得到广泛证实[85],其主要包括甜菜碱、苦参碱等。甜菜碱化学名称为三甲胺乙内酯,是一种广泛存在于动物、植物和微生物体内的天然生物碱。甜菜碱被认为是缓解家禽慢性热应激和提高肉品质的有前景的抗氧化剂[86]。有研究发现,在母猪妊娠期间补充3 g/kg的甜菜碱增强了母体绒毛膜尿囊和仔猪血浆与肝脏中抗氧化酶(CAT和GPx等)活性;同时,甜菜碱的添加能够减缓双酚A诱导的母猪和胎儿的氧化应激状态[87]。另外,苦参碱作为一种生物碱也具有一定的抗氧化作用,卢金霞等[88]证实不同质量浓度(≤100 μg/mL)苦参碱均可提高奶牛BMECs的抗氧化能力,其中50 μg/mL苦参碱效果最明显。

植物精油是一种最常见的饲用挥发油类,在畜禽生产中被广泛应用于促进动物生长和改善动物健康。本实验室之前的研究发现,饲粮中添加200 mg/kg的复合植物精油(3.0%桉树精油、2.0%香芹精油、0.5%百里香精油、0.1%柠檬精油、0.1%大蒜精油、5.0%薄荷精油)具有增强断奶后仔猪健康、促进生长以及提高机体抗氧化功能和免疫力的功效[89]。并且,饲粮中添加50 mg/kg复合植物精油(肉桂油:牛至油:百里香油=1:1:1)可以在一定程度上缓解由LPS刺激引起的仔猪氧化应激[90]。

人参、西洋参、三七主要的有效成分均为皂苷类化合物,已有研究表明人参皂苷类化合物具有清除自由基的作用[91],三七皂苷类化合物的抗氧化作用也已被广泛应用到食品和化妆领域[92]。

近年学术期刊报道了大量关于桑叶、金银花、迷迭香提取物对畜禽抗氧化能力的研究,桑叶及其提取物中含有多种活性成分,如黄酮类、多糖类、生物碱类、多酚类化合物等[93];金银花富含有机酸类、黄酮类、挥发油类、三萜皂苷类、环烯醚萜苷类等多种功能活性成分[94];迷迭香富含精油类、酚酸类、黄酮类、萜类化合物等[95];三者因具有解热、抗氧化、抗炎、抑菌、增强免疫等多种功效也被广泛应用于饲料添加剂中。

目前,生物碱类、挥发油类、皂苷类、桑叶、金银花、迷迭香等缓解畜禽抗氧化能力的效果虽已得到广泛证实,但是对母畜乳腺抗氧化能力的影响仍鲜有报道。

4 小 结

氧化应激是影响哺乳动物乳腺炎的主要因素之一,其不仅使母畜泌乳量大大降低,而且还会使乳汁中成分改变。在养殖生产过程中,除通过改善母畜环境管理水平减少氧化应激外,还可通过饲粮营养素对其进行调控。目前,缓解氧化应激的营养物质主要包括维生素(维生素A、维生素C和维生素E)、微量元素(硒和锌)和植物提取物等。

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Effects of Oxidative Stress on Mammary Gland Function of Female Animals and Its Nutritional Regulation Strategy

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Abstract: The heat stress caused by the high temperature environment often leads to the increase of harmful free radicals, causing oxidative damage in the mammary gland tissue of female animals. Decreased lactation of female animals caused by oxidative stress is one of the important limiting factors affecting early nutrient intake of piglets. This article summarizes the major causes of oxidative stress of female animals and their effects on mammary gland tissue of female animals, expecting to provide a theoretical support to alleviate female oxidative stress and improve mammary gland function of female animals. [***Chinese Journal of Animal Nutrition***, **2020, 32(12):5587-5595**]

Key words: heat stress; oxidative stress; mammary gland; lactation; nutritional regulation

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Heng et al. Nutrition & Metabolism (2020) 17:10
https://doi.org/10.1186/s12986-019-0424-x

Nutrition & Metabolism

RESEARCH Open Access

Excessive BCAA regulates fat metabolism partially through the modification of m^6A RNA methylation in weanling piglets

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Abstract

Background: Fat percentage and distribution in pigs are associated with their productive efficiency and meat quality. Dietary branched-chain amino acids (BCAA) regulate fat metabolism in weanling piglets with unknown mechanism. It is reported that N6-methyl-adenosine (m^6A) is involved in fat metabolism in mice. The current study was designed to investigate the relationship between dietary branched-chain amino acids and fat metabolism through N6-methyl-adenosine (m^6A) in weanling piglets.

Methods: A total of 18 healthy crossbred weaned piglets (Duroc × Landrace × Large White, 10.45 ± 0.41 kg) were divided into 3 treatments and were fed the low BCAA dose diet (L-BCAA), the normal dose BCAA diet (N-BCAA), or the high dose BCAA (H-BCAA) diet for 3 weeks.

Results: Our results show that compared with the N-BCAA group, the L-BCAA group had higher concentration of serum leptin ($P < 0.05$), while the H-BCAA group had lower concentration of serum adiponectin ($P < 0.05$). Fatty acid synthesis in pigs from the H-BCAA group was lower than those from the N-BCAA group with the down-regulation of lipogenic genes (ACACA, FASN, PPAR-r, SREBP-1c in ventral and dorsal fat, SREBP-1c in liver) and up-regulation of lipolysis genes (HSL, ATGL, CPT-1A, FABP4 in ventral fat, HSL in liver) ($P < 0.05$). Similarly, fatty acid synthesis in pigs from the L-BCAA group was also lower than those from the N-BCAA group with the decrease of lipogenic genes (ACACA in ventral, ACACA and FASN in dorsal fat, ACACA, FASN, SREBP-1c in liver) and the increase of lipolysis genes (ATGL, CPT-1A CD36, FABP4 in ventral fat and HSL, ATGL, CPT-1A in dorsal fat, CPT-1A) ($P < 0.05$). Feeding H-BCAA diet significantly reduced total m^6A levels in ventral and dorsal fat and liver tissues ($P < 0.05$). The decrease of total m^6A is associated with down-regulation of METTL3, METTL14 and FTO in dorsal fat and METTL3 and FTO in liver ($P < 0.05$). Decreased m^6A modification of ACACA and FASN in ventral and dorsal adipose tissues was observed in pig fed with excessive BCAA.

Conclusion: These results suggest that insufficient or excessive BCAA decreased the fat deposition by increasing lipolysis and deceasing lipogenesis in adipose and liver tissues. Dietary excessive BCAA might regulate the process of lipid metabolism partly through the m^6A RNA methylation.

Keywords: Branched-chain amino acids, Lipid metabolism, m^6A RNA methylation, Weanling piglets, Low-protein diet

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Introduction

Feeding pigs with protein-restricted diet is a strategic way to save the cost of protein ingredients and decrease the emission of nitrogen in animal husbandry [1]. However, pigs fed with low-protein diet tend to deposit thicker back fat during growing and fastening periods, which eventually affect carcass characteristics [2, 3]. Decreasing dietary crude protein level by 2–4% from the NRC (1998) recommendation is the most classical method to make protein-restricted diets for pigs. In order to compensate the deficiency of essential amino acids, four crystalline amino acids (L-lysine, DL-methionine, L-threonine, and L-tryptophan) are usually supplemented to the low protein diets [1]. However, there might be a shortage of other functional amino acids which participated in protein synthesis and lipid metabolism in low-protein diets. Branched chain amino acids (BCAAs) are considered as important functional amino acids to improve carcass characteristics of pigs due to its potential to enhance protein synthesis through activating the Sestrin2/mTORC1 (mammalian target of rapamycin complex 1) pathway [4, 5].

Recent studies have shown that BCAAs also play essential roles in energy homeostasis and lipid metabolism [6]. Growing evidence have found that shortage of BCAAs reduces fat deposition in mice. A significant reduction of ventral fat mass is observed in mice consuming a leucine-deficient diet, which is partly caused by the increase of energy expenditure [7]. Furthermore, expression of genes involved in lipogenesis and lipolysis is decreased and increased in these mice, respectively [7]. Similarly, when mice are fed isoleucine- or valine-deficient diets, ventral and whole body fat mass are also decreased along with the up-regulated lipolytic genes [8]. Interestingly, excessive BCAAs also inhibit fat accumulation. Decreased body fat mass is observed in rats fed a high level of leucine diet [9]. Leucine over-supplementation also suppresses fat synthesis in murine adipocytes in vitro [10]. In recent years, studies in growing pigs have demonstrated that different dietary BCAA ratios modulate lipid metabolism in intramuscular fat and adipose tissues, indicating their vital role in fat metabolism in pigs [11, 12].

Lipogenesis and lipolysis are well-regulated pathways and a number of genes have been shown to be involved in these processes. The Fat Mass and Obesity-associated (FTO) is one of the genes associated with body mass index and obesity in children and adult [13]. FTO has been originally reported to contribute to human risk of obesity, mainly through the regulation of food intake [14]. Recently, some studies have demonstrated that FTO knockout in mouse significantly disrupts the progress of adipogenesis, while FTO overexpression in mouse increases adipogenic differentiation in primary adipocytes and mouse embryonic fibroblasts [15]. In addition, an intimate relationship between FTO expression and fat deposition has also been observed in pigs [16]. It is worth mentioning that FTO is also an important enzyme involved in N6-Methyladenosine (m^6A) RNA modification [17]. m^6A modification, which promotes mRNA stability, splicing, export, and translation, is considered as the most abundant post-transcriptional modification in eukaryotic mRNA and long noncoding RNA [18]. The biological process of m^6A is reversible, which is regulated by methyltransferases and demethylases [19]. Methyltransferases, which add methyl groups to m6A, include Wilms' tumor 1-associating protein (WTAP), methyltransferase like 3 (METTL3), and methyltransferase like 14 (METTL14). These methyl groups from m^6A can be removed by demethylases [FTO and α-ketoglutarate-dependent dioxygenase alkB homolog 5 (ALKBH5)]. The execution of the function of m^6A requires the recognition of m^6A by readers like: YTH domain family (YTHDF) proteins and the heterogeneous nuclear ribonucleoprotein (HNRNP) proteins.

In the current study, we hypothesized that dietary BCAAs might dose-dependently regulate fat metabolism through m^6A methylation partially through the regulate of the FTO. To test our hypothesis, weanling pigs were assigned to one of the following diets: BCAA-deficient, –normal, or -excessive diets. Changes of rate-limiting enzymes involved in lipolysis, lipogenesis and m^6A RNA modification in subcutaneous adipose tissue, ventral adipose tissue, and liver were determined in this study. These results provide important information regarding the relationship between dietary BCAAs and carcass characteristics in pigs and its underlying mechanism. Our study also extends our knowledge that m^6A methylation might act as a novel and critical bridge between dietary nutrients and obesity in human.

Materials and methods

Animals and experimental diets

All procedures outlined in this study were conducted under the protocol (SCAU-AEC-2010-0416) approved by the South China Agricultural University Animal Care and Use Committee. In our experiments, a total of 18 healthy crossbred weaned piglets (Duroc × Landrace × Large White, 10.45 ± 0.41 kg) were divided into 3 treatments using a completely randomized design. All piglets were kept in metabolic cages (1.40 × 0.68 × 0.90 m^3). The formula of three treatments are shown in Table 1. Pigs in the L-BCAA group were fed the low-protein (LP) diet containing 17.05% crude protein (CP) supplemented with L-lysine, L-methionine, L-threonine, and L-serine. N-BCAA and H-BCAA groups were designed based on the L-BCAA group. In N-BCAA group, extra crystal BCAAs were supplemented as recommended by the National Research Council (NRC) (2012) (0.13% L-isoleucine,

Table 1 Composition of the experimental diets (As-Fed basis)

Items	Treatments[a]		
	L-BCAA	N-BCAA	H-BCAA
Ingredient (%)			
Corn (7.8%, CP)	69.24	69.38	69.50
Soybean meal (47%, CP)	9.45	9.15	8.55
Concentrated soybean protein (65.2%, CP)	5.00	5.00	5.00
Fish meal (62.5%, CP)	4.00	4.00	4.00
Whey powder (3.8%, CP)	5.00	5.00	5.00
Soybean oil powder	1.50	1.50	1.50
L-lysine HCl	0.66	0.66	0.67
$_{D,L}$-methionine	0.30	0.30	0.31
L-threonine	0.30	0.30	0.32
L-tryptophan	0.08	0.08	0.09
L-leucine	0	0.09	0.82
L-isoleucine	0	0.13	0.51
L-valine	0	0.23	0.70
L-alanine	1.44	1.15	0
Salt	0.30	0.30	0.30
Calcium formate	0.60	0.60	0.60
Dicalcium phosphate	1.13	1.13	1.13
Premix[b]	1.00	1.00	1.00
Calculated Nutrient Content (%)			
Crude protein	17.45	17.45	17.45
Digestive energy (MJ/kg)	14.68	14.70	14.64
Total Ca	0.80	0.80	0.80
Total P	0.66	0.66	0.66
Available P	0.44	0.44	0.44
Lys	1.36	1.35	1.35
Met + Cys	0.75	0.75	0.74
Threonine	0.79	0.79	0.79
Tryptophan	0.22	0.22	0.22
Leucine	1.25	1.35	2.03
Isoleucine	0.55	0.69	1.04
Valine	0.62	0.86	1.29

[a] *L-BCAA* Low dose BCAA diet, *N-BCAA* Normal dose BCAA diet, *H-BCAA* High dose BCAA diet. [b] Provided per kilogram of diet (as-fed basis): VA, 13000 IU; VD_3, 40 00 IU; VE, 39.4 mg; VB_1, 6.2 mg; VB_2, 11.2 mg; VB_6, 12.2 mg; VB_{12}, 4 mg; VK, 4.0 mg; niacin, 45.0 mg; folate, 2.2 mg; pantothenic acid, 25.0 mg; biotin, 0.2 mg; choline chloride, 500.0 mg; Cu, 35.0 mg; Fe, 105.0 mg; Mn, 25.0 mg; Zn, 1600.0 mg; I, 0.3 mg; Se, 0.6 mg; Co, 0.3 mg

0.09% L-leucine, and 0.23% L-valine) to meet the requirement of standardized ileal digestible amino acids (SID AAs). In the H-BCAA diet, BCAAs were supplemented to reach the 150% SID AA requirement according to the NRC (2012). Pigs had free access to water via a nipple drinker throughout the process of this experiment.

Sample collection

Blood samples were collected from pigs in heparin-free vacutainer tubes at the end of the experiment (fasting state). After blood collection, all samples were centrifuged at 3000 g for 15 min at 4 °C. The serum was obtained and stored at − 80 °C immediately for later analysis. All piglets were sacrificed by electrocution immediately after blood sampling. Ventral, subcutaneous adipose and dorsal subcutaneous adipose were excised from the left side of the carcasses between the sixth and seventh ribs. Liver samples were consistently dissected from right side of whole liver. Adipose and liver tissues were immediately frozen in liquid nitrogen and then stored at − 80 for further analysis.

Serum biochemical analysis

The concentrations of total cholesterol (TC), high-density lipoprotein-cholesterol (HDL-C), low-density lipoprotein-cholesterol (LDL-C), glucose and triglyceride in serum were measured using corresponding commercial available kits (Nanjing Jiancheng Biochemical Reagent Co., Nanjing, China) through the automatic microplate reader (Thermo Scientific™ Multiskan™ GO, USA). In addition, the concentrations of leptin, insulin and adiponectin in serum samples were measured by the commercial ELISA kits purchased from Cusabio Biotech Co., Ltd. (Wuhan, China).

RNA extraction and purification

Cytoplasmic RNA was isolated from ventral subcutaneous adipose, dorsal subcutaneous adipose, and liver of piglets using the cytoplasmic & nuclear RNA purification kit according to the manufacturer's protocol (NORGEN, Canada, North American). The concentration of extracted RNA was measured by Nano Drop spectrophotometer (Nano Drop Technologies, Wilmington, DE, USA). We also checked the integrity of mRNA by 1% agarose gel electrophoresis. The mRNA was reverse-transcribed to complementary DNA (cDNA) with the PrimeScript 1st Strand cDNA Synthesis Kit (Takara, Dalian, Liaoning, China) according the manufacturer's protocol. After that, the synthesized cDNA was stored at − 20 °C for further real-time PCR analysis.

Gene expression using RT-PCR

The real-time polymerase chain reaction (RT-PCR) was conducted using an ABI Prism 7500 sequence detection system (Applied Biosystems, Carlsbad, CA). This procedure was performed in a 20 μL reaction volume, containing 10 μL SYBR Green PCR Master Mix (Takara, Dalian, Liaoning, China), 2 μL cDNA, 0.8 μL of each PCR primer (10 μM), 0.4 μL ROX (Dalian, Liaoning, China), and 6 μL dd H_2O. The cycling conditions for polymerase chain reaction were as follows: (1) incubation for 5 min at 94 °C, followed by (2) 40 repeated cycles of 94 °C for 30 s, (3) annealing at 60 °C for 30 s and extension at 72 °C for 20 s. The mRNA expression level of the target genes was calculated through the $2^{-\Delta\Delta Ct}$ method. Gene-specific primer sequences used for the RT-PCR detection are listed in Table 2, which were synthesized by Sangon Biotech Co. Ltd. (Shanghai, China).

Western blotting analysis

Western blotting analysis was conducted to detect relative protein levels for METTL3, METTL14, FTO, ALKBH5 and YTHDF2 which are associated with RNA methylation. First, all proteins samples were homogenized on ice with RIPA Lysis Buffer (Beyotime, Shanghai, China), which contains 150 mM NaCl, 50 mM Tris-HCl (pH = 7.4), 1% sodium deoxycholate, 1% Triton X-100, 0.1% SDS and some specific protease inhibitors. A BCA Protein Assay Kit (Beyotime, Shanghai, China) was used to detected total protein concentrations of each sample. Subsequently, a total of 30 μg of protein from each tissue was separated by electrophoresis on SDS-PAGE gels. After transferring to nitrocellulose membranes, all strips were blocked in 5% skimmed milk for 1 h at room temperature. Next, blots were incubated with the following primary antibodies at 4 °C overnight with gently shaking: METTL3, ALKBH5 and MTTL14 (1:1000; Abcam, Cambridge, MA, USA), FTO and β-actin (1: 1000; Santa Cruz Biotechnology, USA), YTHDF2 (1: 1000, Millipore, Bedford, MA, USA). Subsequently, the membranes were incubated with the corresponding secondary antibody (1: 5000 dilution) (Thermo Fisher Scientific, Rockford, IL, USA) for 1 h at room temperature. Finally, proteins on membrane were developed by the ECL Plus chemiluminescence detection kit (Applygen Technologies, Beijing, China) and analyzed by Image Processing Software (Image Pro Plus 6.0) (Rockville, MD, USA).

Measurement of m^6A content

The total content of m^6A in adipose and liver tissues were determined by an EpiQuik™ m^6A RNA methylation quantification kit (Epigentek Group Inc. USA) following the recommended procedures. The total content of m^6A was calculated using the formula: $m^6A\ \% = \{[(OD_{Sample} - OD_{NC})/S] \div [(OD_{PC} - OD_{NC})]\} \times 100\%$, and S, PC and NC represents the total amount of inputting RNA, positive and negative controls, respectively.

m^6A immunoprecipitation QPCR of target genes

In order to measure the m^6A modification of genes involved in lipogenesis and lipolysis, m^6A immunoprecipitation was performed. Briefly, m^6A antibody (Abcam, Cambridge, MA, USA) and Normal rabbit IgG (Abclonal, Wuhan China) were conjugated to protein A/G mixed magnetic beads (Bio-rad Laboratories, Hercules, CA, USA) at 4 °C overnight. RNA was fragmented and incubated with the magnetic beads in immunoprecipitation buffer (10 mM Tris, 0.5% NP-40, and 150 mM NaCl) supplemented with RNase inhibitor at 4 °C for 3 h. After washing twice with IP buffer, mRNA was eluted from the beads by elution buffer (5 mM Tris·HCl, 1 mM EDTA, and 0.05% SDS). After extraction and precipitation, the input RNA and eluted RNA were reverse transcribed, and its abundance was determined by RT-PCR.

Statistical analysis

Data of this experiments were analyzed with one-way ANOVA according to the procedure GLM of SAS (SAS Institute, Cary, NC, USA). Differences among treatment

Table 2 Primer sequences used in quantitative real-time PCR assay

Primers	Accession No.	Sequences (5′ → 3′)	Product size (bp)
PPAR-γ	NM_214379.1	F-AACATTTCACAAGAGGTGACCA	213
		R-GATCTCGTGGACGCCATACT	
SREBP-1c	NM_214157.1	F-AGCGGACGGCTCACAATG	121
		R-CGCAAGACGGCGGATTTA	
ACACA	NM_001114269.1	F-ACATCCCCACGCTAAACA	186
		R-AGCCCATCACTTCATCAAAG	
FASN	NM_001099930.1	F-GCTTGTCCTGGGAAGAGTGTA	115
		R-AGGAACTCGGACATAGCGG	
CD36	NM_001044622.1	F-ACCCTGAGACCCACACAGTC	119
		R-TACAGCTGCCACAGCCAGAT	
FABP4	NM_001002817.1	F-TGAAAGGTGTCACGGCTAC	102
		R-TCGGGACAATACATCCAACAGAG	
CPT-1A	XM_021091195.1	F-ATTACGACGGCAGGCTGTTGAAG	133
		R- AATAGGCTTGGCGACACTTGGC	
HSL	NM_214315.3	F-GCCCGAGACGAGATTAGC	143
		R-ATGAAGGGATTCTTGACGATG	
ATGL	no. EU373817	F-CGCCAGCATCATCGAGGTGTC	94
		R-GCAGCCACGGATGGTCTTCAC	
DGAT1	NM_214051.1	F-GCTTCAGCCTTCTTCCACGAGTAC	163
		R-CGATGATGAGCGACAGCCACAC	
METTL3	XM_003128580.5	F-CCAGCACAGCTTCAGCAGTTCC	129
		R-TGGAGATGGCAAGACGGATGGAG	
METTL14	XM_003129231.6	F-GAGGAAGAGGTGGAACTTCTGCTG	96
		R-CTCCTCCACGGCCTCCTCTG	
WTAP	NM_001244241.1	F-GCGGGAATAAGGCCTCCAAC	136
		R-TGTGAGTGGCGTGTGAGAGA	
FTO	NM_001112692.1	F-GCAGAGCCGCCTACAACCTAAC	142
		R-ACCGCTGACCTGTCCACCAG	
ALKBH5	XM_021067995.1	F-GCAAGGTGAAGAGCGGCATCC	128
		R-GTCCACCGTGTGCTCGTTGTAC	
YTHDF1	XM_021078235.1	F-GTCTACCTGCTCTTCAGCGTCAAC	139
		R-AGATCCACTTCACGTCGAACTTGC	
YTHDF2	XM_005665152.3	F-CCACCTCCACCACAGCCTACTC	140
		R-CCAGCCTGAGACTGTCCTACTCC	
YTHDF3	XM_021089300.1	F-ATTGAGCAAGGCATGACTGGACTG	140
		R-GGCGCTGCACTGCTAACTGG	
β-actin	397,563	F-TGCGGGACATCAAGGAGAAG	176
		R-AGTTGAAGGTGGTCTCGTGG	

means were determined using Student-Newman-Keuls (SNK) multiple range test. All results are presented as means with their standard error of mean (SEM). P value less than 0.05 was considered as significant and effects were considered as tendency when $0.05 \leq P \leq 0.10$.

Result

Lipid-related hormone levels in serum

Plasma hormone levels of insulin, leptin and adiponectin are shown in Table 3. Compared with pigs fed the N-BCAA diet, plasma leptin levels were significantly increased

Table 3 Lipid-related hormone levels in serum

Projects	Diets			P-value
	L-BCAA	N-BCAA	H-BCAA	
Insulin	5.03 ± 0.28	5.13 ± 0.55	6.07 ± 0.60	0.31
Leptin	1.02 ± 0.09[a]	0.44 ± 0.05[b]	0.32 ± 0.02[b]	< 0.01
Adiponectin	15.70 ± 1.23[a]	16.26 ± 3.67[a]	6.63 ± 0.23[b]	0.02

Serum hormone parameters. Diet treatments: *L-BCAA* Low dose BCAA diet, *N-BCAA* Normal dose BCAA diet, *H-BCAA* High dose BCAA diet. Values are means of four pens of four pigs per diet. [a-b] Mean values within a line with different superscript letters were significantly different ($p < 0.05$)

in pigs from the L-BCAA groups ($P < 0.05$). In comparison with the N-BCAA diet, plasma adiponectin levels did not change in the L-BCAA group, but significantly increased in the H-BCAA group ($P < 0.05$). There was no difference in plasma insulin level among different groups ($P > 0.05$).

Serum biochemical parameters

Serum biochemical parameters are shown in Table 4. Compared with the L-BCAA group, serum concentrations of T-CHO were lower in the H-BCAA group ($P < 0.05$). Both serum concentrations of HDL-C and LDL-C were obviously lower in pigs fed the L-BCAA diet ($P < 0.05$) compared with those fed the N-BCAA diet. However, no difference was found in plasma TG and glucose levels among different BCAA dose groups ($P > 0.05$).

Expression of genes involved in fat metabolism in adipose and liver tissues

Figure 1 shows the expression level of genes associated with lipid metabolism in adipose and liver tissues. In ventral subcutaneous adipose tissue, the mRNA level of ACACA in L-BCAA treatment was lower than that in the N-BCAA treatment ($P < 0.05$) (Fig. 1a). Also, the mRNA expression of ACACA, FASN, PPAR-γ and SREBP-1c were lower in the H-BCAA group when compared with the N-BCAA group ($P < 0.05$) (Fig. 1a). Furthermore, the mRNA expression of ATGL and CPT-1A involved in fat hydrolysis ($P < 0.05$) (Fig. 1b) as well as FABP4 and CD36 ($P < 0.05$) (Fig. 1c) involved in fatty acid transport were significantly increased in piglets fed the L-BCAA diet compared with those fed the N-BCAA diet. Similarly, the mRNA expression of HSL, ATGL, CPT-1A ($P < 0.05$) (Fig. 1b) and FABP4 ($P < 0.05$) (Fig. 1c) were higher in the H-BCAA group than the N-BCAA group. In dorsal subcutaneous adipose tissue, the L-BCAA group significantly inhibited ACACA and FASN mRNA expression level ($P < 0.05$) (Fig. 1d) when compared with the N-BCAA group. Also, the H-BCAA group have lower mRNA expression of ACACA, FASN, DGAT1, PPAR-r and SREBP1c than the N-BCAA group ($P < 0.05$) (Fig. 1d). In addition, when compared with the N-BCAA group, pigs from the L-BCAA group have significantly higher mRNA expression of HSL, ATGL, CPT-1A ($P < 0.05$) (Fig. 1e) and FABP4 ($P < 0.05$) (Fig. 1f). In liver tissue samples, pigs fed the L-BCAA diet had lower expression of lipogenic genes (ACACA and SREBP-1c) ($P < 0.05$) (Fig. 1g) and higher expression of lipolysis genes (CPT-1A) ($P < 0.05$) (Fig. 1h) than those fed the N-BCAA diet. While pigs fed with H-BCAA diet have lower level of SREBP-1c ($P < 0.05$) (Fig. 1g) and FABP4 ($P < 0.05$) (Fig. 1i).

Table 4 Lipid-related biochemical parameters in serum

Projects	Diets			P-value
	L-BCAA	N-BCAA	H-BCAA	
TCHO	8.53 ± 0.28[a]	7.63 ± 0.75[ab]	6.47 ± 0.44[b]	0.06
HDL-C	1.48 ± 0.11[b]	2.29 ± 0.20[a]	1.60 ± 0.25[b]	0.04
LDL-C	1.30 ± 0.12[b]	2.72 ± 0.71[a]	0.91 ± 0.25[b]	0.03
Glucose	5.85 ± 0.15	4.87 ± 0.86	3.54 ± 0.98	0.15
TG	0.87 ± 0.05	0.76 ± 0.08	0.86 ± 0.07	0.42

Serum biochemical parameters. Diet treatments: *L-BCAA* Low dose BCAA diet, *N-BCAA* Normal dose BCAA diet, *H-BCAA* High dose BCAA diet, *TCHO* Total cholesterol, *HDL-C* High-density lipoprotein-cholesterol, *LDL-C* Low density lipoprotein-cholesterol, *TG* Triglyceride. Values are means of four pens of four pigs per diet. [a-b] Mean values within a line with different superscript letters were significantly different ($p < 0.05$)

Total methylation levels in adipose and liver tissues

As shown in (Fig. 2), in ventral subcutaneous adipose tissue and dorsal subcutaneous adipose tissue, compared with the N-BCAA group, total amount of m^6A was significantly reduced in the H-BCAA group ($P < 0.05$) (Fig. 2a and b), In liver, compared with the N-BCAA group, total amount of m^6A were higher in the L-BCAA group ($P < 0.05$) (Fig. 2c).

mRNA expression of m^6A related enzymes

The expression of m6A related enzymes are listed in (Fig. 3). In ventral subcutaneous adipose tissue, the mRNA levels of FTO and ALKBH5 ($P < 0.05$) (Fig. 3a, b, and c) were significantly increased in the H-BCAA group when compared with the N-BCAA group. Compared with the N-BCAA group, L-BCAA group increased mRNA expression of METTL3, MELLT14, WTAP as well as YTHDF1 and decreased mRNA expression of YTHDF2 and YTHDF3 in the ventral subcutaneous adipose tissue (Fig. 3a, b, and c). In dorsal subcutaneous adipose, piglets fed with the H-BCAA diet had lower mRNA expression of METTL3, METTL14, WTAP, FTO, ALKBH5, YTHDF2 and YTHDF3 when compared with pigs fed with the N-BCAA diet (Fig. 3d, e and g). However, piglets fed with the L-BCAA diet have higher mRNA levels of METTL14, FTO, ALKBH5, and YTHDF1 than the piglets fed with the N-BCAA diet (Fig. 3d, e and g). In liver tissue, piglets fed the H-BCAA diet showed higher mRNA levels for METTL14, WTAP, FTO, YTHDF2 ($P < 0.05$) (Fig. 3g) compared to those

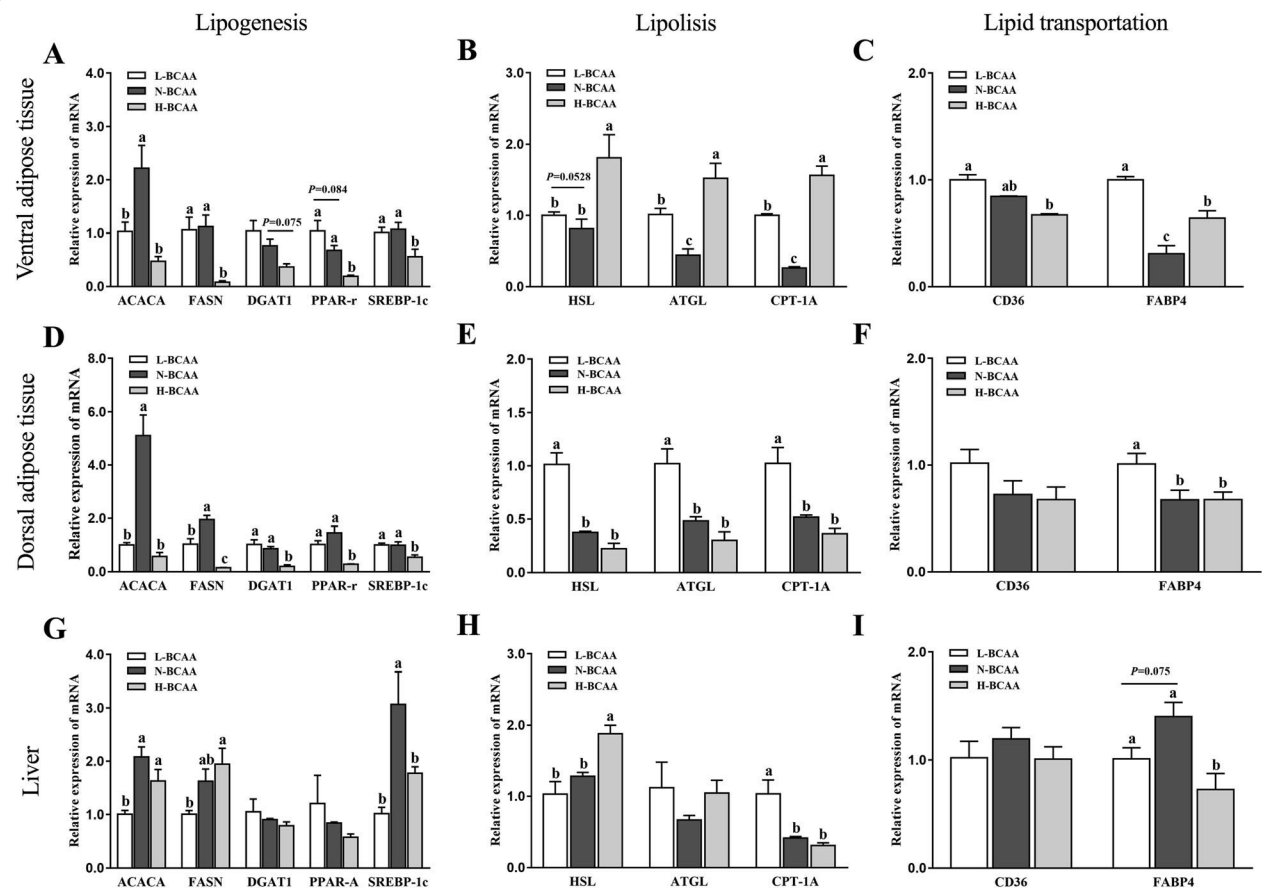

Fig. 1 Effect of dietary BCAA level on the expression of genes involved in fat metabolism in ventral subcutaneous adipose (**a**, **b**, **c**), dorsal subcutaneous adipose (**d**, **e**, **f**) and liver (**g**, **h**, **i**). Data are shown as mean ± SEM ($n = 4$). Different letters indicate a significant difference among different treatments ($P < 0.05$)

fed with the N-BCAA diet. In addition, compared with the N-BCAA group, pigs fed the L-BCAA group significantly decreased FTO and ALKBH5 expression, but increased METTLE3, METLE14, YTH family protein 1, 2, and 3 expression in liver tissue.

Protein abundance of m^{6}A related enzymes

The proteins abundance of METTL3, METTL14, FTO, ALKBH5 and YTHDF2 in adipose and liver tissues are presented in (Fig. 4). In ventral subcutaneous adipose tissue, protein levels of METTL14 and ALKBH5 were

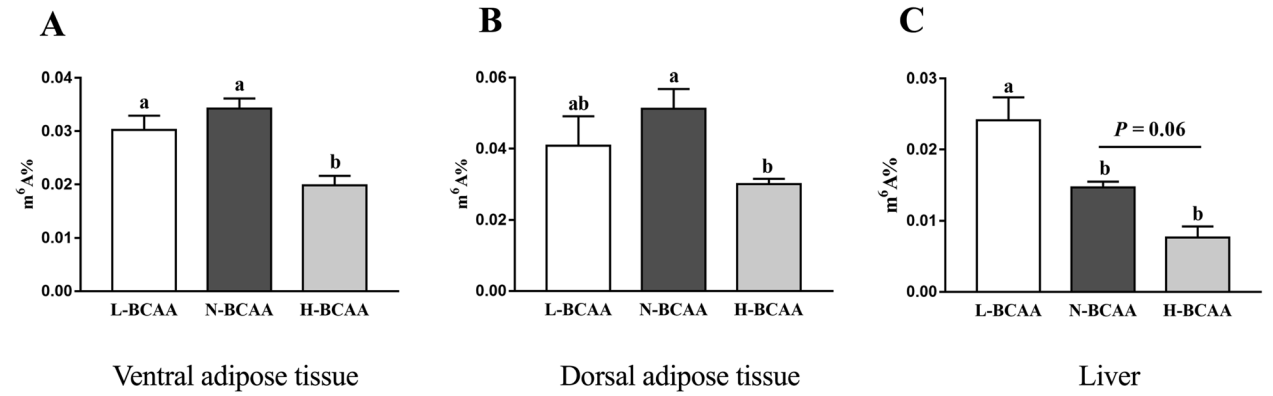

Fig. 2 Effect of dietary BCAA level on m6A RNA methylation in ventral subcutaneous adipose (**a**), dorsal subcutaneous adipose (**b**) and liver (**c**). Data are shown as mean ± SEM ($n = 6$). Different letters indicate a significant difference among different treatments ($P < 0.05$)

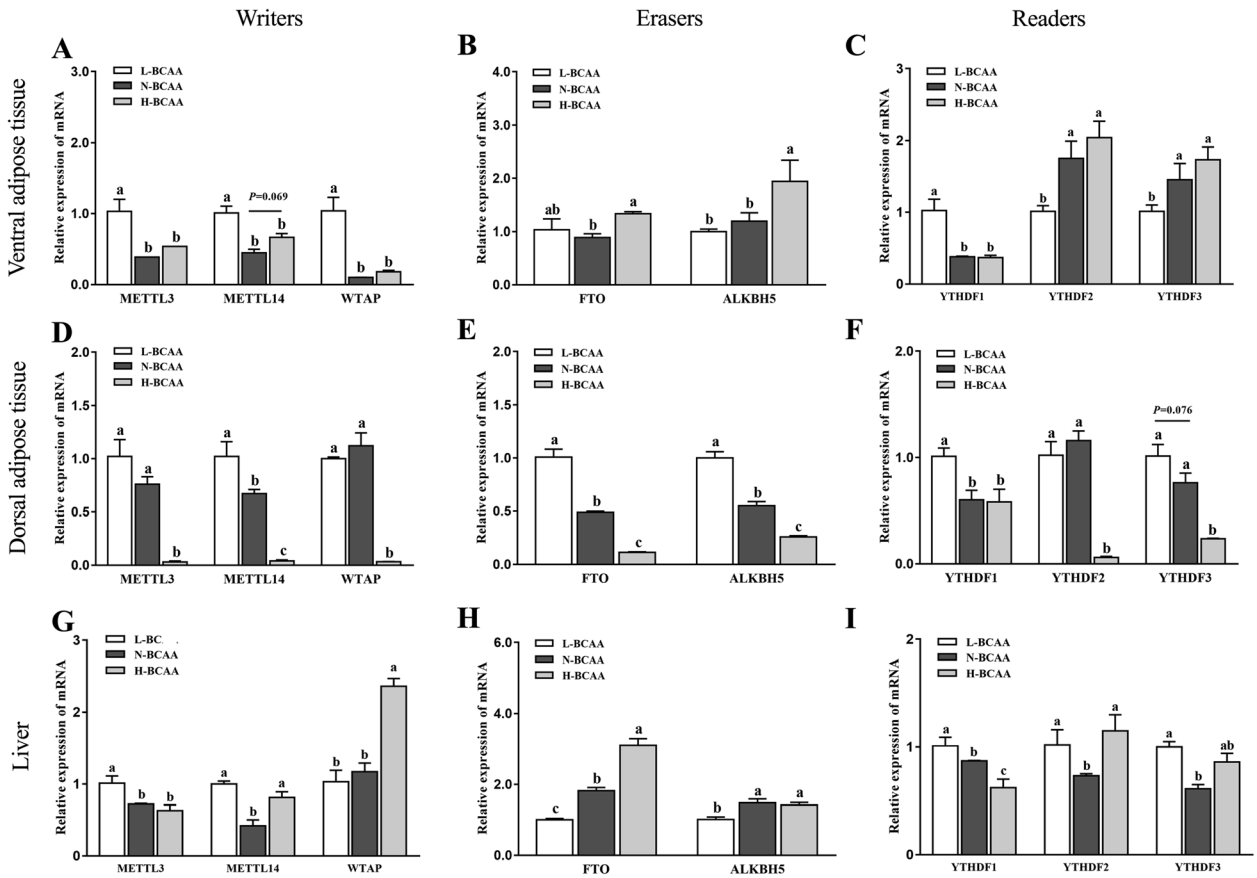

Fig. 3 Effect of dietary BCAA level on the expression of genes related to m^{6}A RNA methylation in ventral subcutaneous adipose (**a**, **b**, **c**), dorsal subcutaneous adipose (**d**, **e**, **f**) and liver (**g**, **h**, **i**). Data are shown as mean ± SEM ($n = 6$). Different letters indicate a significant difference among different treatments ($P < 0.05$)

higher in the L-BCAA group than the N-BCAA group. The protein levels of MELLT14, FTO and YTHDF2 were higher in the H-BCAA group than the N-BCAA group. ($P < 0.05$) (Fig. 4a). In dorsal subcutaneous fat tissue, the protein amounts of METTL3 and METTL14 were obviously decreased ($P < 0.05$) (Fig. 4b) in the H-BCAA group, when compared with the N-BCAA group. Notably, the protein abundance of ALKBH5 in the L-BCAA group was higher than that in the N-BCAA group ($P < 0.05$) (Fig. 4b). In liver tissue, compared with the N-BCAA group, pigs fed the L-BCAA diet had higher protein levels of METTL3 and METTL14 ($P < 0.05$) (Fig. 4c), while pigs fed the H-BCAA diet inhibited the protein abundance of METTL3 ($P < 0.05$) (Fig. 4c).

m^{6}A modification on lipid metabolism associated genes in adipose tissue

The effect of excessive BCAA on m^{6}A modification of genes involved in lipogenesis and lipolysis in adipose tissues is presented in Fig. 5. Compared with the N-BCAA group, the H-BCAA group significantly decreased the m^{6}A modification of ACACA and FASN in ventral adipose tissue ($P < 0.05$) and ACACA, FASN and DGAT1 in dorsal adipose tissue ($P < 0.05$).

Discussion

BCAAs are traditionally considered as functional amino acids that regulate protein synthesis via mTORC1 [6]. Recently, BCAAs have been reported to participate in fatty acid metabolism [7]. However, the underlying mechanism is still unclear. In the current research, the BCAA dose-dependent experiment was conducted to explore the effect of dietary BCAA deficient or excess on fat metabolism in fat and liver and its related mechanism.

Lipid related hormones are affected by different dietary BCAA levels in our study. Adipose tissue is not only an organ for energy storage, but also an endocrine organ which secretes different biologically active molecules into the circulation. Leptin and adiponectin are the two most important fat-derived hormones that participate in energy metabolism [20, 21]. Plasma leptin and adiponectin concentrations are positively and negatively correlated with body fat storage, respectively [22, 23]. In our study, piglets fed the BCAA deficient diet significantly

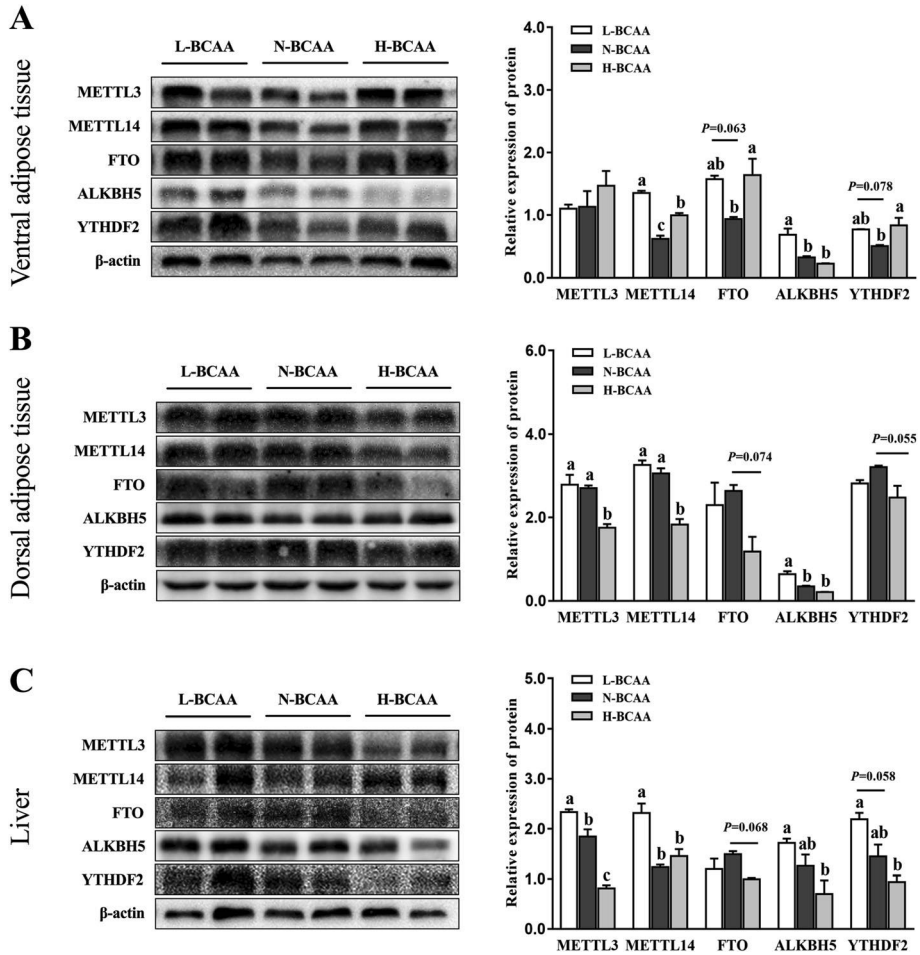

Fig. 4 Effect of dietary BCAA level on METTL3, METTL14, FTO, ALKBH5 and YTHDF2 protein level in ventral subcutaneous adipose (**a**), dorsal subcutaneous adipose (**b**) and liver (**c**). Data are shown as mean ± SEM ($n = 3$). Different letters indicate a significant difference among different treatments ($P < 0.05$)

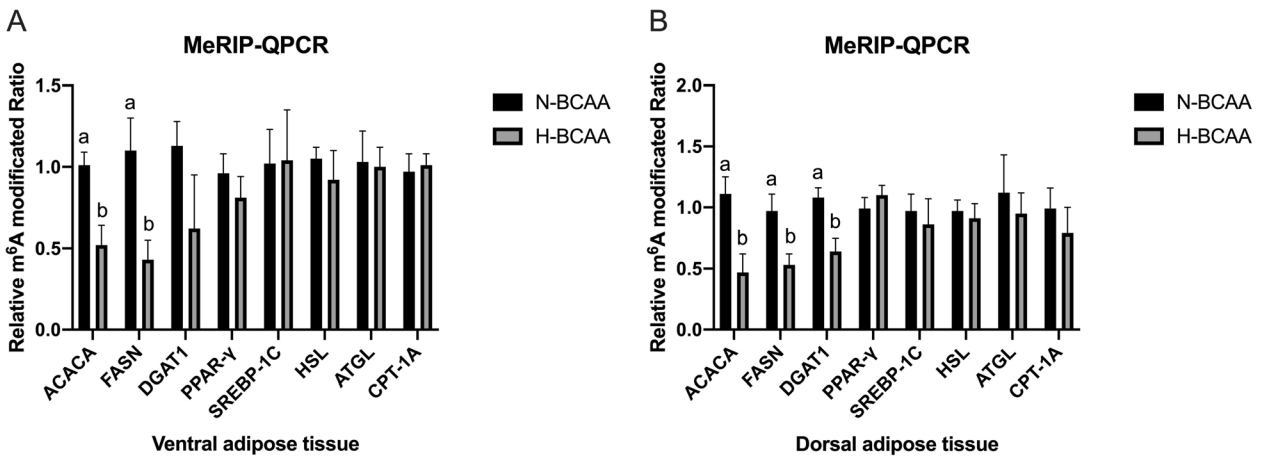

Fig. 5 Effect of excessive BCAA on m^6A modification of genes involved in lipogenesis and lipolysis in ventral (**a**) and dorsal adipose tissues (**b**). Data are shown as mean ± SEM ($n = 3$). Different letters indicate a significant difference among different treatments ($P < 0.05$)

increased the level of leptin, while a lower level of adiponectin was observed from the piglets fed the BCAA excessive diet. In consistent with our results, Li, Wei [24] also found plasma leptin concentration decreases in the BCAA-deficient group. Although the relationship between plasma adiponectin and dietary BCAA level is unclear, an intimate relationship was found between them in the current study. Duan, Li [12] reported that plasma adiponectin concentrations change with different dietary BCAA ratios. Furthermore, leucine metabolite β-hydroxy-β-methylbutyrate regulates fat metabolism partly through adiponectin. Together, these results indicate that unbalanced BCAA might disrupt the whole-body energy metabolism through the modification of leptin and adiponectin secretion. In addition, HDL-C and LDL-C that participated in fat metabolism are also regulated by different dietary BCAA concentrations in our study.

To further study the effect of dietary BCAA on fat metabolism in piglets, genes related to lipolysis and lipogenesis were analyzed in our study. Lipogenesis consists of a series of enzymes, such as ACACA, FASN, DGAT1, SREBPs and PPARs. First of all, acetyl CoA (converted from glucose) is carboxylated into malonyl-CoA by acetyl-CoA carboxylase (ACACA) [25]. Subsequently, fatty acids are synthesized by fatty acid synthase (FASN) with acetyl-CoA, malonyl-CoA and NADPH [26]. Finally, three free fatty acids and one glycerol are combined into a triglyceride under diacylglycerol acyltransferase (DAGT) catalysis as the final step of lipogenesis [27]. This lipogenesis process is regulated by several important regulators. For instance, sterol regulatory element–binding proteins (SREBPs), a membrane-bound transcription factor, can strongly increase the expression of genes involved in synthesis and uptake of fatty acids [28]. Furthermore, nuclear receptor peroxisome proliferator-activated receptors (PPARs) also enhance fatty acid synthesis by increasing the expression of fatty acid transport protein and acyl-CoA synthetase [29]. Lipolysis is also a well-regulated process, which includes fatty acid mobilization and oxidation. Free fatty acids are released from triacylglycerol by hormone-sensitive lipase (HSL) and adipose triglyceride lipase (ATGL) [30, 31] and are then transported into the cell by FABP4 and FAT/CD36 [32–34]. After the production of acyl CoA from free fatty acid, the step-limiting enzyme carnitine Palmitoyl Transferase-1 (CPT1) transports acyl-CoA into mitochondria for β-oxidation [35].

In this study, decreased lipogenesis and enhanced lipolysis gene expression was observed in piglets fed both BCAA deficient and excess diets. The inhibition of fat synthesis in adipose tissue have also been reported by many other groups using animal models or cell lines in BCAA deficient situation [7, 8, 36, 37] or excessive situation [9, 10, 38, 39]. Similar effects are also observed in the liver. In broiler chicken, low BCAA decreased the lipogenesis and increased lipolysis in the liver partly through AMPK-mTOR-FoxO1 pathway [40]. In addition, a research conducted in mice reveals that supplementation of BCAA decreases the hepatic TG and lipid droplet size and inhibits the expression of lipogenesis gene [41]. Three interesting observations in the current study are worth noting. First of all, more lipid metabolism genes were changed in piglets fed BCAA excess diet than deficient diet. For example, genes participated in lipogenesis (ACACA, FASN, DAGT1, PPAR-γ and SREBP-1c) were significantly decreased both in fat tissues when piglets fed BCAA excess diet, while only ACACA was down-regulated when piglets fed BCAA deficient diet. In addition, the degree of the decrease in gene expression was greater in the BCAA excess group than in the BCAA deficient group. Finally, the effects of BCAA on gene expression were tissue dependent. For instance, although both ventral subcutaneous adipose tissue and dorsal subcutaneous adipose tissue are belonging to fat, pigs fed BCAA deficient diet only increased the lipolysis genes (HSL, ATGL, and CPT-1A) in ventral subcutaneous adipose tissue, but not in dorsal subcutaneous adipose tissue. However, the underlying mechanism of this phenomenon is still unknown and needs further research.

To test the possible role of m^6A RNA modification in this experiment, the total m^6A methylation level and critical enzymes involved in m^6A RNA modification were tested in our study. We found that only high level of BCAA regulated the m^6A RNA methylation in adipose tissues. Interestingly, our study also observed that BCAA dose-dependently decreased the m^6A RNA methylation in the liver. FTO, a m^6A demethylase, has been found to have positive correlation with fat deposition [42]. The expression of FTO in subcutaneous fat and liver were decreased in the H-BCAA group, which is consistent with the inhibition of the fatty acid deposition in this research. Intriguingly, we observed that down-regulated FTO is accompanied with the decrease of the total m^6A methylation levels in subcutaneous fat and liver in piglets. The seemingly contradictory results may imply that in addition to FTO, other methyltransferases and demethylases might be involved in the process and need further investigation. We further measured other enzymes and found methyltransferases METTL3 (liver and dorsal subcutaneous adipose tissue), MELLT14 (dorsal subcutaneous adipose tissue) also significantly decreased in the H-BCAA group. The decreased expression of methyltransferases may explain the decreased level of m^6A methylation. Interestingly, in this study, although excess BCAA diet inhibited lipogenesis in ventral subcutaneous adipose tissue, the expression of FTO was unexpected up-regulated for unknown reason. Collectively, the present study indicates: 1. high dose

BCAA treatment decrease the level of m^6A RNA methylation in adipose tissue and liver, 2. The effect of BCAA on demethylase and methyltransferase is tissue specific. m^6A RNA methylation is commonly considered to be regulated by dietary methyl donors like: methionine, choline and betaine [43]. To our knowledge, our study was first to report that BCAAs affect m^6A RNA methylation which might further regulate fat metabolism. Furthermore, decreased m^6A modification of ACACA and FASN in ventral and dorsal adipose tissues was observed in pig fed with excessive BCAA. Future studies are needed to clarify the changes of m^6A methylation level of other lipid metabolism associated genes which might participated in fat metabolism.

Conclusion

BCAA deficient or excess diet decrease the fat deposition by increasing lipolysis and lipogenesis in adipose tissue and the liver. m^6A RNA methylation is partially involved in the regulation of fat metabolism in diet supplemented with excessive but not deficient BCAA.

Abbreviations

AAs: Amino acids; BCAA: Branched-chain amino acids; CP: Crude protein; H-BCAA: High dose BCAA; HDL-C: High-density lipoprotein-cholesterol; HNRNP: Heterogeneous nuclear ribonucleoprotein proteins; L-BCAA: Low BCAA dose diet; LDL-C: Low-density lipoprotein-cholesterol; LP: Low-protein; m^6A: N6-methyl-adenosine; N-BCAA: Normal dose BCAA diet; NRC: National Research Council; RT-PCR: Real-time polymerase chain reaction; SID: Standardized ileal digestible; TC: Total cholesterol

Authors' contributions

JH, SZ, WG and FC designed the experiment and supervised the project. JH, ZW, MT and JC performed the experiments and conducted the lab work. JH, ZW and HS conducted the statistical analysis. JH and SZ wrote the paper. All authors read and approved the final manuscript.

Funding

This study was supported by National Natural Science Foundation of the P. R. of China (No. 31802067 and 31872364) and the Natural Science Foundation of Guangdong Province (No. 2018A030310201) and the National Key R&D Program of China (No. 2018YFD0500600 and 2018YFD0501000).

Availability of data and materials

All data used in the current study are available from the corresponding author on reasonable request.

Ethics approval and consent to participate

All animal use and care protocols were approved by the Committee of the South China Agricultural University Animal Care and Use (20110107–1).

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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Received: 6 September 2019 Accepted: 29 December 2019
Published online: 23 January 2020

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特发此证，以资鼓励。

中共华南农业大学委员会

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二〇二四年七月

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2024年6月18日