NUTRITIONAL MANAGEMENT FOR THE ENERGY METABOLISM IN ANIMALS

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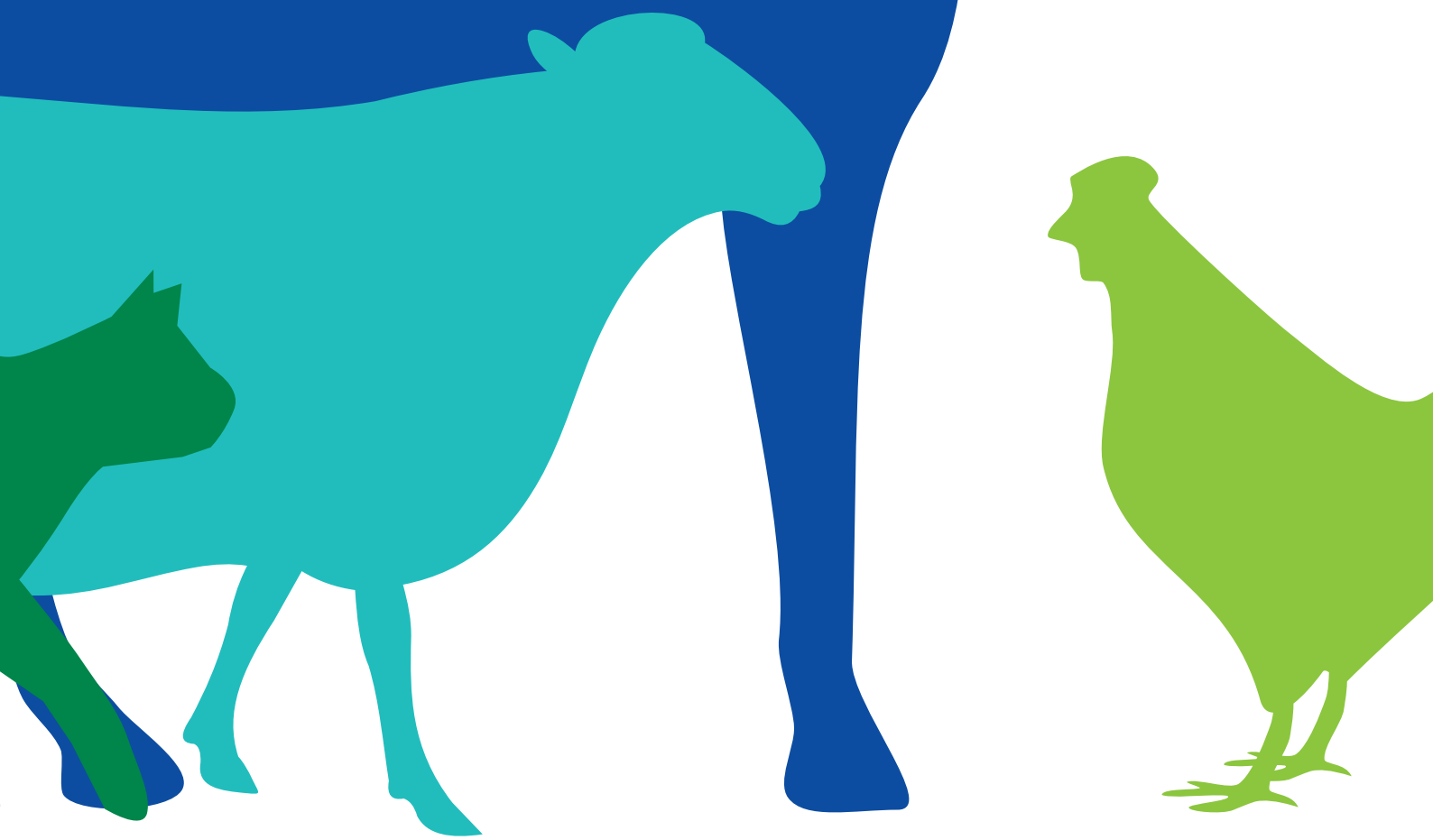

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NUTRITIONAL MANAGEMENT FOR THE ENERGY METABOLISM IN ANIMALS

Topic Editors:
Jing Wang, University of California, Los Angeles, United States
Tarique Hussain, Nuclear Institute for Agriculture and Biology, Pakistan
Yehui Duan, Chinese Academy of Sciences (CAS), China

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EDITORIAL
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Editorial: Nutritional Management for the Energy Metabolism in Animals

Jing Wang[1]*, *Tarique Hussain*[2] *and Yehui Duan*[3]

[1] Hunan Provincial Key Laboratory of Animal Nutritional Physiology and Metabolic Process, College of Animal Science and Technology, Hunan Agricultural University, Changsha, China, [2] Animal Sciences Division, Nuclear Institutes for Agriculture and Biology, Faisalabad, Pakistan, [3] Institute of Subtropical Agriculture, Chinese Academy of Sciences (CAS), Changsha, China

Keywords: energy metabolism, energy disorder, nutritional management, microbiome, mitochondria

Editorial on the Research Topic

Nutritional Management for the Energy Metabolism in Animals

Energy metabolism is the process of generating energy (ATP) from nutrients, including both aerobic respiration (oxygen present), anaerobic respiration (fermentation) as well as fatty acid and amino acid metabolism (1). Energy homeostasis is central to animals to maintain normal function and production.

The Research Topic includes 14 papers, one review, and 13 research articles, concerning energy metabolism and its nutritional management, as well as the effect of gut microbiome and host mitochondria on energy homeostasis at various environmental situations and physiological stages of animals.

One study, Du et al. investigate the effect of different dietary energy levels on the rumen bacteria and meat quality in yak. They suggest that muscle quality of longissimus pectoris of yak fed with high dietary energy level was better, evidenced by the significantly increased water content and crude fat content. Additionally, the high energy diet also elevated the abundance of bacteria related to carbohydrate metabolism in the rumen. They draw the conclusion that high energy diet improved the meat quality of yak mainly by affecting the ruminal amyl lytic bacteria abundance to provide substrates for fatty acid synthesis.

Animals adapt to various changing environment by adjusting their development, metabolism and behavior to improve their chances of survival and reproduction (2). In this Research Topic, Kong et al. use the metabonomics and blood biochemical indexes to investigate the metabolic changes of dairy cows in different high-altitudes. With the increasing of altitude, the different metabolites are mainly enriched in amino acid metabolism and sphingolipid metabolism. And sphingolipid metabolism showed a negative correlation with increased altitude. Meanwhile, they (Kong et al.) uncover the specific mammary metabolic mechanism in hypoxic dairy cows. The results reveal that hypoxia exposure was associated with the elevation of AGPAT2-mediated glycerophospholipid metabolism. These intracellular metabolic disorders consequently lead to the lipid disorders associated with apoptosis of bovine mammary epithelial cells. A certain key nutrient deficiency also affects the metabolism and reproduction. A study of Qian et al. shows that vitamin E deficiency in the early post-partum period of cow will significantly down-regulate the apolipoprotein A3, serum amyloid protein A4 and pantetheinase-1 protein abundance in plasma, among which pantetheinase-1 is closely related to dairy cow subclinical vitamin E deficiency and can be a potential biomarker.

Natural materials of animal origin are involved in regulating nutrient metabolism (3, 4). The paper by Mesgaran et al. indicate that rumen-protected l-carnitine plays a role in supporting production, enhancing liver metabolism and regulating health biomarkers of high-yielding dairy cows during perinatal period. Based on these findings, the authors suggest that the effects of

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Domenico Bergero, University of Turin, Italy

***Correspondence:**
Jing Wang
jingwang023@hunau.edu.cn

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perinatal feeding of l-carnitine on the uterus should be further studied to determine its impact on offspring performance and health. Wang X. et al. find that estrogen promotes glycogen synthesis and storage, and maintains energy homeostasis by enhancing extracellular glucose uptake and regulating autophagy. They suggest that estrogen is necessary to protect cells from apoptosis and enhance the immune potential of PMNs. However, the mechanism by which estrogen regulates glucose metabolism remains unclear.

In addition to animal derived natural materials, plant extracts and probiotics also show the strong ability to interfere the animal nutritional metabolism. Four studies about plant extracts are presented in this Research Topic. Based on the beneficial effect of resveratrol on intestinal injury, the authors (Huang et al.) find that resveratrol can improve intestinal injury induced by deoxynivalenol through mitochondrial autophagy in weaned piglets. Yang et al. find that dietary supplementation of *Eucommia ulmoides* leaf extract (ELE) in finishing pigs improves the carcass traits and reduces the lipid levels by activating the AMPK-ACC pathway to regulate lipid metabolism. Results from Wang Q. et al. indicate that feeding *Phragmites australis* shoot remainder (PSR) silage could improve the growth performance, alter the rumen bacteria diversity and the corresponding function. They demonstrate that PSR silage could partially substitute (30%) corn silage for beef cattle breeding. The study of Afzal et al. elaborates that dietary supplementing with 3.5% *Moringa oleifera* leaf powder (MOLP) improves the antioxidant status, milk yield, and reproductive performance in goats. Moreover, Han et al. suggest that maternal dietary supplementation with *Bacillus subtilis* protease and *Bacillus subtilis* improves the reproductive performance and overall health indicators of sows, as well as the growth and development of their offspring.

In recent years, the nutrition and metabolism of special economic animals and pets have also attracted much attention. Bao et al. find that metabolizable energy intake (MEI), caloric production (HP) and retained energy (RE) of male Sika deer decreased significantly as the apparent digestibility of carbon and nitrogen increased with the decrease of feed intake. Particularly, they calculate the net N requirement for maintenance (NNm) and net protein requirement for maintenance (NPm) of growing male sika deer, fill the gap in net energy and protein requirements and serve as basic data for establishing the nutritional standards for sika deer breeding in China. Obesity is troubling the health of pet dogs. Lyu et al. compare the inflammatory response and fecal metabolome of dogs fed a high-fat vs. a high-starch diet, and suggests a high-starch diet is more suitable for feeding pet dogs. The high-starch diet promotes the lipid metabolism, antioxidant effects, protein biosynthesis and catabolism, mucosal barrier function and immune regulation compared to a high-fat diet in healthy lean dogs. Additionally, Li has conducted an interesting review on a state-of-the-art overview on recent advances in systems biology in canine cardiac disease. He discusses this topic based on three aspects: (1) the changes occurring in each of the three components of energy metabolism in myxomatous mitral valve disease (MMVD) and heart failure (HF); (2) the changes in circulating and myocardial glutathione, taurine, carnitines, branched-chain amino acid catabolism and tryptophan metabolic pathways; (3) the potential role of the gut microbiome in MMVD and HF. He emphasizes that systems biology and high-throughput multi-omics techniques are likely to be used for canine MMVD and HF, and that as new techniques emerge, it will be possible to provide breakthrough nutritional interventions for the treatment of pet dogs with heart disease such as MMVD.

Therefore, the Research Topic provides a review of the articles on the nutritional management of animal nutrition metabolism, and lists some of the authors' new research inspirations based on the current research results and some of the significant contributions of the researches.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct, and intellectual contribution to the work and approved it for publication.

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ORIGINAL RESEARCH
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Comparative Analysis of Metabolic Differences of Jersey Cattle in Different High-Altitude Areas

Zhiwei Kong[1,2,3], *Bin Li*[4]*, *Chuanshe Zhou*[5]*, *Qinghua He*[1]*, *Yuzhong Zheng*[3] and *Zhiliang Tan*[5]

[1] Department of Food Science and Engineering, College of Chemistry and Environmental Engineering, Shenzhen University, Shenzhen, China, [2] Key Laboratory of Optoelectronic Devices and Systems of Ministry of Education and Guangdong Province, College of Optoelectronic Engineering, Shenzhen University, Shenzhen, China, [3] School of Food Engineering and Biotechnology, Hanshan Normal University, Chaozhou, China, [4] Institute of Animal Husbandry and Veterinary, Tibet Autonomous Regional Academy of Agricultural Sciences, State Key Laboratory of Hulless Barley and Yak Germplasm Resources and Genetic Improvement, Lhasa, China, [5] CAS Key Laboratory for Agro-Ecological Processes in Subtropical Region, National Engineering Laboratory for Pollution Control and Waste Utilization in Livestock and Poultry Production, Hunan Provincial Key Laboratory of Animal Nutritional Physiology and Metabolic Process, Institute of Subtropical Agriculture, The Chinese Academy of Sciences, Changsha, China

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Edited by:
Jing Wang,
University of California, Los Angeles, United States

Reviewed by:
Duanqin Wu,
Chinese Academy of Agricultural Sciences, China
Bo Lin,
Guangxi University, China

***Correspondence:**
Bin Li
xukesuolibin@163.com
Chuanshe Zhou
zcs@isa.ac.cn
Qinghua He
qinghua.he@szu.edu.cn

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In high-altitude area, hypoxia is a serious stress for humans and other animals, disrupting oxygen homeostasis and thus affecting tissue metabolism. Up to now, there are few reports on the metabolic changes of dairy cows at different altitudes. In this experiment, metabonomics technology and blood biochemical indexes were used to study the metabolic changes of dairy cows in different altitudes. The results showed that the different metabolites were mainly enriched in amino acid metabolism and sphingolipid metabolism, and sphingolipid metabolism showed a negative correlation with increased altitude. The results of this study will enrich the hypoxia-adaptive mechanism of dairy cows in high-altitude areas and provide a theoretical basis for the nutritional regulation of performance and disease treatment of dairy cows in high-altitude areas.

Keywords: metabonomics, high altitude, Jersey cattle, difference, hypoxia

INTRODUCTION

From the medical point of view, there is no obvious geological boundary between high and low altitudes, but from the evidence of plateau-related diseases, it is generally believed that high altitude (HA) refers to the altitude of 1,500 m or above the average sea level (1). It can be divided into three levels: high altitude (1,500–3,500 m), extremely high altitude (3,500–5,500 m), and extreme altitude (>5,500 m) (2). The main challenge faced by vertebrates at high altitude is that the reduction of oxygen partial pressure (PO_2) limits the aerobic metabolic rate, which leads to metabolic adaptation to reduce oxygen demand (3). At present, the research on animal metabolism adaptability at high altitude mainly includes rats (4), pigs (5), donkeys, and sheep (6). There are few reports on the metabolic adaptation of dairy cows in high-altitude areas, and the description of metabolic differences of dairy cows between different altitudes is less.

As an important breed of dairy cattle in countries with developed animal husbandry, Jersey cattle has the characteristics of rough feeding tolerance, strong disease resistance, and strong adaptability (7). In addition, Jersey cattle's milk fat color is yellow, its fat globules are large and easy to separate, and it is an ideal raw material for processing high-quality

cream (8). Butter (mainly composed of milk fat) provides rich nutrients for residents in high-altitude areas and is an indispensable life product. What is more, it was found that the adaptability of Jersey cattle was the best among the different breeds introduced to high-altitude areas. Therefore, there is a lot of interest in Jersey cattle adapting to high altitudes.

Recent studies have found that people from low altitude to high altitude will cause significant remodeling of tissue metabolism, as well as changes in the level of circulating metabolism (9, 10). Hypoxia can inhibit the oxidative metabolism of heart (11) and skeletal muscle (12), reduce the ability of fatty acid oxidation (13), and increase glycolysis (14) in rodents and non-plateau native people. Based on miRNA and proteomics, we found that Jersey cattle adapt to high-altitude hypoxia by regulating inflammatory homeostasis (15). However, the specific metabolic adaptation mechanism of Jersey cattle transferred from low altitude to high altitude is still unclear. Metabonomics based on mass spectrometry (MS) is a method to study the overall changes of small-molecule metabolites, reflecting the physiological activities in organisms (16). In this study, metabonomics was applied to explore the metabolic changes of Jersey cattle in different altitudes. This will enrich the adaptive mechanism of Jersey cattle at high altitude and lay a theoretical basis for subsequent nutritional regulation.

MATERIALS AND METHODS

This study was carried out based on the animal protection and use guidelines of the Animal Protection Committee, Institute of Subtropical Agriculture, Chinese Academy of Sciences (protocol ISA-201809).

Animals and Experimental Design

Eighteen multiparous Jersey cattle (400 ± 28 kg) were selected and randomly divided into three groups (six cattle in each group) in Shenyang [altitude 50 m; high-altitude-free (GJ) group], Nyingchi [altitude 3,000 m; high-altitude (CJ) group], and Lhasa [altitude 3,650 m; extremely high-altitude (XJ) group] for 60 days in the autumn. Six animals were randomly selected from each group for metabonomics analysis and other measures. Based on the Chinese Feeding Standard of Dairy Cow (MOA, 2004), basic diets meeting the nutritional requirements of energy, protein, minerals, and vitamins are prepared (**Table 1**). The same TMR diet was fed *ad libitum*.

Measurement of Blood Oxygen Saturation

After the vulva of the cow was opened, and the liquid was dried with an absorbent paper. The blood oxygen saturation was measured with Nonin Avant 9600 (Nonin Medical, Inc., Plymouth, MN, USA) blood oxygen saturation detector. The sensor probe was close to the vulva skin to measure the blood oxygen saturation (BOS). The average of the three measurements is taken as the measurement value.

Blood Sample Preparation

Before the morning feeding on the last day of the experiment, all cows were punctured through the caudal vein to take blood samples. The blood samples in the anticoagulant tube were centrifuged at 3,000 rpm for 10 min at 4°C. The plasma was collected and stored in a refrigerator at −80°C for metabonomics analysis. The blood samples collected by a non-anticoagulant tube were centrifuged at 2,500 rpm for 5 min. The serum was collected and stored in a refrigerator at 4°C for determination of biochemical indexes.

TABLE 1 | The difference of plateau adaptability of dairy cows at different altitudes.

Items	Treatment			SEM	*P*-value
	GJ	CJ	XJ		
BOS (%)	90.08	84.55	72.48	1.541	0.001
NO (μmol/l)	168.03	202.62	184.45	6.571	0.006
NOS (U/ml)	1,607.45	1,863.94	1,744.45	44.636	0.004
EPO (mU/ml)	4,838.19	5,254.33	5,689.11	144.903	0.003
HSP70 (ng/ml)	19.34	19.81	20.13	0.233	0.039
HIF-1 (ng/l)	138.33	165.44	193.66	13.501	0.018
VEGF (ng/ml)	167.05	185.44	207.32	7.659	0.006

BOS, blood oxygen saturation; NO, nitric oxide; NOS, nitric oxide synthase; TRP, total reactive protein; EPO, erythropoietin; HSP70, heat shock protein 70; VEGF, vascular endothelial growth factor; HIF, hypoxia-inducible factor; GJ, high-altitude-free group; CJ, Nyingchi (altitude 3,000 m; high-altitude group); XJ, Lhasa (altitude 3,650 m; extremely-high-altitude group).

High-Altitude Adaptation Index Determination

The levels of nitric oxide (NO), nitric oxide synthase (NOS), total reactive protein (TRP), erythropoietin (EPO), heat shock protein 70 (HSP70), vascular endothelial growth factor (VEGF), and hypoxia-inducible factor (HIF) in serum were measured by ELISA kit.

Metabolite Extraction

Firstly, the 100 μl plasma obtained by centrifugation was mixed with 300 μl methanol (including internal standard 1 μg/ml), vortexed for 30 s, sonicated in ice bath for 10 min, and incubated at −20°C for 1 h to precipitate protein. Secondly, to process the sample, it was centrifuged at 12,000 rpm for 15 min at 4°C. Finally, the supernatant was transferred to a liquid chromatography–mass spectrometry (LC/MS) sample bottle at −80°C for storage and standby and used for the analysis of UHPLC-QE Orbitrap/MS. Quality control (QC) samples were prepared by mixing the same supernatant from all samples.

Liquid Chromatography With Tandem Mass Spectrometry Analysis

A liquid chromatography with tandem mass spectrometry (LC–MS/MS) analysis was performed using a UHPLC system (1290, Agilent Technologies, Santa Clara, CA, USA) coupled with a UPLC HSS T3 column (2.1 mm × 100 mm, 1.8 μm) with Q Exactive (Orbitrap MS, Thermo Fisher Scientific, Waltham, MA, USA). Mobile phase A was positive in 0.1% formic acid aqueous solution and negative in 5 mmol/l ammonium acetate aqueous solution, and mobile phase B was acetonitrile. The

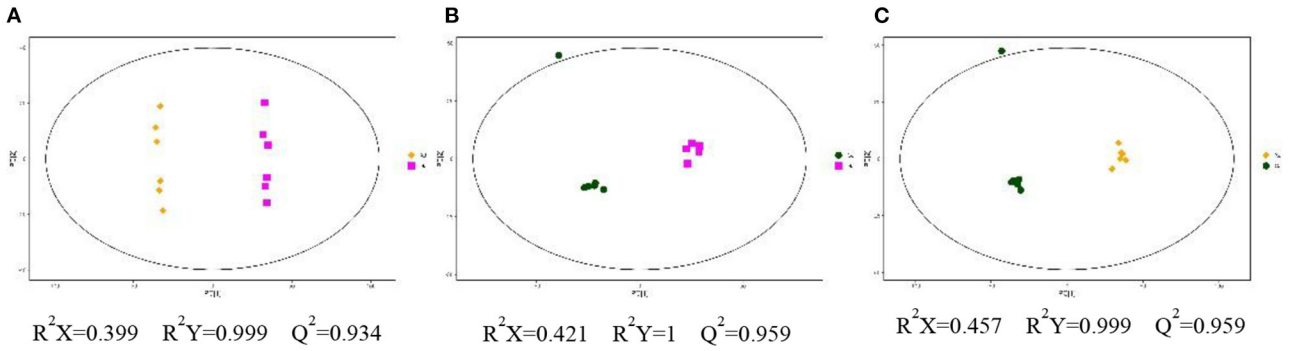

FIGURE 1 | PLS-DA score plot of the three groups in plasma of Jersey cattle using the identified metabolites in positive ionization mode. **(A)** The difference of CJ vs. GJ; **(B)** the difference of XJ vs. GJ; **(C)** the difference of CJ vs. XJ.

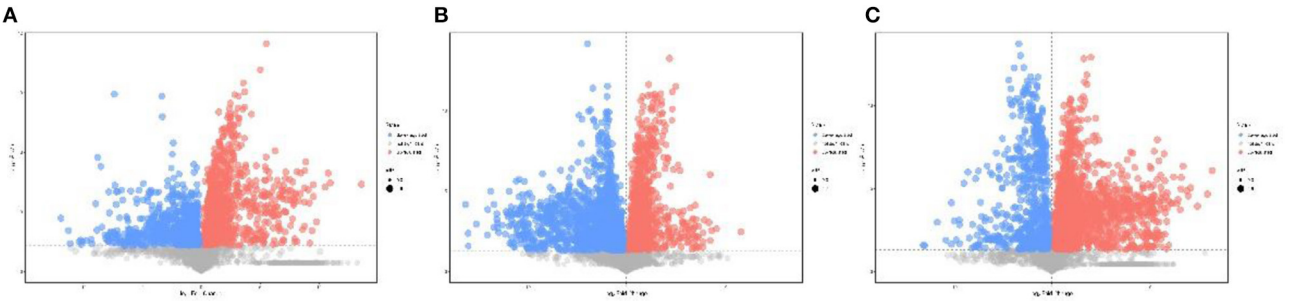

FIGURE 2 | Volcanic plots of the three groups in plasma of Jersey cattle using the identified metabolites in positive ionization mode. **(A)** The results of CJ vs. GJ; **(B)** the results of XJ vs. GJ; **(C)** the results of CJ vs. XJ.

elution gradient was set as following: 0 min, 1% B; 1 min, 1% B; 8 min, 99% B; 10 min, 99% B; 10.1 min, 1% B; and 12 min, 1% B. The flow rate was 0.5 ml/min. The injection volume is 3 μl. In LC/MS experiments, QE mass spectrometer can obtain MS/MS spectra on an information-dependent basis (IDA). In this mode, the acquisition software (Xcalibur 4.0.27, Thermo Fisher Scientific, Waltham, MA, USA) continuously evaluates full-scan measured MS data while collecting and triggering MS/MS spectral acquisition according to pre-selected criteria. ESI source conditions were set as follows: sheath gas flow rate of 45 arb, auxiliary gas flow rate of 15 arb, capillary temperature of 400°C, full MS resolution of 70,000, MS/MS resolution of 17,500, impact energy of 20/40/60 eV, and injection voltage of 4.0 kV (positive) or −3.6 kV (negative).

Statistical Analysis

ProteoWizard was used to convert the original data into mzXML format and processed by MAPS software (version 1.0). A data matrix consisting of retention time (RT), mass/charge ratio (M/Z), and peak strength was generated. The internal MS2 database was used for metabolite identification. The card value standard of differential metabolites was that a p-value of Student's t-test was <0.05. Meanwhile, the variable importance in the projection (VIP) of the first principal component of OPLS-DA model is >1.

RESULTS

Physiological and Biochemical Characteristics

The difference of plateau adaptability of dairy cows at different altitudes is shown in **Table 1**. Compared with cows at low altitude (GJ), blood oxygen saturation of cows at high altitude (CJ) and extremely high altitude (XJ) was significantly lower ($P < 0.05$), and blood oxygen saturation of cows at extremely high altitude was significantly lower ($P < 0.05$) than that of cows at high altitude. In addition, the level of NO, NOS, EPO, HSP70, HIF-1, and VEGF decreased ($P < 0.05$) significantly with the increase of altitude.

Overview of Differential Metabolomic Profiles

In positive ionization mode, compared with the GJ group, a total of 105 and 103 differential metabolites (VIP > 1, $p < 0.05$) were found in the CJ and XJ groups (**Supplementary Table 1**). In addition, 124 differential metabolites were identified in the CJ group compared with the XJ group (**Supplementary Table 1**). Partial least-squares discriminant analysis (PLS-DA) was performed to obtain a global overview of the differences in metabolites among the three groups (**Figure 1**). The R^2Y and Q^2 values of the PLS-DA models are all above 0.93. The above

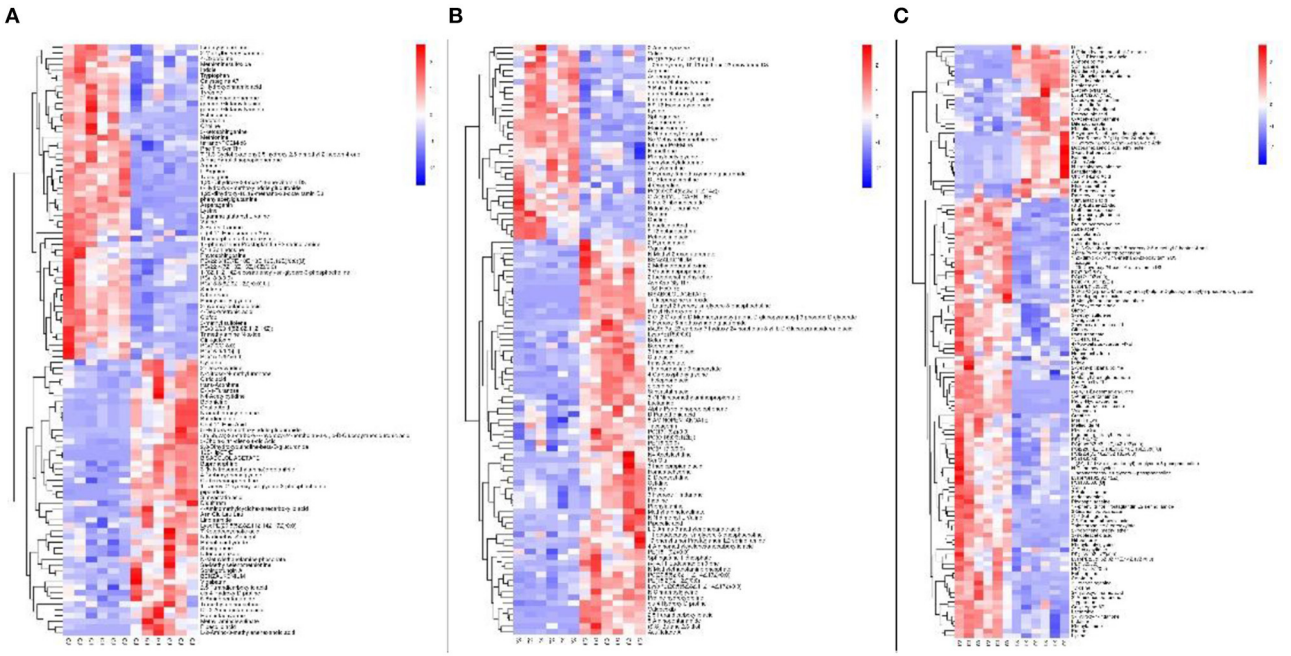

FIGURE 3 | Heatmap of hierarchical clustering analysis for the three groups in palsma of Jersey cattle using the identified metabolites in positive ionization mode. **(A)** The clustering results of CJ vs. GJ; **(B)** the clustering results of XJ vs. GJ; **(C)** the clustering results of CJ vs. XJ.

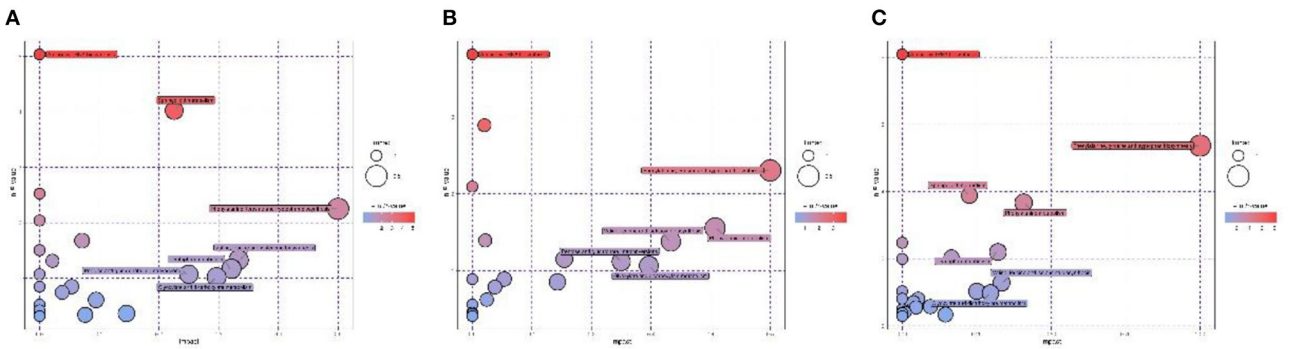

FIGURE 4 | Pathway analysis of the identified metabolites in positive ionization mode in plasma of Jersey cattle exposed to different altitudes. **(A)** The pathway analysis results of CJ vs. GJ; **(B)** the pathway analysis results of XJ vs. GJ; **(C)** the pathway analysis results of CJ vs. XJ.

results indicate that exposure to various altitudes can interfere with the metabolism of dairy cows, which is also supported by the observation results of volcanic plots (**Figure 2**).

Comparison of Metabolomic Profiles Among the Three Altitudes

In positive ionization mode, the heat map constructed from the 105, 103, and 124 differential metabolites revealed two diverse metabolomic profiles of various altitudes (**Figure 3**). Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analysis indicated that up-regulated metabolites in the CJ group were mainly enriched in aminoacyl-tRNA biosynthesis; sphingolipid metabolism; phenylalanine, tyrosine, and tryptophan biosynthesis; and valine, leucine, and isoleucine biosynthesis (**Figure 4A**). Up-regulated metabolites in the XJ group were enriched in aminoacyl-tRNA biosynthesis; phenylalanine, tyrosine, and tryptophan biosynthesis; phenylalanine metabolism; and valine, leucine, and isoleucine biosynthesis (**Figure 4B**). In addition, compared with the XJ group, the up-regulated pathways were mainly aminoacyl-tRNA biosynthesis; phenylalanine, tyrosine, and tryptophan biosynthesis; sphingolipid metabolism; and phenylalanine metabolism in the CJ group (**Figure 4C**). The details of all enriched pathways are shown in **Supplementary Table 2**.

Key Metabolite Identification

Metabolites that could be utilized to distinguish the CJ group from the XJ group were identified on the basis of VIP >

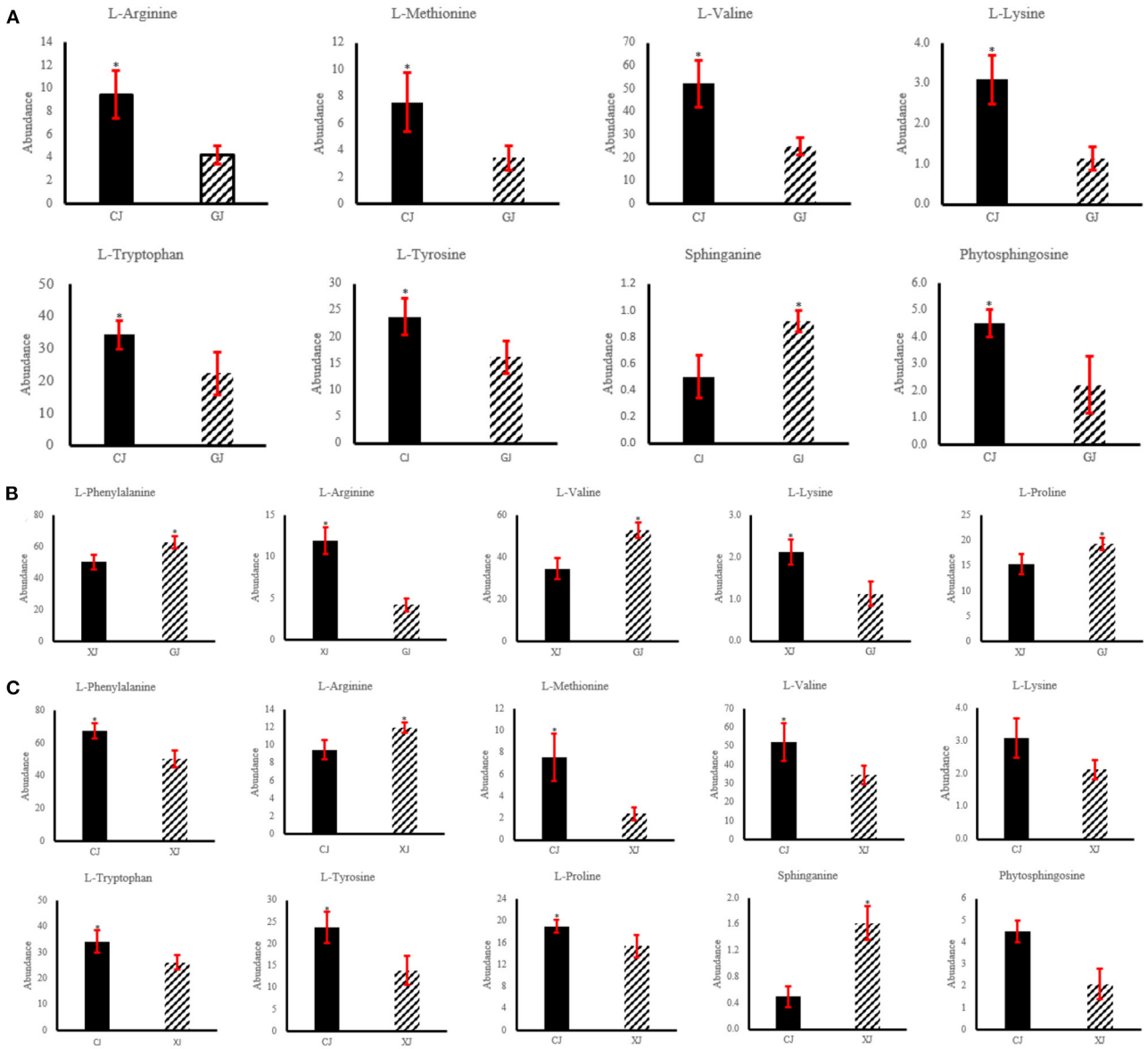

FIGURE 5 | Differential metabolite abundances in plasma of Jersey cattles at different altitudes. **(A)** The abundance of identified differential metabolites for CJ VS GJ in positive ionization mode. "CJ and GJ" represent high altitude and control group, respectively; **(B)** the abundance of identified differential metabolites for XJ VS GJ in positive ionization mode. "xJ and GJ" represent extremely high altitude and control group, respectively; **(C)** the abundance of identified differential metabolites for CJ VS XJ in positive ionization mode. $n = 6$, $^*p < 0.05$.

1, FC > 1, and $p < 0.05$. L-methionine, L-tryptophan, L-arginine, and L-lysine in the aminoacyl-tRNA biosynthesis pathway; L-tyrosine in phenylalanine; tyrosine and tryptophan biosynthesis and L-valine in valine; leucine; and isoleucine biosynthesis showed higher abundance ($P < 0.05$) in the CJ group than in the GJ and XJ groups (**Figure 5** and **Table 2**). Phytosphingosine in sphingolipid metabolism showed higher abundance ($P < 0.05$) in the CJ group than in the GJ and XJ groups (**Figures 5A,C** and **Table 2**), while sphinganine in sphingolipid metabolism and L-proline in aminoacyl-tRNA biosynthesis and L-phenylalanine in phenylalanine, tyrosine, and tryptophan biosynthesis showed lower abundance ($P < 0.05$) in the XJ group than in the GJ group (**Figure 5B** and **Table 2**). In addition, L-proline in aminoacyl-tRNA biosynthesis and L-phenylalanine in phenylalanine, tyrosine, and tryptophan biosynthesis showed higher abundance ($P < 0.05$) in the CJ group than in the XJ group (**Figure 5C** and **Table 2**).

DISCUSSION

The oxygen partial pressure in the atmosphere is 60% that of the sea level. The dairy cows imported into the area under low oxygen environment have a higher incidence rate of altitude sickness. Since the beginning of the last century, the introduction of

TABLE 2 | Differentially expressed metabolites of different groups.

Group	MS2 name	VIP	*P*-value	Fold change
CJ vs. GJ	L-Methionine	1.6063	0.0003	2.1971
	L-Arginine	1.8007	0.0000	2.8363
	L-Valine	1.6853	0.0001	2.0993
	L-Lysine	1.7777	0.0000	2.7309
	L-Tryptophan	1.5732	0.0006	1.5375
	L-Tyrosine	1.3827	0.0056	1.4700
	Phytosphingosine	1.3112	0.0424	2.0365
	Sphinganine	1.3867	0.0119	0.5381
XJ vs. GJ	L-Arginine	1.5491	0.0001	2.2398
	L-Valine	1.1395	0.0213	1.3945
	L-Lysine	1.4544	0.0003	1.8684
	L-Proline	1.0817	0.0198	0.7967
	L-Phenylalanine	1.1146	0.0189	0.8009
CJ vs. XJ	L-Phenylalanine	1.3532	0.0008	1.3407
	L-Arginine	1.0112	0.0019	1.9071
	L-Methionine	1.5442	0.0003	3.1485
	L-Valine	1.3466	0.0013	1.5054
	L-Lysine	1.3119	0.0012	1.4616
	L-Tryptophan	1.2364	0.0072	1.3042
	L-Tyrosine	1.5141	0.0003	1.7095
	L-Proline	1.1753	0.0080	1.2348
	Phytosphingosine	1.2049	0.0359	2.1468
	Sphinganine	1.5114	0.0000	0.3056

GJ, high-altitude-free group; CJ, Nyingchi (altitude 3,000 m; high-altitude group); XJ, Lhasa (altitude 3,650 m; extremely-high-altitude group).

Holstein dairy cows from the mainland in Tibet has failed. Later, due to the strong adaptability of Jersey cattle, we introduced Jersey cattle and achieved good results. Our previous research explored its adaptive mechanism from the miRNA and proteome level (15). This paper further elaborated its good adaptive mechanism from the metabolic level, as well as the adaptive differences at different altitudes.

The results showed that the blood oxygen saturation of Jersey cattle in high-altitude and very-high-altitude areas decreased with the increase of altitude, which may be due to the functional damage of lung tissue caused by high altitude, resulting in the decrease of lung oxygen supply and the obstruction of carbon dioxide exhalation, which eventually led to more severe hypoxia in dairy cows with the increase of altitude (17). Hypoxia can promote the production of vasodilator *in vivo*, and vasodilator can inhibit the formation of vascular injury and pulmonary hypertension caused by hypoxia (18). NO is a very effective vasodilator (19), which can promote vasodilation, increase blood flow velocity, and prevent vascular remodeling caused by hypoxia (20). Studies have shown that hypoxia can increase the expression of NOS, which can be used to explain the findings of this experiment that the level of NOS increases with altitude (21). In addition, the level of NO also increased with the elevation, which may be due to the increase of the expression of nitric oxide, which is the core mechanism of mammalian adaptation to hypoxia (22, 23). However, the level of NO and NOS was higher in the CJ group than in the XJ group, which might resulted from downregulation of arginine and proline metabolism leading to the production of oxidative stress (24). The results showed that the expression of HIF was up-regulated under hypoxia (25), which was consistent with the results of this experiment. HIF can increase the expression of EPO after it enters the cells (26), so it can adapt to high-altitude hypoxia by increasing the oxygen-carrying capacity of the body (27). Therefore, the higher the altitude is, the higher the level of EPO is, as was shown in our results. VEGF is recognized as the most typical target gene of HIF-1 α (28). In this study, it was found that HIF-1 was up-regulated with the increase of altitude, which may be because HIF-1 regulates VEGF to promote the formation of blood vessels to adapt to hypoxia (29). In addition, studies have shown that Hsp70 can protect against tissue hypoxia and organ damage by degrading HIF-1 α activity under hypoxia (30), which leads to the increase of HSP70 level with altitude. To sum up, we can find that dairy cows will show varying degrees of adaptive physiological response with the increase of altitude.

In this study, we also predicted the involved pathways affected by altitude differences by using KEGG analyses. The differential metabolites involved in aminoacyl-tRNA biosynthesis were L-arginine, L-tryptophan, L-lysine, L-methionine, and L-proline. In this study, we found that the level of L-arginine increased with altitudes, and its rule of change is the same as that of NO (31), which might resulted from NO that could be synthesized from L-arginine (32). During hypoxia exposure, L-tryptophan levels in tissues increased significantly, which may be caused by blocked energy metabolism (33). Previous studies have shown that hypoxia can lead to the increase of lysine and methionine concentration (34), and it is consistent with the results of this experiment that lysine and methionine concentration increases at high altitude and very high altitude, which may be due to a disturbance of osmotic balance associated with hypoxia (35). A recent metabolic study has shown that a variety of amino acids, including proline, may be involved in the regulation of intracellular osmotic pressure during environmental hypoxia and may act as osmotic fluid (36), which might indicate that proline adapts to hypoxia as an osmolyte. These results suggested that dairy cows adapted to different altitudes by regulating the metabolic pathway of aminoacyl-tRNA biosynthesis.

In the present work, the pathway of valine, leucine, and isoleucine biosynthesis was both up-regulated in the CJ and XJ groups. Studies found that hypoxia contributed to the accumulation of L-valine (37), which was consistent with the results in the CJ group. However, there was lower abundance of L-valine in the XJ group, which might resulted from the application of valine to avoid mitochondrial damage or convert to other amino acids (38, 39). The differentially expressed metabolites of phenylalanine, tyrosine, and tryptophan biosynthetic signaling pathways in the CJ and XJ groups were tyrosine and phenylalanine, respectively. In the CJ group, the level of tyrosine increased, which might be due to the replenishment of insufficient energy supply to adapt to hypoxia stress (33). However, in the XJ group, the level of phenylalanine was reduced, which may be due to the more severe immune and inflammatory responses in cows at extremely high altitude

(15), and phenylalanine needs to be converted into tyrosine to regulate oxidative stress, immune response, and inflammation, thus protecting the body from damage (40). These results suggest that dairy cows adapt to high-altitude hypoxia by upregulating phenylalanine metabolism and phenylalanine, tyrosine, and tryptophan biosynthetic signaling pathways.

Phytosphingosine and sphingosine are two important metabolites involved in sphingolipid metabolism. The results of this study showed that sphingolipid metabolism pathway was up-regulated under hypoxia. In the presence of hypoxia, elevated levels of plant sphingosine in the blood of cows in the CJ group (41) were found in this experiment, which might be caused by changes in key enzymes regulating sphingosine metabolism (42). The decreased level of sphingosine may be due to the conversion of blood sphingosine into plant sphingosine (43), which regulates angiogenesis in response to hypoxia stress (44). Additionally, we found that the sphingolipid metabolism pathway was up-regulated in dairy cows at high altitude compared with those at extremely high altitude, suggesting that sphingolipid metabolism might be negatively correlated with the adaptability to elevated altitude (42).

CONCLUSION

In this experiment, we detected the related indexes of high-altitude adaptation in Jersey dairy cows in the GJ, CJ, and XJ groups, which replenished the basic data of blood biochemical indexes of Jersey dairy cows from different altitudes. At the same time, it was found that Jersey cows can adapt to high-altitude hypoxia mainly through up-regulation of amino acid metabolism and sphingolipid metabolism. Additionally, it was found that the metabolism of sphingolipid was negatively correlated with the ability to adapt to hypoxia induced by elevated altitudes.

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 author/s.

ETHICS STATEMENT

The animal study was reviewed and approved by the animal protection and use guidelines of Animal Protection Committee, Institute of Subtropical Agriculture, Chinese Academy of Sciences.

AUTHOR CONTRIBUTIONS

CZ, ZK, QH, and ZT made significant contributions in the conceptualization of the study. ZK, BL, and YZ made significant contributions in the analysis of data. ZK made significant contributions in the data curation and original draft preparation. CZ and ZT made significant contributions in the review and editing of the manuscript. CZ and BL made significant contributions in the funding acquisition. All authors have read and approved the final manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fvets.2021.713913/full#supplementary-material

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Energy and Protein Requirements for the Maintenance of Growing Male Sika Deer (*Cervus nippon*)

*Kun Bao, Xiaoxu Wang, Kaiying Wang, Guangyu Li and Hanlu Liu**

Institute of Special Animal and Plant Sciences, Chinese Academy of Agricultural Sciences, Changchun, China

The objective of this study is to study the effects of dietary intake levels on energy metabolism, carbon (C), and nitrogen (N) balance and to determine the maintenance requirements of energy and protein for male sika deer during their growing period. A total of 16 1-year-old male sika deer with similar body weight (BW) (63.25 ± 2.42 kg) were selected, with four animals per feed intake level. The feeding levels of the four groups for deer were 40, 60, 80, and 100% of the recommended amount, respectively. The nutrient digestibility and methane production were measured through digestion trials and respiratory trials. A 4 × 4 Latin Square design was performed in a respirometry trial. The results show that the apparent digestibility of C and N gradually increased as the level of feed intake decreased. Furthermore, with a decrease in feed intake level, the metabolic energy intake (MEI), heat production (HP), and retained energy (RE) of male sika deer significantly decreased ($P < 0.01$). The requirements of metabolic energy for maintenance (MEm) and net energy for maintenance (NEm) of growing deer are 251.17 and 223.62 kJ $kg^{-1}BW^{0.75}d^{-1}$, respectively, as estimated according to the logarithmic regression equations between HP and MEI. The net N requirement for maintenance (NNm) and net protein requirement for maintenance (NPm) of growing male sika deer based on the linear relationship between retained nitrogen (RN) and daily nitrogen intake (NI) were 251.8 mg $kg^{-1}BW^{0.75}d^{-1}$ and 1.57 g $kg^{-1}BW^{0.75}d^{-1}$, respectively. The NEm and NPm values obtained from this experiment fill the gap in net energy and protein requirements and serve as basic data for establishing the nutritional standards forsika deer breeding in China.

Keywords: carbon and nitrogen balance, maintenance requirement, methane emission, net energy, net protein

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Yehui Duan,
Chinese Academy of Sciences, China

Reviewed by:
Peng Liao,
Chinese Academy of Sciences, China
Xuezhuang Wu,
Anhui Science and Technology University, China
Qingkui Jiang,
Rutgers University, Newark, United States

****Correspondence:***
Hanlu Liu
liuhanlu2003@163.com

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INTRODUCTION

Sika deer (*Cervus nippon*) produce traditional Chinese medicine velvet antler and thus they are important ruminants in China. The nutritionals level of domestic sika deer are mainly drawn from foreign nutritional standards, such as NRC (1). However, since China has a vast territory, rich pasture resources, and many deer species, it is actually unreasonable to deal with all situations using a foreign standard. Moreover, the nutritional requirements for male sika deer during their growing period have not been fully determined in China, which limits the efficient development of sika deer industry. Therefore, it is essential to study the nutritional requirements of sika deer to improve production performance and ensure efficient utilization of feed.

To study the energy and protein maintenance requirements of 1-year-old male sika deer, carbon-nitrogen (C-N) balance method was used in this study, as well as the measurement of methane emissions through an open-circuit respiration measurement system. The C-N balance method has been used to calculate retained energy (RE), assuming that all energy is retained in the form of fat or protein (2). Therefore, this study further explores the effects of feeding levels on energy metabolism, C-N balance, and methane emission and uses the C-N balance method to determine the maintenance energy and protein requirements of male sika deer during the growing period.

MATERIALS AND METHODS

The study was carried out in the antler deer breeding base of the Institute of Special Animal and Plant Science, CAAS. All experiments were performed in accordance with the Animal Care and Use Guidelines of the Institute of Special Animal and Plant Science (Jilin, China).

Animals and Treatments

A total of 16 1-year-old male sika deer with similar body weight (BW; 63.25 ± 2.42 kg) were selected, with four animals per intake level. The deer were divided into four treatment groups, and the experiment was carried in four experimental stages. Each stage lasted for 12 days. The feeding levels of the four groups for deer were 40, 60, 80, and 100% of the recommended amount, respectively, following the nutrition requirements of feed for deer (1). To reduce the inaccuracy of the test data caused by the deer' body conditions, the deer were given a rest for 5 days and fed a normal nutrition level diet after each stage. The deer were fed two equal meals at 06:30 and 15:30 daily, and they can drink freely. The dietary composition and nutritional content of basal diets are shown in **Table 1**.

TABLE 1 | Composition and nutritive levels of control diet.

Parameter	Concentration
Composition (%)	
Corn flour	22
Soybean meal	12
Lucerne	50
Distillers dried grains with soluble (DDGS)	4
Corn germ meal	5.5
Molasses	5
NaCl	0.5
Conjugated linoleic acid	0.5
Additives*	0.5
Total	100
Measured nutrient concentration(dry matter)	
Gross energy (GE,MJ/kg)	14.03
Crude protein (CP, %)	15.80
Neutral detergent fiber (NDF, %)	41.54
Ether extract (EE, %)	3.31
Acid detergent fiber (ADF, %)	16.16
Ca (%)	0.76
P (%)	0.50

**Contained the following per kg of premix: Mg, 76 mg; Cu, 36 mg; Zn, 43 mg; Fe, 53 mg; vitamin A,2484 IU; vitamin D_3,496.8 IU; vitamin K_3, 0.23 mg; vitamin B_1,10.092 mg; vitamin B_2,0.69 mg; vitamin B_{12},1.38 mg; folic acid, 0.023 mg; nicotinic acid, 1.62 mg; calcium pantothenate, 1.15 mg; $CaHPO_4$,5.17 g; $CaCO_3$,4.57 g.*

Digestibility Trials

The digestibility of nutrients was measured by digestion trials. Deer were weighed at the beginning and end of the collection period. The feed refusals and feces were collected and weighed every day. Feces were collected every day for 3 days, stored at −20°C, then mixed and sampled again before chemical analysis, and dried at 65°C. Urine was collected in a bucket containing 20 mL of 10% concentrated sulfuric acid to avoid loss of nitrogen from urine. All the collected urine was weighed, and 3% of the daily urine output was sampled for analysis.

Gas Metabolism

Four open-circuit respiration calorimetry chambers with a volume of 17.82 m^3 (450 × 180 × 220 cm) were used in this study. In short, air conditioners and heaters were used to regulate the respiration chambers to maintain a constant temperature and humidity. A vacuum pump was used to continuously extract gas from the respiration chambers. The gas concentration in each respiration chamber was measured using an analyzer at a 3-min interval. O_2 was measured with a zirconia sensor, while CO_2 and CH_4 were measured with a non-dispersive infrared sensor in the analyzer.

The concentration of CH_4, CO_2, and O_2 was measured according to the method proposed by Tovar-Luna (3). Air was first analyzed for CH_4, followed by CO_2 and O_2. Before each test, analyzers were calibrated with standard gas mixtures (19.5% and 20.5% O_2, 0.0% and 1.5% CO_2, and 0.0% and 0.3% CH_4). The temperature and humidity in the calorimetry room were maintained at 20–23°C using an air conditioner at 50–55% using a dehumidifier, respectively (Whirlpool, Benton Harbor, MI).

An open-circuit respiratory heat measurement system was utilized at Deer Breeding Base of the Institute of Special Animal and Plant Science, CAAS (Jilin, China). A 4 × 4 Latin Square design was performed. Four deer were selected, with one deer put into one metabolism bin. After the 24-h adaptation period, CH_4 and CO_2 production of the individuals was measured for 24 consecutive hours. To avoid stress response in the deer in the metabolic cage, all animals had been trained previously. To reduce the inaccuracy of the test data caused by the deer' body conditions, the deer were given a rest for 5 days and fed a normal nutrition level diet after each stage.

Chemical Analyses

The content of dry matter (DM), ash, Ca, and P in the feed, orts, and feces were analyzed by the method of AOAC (4). The concentrations of neutral detergent fiber (NDF) and acid detergent fiber (ADF) were determined according to the method described by Van Soest et al. (5). A bomb calorimeter (IKA C200,

TABLE 2 | Effect of feed intake on body weight, dry matter intake (DMI), and energy balance in the digestion and respirometry tiral.

Item[a]	Feed level				*P*-value
	100%	80%	60%	40%	
DMI (kg d^{-1})	1.90 ± 0.12[a]	1.72 ± 0.25[b]	1.14 ± 0.13[c]	0.76 ± 0.06[d]	<0.001
BW[1] (kg)	64.26 ± 3.87	62.15 ± 4.36	59.83 ± 3.83	56.43 ± 4.17	<0.001
GE[1] intake (kJ $kg^{-1}BW^{0.75}d^{-1}$)	1174.51 ± 109.56[a]	1080.20 ± 103.21[b]	743.48 ± 78.31[c]	517.89 ± 57.62[d]	<0.001
Fecal energy (FE) (kJ $kg^{-1}BW^{0.75}d^{-1}$)	385.24 ± 15.87[a]	339.32 ± 21.56[b]	206.09 ± 20.31[c]	118.79 ± 12.57[d]	<0.001
Urinary energ y (UE) (kJ $kg^{-1}BW^{0.75}d^{-1}$)	36.40 ± 3.51[a]	34.01 ± 2.87[b]	23.35 ± 3.56[c]	16.87 ± 2.91[d]	<0.001
Methane energy (kJ kg^{-1} $BW^{0.75}d^{-1}$)	76.34 ± 7.31[a]	73.04 ± 8.52[a]	55.91 ± 7.63[b]	43.01 ± 5.45[c]	0.035
Methane energy/GE (%)	6.48 ± 1.13[a]	6.69 ± 0.89[a]	7.52 ± 1.25[b]	8.32 ± 1.37[c]	0.631
DE[1] (kJ kg^{-1} $BW^{0.75}d^{-1}$)	769.27 ± 20.51	745.88 ± 25.87	537.39 ± 24.01	399.10 ± 18.75	<0.001
MEI[1] (kJ kg^{-1} $BW^{0.75}d^{-1}$)	752.87 ± 35.05[a]	686.87 ± 28.67[a]	434.04 ± 20.55[b]	286.23 ± 18.96[c]	<0.001
DE/GE (%)	65.50 ± 7.82[a]	68.98 ± 8.31[a]	72.27 ± 7.69[b]	77.18 ± 7.53[c]	0.023
ME/GE (%)	64.10 ± 6.55	65.46 ± 7.69	69.18 ± 5.81	74.27 ± 7.13	0.045
ME/DE (%)	95.38 ± 9.66	95.29 ± 8.75	94.89 ± 8.94	94.44 ± 9.02	0.532

[1]*BW, body weight; GE, gross energy; DE, digestible energy; ME, metabolisable energy; MEI, ME intake.*
[a–d]*In the same row, values without a different superscript differ (P < 0.05).*

Germany) was used to measure gross energy (GE) in diets and feces. The GE of urine samples was measured with the method described by Deng et al. (6, 7). Carbon and nitrogen content in the feed, orts, feces, and urine was estimated in a C-N analyzer (Elementary Vario MAX CN, Germany).

Data Calculation

Metabolizable Energy

The content of metabolizable energy (ME) in the diet was calculated based on the data obtained from the digestion trials. The difference between GE intake and fecal energy was thought to be digestible energy (DE). The ME of the diet with four feeding levels was obtained by subtracting CH_4 energy and urinary energy from DE. Energy equivalent of CH_4 was 39.54 kJ L^{-1} (8).

Carbon and Nitrogen Balance

In the C-N method, it is assumed that all energy is retained in the form of fat or protein (9), and the RE is determined based on the analysis of the C-N balance. C balance is the total amount of C retained in the body, and the amount of C retained in fat can be calculated by subtracting the amount of C retained in protein determined by N balance from the C balance. Assuming that fat has an energy equivalent of 39.76 kJ g^{-1} and contains 0.767 C and protein has an energy equivalent of 23.86 kJ g^{-1} and contains 0.16 N and 0.52 C, the RE in fat (REfat) and protein (REprotein) can be calculated. Energy retained as fat (REf) and protein (REp) can be calculated as follows:

REp = N balance (g) × 6.25 × 23.86;
REf = (C balance (g) - N balance (g) ×6.25×0.52) × 1.304 × 39.76 (2).
RE can be calculated as RE = REp + REf according to Brouwer (8).

Requirements of Energy and Protein for Maintenance

The difference between the metabolic energy intake (MEI) and the retained energy was thought to be heat production (HP). According to the method described by Lofgreen and Garrett (10), the antilog of the linear regression intercept between the HP and MEI logarithms was used to estimate the net energy demand for maintenance (NEm, kJ kg^{-1} $BW^{0.75}$) of male sika deer. According to the method proposed by Galvani et al. (11), the ME requirement for maintenance (MEm, kJ $kg^{-1}BW^{0.75}$) was calculated by iterating the semi-logarithmic linear regression equation until HP was equal to MEI. The maintenance efficiency (km) of ME was calculated as NEm/MEm.

The net protein requirement for maintenance was estimated by a linear regression equation between the daily retained N (RN; mg kg^{-1} $BW^{0.75}$) and the daily N intake (NI, g kg^{-1} $BW^{0.75}$). The intercept of this regression equation represents the loss of endogenous and metabolic N. The result of multiplying the loss by factor 6.25 was assumed to be the net protein requirement for maintenance (NPm, g kg^{-1} $BW^{0.75}$).

Statistical Analysis

Data were presented as means ± SD. The effects of feeding levels on the apparent digestibility of nutrients, energy values, energy balance, carbon-nitrogen balance, CH_4, and CO_2 emissions were analyzed using PROC GLM of SAS 8.0 (SAS Institute, Inc.; Cary, NC). The differences among the treatments were considered statistically significant with $P < 0.05$.

RESULTS

DM Intake and Energy Balance

The effects of feed intake on BW, dry matter intake (DMI), and energy balance in the digestion and respirometry trials are present in **Table 2**. Different feeding levels had a significant impact on DM intake ($P < 0.05$). The feed levels significantly affected the BW of deer. The BW decreased significantly with the decrease of feed levels. As feed intake increased, total energy intake (GEI), fecal energy (FE), urine energy (UE), DE, ME, and methane energy significantly decreased ($P < 0.05$), while DE/GE,

TABLE 3 | Effect of feed intake on daily methane (CH_4) and carbon dioxide (CO_2) emissions.

Item[1]	Feed level				*P*-value
	100%	80%	60%	40%	
CH_4 emission					
L d^{-1}	61.26 ± 6.13[a]	50.15 ± 6.03[b]	41.82 ± 3.97[c]	30.53 ± 3.66[d]	0.012
L kg^{-1} $BW^{0.75}d^{-1}$	2.70 ± 0.38[a]	2.27 ± 0.42[a]	1.89 ± 0.35[b]	1.77 ± 0.31[c]	0.021
L kg^{-1}DMI	29.24 ± 3.59[a]	31.16 ± 3.15[a]	35.81 ± 3.52[b]	48.07 ± 5.03[c]	0.035
CO_2 emission					
L d^{-1}	610.50 ± 50.21[a]	530.25 ± 48.33[b]	480.36 ± 36.53[c]	389.55 ± 36.21[d]	<0.001
L kg^{-1} $BW^{0.75}d^{-1}$	26.92 ± 3.27[a]	23.94 ± 2.46[b]	22.33 ± 2.08[b]	18.93 ± 1.88[c]	0.038
L kg^{-1}DMI	308.32 ± 29.56[a]	321.28 ± 28.57[b]	430.06 ± 35.41[c]	512.57 ± 49.32[d]	<0.001

[1]*BW, body weight; DMI, dry matter intake.*
[a–d]*In the same row, values without a different superscript differ ($P < 0.05$).*

methane energy/GE significantly increased ($P < 0.05$). There was no significant difference between ME/GE and ME/DE when the deer experienced different levels of diets ($P > 0.05$).

CH_4 and CO_2 Emissions

The effects of feed intake level on CH_4 and CO_2 emissions are shown in **Table 3**. Feed intake level significantly affected CH_4 emission. CH_4 emission (L d^{-1}; L kg^{-1} $BW^{0.75}$ d^{-1}) decreased ($P < 0.05$) as the feeding level decreased in the growing period. CH_4 emission (L kg^{-1} DMI) showed an opposite trend ($P < 0.05$).

At the same time, feed intake level also produced significant effects on CO_2 emission. The CO_2 emission (L d^{-1}; L kg^{-1} $BW^{0.75}$ d^{-1}) decreased ($P < 0.05$) as the feeding level decreased. However, CO_2 emission (L kg^{-1} DMI) showed an opposite trend ($P < 0.01$).

C-N Balance

Table 4 shows the effects of feed intake level on daily C-N balance, retained energy, and heat production, fecal C, urinary, and retention C were significantly affected by the level of feed intake ($P < 0.05$). The above indicators showed a significant downward trend. The content of CO_2-C and CH_4-C significantly decreased ($P < 0.05$) as feed intake level decreased, but the apparent digestibility of C (digestible C) was not affected by the feed intake level ($P > 0.05$).

RE and HP significantly decreased with a decrease in feed intake ($P < 0.05$), so did the energy retention components REp and REf. NI, FN, UN, RN, and protein deposited significantly decreased ($P < 0.05$) as feed intake level decreased, but the apparent digestibility of N had no difference at different feeding levels ($P > 0.05$).

Nutrient Apparent Digestibility

The effects of feed intake level on the apparent digestibility of dry matter (DM), organic matter (OM), acid detergent fiber (ADF), and neutral detergent fiber (NDF) are shown in **Table 5**. As the feed intake decreased, the digestibility of DM, OM, ADF, and NDF significantly increased ($P < 0.05$).

Requirements of Energy and Net Protein for Maintenance

The estimated values of MEm, NEm, and NEm/MEm (Km) are shown in **Table 6**, and the linear relationship between logHP and MEI is also shown in **Figure 1**. The NEm value of the male sika deer was determined to be 223.62 kJ kg^{-1} $BW^{0.75}d^{-1}$ by calculating the antilog of the regression intercept, and the MEm value was calculated to be 251.17 kJ kg^{-1} $BW^{0.75}d^{-1}$ through iteration of the regression equation between Log HP on MEI until HP is equal to MEI. Meanwhile, the Km (NEm/MEm) value was calculated to be 0.89.

The linear relationship between RN and NI is shown in **Table 7**, **Figure 2**. Endogenous and metabolic loss of N was calculated as 251.8 mg kg^{-1} $BW^{0.75}d^{-1}$ by estimating the intercepts of the linear regression of RN on NI. The NPm value was calculated to be 2.045 g kg^{-1} $BW^{0.75}d^{-1}$ for growing male sika deer.

DISCUSSION

Energy Balance and C-N Balance

The CH_4 emission rate is a key factor used to assess the potential degree of global warming and to estimate enteric CH_4 estimation (12). The results of this study show that the CH_4 emission rate increased as the feeding level decreased for male sika deer during their growing period. It may be an important strategy to increase feeding during the above maintenance period to reduce enteric CH_4 emission (13), which is consistent with the results of this study. For Dorper crossbred ram lambs, CH_4 energy/GE increased but DE/GE, ME/GE, and ME/DE decreased in their growing period as the feeding level increased (6). These are consistent with the results for growing male sika deer in this study, except for ME/DE, which was not affected by feed intake. In this study, the ME/DE of male sika deer was 95.01%, which was higher than that of lamb (6), broiler (14), and sheep (15). This may be related to different dietary components and interspecies differences.

Flatt (16) found that DE and CH_4 energy of cows decreased but their urinary energy remained unchanged during their pregnancy. In contrast, Ferrell et al. (17) reported that any

TABLE 4 | Effect of feed intake on carbon (C) and nitrogen (N) balances (g kg^{-1} $BW^{0.75}$ d^{-1}), heat production (HP) (kJ kg^{-1} $BW^{0.75}$ d^{-1}), and retained energy (RE) (kJkg^{-1} $BW^{0.75}$ d^{-1}) by sika deer.

Item[1]	Feed level				*P*-value
	100%	80%	60%	40%	
C (g kg^{-1} $BW^{0.75}d^{-1}$)					
Intake	50.52 ± 6.03[a]	42.69 ± 4.31[b]	35.21 ± 5.38[c]	24.75 ± 3.51[d]	<0.001
Fecal	24.22 ± 3.56[a]	19.51 ± 2.43[b]	14.34 ± 2.65[c]	9.13 ± 2.41[d]	0.011
Urinary	1.82 ± 0.09[a]	1.53 ± 0.21[b]	1.21 ± 0.18[c]	0.96 ± 0.25[d]	0.013
CO_2-C	14.67 ± 2.56[a]	13.05 ± 1.08[a,b]	12.17 ± 2.56[b]	10.32 ± 2.37[c]	0.032
CH4-C	1.56 ± 0.23[a]	1.31 ± 0.32[a,b]	1.09 ± 0.33[b]	1.02 ± 0.19[c]	0.021
Retention C	8.25 ± 0.35[a]	7.27 ± 0.56[a,b]	4.40 ± 0.57[b]	2.32 ± 0.27[c]	
Apparent C digestibility (%)	52.06 ± 4.82	54.30 ± 4.32	59.27 ± 6.03	63.11 ± 7.38	0.2232
N (g kg^{-1} $BW^{0.75}d^{-1}$)					
Intake (NI)	2.12 ± 0.35[a]	1.96 ± 0.26[b]	1.34 ± 0.31[c]	0.93 ± 0.17[d]	0.018
Fecal (FN)	1.10 ± 0.12[a]	0.98 ± 0.10[b]	0.54 ± 0.11[c]	0.32 ± 0.05[d]	0.022
Urinary (UN)	0.63 ± 0.11[a]	0.61 ± 0.09[a,b]	0.41 ± 0.08[b]	0.30 ± 0.04[c]	0.017
Retention N(RN)	0.38 ± 0.08[a]	0.30 ± 0.04[a,b]	0.24 ± 0.05[b]	0.20 ± 0.02[c]	0.020
Apparent N digestibility (%)	48.04 ± 4.13	50.10 ± 4.87	59.70 ± 5.61	65.72 ± 5.88	0.235
Protein deposited (g kg^{-1} $BW^{0.75}d^{-1}$)	2.38 ± 0.21[a]	1.88 ± 0.36[b]	1.50 ± 0.32[c]	1.38 ± 0.22[d]	0.031
Rep (kJ kg^{-1} $BW^{0.75}d^{-1}$)	56.66 ± 4.87[a]	44.74 ± 3.96[b]	35.79 ± 3.96[c]	32.81 ± 4.57[d]	0.043
REf (kJ kg^{-1} $BW^{0.75}d^{-1}$)	363.71 ± 30.52[a]	326.38 ± 29.56[b]	187.69 ± 25.31[c]	83.21 ± 9.34[d]	0.012
RE (kJ kg^{-1} $BW^{0.75}d^{-1}$)	420.31 ± 40.31[a]	371.08 ± 32.31[b]	223.48 ± 30.67[c]	116.02 ± 13.52[d]	0.018
HP (kJ kg^{-1} $BW^{0.75}d^{-1}$)	332.50 ± 27.86[a]	315.76 ± 28.78[a,b]	210.56 ± 25.36[b]	170.21 ± 19.34[c]	0.013

[1]*HP, heat production; RE, retained energy; REp, RE for protein; REf, RE for fat; NI, nitrogen intake; FN, fecal nitrogen;UN, urinary nitrogen; RN, retained nitrogen.*
[a–d]*In the same row, values without a different superscript differ (P < 0.05).*

TABLE 5 | Effect of feed intake level on DM, ADF and NDF intake, excretion and apparent digestibility by sika deer.

Item[1]	Feed level				*P*-value
	100%	80%	60%	40%	
DM (g $kg^{-1}BW^{0.75}d^{-1}$)					
Intake	83.74 ± 9.78[a]	77.69 ± 8.46[b]	53.00 ± 6.21[c]	36.93 ± 3.58[d]	<0.001
Fecal	41.69 ± 5.65[a]	35.88 ± 4.52[ab]	22.46 ± 3.51[b]	14.75 ± 2.42[c]	<0.001
Apparent DM digestibility (%)	50.21 ± 6.03[a]	53.82 ± 6.37[ab]	57.63 ± 6.84[b]	60.05 ± 6.82[c]	0.035
OM (g $kg^{-1}BW^{0.75}d^{-1}$)					
Intake	71.18[a]	66.03[a]	45.05[b]	31.39[c]	0.038
Fecal	33.54[a]	30.17[a]	18.19[b]	12.11[c]	0.042
Apparent OM digestibility (%)	52.88[a]	55.31[a]	59.63b[c]	61.4[2]	0.022
ADF (g $kg^{-1}BW^{0.75}d^{-1}$)					
Intake	13.53 ± 2.58[a]	12.55 ± 2.41[a]	8.56 ± 1.34[b]	5.97 ± 1.06[c]	<0.001
Fecal	9.45 ± 2.41[a]	8.46 ± 1.85[b]	5.52 ± 1.10[c]	3.58 ± 0.96[d]	<0.001
Apparent ADF digestibility (%)	30.12 ± 5.61[a]	32.56 ± 4.23[b]	35.43 ± 4.36[bc]	40.05 ± 4.31[c]	0.033
NDF (g $kg^{-1}BW^{0.75}d^{-1}$)					
Intake	34.78 ± 2.56[a]	32.27 ± 3.52[ab]	22.02 ± 3.15[b]	15.34 ± 2.06[c]	<0.01
Fecal	24.51 ± 3.87[a]	21.55 ± 2.69[b]	13.89 ± 2.06[c]	8.99 ± 1.87[d]	<0.001
Apparent NDF digestibility (%)	29.53 ± 2.45[a]	33.21 ± 4.57[b]	36.91 ± 4.05[bc]	41.37 ± 5.04[c]	0.021

[1]*BW, body weight; DM, dry matter; OM, organic matter; ADF, acid detergent fiber; NDF, neutral detergent fiber.*
[a–d] *In the same row, values without a different superscript differ (P < 0.05).*

difference in energy digestibility of heifers due to pregnancy was insignificant compared to differences in feeding levels. The current data of male sika deer during their growing period suggest that the methane energy, urinary energy, fecal energy, and DE increased as the feed intake level increased. The results of this study are consistent with those reported above.

TABLE 6 | Estimates of heat production (HP, kJ $kg^{-1}BW^{0.75}d^{-1}$) and metabolisable energy (ME) intake (MEI, kJ $kg^{-1}BW^{0.75}d^{-1}$) in the equation to predict net energy requirement for maintenance of sika deer.

BW, kg	Equation	RMSE[a]	R^2	Number of deer	*P*-value	NEm (kJ kg^{-1} $BW^{0.75}d^{-1}$)	MEm (kJ kg^{-1} $BW^{0.75}$ d^{-1})	Km
56.43–64.26	LogHP = 2.3495(± 0.0212) + 0.0005(± 0.0002) × MEI	0.0831	0.9246	12	<0.001	223.62	251.17	0.89

[a] *RMSE, root mean square error; Km, the efficiency of ME utilization for maintenance was computed as NEm/Mem.*

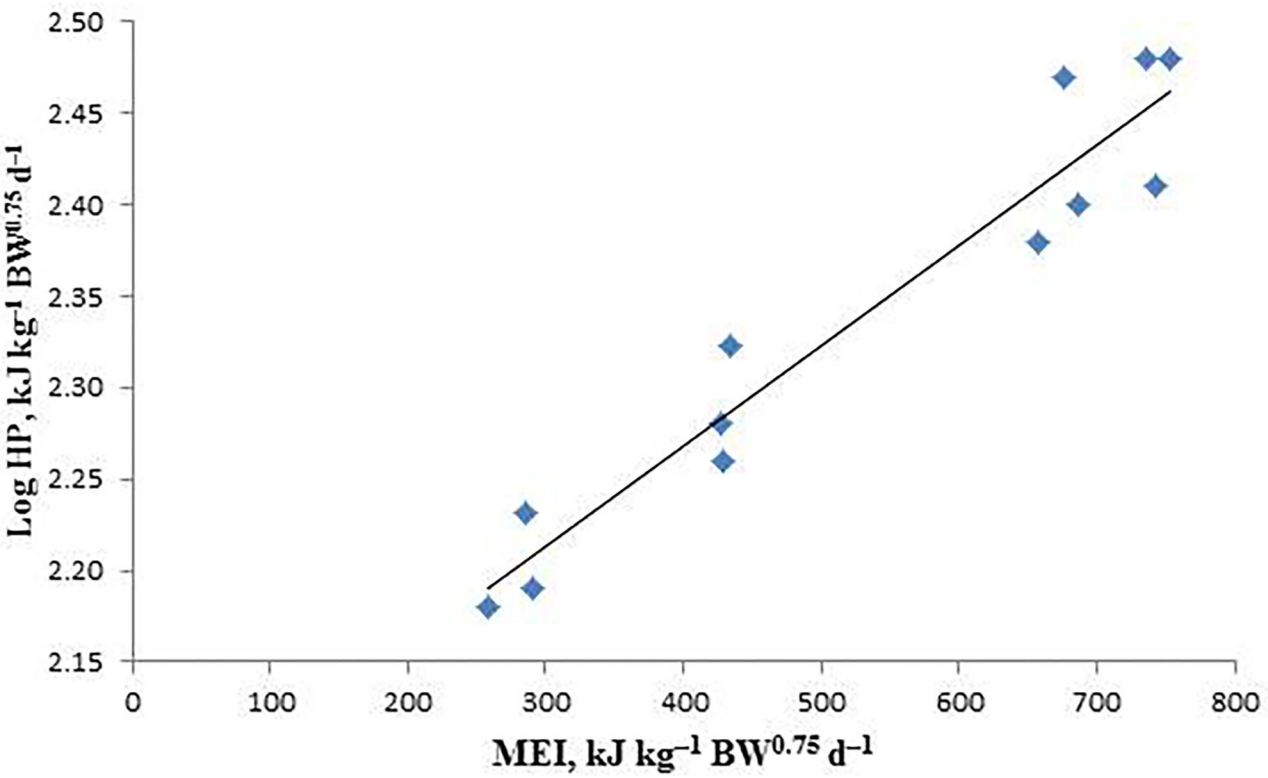

FIGURE 1 | The relationship between the logarithm of heat production (HP) and metabolizable energy intake (MEI) of sika deer in the indirect calorimetry method. LogHP = 2.3495(± 0.0212) + 0.0005(± 0.0002) × MEI; $R^2 = 0.9246$, $P < 0.001$, $n = 12$.

Blaxter (18) found that because the C-N balance method involved more analysis and measurement than the energy balance method, errors of about 30% may be encountered. As revealed by Kishan et al. (19), for male buffaloes and crossbred cattle, energy levels affected the excretion of C and N in urine, the C in urine was significantly related to DE intake ($P < 0.01$), but the urinary, CO_2, and CH_4 carbon outgo were not affected. There is also a correlation between N excretion and urinary C content (20). These are consistent with the results of this study. Furthermore, the results of this study show that the apparent digestibility of C was 52–63%, which is consistent with the results reported by Blaxter and Wainman (20). There is a certain difference between RC and RN calculated using the C-N balance method in this study. Graham (21) found that the RN calculated using the comparative slaughter method decreased as the number of gestation days increased. In this study, RN and RE decreased as the levels of feeding decrease. This is consistent with the results of Zhang et al. (15), Singh et al. (22), and George et al. (23).

Ferrell (24) found that energy intake affected HP because of the metabolic activity of visceral organs. As the MEI of growing cattle increased, HP increased exponentially (25–27). As indicated by the results of Taylor and Turner (28), HP increased as the nutrient level increased, which is mainly due to the increased metabolism associated with energy retention. Analysis of energy metabolism for growing cattle indicated that HP increased exponentially with an increase in MEI level (25). It has been well-established that HP will increase significantly during pregnancy (29). In this study, MEI and HP gradually decreased, accompanied by a decrease in feeding levels, which may be due to the thermal effect of reduced feed. Meanwhile, ME intake decreased, leading to a decrease in HP (26, 30, 31).

TABLE 7 | Estimates of retained N (RN, g kg−1 BW0.75 d−1) and N intake (NI, g kg^{-1} $BW^{0.75}$ d^{-1}) in the equation to predict net protein requirement for maintenance (NPm, g kg^{-1} $BW^{0.75}$ d^{-1}).

BW, kg	Equation	RMSE[a]	R^2	Number of deer	*P*-value	NNm (mg $kg^{-1}BW^{0.75}$ d^{-1})	NPm (g kg^{-1} $BW0^{.75}$ d^{1})
56.43–64.26	RN = −0.2518(± 0.1352) +0.407(± 0.0524) × NI	0.1235	0.8479	12	<0.001	251.8	1.57

[a]*RMSE, root mean square error; NNm, net N requirement for maintenance (mg kg^{-1} $BW^{0.75}$ d^{-1}) calculated as the intercept of this regression; NPm = NNm × 6.25; BW, body weight.*

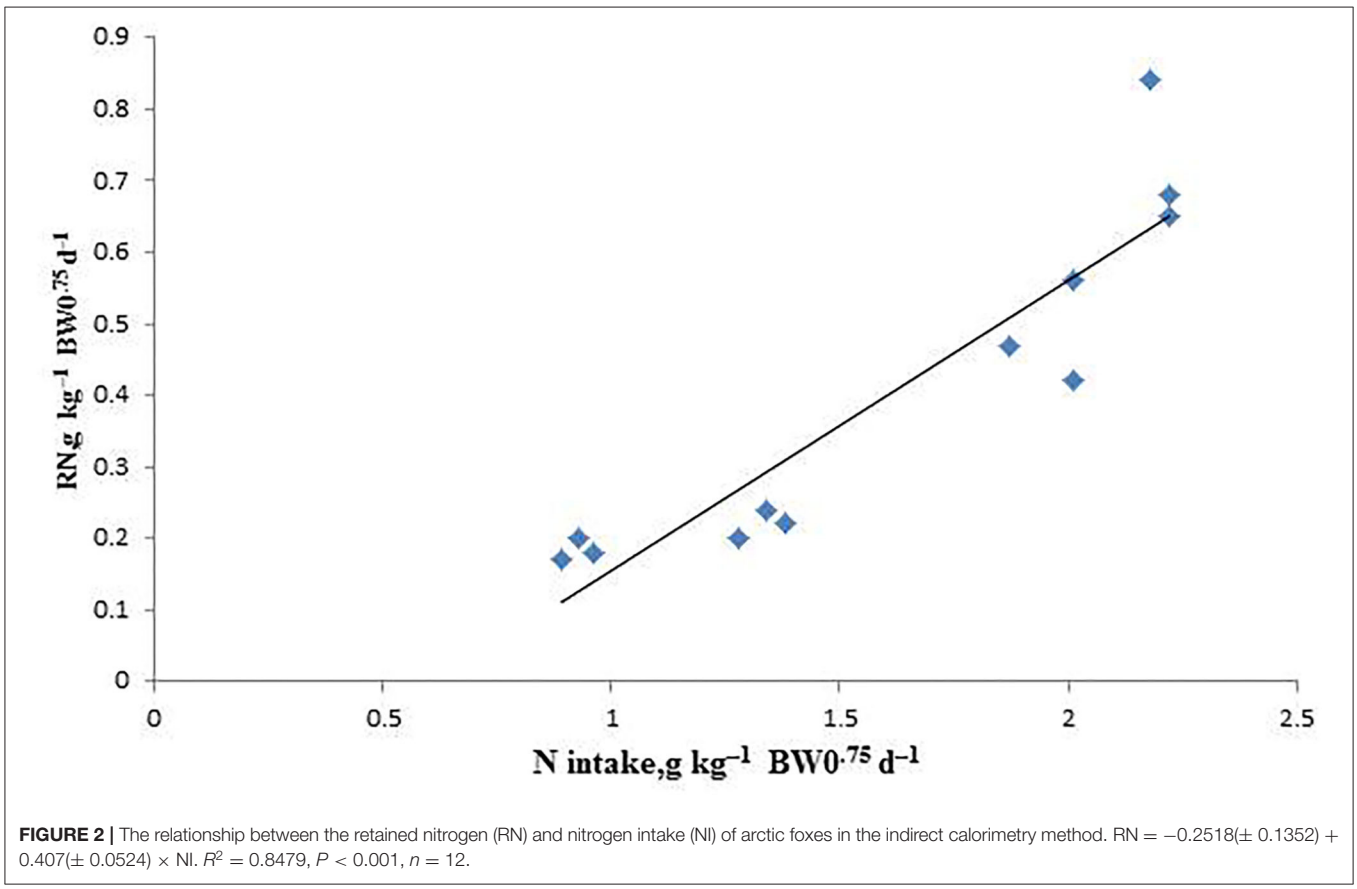

FIGURE 2 | The relationship between the retained nitrogen (RN) and nitrogen intake (NI) of arctic foxes in the indirect calorimetry method. RN = −0.2518(± 0.1352) + 0.407(± 0.0524) × NI. $R^2 = 0.8479$, $P < 0.001$, $n = 12$.

These are consistent with the findings in growing and fattening pigs reported by Zhang et al. (32).

The REp value is much lower than REf value. When deer were fed at 40% of the intake level, REp and REf reached their minimum values. These results are consistent with the findings in Hu sheep (15) and in arctic foxes (33). In this study, the decrease in the feed intake of male sika deer led to the reduction of NI, FN, and UN. As the feed intake decreased, RN gradually decreased from 0.38 to 0.20 g kg^{-1} $BW^{0.75}d^{-1}$. This is consistent with the findings of Singh et al. (22).

Nutrient Digestibility

The digestibility of nutrients in the rumen is the competition result between digestion and passing rate. Among them, the passing rate is positively correlated with dry matter intake (34). Degen and Young (35) found a correlation between increased digesta passage rates and increased feed intake. In this study, the digestibility values of C, N, DM, OM, ADF, and NDF increased significantly with a decrease in diet intake, indicating that dietary restriction can improve the digestibility and utilization of nutrients. The deer body promotes nutrient digestibility and utilization to meet maintenance requirements while feed intake is less. Deng et al. (6) found that the apparent energy digestibility (DE/GE) and metabolic rate (ME/GE) of lambs fed *ad libitum* were lower than lambs fed in a restricted manner. The results on male sika deer in this study are consistent with the above research results.

Energy and Protein Requirements for Maintenance

The logarithmic relationship between MEI and HP is often used to calculate NEm. HP is equal to NEm in the case of zero MEI (10, 26). Similarly, MEm can also be calculated by extrapolating HP where it is equal to MEI. The values of NEm and MEm calculated by the regression equation were 223.62 and 251.17 kJ kg^{-1} $BW^{0.75}d^{-1}$, respectively. The Km was calculated to be 0.89

in this study. Li et al. (36) studied the energy metabolism of adult male sika deer and determined that the requirement of MEm of adult sika deer was 516 kJ kg^{-1} $BW^{0.75}d^{-1}$. The result is greater than that in this study. The reason may be the different ages of the deer selected for experiments and the different physiological states of each period. Adult deer need more energy to maintain their growth and antler. In contrast, for deer in the growing period, more energy is used for the development and growth of their own bodies, and thus less MEm was observed in them. The Km value in this study is higher than that (0.707) reported by Li (36) in adult deer and that of lambs and sheep (14, 15, 32). This may also be related to the age of the selected deer. Luo et al. (37) reported that the difference in MEm seems to depend mainly on the change in NEm rather than Km, which can explain the difference in MEm requirements. The values of NEm and MEm vary from species to species. For animals, the species, physiological stages, environmental temperature, and feed composition also affect the values of NEm and Mem (14, 38). MEm was 768 kJ kg^{-1} $BW^{0.75}d^{-1}$ and 501 kJ kg^{-1} $BW^{0.75}d^{-1}$ at 18 and 24°C, respectively for adult female mink (38). In this study, male sika deer with a bodyweight of 56.43–64.26 kg were selected. Meanwhile, the temperature in the respiration chamber was 22°C, and the male sika deer in Northeast China during their growing period was chosen. These may be the main reasons why the NEm and MEm values are different from those in previous reports (36).

ARC (39) assumed that NPm equal to the amount of protein that can offset the loss of urine, feces, and dermal N, except for growing lambs because they do not consider dermal loss ARC. For lambs and sheep (15, 40), the regression equation between RN and daily NI is an effective method to obtain NPm through N-balance trails. The intercept of the regression equation represents the endogenous and metabolic N loss. According to the regression equation between the daily NI and RN of the male sika deer during their growing period, the values of NNm and NPm were estimated to be 251.8 mg kg^{-1} $BW^{0.75}d^{-1}$ and 2.045 g $kg^{-1}BW^{0.75}d^{-1}$, respectively. Chizzotti et al. (26) reported that the NPm estimated according to the relationship between RN and daily NI using the comparative slaughter method is greater than the NPm determined based on the relationship between RN and daily NI using the N-balance method. This discrepancy may be due to losses of N that are not accounted for by the N balance method. The scurf protein represents about 20% of the maintenance requirement of the ARC system. However, no data was present in growing male sika deer. The N balance trails can reduce animal injuries and meet animal welfare requirements and is an important method for estimating net protein requirement.

CONCLUSIONS

In conclusion, the MEm and NEm values of growing male sika deer were estimated to be 223.62 and 251.17 kJ kg^{-1} $BW^{0.75}d^{-1}$, respectively, according to the logarithmic regression between the HP and MEI The NNm and NPm values of growing male sika deer were estimated to be 251.8 mg and 2.045 g $kg^{-1}BW^{0.75}d^{-1}$, respectively, based on the linear regression relationship between daily NI and RN. These results fill the gap in the research on the net energy and protein requirements of male sika deer and provide basic data for determining the nutritional requirements of sika deer in China.

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 author/s.

ETHICS STATEMENT

The animal study was reviewed and approved by Animal Care and Use Guidelines of the Institute of Special Animal and Plant Science.

AUTHOR CONTRIBUTIONS

KB: conceived the study, managed the animals, and oversaw the statistical analysis and manuscript preparation. XW: sample collection. KW: statistical analysis and drafted the manuscript. GL: statistical analysis and manuscript preparation. HL: manuscript preparation. All authors contributed to the article and approved the submitted version.

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Dietary Energy Levels Affect Carbohydrate Metabolism-Related Bacteria and Improve Meat Quality in the *Longissimus Thoracis* Muscle of Yak (*Bos grunniens*)

Mei Du[1,2], *Chao Yang*[1], *Zeyi Liang*[1,2], *Jianbo Zhang*[1,2], *Yayuan Yang*[2], *Anum Ali Ahmad*[1,3], *Ping Yan*[1]* *and Xuezhi Ding*[1,2]*

[1] Key Laboratory of Yak Breeding Engineering, Lanzhou Institute of Husbandry and Pharmaceutical Sciences, Chinese Academy of Agricultural Sciences, Lanzhou, China, [2] Key Laboratory of Veterinary Pharmaceutical Development, Ministry of Agricultural and Rural Affairs, Lanzhou Institute of Husbandry and Pharmaceutical Sciences, Chinese Academy of Agricultural Sciences, Lanzhou, China, [3] State Key Laboratory of Grassland Agro-Ecosystems, School of Life Sciences, Lanzhou University, Lanzhou, China

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Tarique Hussain,
Nuclear Institute for Agriculture and Biology, Pakistan

Reviewed by:
Zhi Yuan Ma,
Institute of Subtropical Agriculture, Chinese Academy of Sciences, China
Kefyalew Gebeyew,
Jigjiga University, Ethiopia

***Correspondence:**
Ping Yan
pingyanlz@163.com
Xuezhi Ding
dingxuezhi@caas.cn

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The effects of different dietary energy levels on the ruminal bacterial population, selected meat quality indices, and their relationship in yak *longissimus thoracis* (LT) muscle were assessed in this study. A total of 15 castrated yaks were randomly assigned to three groups with low- (NEg: 5.5 MJ/Kg, LE), medium- (NEg: 6.2 MJ/Kg, ME), and high- (NEg: 6.9 MJ/Kg, HE) dietary energy levels and occurred in the cold season (March to May). All yaks from each treatment group were humanely slaughtered and sampled on the day of completion of their feeding treatment. The results showed that the water content and crude fat levels of the LT muscle were markedly elevated in the HE group ($P < 0.05$), while the shear force was drastically reduced ($P = 0.001$). Methionine, aspartic acid, and glycine levels in the LT muscle were higher in the LE group compared with the ME and HE groups ($P < 0.05$). The glutamic acid level in the ME group was greater in comparison to the LE and HE groups ($P < 0.05$), while the histidine level in the ME group was higher than that in the HE group ($P < 0.05$). Additionally, the HE diet significantly elevated ($P < 0.05$) the abundance of carbohydrate metabolism-associated bacteria including *Prevotella_1*, *Lachnospiraceae_NK4A136_group, U29_B03*, *Ruminiclostridium_6*, *and Ruminococcaceae_UCG_013* in the rumen. The results of the Spearman's rank correlation analysis showed that the abundance of *uncultured_bacterium_f_vadinBE97* and *uncultured_bacterium_f_Lachnospiraceae* showed a significant influence on the indicator of IMF and SF. In conclusion, a high dietary energy level improved the meat quality in the LT muscle of yak mainly by increasing the relative abundance of ruminal amylolytic bacteria to provide substrates for fatty acid synthesis.

Keywords: yak, dietary energy level, meat quality, *longissimus thoracis*, carbohydrate metabolism related bacteria

INTRODUCTION

Yak (*Bos grunniens*), is known as "the treasure of the plateau" and is mainly distributed in the area of Qinghai Tibetan Plateau at an altitude of above 3,000 m (1). It provides more than 90% of the milk and about 50% of the meat consumed in this region (2). Yak meat is a semi-wild natural green food and known as the crown of beef. It is rich in protein, amino acids, carotene, calcium, phosphorus, and other trace elements. However, the yak cannot obtain enough feed supply due to the long-term extensive grazing, breeding mode, and lack of forage biomass during the cold season. This malnutrition state results in a long growth cycle and poor meat quality such as low level of tenderness, intramuscular fat content, and taste (3). With the global rise in the importance of green food and growing consumer demand, the production of high-quality yak meat is of extensive concern domestically and internationally. The use of supplementary feeding could be an effective way to reduce the gap between the production of high-quality yak meat and increased market demand (4). Liu et al. (5) reported that fattening yaks with a total mixed diet in the cold season can improve the yield and quality of the yak meat. Zhang et al. (4) reported that supplementing a diet with high-protein in early-weaned yaks could increase intramuscular fat accumulation. Similarly, Kang et al. (6) demonstrated that the growth performance, meat production, and meat quality of yak could be improved by increasing the dietary energy concentration. These results suggested that the dietary energy concentration has a positive impact on the growth performance, carcass characteristics, meat production, and meat quality of livestock.

Rumen microbiota plays a pivotal role in feed digestion and acts synergistically to degrade plant structural and non-structural carbohydrates into volatile fatty acids (VFAs) and microbial proteins (MCP) (7). The end products of rumen fermentation (VFA) are used as the substrate for fueling other animal tissues (including liver, fat, and muscle) and MCP are required by the host to produce meat and milk (8). The change of rumen bacterial community structure was reported to be closely related to the composition of the diet (9). Dietary components affect rumen fermentation and the structure of the rumen microbial population (9). It has been reported that high-energy diets increased rumen amylolytic and propionate-producing bacteria populations such as *Prevotella*, *Ruminobacter amylophilus*, *Succinimonas amylolytica*, and *Bifidobacterium* (10). Feeding high-starch and high-grain diets decreases some fibrolytic bacteria, i.e., *Ruminococcus flavefasciens*, *Fibrobacter succinogenes*, and *Butyrivibrio fibrisolvens* (11, 12). Lin et al. (13) demonstrated that sheep had a capacity to remodel the structure of the microbiota to adapt to a high-grain diet for a long time; *Ruminococcus*, *Prevotella*, and *Bifidobacterium* were tolerant to the diet with stable proportions in each treatment in a sheep model. Thus, diet is a main factor affecting rumen microbial diversity, and its nutritional levels or nutrients composition have a significant effect on rumen microbial communities, which may be due to the bacterial preference for feed ingredients, specific metabolites, and rumen environment (14). In addition, improving dietary energy levels is an effective approach to facilitate the growth performance in yaks and the utilization of dietary energy sources depends on the digestion of feed nutrients by rumen microorganisms (15). However, the details of how dietary energy level regulates ruminal microbiota remain unclear.

Intramuscular fat (IMF) and its fatty acid composition play pivotal parts in determining the meat grade for human consumption (16). It has been widely recognized that the meat grade and flavor are intimately linked to the degree of IMF and fatty acid profiles (17). Several studies highlighted that the IMF content in animal meat could be enhanced by providing a high-energy diet (18). The fat content and fatty acids in ruminants are mainly affected by the dietary nutrition and bacterial metabolism in the rumen (19). However, little information is available about the mechanism by which modulatory effects of rumen microbiota contribute to improving the muscle fatty acid profile, amino acids composition, and other quality parameters of yak fed with different levels of dietary energy. Thus, the aim of this study was to investigate the effects of dietary energy levels on the meat quality, rumen bacteria populations, and the relationship between rumen bacteria and meat quality parameters. Moreover, rumen bacteria contributing in the improvement of meat quality on the yak *longissimus thoracis* (LT) muscle would be identified.

MATERIALS AND METHODS

Experimental Procedure and Sample Collection

This experiment was performed at Hongtu Yak Breeding Cooperatives of Tibetan Autonomous Prefecture in Gansu Province, China. A total of 15 adult castrated yaks (initial BW, 276.1 ± 3.5 kg) originating from the local herders were randomly allotted to different energy level treatments, i.e., low energy level (LE: 5.5 MJ/kg), medium energy level (ME: 6.2 MJ/kg), and high energy level (HE: 6.9 MJ/kg). The basic diet composed mainly of 40% oats silage, 40% micro-storage of corn straw, and 20% highland barley hay. Energy levels of three diets met or exceeded the estimated requirements for a 275 kg finishing beef cattle with an average daily gain of 1 kg in the Feeding Standard of Beef Cattle (NY/T 815-2004). The details of the ingredient and nutritional composition for energy diets have been presented in our previous study (20) and briefly summarized in **Supplementary Table S1**.

All the yaks underwent an acclimatization period of 15 days before study commencement, whereby the designated dietary regimens were implemented accordingly for 60 days. Throughout the study, the animals were individually fed twice daily *ad libitum* and had free access to water provisions. After 60 days, the animals were fasted for 12 h and consequently, humanely slaughtered by electrical stunning. The LT (12th−13th rib) samples were rapidly removed from the carcass regions in quadruplicate, placed within a sterile vacuum packaging, and ultimately stored at 4°C. In addition, LT samples that are required

to determine the amino acid/fatty acids (FA) content were stored at −80°C.

Analytic Methods for Chemical Composition and Amino Acids

The chemical compositions for the LT samples were determined according to Chinese recommended standardized protocols. Water content was determined in line with GB/T 5009.3-2010 through the direct-drying methodology. The crude protein content (Kjeldahl N × 6.25) was determined in line with GB/T 5009.5-2010 through the Kjeltec Auto Analyzer®. The IMF content was determined in line with GB 5009.6-2003 using a Soxhlet Extractor. The crude ash content was determined in line with GB/T 9695.18-2008. The amino acid concentrations were determined based on the previously reported method (GB/T5009.124-2003) (21) using a liquid chromatography (LC, u3000, Thermo Fisher™).

Meat Quality

The pH value of the LT was determined at 1 h and 24-h post-mortem using a portable pH-meter (PHBJ-260, purchased from INESA Scientific Instrument Co., Ltd.). The pH meter was fitted with a spear tip pH electrode and an automatic temperature compensation probe, and it was calibrated with pH 4.01 and 7.00 buffers in advance. A portable colorimeter (Minolta CR400, Konica Minolta, Japan) equipped with an 8-mm aperture, 10° viewing angle, and D65 illuminant was used to determine the meat color. For each meat sample, five different positions were selected to determine the brightness value (L^*), redness value (a^*), and yellowness value (b^*). Chroma value (H^*) and color saturation value (C^*) were calculated based on the L^*, a^*, and b^* values.

Cooking loss and shear force (SF) were determined according to a procedure adapted from Honikel (22) and Oillic (23). For cooking loss determination, five thawed LT samples that underwent external fat trimming and light blotting for moisture removal were weighed and this readout was recorded as the initial weight. Consequently, the LT samples were heated using a water bath at 80°C up to an internal temperature of 70°C, which was monitored using an internal thermocouple (Eirelec Ltd.™, Ireland). All LT samples were cooled to room temperature, residual moisture was removed using a tissue paper, and weights were measured and recorded as the final weight.

Preparation loss was represented by the final weight/initial weight (%). The prepared LT samples were sliced into five cubes (6 cm * 3 cm * 3 cm) and the SF of each cube was determined using a Warner-Bratzler shear apparatus. For water loss analysis (pressing loss), a 10 g LT sample from each animal—wrapped with 12 layers of filter paper—was pressed by a force of 10 kg/cm^2 for 5 min. Residual moisture was lightly removed and the sample weight was quickly recorded as the final weight. Data were presented as a percentage of the final weight/10.

Rumen 16s rDNA Sequence Data Analysis

The raw data of the 16s rDNA sequence for rumen bacteria of yaks in the LE, ME, and HE groups were obtained from our previous study (24), which have been deposited in the European Nucleotide Archive (ENA) at EMBL-EBI under the accession number PRJEB34298. The previous analysis was conducted 4 years ago, and the representative sequences of operational taxonomic units (OTUs) were annotated by using the GreenGene database (25) (uploaded in 2013) which led to 30% of the high-quality reads on the genus level not being annotated. Thus, in this study, we re-analyzed the sequence data using an updated database and the details for the analytic methods are as follows. Raw paired-end reads were merged using the FLASH (version 1.2.11) software to generate the contigs and then assigned to each sample according to the unique barcodes (26). The contigs underwent quality control through trimming and filtering by Trimmomatic (version 0.33) with a criterion, i.e., sequences with an average quality <20 over a 50-bp sliding window were rejected (27), then chimeras were identified and removed by the UCHIME (version 8.1) software (28) to obtain high-quality sequences. The generated high-quality sequences were clustered into OTUs by USEARCH (version 10.0) at 97% similarity levels, and the OTUs were filtered when an abundance of <0.005% (29) was observed. We selected the sequences with the maximum abundance in each OTU as the representative sequences using the QIIME (version 1.9.0) software, and the representative sequences of the OTUs were compared with the SILVA database (version 132) using the RDP classifier with a 0.80 confidence threshold (30). After that, Chao1, Shannon and Simpson indices, and Good's coverage were subsequently calculated using QIIME with the default parameters (31). Principal coordinate analysis (PCoA) was performed using Bray-Curtis distance. To evaluate the effect of the dietary treatment on the microbial taxa, we used the linear discriminant analysis effect size (LEfSe) to identify different taxa using a critical value of LDA Score > 2.0 and P-value < 0.05 (32). To further understand the specific functions of each group of bacteria, PICRUSt2 software (33) was employed for comparing species make-up from the 16S sequencing datasets. The newly determined functional gene compositions were consequently predicted through the Kyoto Encyclopedia of Genes and Genomes (KEGG) database at level 3. Redundancy analysis (RDA) was used to analyze the relationships between differential bacteria and rumen fermentation parameters (TVFA, acetate, propionate, butyrate, valerate, and pH) on OmicStudio (LC-Bio Technology Co., Ltd., Hangzhou, China).

Statistics and Analyses

The SPSS version 24.0 (SPSS Inc., Chicago, IL, USA) was used to analyze the data. The data of meat quality and the concentration of amino acids were analyzed by a one-way analysis of variance (ANOVA) followed by Duncan's *post-hoc* testing procedure for multiple comparisons. Alpha diversity was analyzed using Kruskal–Wallis test in the SPSS 24.0 software. Results were presented as means ± SEM. P-values <0.05 were regarded as statistically significant. Correlation networks based on Spearman's rank correlation analysis between the relative abundance of key bacteria associated with carbohydrate metabolism and the meat quality indices (SF, IMF, SFA, MUFA, and PUFA) in the LT showing $|r| > 0.60$ and P-value < 0.05 were considered as a significant correlation.

RESULTS

Effects of Different Energy Levels on the Meat Chemical Analysis and Meat Quality of the *Longissimus Thoracis* Muscle

Effects of different dietary energy levels on the LT muscle chemical composition and meat quality of yaks are shown in **Tables 1**, **2**, respectively. The LT water and IMF content were significantly promoted ($P < 0.05$) in the HE group, compared to the LE and ME groups. However, dietary energy levels did not influence ($P > 0.05$) LT crude protein and ash content. No differences ($P > 0.05$) in the pH_{1h}, pH_{24h}, and meat color indices in the muscle were found among the treatments. Compared to the LE group, cooking loss and water loss of LT significantly decreased ($P < 0.05$) in the HE group, but were comparable to the ME group. The shear force was markedly reduced ($P < 0.05$) in response to the increasing dietary energy levels.

Effects of Different Energy Levels on the Amino Acids Profile and Fatty Acid Composition of the *Longissimus Thoracis* Muscle

The essential amino acids (EAAs) and non-essential amino acids (NEAAs) levels in the LT muscle of yak are listed in **Table 3**. Methionine levels in the ME and HE groups were significantly decreased ($P < 0.05$), compared with the LE group. While, the other EAA levels were unaffected ($P > 0.05$) by the dietary energy levels. The aspartic and glycine levels in the ME and HE groups were significantly elevated ($P < 0.05$) in comparison to the LE group. Compared to the ME group, the level of glutamic acid in the LE and HE groups was lower, whereas the level of histidine in the ME group was higher than the HE group but comparable to the LE group.

The fatty acid composition of the LT muscle is listed in **Supplementary Table S2**, which was previously published (18). Briefly, no differences were observed ($P > 0.05$) in the total monounsaturated fatty acid (MUFA) and polyunsaturated fatty acids (PUFA) between the LE and ME groups, while the proportions of saturated fatty acids (SFA) in the ME and HE groups were significantly higher ($P < 0.05$) than the LE group. The concentrations of SFA in the HE group was markedly elevated compared to the ME group. In this study, MUFA and PUFA levels in the HE group were markedly elevated compared to the other groups ($P < 0.05$).

Diversity Taxonomy and Function Prediction of Rumen Bacteria

In this study, the unannotated reads were only 0.30% at the genus level after re-annotating the OTU based on the SILVA database compared to 33.83% unannotated reads of the previous study annotated by the GreenGene Database (**Supplementary Table S3**). Microbial abundance and heterogeneity were assessed through the alpha diversity indices. Alpha diversity measures revealed that different dietary energy levels have a little effect on ($P > 0.05$) the number of OTUs, the ACE and Chao1 estimator, and the Shannon index (**Figure 1A**). The PCoA based on the Bray-Curtis distance showed that the HE group presented a degree of diversity discrepancy with the other two groups (**Figure 1B**), indicating that the rumen bacterial community changed significantly with the increase of dietary energy. In addition, 19 phyla were discovered in the rumen for all groups (**Figure 1C**). *Firmicutes*, *Bacteroidetes*, *Kiritimatiellaeota*, and *Tenericutes* were the dominant phyla across all groups. *Firmicutes* and *Bacteroidetes* were the most dominant phyla, accounting for 88% of the total population. Concomitantly, 212 classifiable genera were detected in all samples. The results of LEfSe analysis at the genus level are shown in **Figures 1D,E**. In detail, the rumen bacteria in the ME group were mainly composed of *Lachnoclostridium_10* and *uncultured_bacterium_o_SAR324_cladeMarine_group_B* at the genus level, while *uncultured_bacterium_f_F082* and *uncultured_bacterium_f_Marinilabiliaceae* were significantly ($P < 0.05$) more abundant in the LE group. In addition, eight bacterial taxa including *uncultured_bacterium_f_Lachnospiraceae*, *uncultured_bacterium_c_MVP_15*, *Lachnospiraceae_NK4A136_group*, *U29_B03*, *uncultured_bacterium_f_vadinBE97*, *Ruminiclostridium_6*, *Prevotella_1*, and *Ruminococcaceae_UCG_013* were overrepresented ($P < 0.05$) in the HE group at the genus level.

PICRUSt2 was used to investigate the possible microbial metabolic pathways. As shown in **Figure 2A**, the mean proportion of starch and sucrose metabolism was the highest in the rumen microbial metabolism pathway, and was significantly higher in the HE group than in the LE group ($P = 0.026$). Meanwhile, carbohydrate metabolism was also higher ($P = 0.024$) in the HE group than in the LE group based on the level 3. The proportion of metabolic pathways at level 3 between the LE and ME groups had no difference ($P > 0.05$), while the abundance of significantly different metabolic pathways between the ME and HE groups were lower than 0.25%, therefore, the results of these two comparisons did not present.

The Relationship Between the SF, IMF of *Longissimus Thoracis*, and the Relative Abundance of Differential Bacteria, Phenotype of Fatty Acid Profile

To illustrate the relationship between the significant taxa and ruminal fermentation parameters that were published previously (the data are presented in **Supplementary Table S4**) (24), and an RDA ranking map was generated (**Figure 2B**). In detail, the bacterial genera *uncultured_bacterium_f_Marinilabiliaceae*, *Lachnoclostridium_10*, and *uncultured_bacterium_f_F082* exhibited a positive relationship with the pH value, and showed a negative relationship with the total VFA, acetate, propionate, butyrate, and valerate. The relative abundance of *Prevotella_1*, *U29_B03*, *Ruminiclostridium_6*, *uncultured_bacterium_f_Lachnospiraceae*, *uncultured_bacterium_c_MVP_15*, *Ruminococcaceae_UCG_013*, *uncultured_bacterium_f_vadinBE97*, and *Lachnospiraceae_*

TABLE 1 | Effects of dietary energy levels on the nutritional components in the *longissimus thoracis* muscle of yak.

Items	Groups			SEM	*P*-value
	LE	ME	HE		
Water content (%)	68.94^{b}	70.30^{ab}	72.04^{a}	0.518	0.035
Protein content (%)	23.60	23.78	24.42	0.393	0.702
IMF content (%)	0.56^{c}	0.92^{b}	1.34^{a}	0.102	0.001
Crude ash content (%)	1.66	1.72	1.84	0.038	0.135

LE, low energy level; ME, medium energy level; HE, high energy level; SEM, standard error of the mean; IMF, Intramuscular fat.
^{a,b,c}Values in the same row with different superscript letters differ significantly ($P < 0.05$).

TABLE 2 | Effect of dietary energy levels on the quality in the *longissimus thoracis* muscle of yak.

Items	Groups			SEM	*P*-value
	LE	ME	HE		
pH_{1h}	6.56	6.62	6.65	0.020	0.145
Ph_{24h}	5.60	5.63	5.57	0.024	0.624
Cooking loss (%)	36.69^{a}	32.42^{ab}	28.91^{b}	1.212	0.017
Driage (%)	29.49^{a}	25.89^{ab}	23.05^{b}	1.069	0.033
Shear force (N/cm^2)	74.50^{a}	66.89^{b}	55.96^{c}	0.233	<0.001
*CIE L**	34.22	34.88	35.29	0.250	0.222
*CIE a**	18.18	18.32	18.82	0.138	0.143
*CIE b**	8.16	7.88	8.24	0.087	0.226
*CIE H**	24.18	23.30	23.65	0.274	0.445
*CIE C**	19.94	19.94	20.54	0.132	0.094

LE, low energy level; ME, medium energy level; HE, high energy level. SEM, standard error of the mean.
^{a,b,c}Values in the same row with different superscript letters differ significantly ($P < 0.05$).

NK4A136_group was positively associated with the total VFA, acetate, propionate, butyrate, and valerate.

The correlation results revealed 20 significant Spearman's correlations between differential bacteria and meat quality traits (SF and IMF) as well as fatty acid profile (SFA, MUFA, and PUFA) ($R > 0.60$, $P < 0.05$, **Figure 2C**). In detail, the SF had a negative relationship with *Ruminiclostridium_6*, *U29_B03*, *uncultured_bacterium_f_vadinBE97*, *uncultured_bacterium_f_Lachnospiraceae*, SFA, and PUFA, and a positive relationship with *uncultured_bacterium_f_F082*. The IMF exhibited a significantly positive correlation with *uncultured_bacterium_f_Lachnospiraceae*, *uncultured_bacterium_f_vadinBE97*, and PUFA. In addition, the SFA was positively correlated with *Ruminiclostridium_6*, *U29_B03*, *Ruminococcaceae_UCG_013*, *uncultured_bacterium_f_Lachnospiraceae*, IMF, MUFA, and PUFA. The PUFA had a positive relationship with *uncultured_bacterium_f_Lachnospiraceae* and *uncultured_bacterium_f_vadinBE97*.

DISCUSSION

In the current study, the effects of dietary energy level on the phenotypic parameters related to the meat quality in the LT muscle of yak were focused. Meanwhile, the relationships between meat quality and ruminal bacteria were illustrated, and the contribution of bacteria on fatty acid synthesis in the LT muscle through generating substrates (mainly volatile fatty acids) was assessed. These findings gained a comprehensive understanding of the regulatory mechanisms of the improvement of the meat quality induced by different dietary energy levels.

It is necessary to have a detailed understanding of the physical and chemical properties (e.g., pH, color), as well as the storage quality of meat to determine the quality of meat after slaughtering (34). The pH value of meat is an essential factor that influences the color, tenderness, cooking loss, shelf-life, and other physicochemical properties (35). After slaughtering, the breakdown of glycogen in the muscles results in the accumulation of a large amount of lactic acid which led to a pH reduction of the meat to an ultimate pH value at 24 h (36). Previous studies demonstrated that the optimal range of pH_{24h} for beef cattle is between 5.4 and 5.6 (37); once the pH_{24h} value is higher than 6.0, the meat tends to be dark, firm, and dry (DFD) (38). In this experiment, dietary energy levels showed no impact on the pH_{24h} of the LT muscle and the pH values of yak meat in each group were within or near to the aforementioned optimal range, suggesting that high-quality yak meat in each group was earned. Cooking loss and water holding capacity are important factors in evaluating the meat quality, which affect the juiciness of cooked

TABLE 3 | Effect of dietary energy levels on the amino acids content in the *longissimus thoracis* muscle of yak.

Items		Groups			SEM	*P*-value
		LE	ME	HE		
EAA (mg/g)	Threonine	10.24	10.10	9.87	0.114	0.434
	Valine	11.77	11.56	11.08	0.155	0.185
	Methionine	0.11[a]	0.09[b]	0.09[b]	0.003	0.006
	Isoleucine	10.52	10.42	9.93	0.146	0.221
	Leucine	19.24	18.83	18.33	0.231	0.292
	Phenylalanine	9.96	9.50	9.59	0.145	0.426
	Lysine	21.52	21.09	20.47	0.258	0.263
NEAA (mg/g)	Aspartic acid	24.94[a]	22.90[b]	23.05[b]	0.327	0.006
	Glutamic acid	36.58[b]	38.82[a]	35.03[b]	0.534	0.004
	Cystine	8.01	7.15	7.73	0.161	0.074
	Serine	9.60	9.28	9.19	0.104	0.243
	Glycine	10.19[a]	9.14[b]	8.90[b]	0.221	0.023
	Histidine	8.59[ab]	9.12[a]	8.34[b]	0.130	0.030
	Arginine	13.96	13.92	13.28	0.162	0.154
	Alanine	13.27	12.86	12.68	0.140	0.220
	Proline	8.21	7.81	7.72	0.110	0.159
	Tyrosine	8.11	7.78	7.58	0.105	0.114

LE, low energy level; ME, medium energy level; HE, high energy level; EAA, essential amino acids; NEAA, non-essential amino acids; SEM, standard error of the mean.
[a,b,c]Values in the same row with different superscript letters differ significantly ($P < 0.05$).

meat (39). In the present study, the significant decreasing trend of cooking loss and water loss were observed with increasing dietary energy levels, which is consistent with the study of Kang et al. (6). Shear force is an important indicator of meat tenderness. The tenderness of the meat is a major characteristic that is highly related to the overall acceptability of consumers of yak meat. It is highly variable and can be affected by many factors, including muscle fiber temperament, connective tissue composition, and protease configuration modulations within the muscle mass (40). In this study, shear forces were decreased with increasing dietary energy levels. It falls outside the optimum range proposed by Miller et al. (41). The discrepancy in the results might be due to the difference in the diet, age, and breed (42). Zeng et al. (43) have demonstrated that a drastic reduction in shear force due to elevated dietary energy levels could be attributed to an enhanced IMF content. IMF affects the modification of muscle fiber condition, the composition, and content of connective tissue in the muscle, and the configuration of protease in muscle, which can affect muscle tenderness (44). Our results showed that high-energy diets resulted in an elevated IMF content, which can reduce collagen cross-linking and contributes to the tenderness of the meat (45). Liu (46) and Hwang et al. (47) reported that the IMF content has a positive correlation with meat tenderness and juiciness. Furthermore, in this trial, the water content and IMF content in the LT muscle elevated with the increase in the dietary energy level. Therefore, we speculated that the difference in yak meat tenderness receiving different dietary energy levels could be explained through the IMF variations and fatty acid composition. It has been reported that the rumen bacteria could indirectly affect the metabolite deposits within the muscle-mass due to their interplays with the host organism (48).

Amino acids are the basic components of animal protein, and the changes in the amino acid composition directly affect the nutritional value of the meat (49). Since EAA could not be synthesized *in vivo*, the difference in the methionine content in the LT muscle for each group might be caused by differences in the dietary methionine content. The rumen microbiome is essential for meat generation, with rumen microbial protein being a major precursor for meat protein (50). The contents of aspartic acid, glycine, glutamic acid, and histidine might be affected by the microbial synthesis in the rumen. *Streptococcus bovis*, *Selenomonas ruminantium*, and *Prevotella bryantii* of rumen microorganisms are reported to be involved in the *de novo* synthesis of amino acids (51). In this study, dietary energy levels significantly affected the relative abundance of *Prevotella.* Generally, the levels of glutamate/glutamine in beef were the highest, accounting for 16.5% of the total amino acids, followed by aspartic (52), which is consistent with the results of the present study. The increased glutamic acid production in the rumen of ruminants can increase glutamic acid synthesis, and glutamine can be converted into glucose *in vivo* (53). Therefore, the increase of glutamic acid content in the LT muscle of yak fed with a medium dietary energy level might be due to the contribution of rumen microorganisms.

In the current study, neither the alpha diversity nor the relative abundances of the main phyla showed significant variations among the different dietary treatments. The relative abundances of the *Firmicutes* and *Bacteroidetes* were observed to be important phyla in the three groups. At the genus level, the

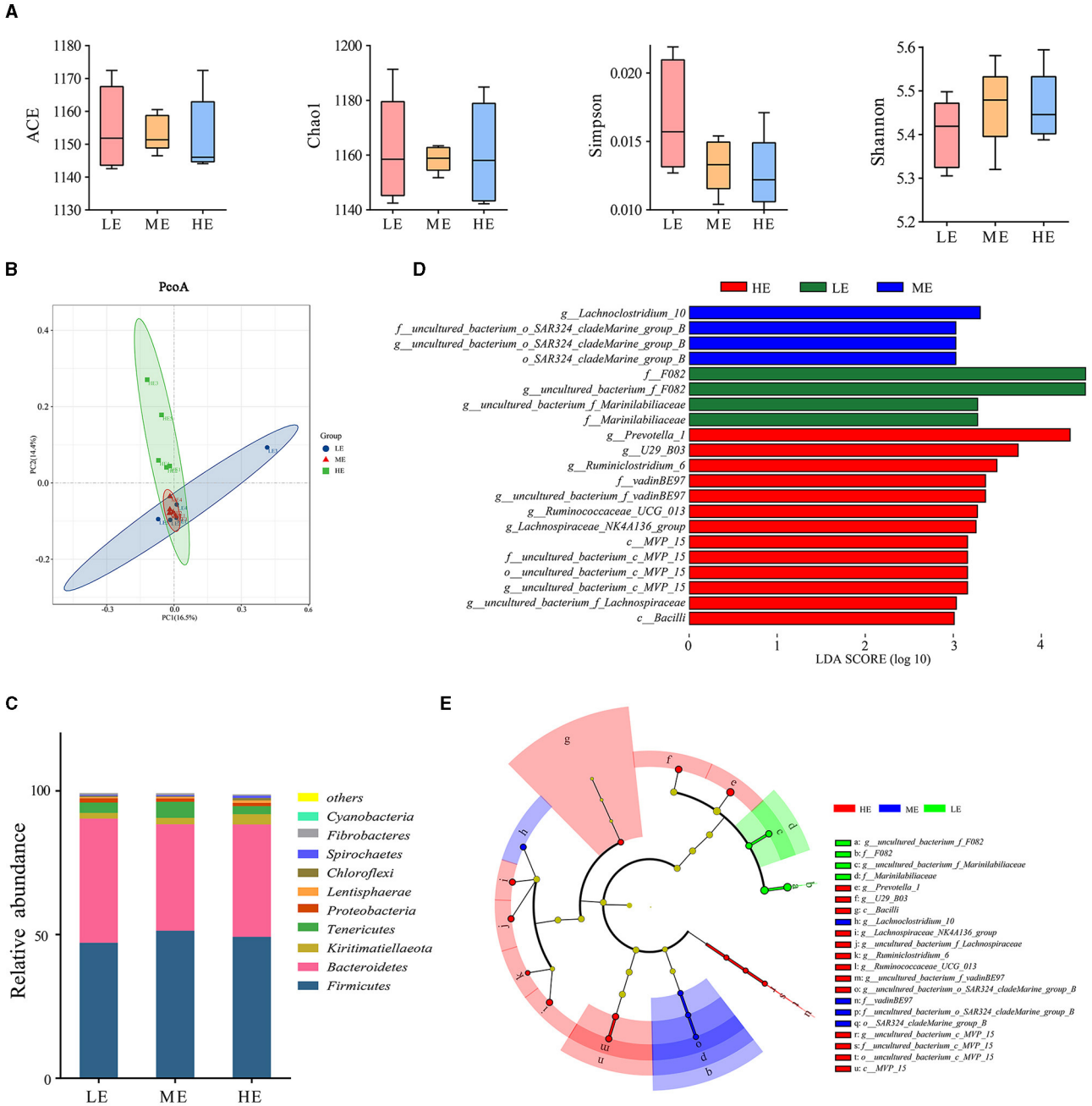

FIGURE 1 | Effect of dietary energy level on the diversity and structure of ruminal bacteria. **(A)** Differences in yak ruminal bacterial diversity and richness between the LE, ME, and HE groups. Box plot showing the alpha diversity of the rumen bacterial communities in yaks given different dietary energy levels. **(B)** Principal-coordinate analysis (PCoA) of bacterial communities based on OTUs. **(C)** Effects of dietary energy levels on the yak rumen bacterial composition at the phylum level. Each bar and color represent the average relative abundance of each phylum, and the 10 most abundant taxa are shown. **(D)** Linear Discriminant Analysis Effect Size (LEfSe). Histogram of the LDA scores for differentially abundant genera among the HE, ME, and LE groups (LDA score ≥ 3). **(E)** A cladogram showing the differences in the relative abundance of the taxa at five levels between the HE, ME, and LE groups.

majority of the genera present in all groups were not affected by the different diets, which is consistent with the results reported by Bi et al. (54). Interestingly, most of the differential bacteria at the genus level belonged to the carbohydrate-degrading bacteria. Rumen microbes degrade carbohydrates into volatile fatty acids (VFAs) to provide 70–80% of the metabolizable energy (55), which are the main substrates for the synthesis of milk fat and body fat (56). Genus *Prevotella_1* is a dominant beneficial bacterial species, which plays a vital role in the degradation of starch, xylan, protein, peptide, hemicellulose, and pectin (57). In the present study, the abundance of *Prevotella_1* increased with the level of starch in the diet, which is consistent with a

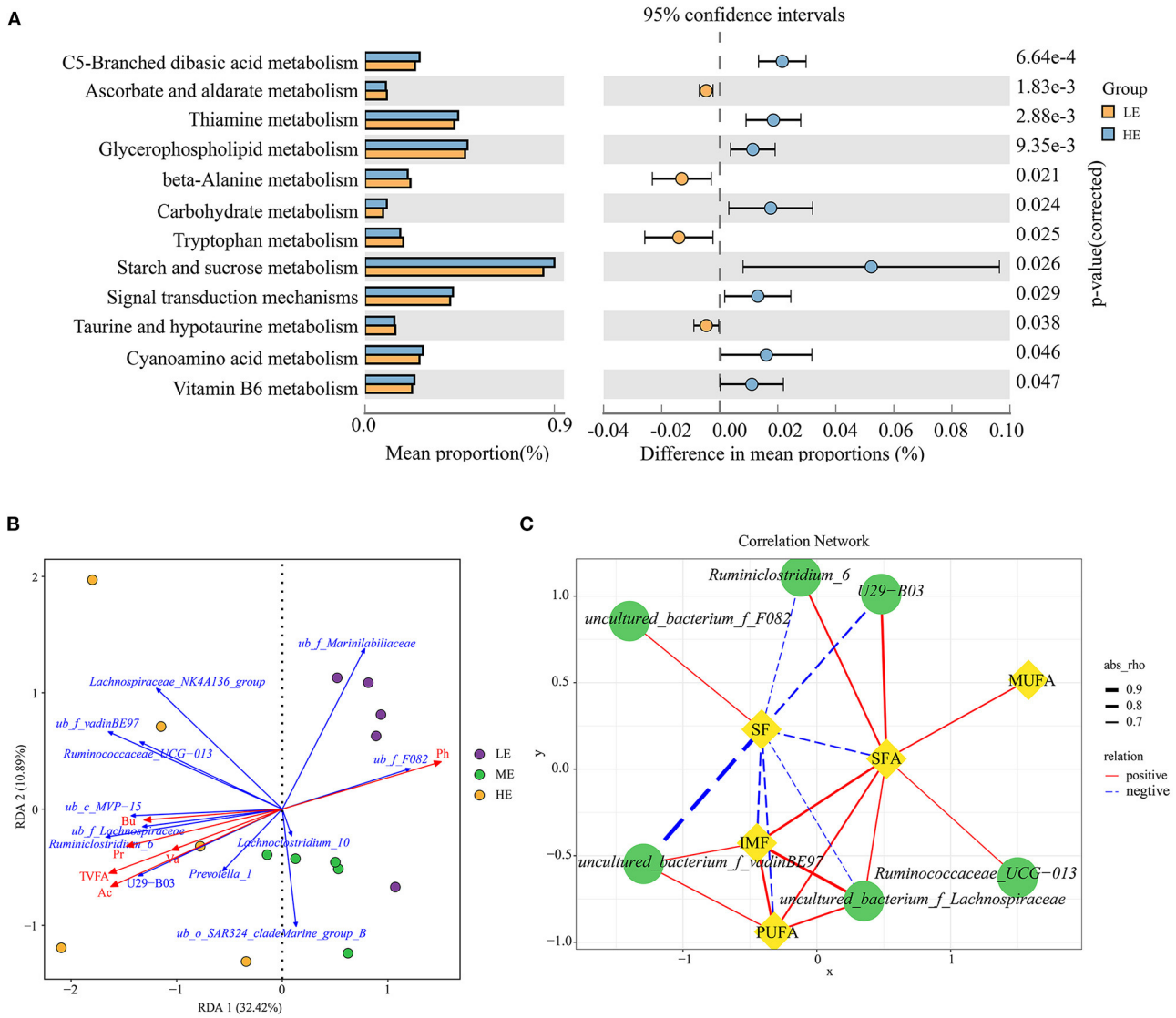

FIGURE 2 | Predicted function analysis of ruminal bacteria and correlation analysis between the phenotypic values and ruminal main taxa. **(A)** Pathways were predicted by PICRUSt2. The significant microbial metabolic pathways between the HE and LE groups (*P*-value < 0.05) are shown. **(B)** Redundancy analysis (RDA) of the sequencing data of the 16S rRNA gene showing relationships between the differential bacteria and environmental factors (TVFA, acetate, propionate, butyrate, valerate, and pH). TVFA, Total VFA; Ac, Acetate; Pr, Propionate; Bu, Butyrate; Va, valerate; ub, uncultured_bacterium. **(C)** Spearman's correlation network showing relationships between the relative abundance of differential bacteria at the genus level and the meat quality indices. Only the strong correlations ($|r| > 0.60$ and *P*-value < 0.05) were showed in the correlation networks.

previous study in Holstein-Friesians bulls (58). In the current study, *Prevotella_1* had the highest LDA score in the HE group, which is mainly involved in the fermentation of starch and production of propionic acid. *U29_B03* is a member of the phylum Bacteroidetes belonging to the family *Rikenellaceae*, and was found to be involved in the degradation of structural carbohydrates (59). In addition, a previous study has reported that the relative abundance of *Rikenellaceae* was elevated in humans and mice when fed a diet with a high resistant starch level (60). Similarly, the relative abundance of *U29_B03* was found to be significantly higher in the HE group than in the ME and LE groups, which indicates that the genus *U29_B03* might be involved in carbohydrate degradation, especially starch metabolism. The genus *uncultured_bacterium_f_vadinBE97*, which belongs to efficient sugar-fermenting families (*vadinBE97*) (61), was significantly higher in the HE group than in the other groups. The bacterial families *Lachnospiraceae* and *Ruminococcaceae* are known to produce butyrate by degrading complex polysaccharides, including starch (62), which supported our results of a dramatic increase in the abundance of four genera (*uncultured_bacterium_f_Lachnospiraceae, Lachnospiraceae_NK4A136_group*, *Lachnospiraceae_10*, *Ruminiclostridium_6*, and *Ruminococcaceae_UCG_013*) which belonged to the families *Lachnospiraceae* and *Ruminococcaceae*. The *uncultured_bacterium_c_MVP_15* is rare in the rumen and its function needs to be studied further; however, its bacterial

phylum (Spirochaetes) has shown that it is primarily responsible for the degradation of starch in a starch-fed reactor (63). In the current study, we observed a decreased abundance of *uncultured_bacterium_f_F082* with increasing dietary energy levels, which was consistent with the results of Zened et al. (11) that uncultured or unclassified bacteria in the rumen were negatively affected by starch addition.

Microbial potential function analysis with PICRUSt2 indicated that the most prominent functional categories were starch and sucrose metabolism and carbohydrate metabolism at the KEGG level 3 metabolic category. As we expected, the proportion of starch metabolism was higher in the HE group than in the LE group when the dietary energy levels increased (corn as the main energy source and the starch content increased with the dietary energy level). Actually, rumen microbial degradation of dietary starch elevated the propionic acid concentration with the increase of starch content. Propionic acid is mainly used in the gluconeogenesis of the liver to synthesize glucose. Propionic acid and glucose are substrates of long-chain fatty acid esterification and fat formation in IMF (64). In the present study, the IMF was found to be significantly positively corrected with SFA and PUFA, probably the high degree of biohydrogenation of unsaturated fatty acids in the rumen and the contribution of SFA to intramuscular fat deposition was greater than that of unsaturated fatty acids (48). Genus *uncultured_bacterium_f_vadinBE97* and *uncultured_bacterium_f_Lachnospiraceae* had a positive influence on the content of IMF, indicating these bacteria underwent a rapid proliferation with the increase in substrates to degrade dietary carbohydrate and provide VFAs to IMF deposition. *Ruminiclostridium_6* and *U29-B03* had a positive influence on the SFA and VFAs had a negative influence on the SF, suggesting that the above-mentioned bacteria may patriciate might participate in carbohydrate metabolism to produce VFAs, thereby facilitating IMF deposition to promote tenderness in the LT muscle. *Ruminococcaceae_UCG-013* genus is famous for its ability to degrade cellulose and hemicellulose in the rumen and produce butyric acid (65). However, in this study, *Ruminococcaceae_UCG-013* had a strong positive correlation with butyric acid and SFA, suggesting that *Ruminococcaceae_UCG-013* was tolerant to a high starch diet. In summary, the aforementioned bacteria mainly degraded dietary starch into VFAs that provided substrates for fatty acid synthesis and, finally, accelerated the fat deposition and enhanced the meat quality in the LT muscle.

It must be taken into account that the exploration of rumen microbial degradation products might be associated with the observed phenotypic differences in the LT muscle. However, the underlying mechanism is still not well-known and more research is needed. Another limitation of this study is that the sample size was too small because yaks are important and expensive for herders, so there is no way to expand the sample size.

CONCLUSIONS

Feeding different energy level diets improved fat deposition, water holding capacity, and tenderness and changed the content of functional amino acids in the LT muscle of yak. Moreover, the carbohydrate metabolism-related bacteria, especially amylolytic bacteria, were positively correlated with the content of IMF but had a negative impact on SF. The results indicated that high dietary energy levels could improve the meat quality in the LT muscle of yak through increasing the abundance of amylolytic bacteria and their fermentation products to provide substrates for fatty acid synthesis.

DATA AVAILABILITY STATEMENT

Supplementary Material and Supporting Data for this article are depositied in the database and can be found at can be found online a https://www.jianguoyun.com/p/DbXaVqwQmrzKCRietoUE. The raw data of 16s rDNA sequence for rumen bacteria of yaks in LE, ME and HE groups were obtained from our previously study (24), and the raw sequence data have been deposited in the European Nucleotide Archive (ENA) at EMBL-EBI under accession number PRJEB34298.

ETHICS STATEMENT

The animal study was reviewed and approved by the procedures of animal experiments complied with Gansu Province Animal Care Committee (Lanzhou, China), and experimental protocols in this study were reviewed and approved by the Animal Care and Use Committee of Lanzhou Institute of Husbandry and Pharmaceutical Sciences, China [SCXK (GAN) 2014-0002]. Written informed consent was obtained from the owners for the participation of their animals in this study.

AUTHOR CONTRIBUTIONS

XD and CY designed the manuscript. MD wrote the manuscript. XD, PY, YY, JZ, AA, and ZL provided the writing guidance and revised the manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fvets.2021.718036/full#supplementary-material

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Multi-Omics Analysis of Mammary Metabolic Changes in Dairy Cows Exposed to Hypoxia

Zhiwei Kong[1,2,3], *Bin Li*[4], *Chuanshe Zhou*[5]*, *Qinghua He*[1]*, *Yuzhong Zheng*[3] and *Zhiliang Tan*[5]

[1] Department of Food Science and Engineering, College of Chemistry and Environmental Engineering, Shenzhen University, Shenzhen, China, [2] Key Laboratory of Optoelectronic Devices and Systems of Ministry of Education and Guangdong Province, College of Optoelectronic Engineering, Shenzhen University, Shenzhen, China, [3] School of Food Engineering and Biotechnology, Hanshan Normal University, Chaozhou, China, [4] State Key Laboratory of Hulless Barley and Yak Germplasm Resources and Genetic Improvement, Institute of Animal Husbandry and Veterinary, Tibet Autonomous Regional Academy of Agricultural Sciences, Lhasa, China, [5] CAS Key Laboratory for Agro-Ecological Processes in Subtropical Region, National Engineering Laboratory for Pollution Control and Waste Utilization in Livestock and Poultry Production, Hunan Provincial Key Laboratory of Animal Nutritional Physiology and Metabolic Process, Institute of Subtropical Agriculture, Chinese Academy of Sciences, Changsha, China

Hypoxia exposure can cause a series of physiological and biochemical reactions in the organism and cells. Our previous studies found the milk fat rate increased significantly in hypoxic dairy cows, however, its specific metabolic mechanism is unclear. In this experiment, we explored and verified the mechanism of hypoxia adaptation based on the apparent and omics results of animal experiments and *in vitro* cell model. The results revealed that hypoxia exposure was associated with the elevation of AGPAT2-mediated glycerophospholipid metabolism. These intracellular metabolic disorders consequently led to the lipid disorders associated with apoptosis. Our findings update the existing understanding of increased adaptability of dairy cows exposure to hypoxia at the metabolic level.

Keywords: metabonomics, lipidomics, hypoxia, lipid metabolism, dairy cow

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Bo Lin,
Guangxi University, China
Hao Xiao,
Guangdong Academy of Agricultural Sciences, China

***Correspondence:**
Chuanshe Zhou
zcs@isa.ac.cn
Qinghua He
qinghua.he@szu.edu.cn

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INTRODUCTION

Mammary gland has the special function of secreting milk and is mainly made up of mammary epithelial cells (MECs). A lot of components in milk can only be synthesized by MECs. The number and activity of mammary epithelial cells reflect the lactation ability of mammary gland (1, 2). Therefore, mammary epithelial cells play an important role in mammary development and lactation. In high-yield dairy cows, a large number of milk production and secretion will cause spikes in energy demand of mammary cells. Thus, its aerobic metabolism activity is significantly enhanced by regulating lipid metabolism (3), leading to hypoxia (determined by arteriomammary venous O_2 and CO_2 level) in the mammary internal environment (4).

Hypoxia is a pathological process involved in a variety of physiological and biochemical changes (5). Hypoxia can cause cell damage, including cell apoptosis, oxidative stress, mitochondrial dysfunction and abnormal lipid metabolism (6). Marques et al. (7) found that increasing lipid storage and inhibiting lipid catabolism could adapt to hypoxia stress. Under hypoxia, lipid storage is mediated by hypoxia inducible factor 1α (HIF1α), and lipid levels are increased by regulating the expression of peroxidase in lipid metabolism (8). The inhibition of hypoxic lipolysis is mainly achieved by reducing the expression of PPAR γ 2 (7) and inhibiting fatty acid β-oxidation (9). As

one of the main substances in milk, milk fat is mainly composed of triglycerides (more than 95%) synthesized from fatty acids and a small amount of other lipids (10, 11). At present, the research on the regulation mechanism of milk fat synthesis mostly focuses on the effects of genetics (12), environment (13), hormone levels (14), and nutritional status (15). However, the mechanism of milk fat synthesis of dairy cow in hypoxic condition has not been elucidated.

Metabolomics based on chromatography and mass spectrometry is a research method to search for the relative relationship between small molecule metabolites and physiological and pathological changes (16). At present, studies on metabolic changes under environmental stress have been carried out in many animals (17, 18). Lipidomics is becoming an important research field based on the development of mass spectrometry and bioinformatics (19). Although some researchers have used these methods to explore the lipid response of dairy cows under biological stress (20), and metabonomics and lipidomics can be used as powerful tools to identify new signaling pathways in hypoxic stress (21, 22), our understanding of lipid responses to hypoxic stressor is still limited. In our previous studies, the results showed that the level of milk fat increased under hypoxia. Therefore, we speculated that the increase of milk fat level was caused by hypoxia through regulating lipid metabolism. In order to test this hypothesis, we first detected the serum biochemical indicators and milk quality, and then constructed a hypoxia stress model of bovine mammary epithelial cells (BMECs) *in vitro*, and finally carried out metabonomics and lipidomics analysis. We found that acylglycerol-3-phosphate acyltransferase 2 (AGPAT2)-mediated glycerophospholipid metabolism played an important role in the process of hypoxia induced increase in lipid synthesis. This will help us to understand the mechanism of hypoxia adaptation more comprehensively.

MATERIALS AND METHODS

The present study was carried out based on the Animal Care and Use Guidelines of the Animal Care Committee, Institute of Subtropical Agriculture, Chinese Academy of Sciences, Changsha, China, with protocol ISA-201710.

Animals and Experimental Design

Twelve multiparous Holstein dairy cows (600 ± 35 kg) were chosen and randomly divided into two groups in Shenyang (GH) (average altitude 50 m). One group (six cattle in each group) was selected and raised in Nyingchi (CH) in autumn (average altitude 3,000 m) for 30 days. Another group was raised in Shenyang at the same time for 30 days. The experimental animals were in good physiological condition, and they were not pregnant cows, but were in early lactation (the third month after delivery). The animals were raised in a single column to reduce the stress caused by excessive exercise.

The TMR basal diet (**Table 1**) is prepared according to the Feeding Standards of Dairy Cattle in China, which can meet the energy, protein, minerals and vitamins required for basic metabolism of dairy cows (23). All dairy cows were freely supplied the same TMR diet.

TABLE 1 | Ingredient composition and nutrition levels of the diet (% of DM).

Items	Content (%)
Diet composition	
Chinese leymus	37.5
Corn silage	22.5
Corn	15.2
Wheat bran	5.3
Soybean meal	9.2
DDGS	8.4
Calcium hydrophosphate	1.4
Premix[a]	0.5
Nutrient composition	
CP	13.1
NDF	39.6
Ca	0.6
P	0.4
NEL[b], MJ/kg DM	5.4

[a]*One kilogram of premix contained mixed vitamins, 800,000 IU; Fe, 1,500 mg; Cu, 1,000 mg; Zn, 11,000 mg; Mn, 3,500 mg; Se, 80 mg; I, 200 mg; and Co, 50 mg.*
[b]*NEL was calculated. DDGS, distillers dried grains with soluble; CP, crude protein; DNF, neutral detergent fiber; NEL, Net energy of Lactation.*

Sample Preparation

On the last day morning, all experimental animals were punctured through the caudal vein and collected blood into K2 EDTA anticoagulant vacuum tube before the feeding. Plasma was separated from blood samples by centrifugation at 3,000 g for 10 min, and then collected and stored at −80°C for subsequent metabolomic analysis. Serum samples were collected from blood at 4,000 rpm for 5 min and stored at −40°C for subsequent detection of biochemical indicators. Milk samples (10 mL) were collected on the morning and evening of the last day of this experiment. The collected milk samples were stored in a −20°C freezer for milk fat level detection.

Analysis of the Serum Biochemical Indicators and Milk Quality

The levels of triglyceride, total cholesterol and high-density lipoprotein were measured by Enzyme-Linked Immunosorbent Assay (ELISA) Kits (Sekisui Diagnostic Ltd., Stamford, CT, USA) through an automatic blood analyzer (Hitachi 7170A, Japan). Milk fat was detected by Basic Unit MilkoScan FT 76150 (FOSS, Denmark).

Cell Culture and Treatment

BMECs were isolated and cultured based on the methods described previously (24). In brief, Bard Magnum biopsy gun and biopsy needle were selected as the tools for udder tissue collection. Fifty to one hundred mg of tissue from 2-year-old late-lactation dairy cows were collected from the midpoint of the upper quarter of the posterior area of the udder 3 h after

milking by No. 12 biopsy needle. The samples was harvested and transferred to the lab immediately. The samples were washed 3 times with DPBS (D8662, sigma, USA), and then transferred to clean cell culture dishes with tweezers. The tissue was cut into 1mm^3 pieces with surgical tweezers. The tissue blocks were evenly daubed on the bottom of the cell culture dish and placed upside down in the carbon dioxide cell incubator for 2–4 h. Then appropriate complete medium [including DMEM/F12 (12400-024; Gibco, USA), 10% fetal bovine serum (FBS; Sigma-Aldrich, St. Louis, MO), penicillin/streptomycin (Baoman Biotechnology, Shanghai, China), and 4 μg/ml prolactin (Sigma-Aldrich)] was added for culture. When the epithelial cells reached about 80% of the bottom of the culture dish, the cells were subcultured with 0.25% trypsin. The cells were purified by differential adhesion and then cryopreserved (25). Purified cells were counted and inoculated in culture dishes. When the cells reached 70–80% confluence, they were treated with hypoxia. The medium was changed every 2–3 d. BMECs were exposed to hypoxia (1% O_2) for 72 h. Cells cultured in an incubator with normoxia (21% O_2) were served as controls.

siRNA Transfection

AGPAT2 siRNA library for various parts of AGPAT2 mRNA and negative control siRNA (NC) were designed and provided by Ribo Bio (Guangzhou, China). The siRNA sequence of AGPAT2 applied was shown as following: siRNA-1: 5′-CTACCGTTGTTATAGGTG-3′ (control), siRNA-2: 5′-GGAGAATCTCAAAGTGTGG-3′, siRNA-3: 5′-TGTCAAGACGAAGCTCTTC-3′. BMECs were transfected with 2 mg of the AGPAT2 siRNA or NC siRNA in 10 mL X-tremeGENE siRNA Transfection Reagent (03366236001, Roche). Twenty-four hours after transfection, the cells were collected and used for subsequent index detection.

Determination of Triglyceride (TAG) Content in Cells

BMECs were pretreated with hypoxia for 72 h. TAG levels were determined as described previously (26). Briefly, the treated cells were firstly digested by trypsin and collected, and then the triglyceride test solution was extracted according to the instructions of the triglyceride detection kit (Applygen, Beijing, China). Finally, the microplate reader (BIORAD, CA, USA) was adjusted to the appropriate wavelength and the triglyceride content was calculated according to the OD value.

Oil Red O (ORO) Staining

BMECs were pretreated with hypoxia for 72 h. The formation of lipid droplets was observed according to the method previously described (27). Briefly, the treated BMECs cells were fixed with 10% neutral formaldehyde fixing solution (Sigma-Aldrich, St. Louis, MO, USA), stained with ORO (Sigma-Aldrich), then rinsed and decolorized with 75% alcohol/60% isopropanol, and finally re-stained with light hematoxylin (Sigma-Aldrich) and sealed with glycerol gelatin for observation and photography under the microscope (Olympus, Tokyo, Japan).

Fluoroboron Dipyrrole (BODIPY) Staining

After hypoxia treatment, the cells were collected and fixed with 4% formaldehyde (Sigma-Aldrich), then rinsed using phosphate buffer, stained by BODIPY493/503 (Thermo Fisher Scientific, MA, USA), and then covered with tablet sealant containing DAPI. Finally, confocal laser microscope was used to take photos and observe the distribution of lipid droplets.

Real-Time PCR

After treatment of hypoxia for 72 h, the level of genes related to milk fat synthesis were determined as described previously (28). In brief, $1 \times 10^6 \sim 1 \times 10^7$ cells were taken from each sample, and the cells were washed with PBS. PBS was removed and 1 mL of RNA extraction reagent Trizol (Invitrogen) was added. RNA was extracted after lysis. Genomic DNA was cleared by DNaseI (Thermo Scientific), and the quality and quantity of RNA obtained were evaluated by Nano Drop 2000 (Thermo Scientific). Primers were designed using Primer 5.0 software, and PCR Primer sequences were shown in **Table 2**. The PCR amplification conditions were as follows: pre-denaturation at 95°C for 30 s; after denaturation at 95°C for 5 s, each gene was annealed at the optimum annealing temperature for 20 s and 72°C for 20 s, a total of 40 cycles were carried out. After PCR reaction, the melting curve was drawn to judge the correctness of the amplified products. The temperature raised from 60 to 95°C at the rate of 0.5°C/5 s. Using β-actin as internal reference, the CT values of each sample were homogenized. Under the condition that the amplification efficiency of each target gene and β-actin was basically the same, the expression levels of related genes were compared and analyzed by $2^{-\Delta\Delta CT}$ using the gene expression level of control group as the reference (29).

Western Blotting Analysis

After treatment of hypoxia for 72 h, the protein isolation and western blotting of BMECs were conducted based on previous methods (30, 31). Briefly, in 10% SDS-polyacrylamide gels, electrophoresis separation was performed for each sample and pre-stained standard (Bio-RAD Laboratories, Berkeley, CA, USA). The polyvinylidene fluoride (PVDF) membranes (Bio-RAD Laboratory) containing isolates were firstly incubated with primary antibodies (see **Table 3**) at 4°C overnight, washed and then incubated in a blocking solution with secondary antibody (1:6,000, Proteintech) at 25°C for 2 h. The images were taken and analyzed by a Alpha Imager 2200 digital imaging system (Digital Imaging System, Kirchheim, Germany).

Metabolomics and Lipidomics Analysis

One hundred microliters of blood samples/BMECs were collected. Three hundred microliters methanol (including internal standard) was added to the sample, and protein precipitation was obtained by vortices. Samples after vortex were then centrifuged at 12,000 rpm for 15 min. Finally, transfer the supernatant to LC-MS loading bottle for storage at −80°C for UHPLC-QE Orbitrap/MS analysis (metabolomics). Cells samples were homogenized with MTBE and sonicated in ice-water bath for 5 min. Then the sonicated samples were centrifuged at a rate of 3,000 rpm at 4°C for 15 min. Three hundred microliters

TABLE 2 | Gene primers.

Gene Name	GeneBank No.	Primer Sequence (5′-3′)	Product Size (bp)
FASN	NM_001012669.1	GCAGCCCCTCAAGCGAACAGT ACCGCCTCCTGCTCTTCCTCACGTAA	123
ACACA	NM_174224.2	CTCTTTGTTTGGTCGTGATTGCTCT CTGGCAAGTTTCACCGCACAC	126
SCD1	NM_173959.4	CCAACAACTCTGCCTTTATGATGC TGACTGACCACCTGCTTGCC	155
CD36	NM_001278621.1	CCCTGCAAAACACAGACCCA ATGGTTATAATGCCTTGCTGATGCT	178
FABP3	NM_174313.2	ACCAAGCCTACCACAATCATCG ACAAGTTTGCCGCCATCCAG	170
LPL	NM_001075120.1	AGCGAGAACATCCCTTTTACCCT GCAATTCTCCAATATCCACCTCCGT	91
PPARG	NM_181024.2	TTCCCAAGAGCTGACCCGAT TCCCCACAGACCCGGCAT	185
SREBP1	NM_001113302.1	ACAGCCCACAACGCCATCGAG CCTCCACTGCCACAAGCCGACAC	248
β-actin	NM_173979.3	ACTGTTAGCTGCGTTACACC TGCTGTCACCTTCACCGTTC	167

FASN, fatty acid synthase; ACACA, acetyl-CoA carboxylases alpha; SCD1, stearoyl coenzyme A1; CD36, Fatty acid synthase subunit beta; FABP3, fatty acid binding protein 3; LPL, lipoproteinlipase; SREBP1, sterol regulatory element binding protein1; PPARG, peroxisome proliferator-activated receptor gamma.

TABLE 3 | Details of the primary antibodies.

Name	No.	Host species	Dilution	Supplier
PPARG	ab45036	Rabbit	1:500	Abcam
SREBP1	14088-1-AP	Rabbit	1:1,000	Proteintech
β-actin	66009-1-Ig	Mouse	1:5,000	Proteintech

PPARG, peroxisome proliferator-activated receptor gamma; SREBP1, sterol regulatory element binding protein1.

of supernatant was taken and dried. Then, the dried samples were reconstituted and centrifuged. Appropriate amount of the recombinant supernatant was put into a new sample bottle for LC/MS analysis (lipidomics).

LC-MS/MS Analysis for untargeted metabolomics: UHPLC system (1290, Agilent Technologies) combined Q exactive (Orbitrap MS, thermo) performed the LC-MS/MS analysis for this trial. The mobile phase A used in the instrument is 0.1% formic acid aqueous solution and acts as a positive. Mobile phase B is acetonitrile, while ammonium acetate aqueous solution acts as negative. The sample size required for the test is 3 μl. The characteristic of the mass spectrum is to obtain the MS/MS spectrum in an information-dependent basis (IDA).

LC-MS/MS Analysis for untargeted lipidomics: the UPLC system used in this test is unique in that it is equipped with kinetex C18 column and Exionlc infinity system. Mobile phase A (positive) is a mixture of water, acetonitrile and ammonium formate, while mobile phase B (negative) is a mixture of acetonitrile, isopropyl alcohol and ammonium formate. The loading quantity is 2 μl. Spectra were obtained using a TripleTOF 5600 mass spectrometer.

For data processing of metabonomics, the original data is converted into mzXML format by proteowizard and processed by internal program. The program is developed by R and based on xcms for peak detection, extraction, comparison, and integration. The MS2 internal database (BiotreeDB) was then used for metabolite annotation. The cutoff value for comments is set to 0.3. For data processing of lipidomics, the raw data files (.wifff format) have been converted to mzXML format through the msConvert program in the Proteowizard. Firstly, the CentWave algorithm in XCMS was used to detect the peak value of MS1 data, and then the obtained MS/MS spectra were matched with LipidBlast library to obtain the lipids screened in the experiment.

Statistical Analysis

SPSS software is mainly applied for data analysis. The levels of triglyceride in cells, relative mRNA expression of genes associated with milk fat synthesis and protein abundance were analyzed using one-way ANOVA. The data are shown as the mean ± SD.

Q Exactive Orbitrap (Thermo Fisher Scientific, USA) and Ultra High Performance Liquid Tandem Chromatography Quadrupole Time of Flight Mass Spectrometry (UHPLC-QTOFMS, AB Sciex, USA) were applied to analyze the data of metabolomics and lipidomics. Principal component analysis is conducted on the normalized original data to observe the reliability of the data. Orthogonal partial least squares discriminant analysis (OPLS-DA) was used to filter out the non-conforming metabolites. Univariate statistical analysis (UVA) was used to screen the differential metabolites [$P < 0.05$, and Variable Importance in the Projection (VIP) > 1] and make the volcanic map. KEGG, PubChem and other authoritative metabolite databases were used to analyze the metabolic pathways of differential metabolites. Self-built database from Shanghai Biotree biotech CO., Ltd. was used to lipidomics analysis.

RESULTS

Serum Lipid Metabolism Related Indexes and Milk Fat Level

As shown in **Figure 1**, the level of serum triglycerides and high-density lipoprotein increased ($P < 0.05$) in hypoxia-stressed dairy cows compared with hypoxia-free dairy cows. In addition, the dairy cows in hypoxia group had higher ($P < 0.05$) milk fat level than that of the control group.

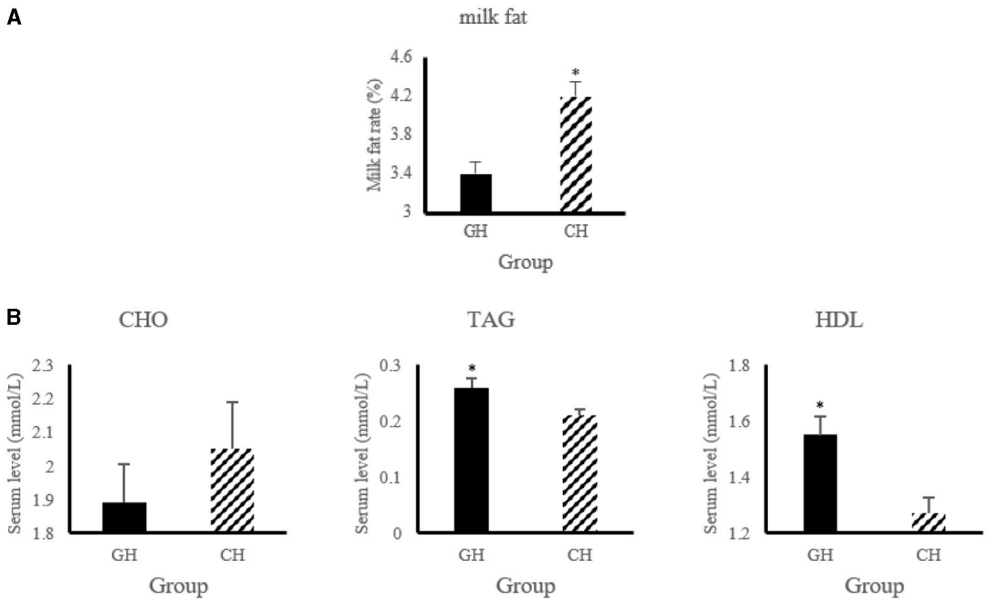

FIGURE 1 | Lipid metabolic changes of altered by hypoxia exposure in milk and blood of dairy cows. **(A)** Changes of milk fat and protein. "GH and CH" represent control, hypoxia group, respectively; **(B)** Changes of serum biochemical indexes. CHO, Cholesterol; TAG, triglycerides; HDL, high-density lipoprotein. $n = 6$, $^*p < 0.05$.

Plasma Metabolomics Analysis of Hypoxic Dairy Cows

As shown in **Figure 2**, we could observe the metabolic changes induced by hypoxia exposure in plasma of dairy cows. In positive ionization mode, 4,083 metabolic characteristics of plasma were extracted to establish PLS-DA model (**Figure 2A**). Goodness of fit (R^2Y) and predictive power (Q^2) are often used to verify PLS-DA model. The R^2Y and Q^2 values of the PLS-DA model are both >0.9, which indicates that the PLS-DA model has good fitting and strong prediction ability. In combination with the results of the volcanic plots (**Figure 2B**), we found that hypoxia exposure interfered with plasma metabolism in dairy cows. Through MS2 spectral matching, 96 potential biomarkers were identified (**Supplementary Table 1**; **Supplementary Material**) in plasma, including amino acids, peptides, nucleosides, nucleotides, and phospholipids. In addition, metaboanalyst (4.0) based on KEGG database was used to analyze the most relevant metabolic pathways of hypoxia exposure changes (**Figure 2C**). From the results, the up-regulated pathways were arginine and proline metabolism, glycine, serine and threonine metabolism, and glycerophospholipid metabolism, while the down regulated pathways were fatty acid metabolism (**Figure 2C**), which were involved in metabolic disturbance.

Hypoxia Contributes to Lipid Synthesis in BMECs

The results of lipid synthesis induced by hypoxia are shown in **Figure 3**. The results of ORO and BODIPY staining indicated that hypoxia could promote the formation of lipid droplets in BMECs (**Figures 3A,B**). The level of TAG in hypoxia group was significantly higher ($P < 0.05$) than that of control group (**Figure 3C**). In addition, compared with the control group, the mRNA expressions of ACACA, FABP3, LPL, PPARG, and SREBP1 increased significantly ($P < 0.05$), while the levels of CD36, FASN and SCD1 decreased ($P < 0.05$) significantly (**Figure 3D**). The protein abundance of PPARG and SREBP1, the key protein of hypoxia cell lipid synthesis, was also increased ($P < 0.05$) significantly (**Figure 3D**).

Cell Metabolomics Analysis

As shown in **Figure 4**, we could observe the metabolic changes induced by hypoxia exposure in BMECs. In positive ionization mode, 3,361 metabolic characteristics of BMECs were extracted to establish PLS-DA model (**Figure 4A**). The R^2Y and Q^2 values of the PLS-DA models are both >0.9, which shows that hypoxia exposure can interfere with BMECs metabolism. The results in volcanic plots (**Figure 4B**) also showed the same condition as shown in **Figure 4A**. Through MS2 spectral matching, 302 potential biomarkers such as amino acids, peptides, nucleosides, nucleotides, and phospholipids were identified (**Supplementary Table 2**; **Supplementary Material**). In addition, the up-regulated pathways including arginine and proline metabolism, glycine, serine and threonine metabolism, and glycerophospholipid metabolism and down-regulated pathways such as fatty acid metabolism (**Figure 4C**) were screened out by metaboanalyst (4.0) based on KEGG database.

Cell Lipidomics Analysis

In the current research, non-targeted HPLC-QTOF-MS was used to investigate the differential expression of lipid metabolites in hypoxic and normoxic BMECs with high sensitivity, specificity and peak resolution (**Figure 5**).

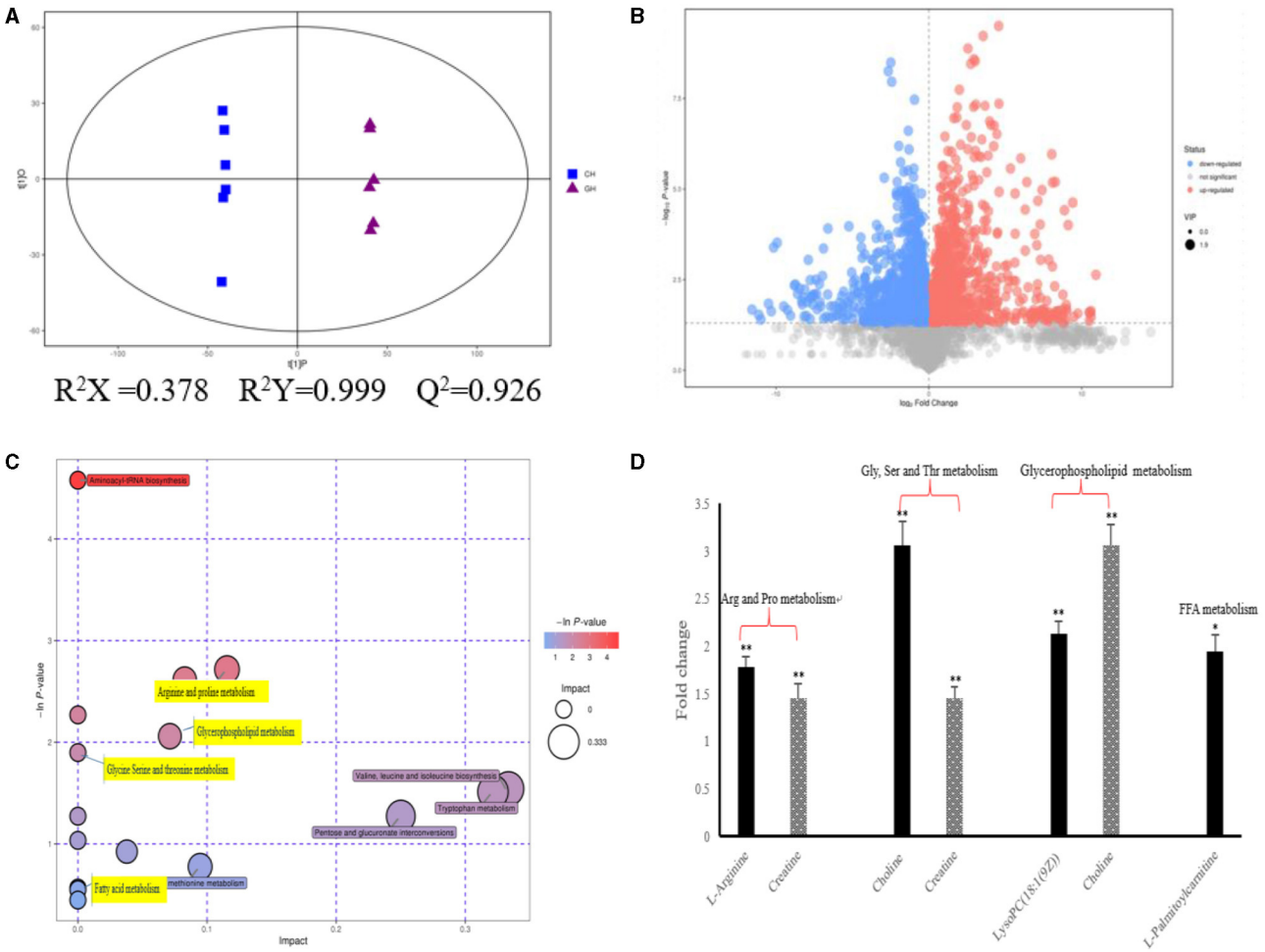

FIGURE 2 | Metabolic changes altered by hypoxia exposure in plasma of dairy cows. **(A)** PLS-DA score plot of the two groups in plasma of dairy cows using the identified metabolites in positive ionization mode. "GH and CH" represent control, hypoxia group, respectively; **(B)** Volcanic plots of the two groups in plasma of dairy cows using the identified metabolites in positive ionization mode; **(C)** Pathway analysis of the identified metabolites in plasma; **(D)** The fold changes of the significantly changed metabolites of plasma in metabolic pathways in hypoxia exposure group relative to the control group. Arg, Arginine; Pro, proline; Gly, Glycine; Ser, serine; Thr, threonine; Val, Valine; Leu, leucine; Ile, isoleucine; FFA, Free Fatty acid. $n = 6$, $^{*}p < 0.05$, $^{**}p < 0.01$.

After signal standardization, lipids identified from positive and negative ionization modes were introduced into SIMCA-P to establish the PLSDA model (**Figure 5A**; **Supplementary Figure 1**; **Supplementary Material**). The R^2Y and Q^2 values of the two PLS-DA models were both >0.8, which indicated that the model had good fitting and strong prediction ability. We have demonstrated that the differential regulation of glycerophospholipid metabolites (**Figures 5B,C**) had a strong correlation with the diagnosis and prognosis of hypoxia, and a total of 541 lipids (**Supplementary Figure 2**; **Supplementary Material**) were screened, in which 27 phosphatidylethanolamines (PEs), 9 phosphatidylserines (PSs), 31 phosphatidylcholine (PCs), and 3 phosphatidylglycerols (PGs) were up-regulated. Oppositely, the levels of 3 PCs decreased (**Figure 5D**). The details of the differential lipids were shown in **Supplementary Table 3**; **Supplementary Material**.

Cell Apoptosis Detection

The changes of cell apoptosis after hypoxia exposure are shown in **Figure 6**. Compared with normoxic BMECs, the apoptosis rate of hypoxic BMECs increased ($P < 0.05$) significantly.

Verification the Role of Glycerophospholipids Metabolism

The effects of glycerophospholipids metabolism on the lipid synthesis induced by hypoxia in BMECs are shown in **Figure 7**. The results of ORO and BODIPY staining showed significantly reduced ($P < 0.05$) lipid droplet formation in shAGPAT2 group compared with that of hypoxia group, while no significant difference ($P > 0.05$) was shown between control and shAGPAT2 group (**Figures 7A,B**). The level of TAG in shAGPAT2 group was significantly decreased ($P < 0.05$) than that in hypoxia group, while no significant difference ($P > 0.05$) was found between control and shAGPAT2 group (**Figure 7C**).

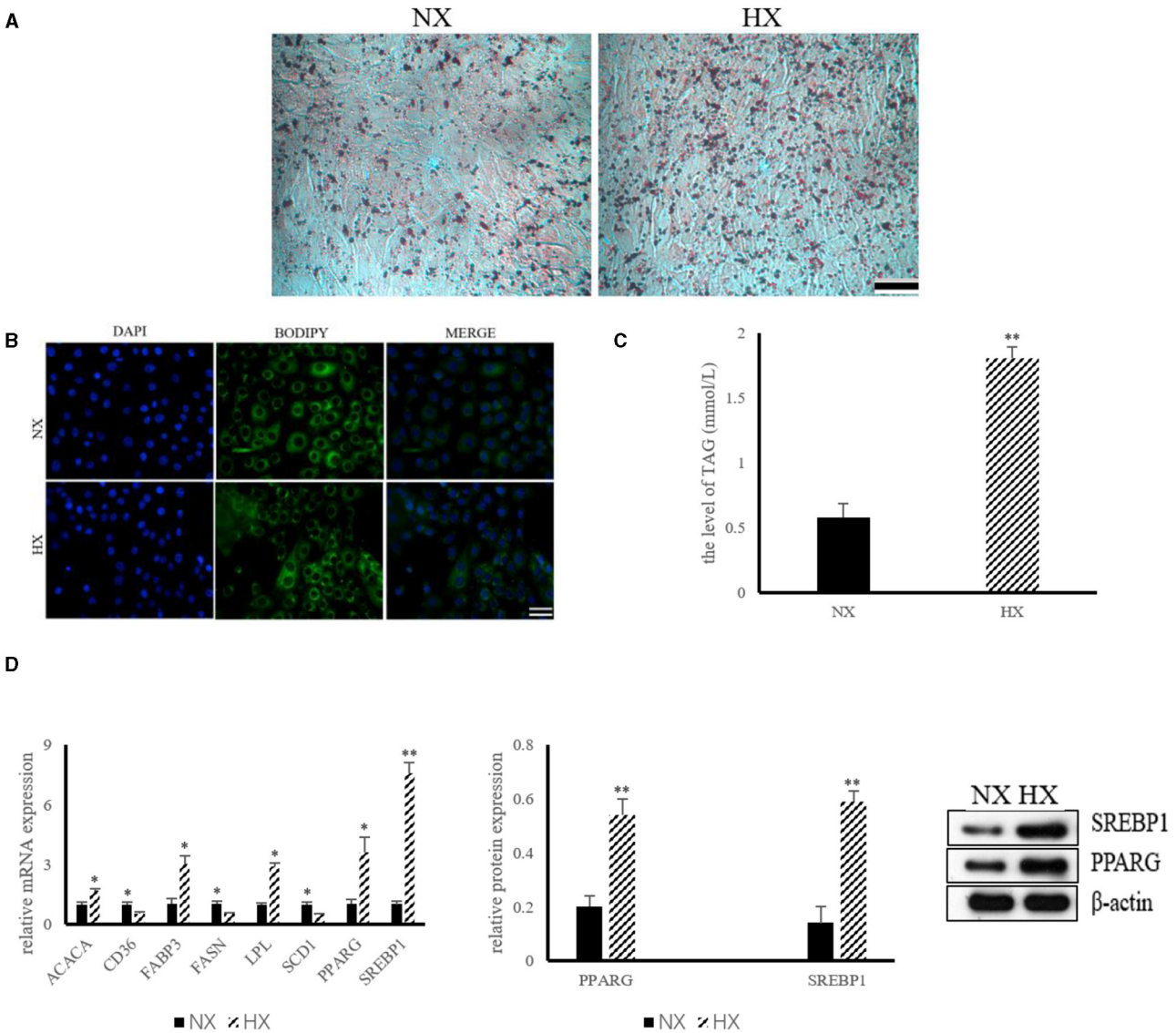

FIGURE 3 | Lipid synthesis by hypoxia exposure in BMECs. **(A)** ORO staining of BMECs under hypoxia. NX, normoxia; HX, hyoxia; **(B)** BODIPY staining of BMECs under hypoxia; **(C)** Level of TAG in BMECs under hypoxia; **(D)** Expression of genes and proteins related to lipid synthesis under hypoxia. $n = 3$, $^{*}p < 0.05$, $^{**}p < 0.01$. FASN, fatty acid synthase; ACACA, acetyl-CoA carboxylases alpha; SCD1, stearoyl coenzyme A1; CD36, Fatty acid synthase subunit beta; FABP3, fatty acid binding protein 3; LPL, lipoproteinlipase; SREBP1, sterol regulatory element binding protein1; PPARG, peroxisome proliferator-activated receptor gamma.

DISCUSSION

In our previous study on the adaptive mechanism of Holstein dairy cows to hypoxia, we found that the milk fat rate increased obviously. At present, the research on its mechanism is not clear, so we intend to explore it in this paper. The most striking finding of our trial was that milk fat rate levels increased significantly in hypoxia condition. Interestingly, we measured several biochemical indexes related to lipid metabolism, such as cholesterol, high density lipoprotein, ApoA1, and ApoB (32), and found that hypoxia could affect the changes of these indexes in varying degrees, indicating that hypoxia promotes the accumulation of serum lipids, which was consistent with the previous description that TG mobilization and lipid peroxidation could be increased by hypoxia exposure (33).

In this study, a non-target metabolomics approach was used to study the metabolic disorders and adaptive mechanisms associated with hypoxia exposure in dairy cows. The results of metabolomics of plasma showed that glycerophospholipids metabolism was significantly up-regulated (34). As an inflammatory mediator, LysoPC regulated the proliferation and apoptosis of endothelial cells (35). In hypoxic dairy cows, the body adapted to hypoxic stress by regulating inflammation (32), which explained why the level

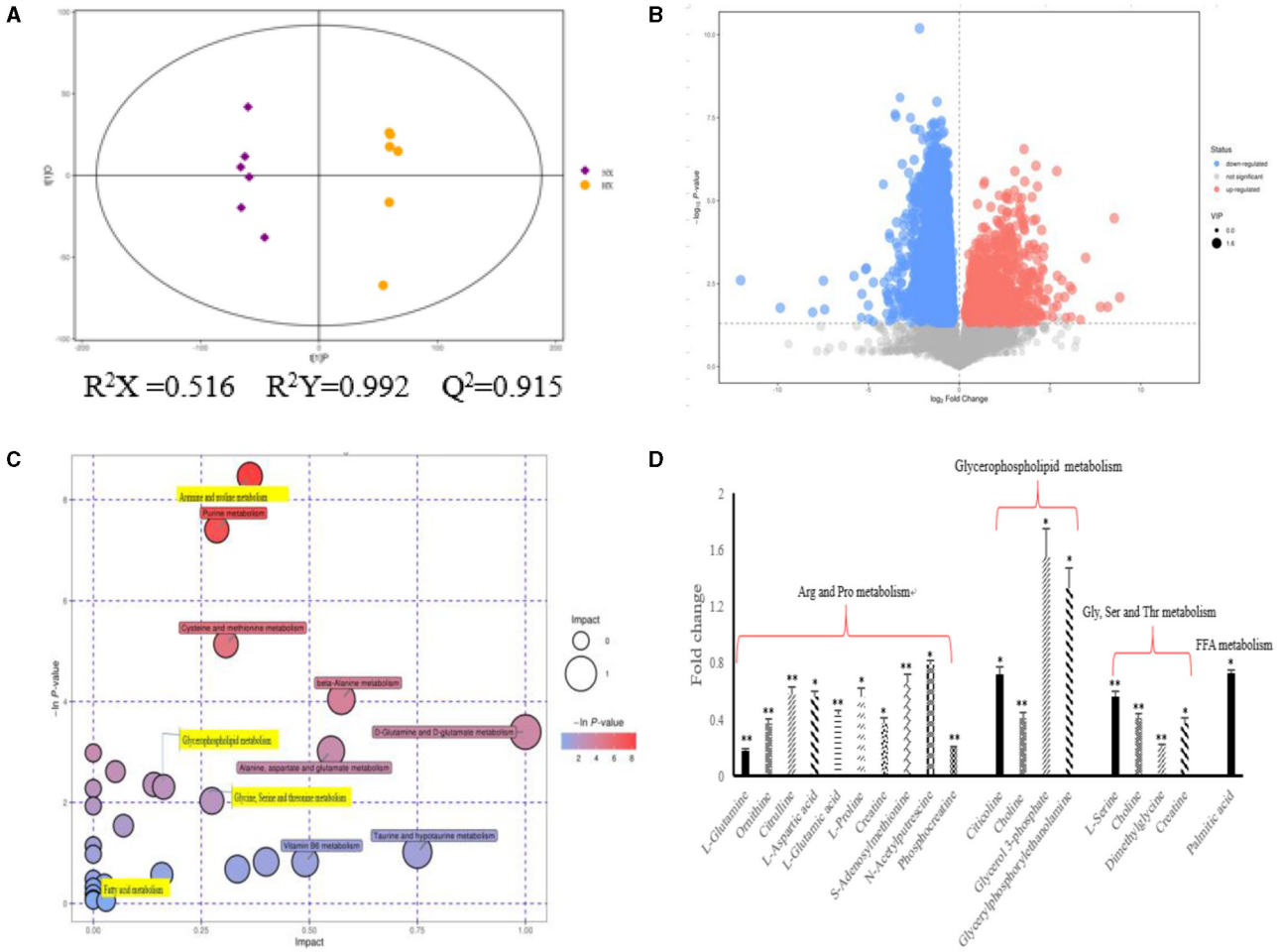

FIGURE 4 | Metabolic changes altered by hypoxia exposure in BMECs of dairy cows. **(A)** Two groups of PLS-DA scores were performed on BMECs using the identified metabolites under positive ionization mode. "NX" and "HX" represent normoxia and hypoxia groups, respectively; **(B)** Volcanic plots of the two groups in BMECs using identified metabolites under positive ionization modes; **(C)** Pathway analysis of identified metabolites in BMECs; **(D)** Fold changes in metabolites significantly altered in the metabolic pathway of BMECs in the HX group compared to the NX group. Arg, Arginine; Pro, proline; Gly, Glycine; Ser, serine; Thr, threonine; Val, Valine; Leu, leucine; Ile, isoleucine; FFA, Fatty acid. $n = 6$, $^{*}p < 0.05$, $^{**}p < 0.01$.

of lysophosphatidic acid (LPA) increased significantly in this experiment.

It was found that the level of free fatty acids (FFA) in plasma was down regulated. Former studies indicate that the level of l-palmitoylcarnitine in plasma can be used as a marker of body FA metabolism. In the present work, we found that the level of l-palmitoylcarnitine was down regulated in plasma metabolomics, which may be due to hypoxia regulating HMG CoA reductase activity to up regulate FA metabolism in hypoxic cows (36).

Phenotypic and metabolomic results of plasma indicate that glycerophospholipid metabolism contributes to hypoxia adaptation. To further investigate whether glycerophospholipid metabolism play an equally important role in mammary epithelial cells, we used metabolomics and lipidomics to verify this.

The results of RT-PCR indicated that hypoxia increased significantly the level of ACACA, FABP3, LPL, PPARG, and SREBP1, which may be due to the up regulation of genes related to FASN and SREBP1 under hypoxia stimulation. SREBP1 regulates FASN, SCD1, and intracellular FABP3 (37, 38). In addition, LPL gene was the key to the uptake and secretion of long-chain fatty acids in milk, and its increased mRNA expression could promote the uptake and transport of long-chain fatty acids, thus promoting milk fat synthesis (39). However, the mRNA levels of CD36 and FASN were significantly decreased, which might be due to that milk fat synthesis was not through the regulation of these genes (40, 41). The results from western blotting indicated that the protein expression of SREBP1 and PPARG increased significantly by hypoxia exposure, which might due to that SREBP1 and PPARG genes were two important factors in regulating mammary milk fat synthesis (42). When migrated from endoplasmic reticulum to Golgi, SREBP1 precursor was hydrolyzed by protease, and then mature SREBP1 with transcriptional activity was released into nucleus

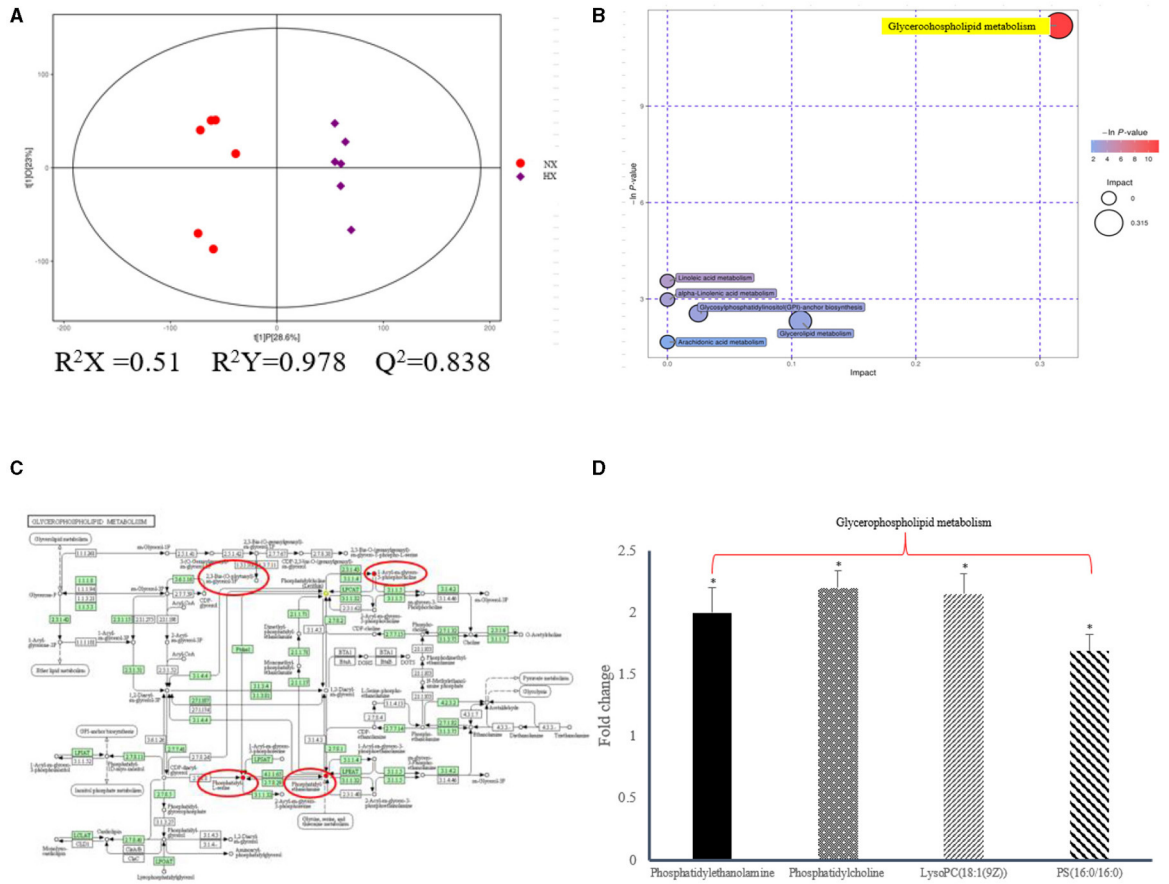

FIGURE 5 | Lipidomic changes altered by hypoixa exposure in BMECs. **(A)** PLS-DA score plot of the two groups of BMECs using the differential metabolites under positive ionization mode. "NX and HX" represent normoxia and hypoxia group, respectively; **(B)** Pathway analysis of the differential metabolites; **(C)** KEGG enrichment analysis of diffential metabolites; **(D)** Fold changes of the significantly changed metabolites in glycerophospholipid metabolic pathways in HX group relative to NX group. $n = 6$, $^*p < 0.05$.

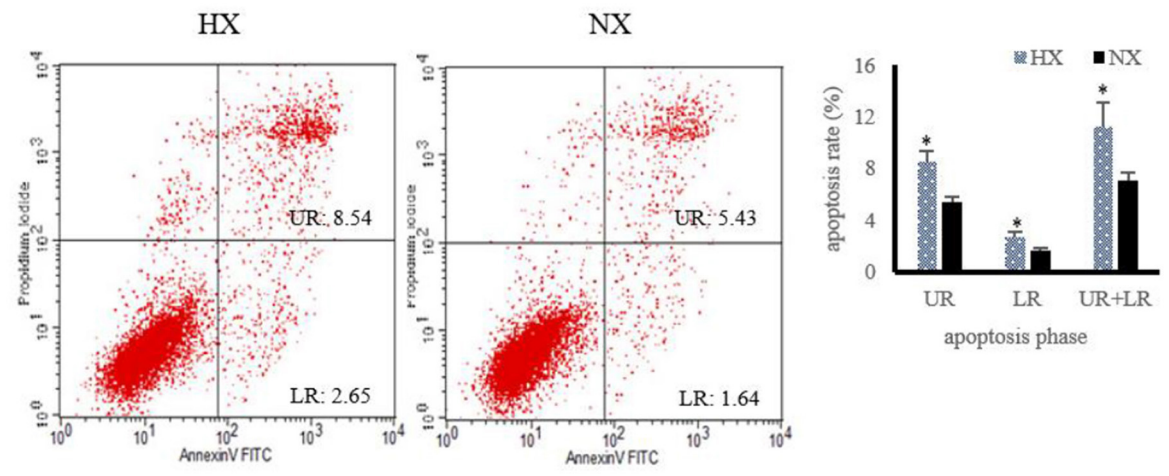

FIGURE 6 | Changes of cell apoptosis after hypoxia exposure ($n = 3$). Apoptosis rate under hypoxia detected by flow cytometry, "HX and NX" represent hypoxia, normoxia group; The data are expressed as mean ± SD. Compared with the control, $^*p < 0.05$ show that the difference is statistically significant.

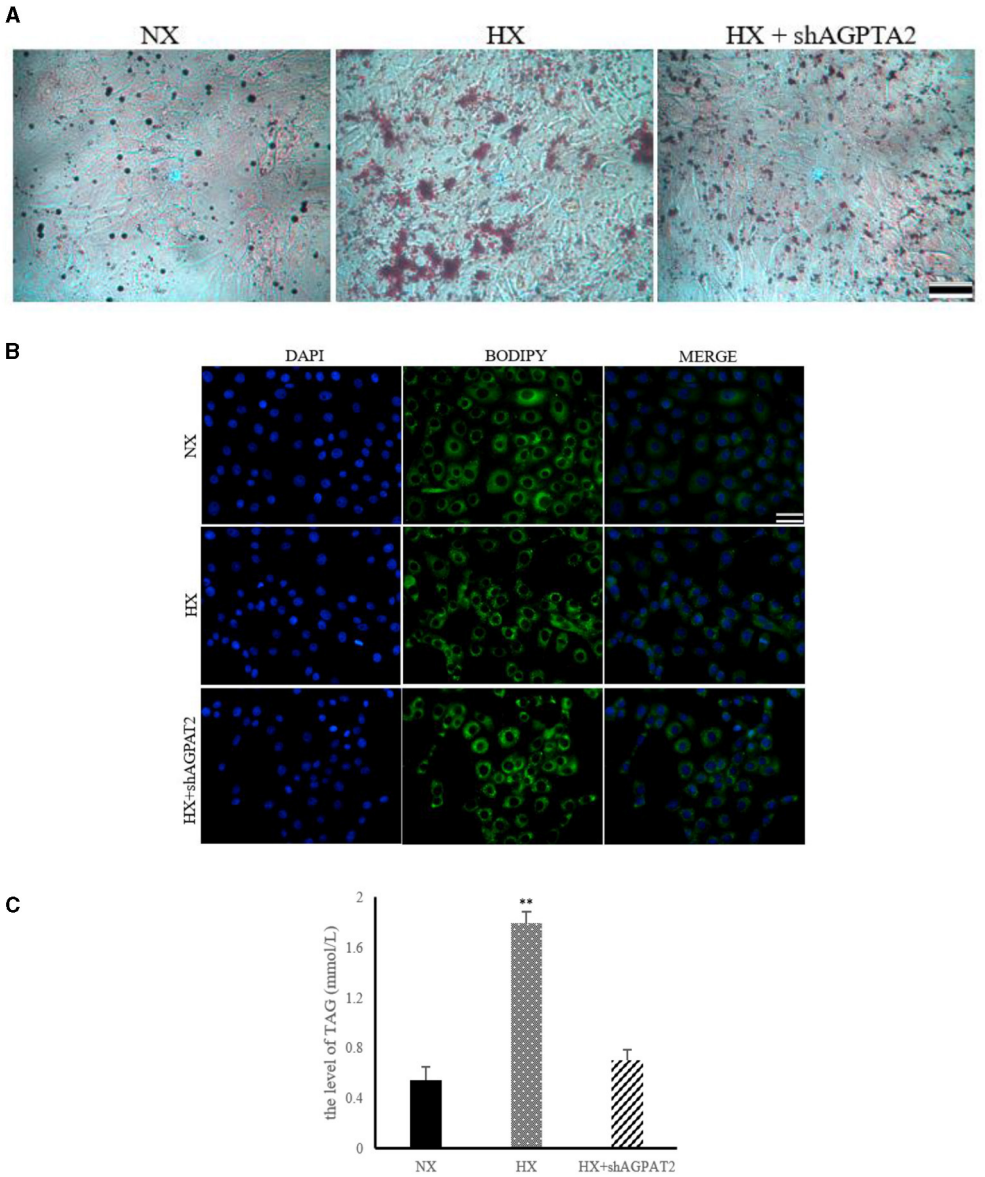

FIGURE 7 | Effect of silencing of AGPAT2 gene on lipid synthesis in BMECs. **(A)** ORO staining of BMECs under silencing of AGPAT2 gene. NX, normoxia; HX, hypoxia; **(B)** BODIPY staining of BMECs under silencing of AGPAT2 gene; **(C)** Level of TAG in BMECs under silencing of AGPAT2 gene; $n = 3$, $^{**}p < 0.01$.

by Golgi (43). In conclusion, these results indicated that hypoxia promoted the synthesis of milk fat in BMECs.

In the metabolomics of BMECs, palmitic acid was found to be the marker of fatty acid metabolism, which was consistent with the previous research results (44). The level of palmitic acid was down regulated (FC = 0.73), probably because most of palmitic acid synthesized in mammary gland was used to synthesize triglycerides, leading to the increased level of triglycerides in mammary epithelial cells in this experiment.

Additionally, glycerophospholipids metabolism was up-regulated (34) in BMECs. As an important intermediate in the phospholipid biosynthesis pathway of cell membrane, citicoline was mainly synthesized *in vivo* and is a choline donor (45). Citicoline was mainly composed of two main components, cytidine and choline. In the results of BMECs in this experiment, the level of choline was down regulated, which was consistent with the change of citicoline level. A significant increase in milk fat level was found in the milk of hypoxic cows in this experiment, which may be caused by the increase in the level of glycerol-3-phosphate in BMECs. Studies found that triglycerides were produced by continuous fatty acylation of glycerol-3-phosphate in eukaryotic cells (46), which confirmed the above conjecture. Glycerophosphate ethanolamine was a direct substrate for the synthesis of phosphatidylethanolamine

and phosphatidylcholine (47). This experiment found that the increased level of glycerophosphate ethanolamine may be due to the increased synthesis of phosphatidylethanolamine and phosphatidylcholine under hypoxic conditions, thus more glycerophosphate ethanolamine was needed to reshape the body's lipid membrane damage (48).

Lipidomics is a new technology to analyze the final products of lipid metabolism and reveal the internal changes of the whole organism. PCs is the main scaffold of biofilm. A total of 31 PCs increased significantly, including 7 containing polyunsaturated fatty acids (PUFA), 10 containing unsaturated fatty acids, and 13 containing saturated fatty acids. Previous study showed that the fluidity of the membrane was determined by the degree of saturation of the fatty acid chain (49). The increase of PCs containing polyunsaturated fatty acids indicated that the fluidity of cell membrane changes with hypoxia treatment, which promoted the release of lipid produced by mammary epithelial cells into milk (50). PSs was a component of cell membrane. Under normal circumstances, it was maintained in the inner lobule through a family of aminophosphatidylcholine translocase and flipping enzyme (51). In apoptotic cells, PS translocated to the outer lobule, resulting in increased expression. In this experiment, the increase of PS caused the increase of PE, because PS and PE were regulated by the same transport enzyme (52). Exposure of one species to the outer lobule inevitably led to exposure of the other. PG was a precursor of cardiolipin, which was an important component of mitochondrial inner membrane (53). The significant increase of PGs indicated that the structure of mitochondrial membrane was destroyed by hypoxia. TAG was the main component of milk fat. High TAG content might reflect high synthesis rate and low turnover rate (54). The increase of TAGs indicated that hypoxia induced the accumulation of TAG in normal mammary epithelial cells, which reflected the effect of hypoxia on TAG anabolism. In addition, SM is a kind of sphingolipid in cell membrane, and its hydrolysis can produce CER, which is involved in apoptosis signaling pathway (55). SM hydrolysis and CER signaling are essential in the process of apoptosis, which leads to the apoptosis increase. In general, the disorder of lipid level is an important evidence of apoptosis after hypoxia exposure.

Glycerophospholipids metabolism mediated by 1-acylglycero-3-phosphate acyltransferase (AGPAT) plays an important role in the synthesis pathway of TAG (56), which is consistent with our findings in this study. In hypoxic mammary epithelial cells, the silencing of AGPAT2 gene caused reduced intracellular TAG synthesis, possibly because the role of AGPAT2 appeared to be to provide a substrate for the synthesis of glycerophospholipids and TAG in the cell culture model. Overexpression of AGPAT2 in adipocytes increased TAG content (57), which was similar to the results of this study. In addition, gene silencing resulted in down-regulation of the expression of a key protein for TAG synthesis, which was similar to the results of development tests in AGPAT2–/– mice, suggesting that AGPAT2 was critical for glycerophospholipids synthesis in adipose tissue (58). In addition, AGPAT2-induced upregulation of glycerophospholipids metabolism was necessary for LDS enrichment and survival under hypoxia (34).

CONCLUSION

In summary, untargeted metabonomics and lipidomics were used in this experiment to study lipid synthesis induced by hypoxia at the metabolic level. The results of metabolomics showed that the metabolism of arginine and proline, glycine, serine and threonine, glycerophospholipids were up-regulated, while the metabolism of fatty acid was down regulated. The results of lipidomics showed that the metabolism of glycerophospholipids was up-regulated by regulating cell apoptosis during hypoxia. In conclusion, we can speculate that Holstein cows adapt to hypoxia exposure mainly by up regulating the glycerophospholipids metabolism. The results of this study are helpful to further understand the mechanism of lipid synthesis related to hypoxia in bovine mammary gland at molecular level.

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 author/s.

ETHICS STATEMENT

The animal study was reviewed and approved by Animal Care and Use Guidelines of the Animal Care Committee, Institute of Subtropical Agriculture, Chinese Academy of Sciences, Changsha, China, with protocol ISA-201710.

AUTHOR CONTRIBUTIONS

CZ, ZK, QH, and ZT: conceptualization. ZK, BL, and YZ: analysis. ZK: data curation and writing—original draft preparation. CZ and ZT: writing—review and editing. CZ and BL: funding acquisition. All authors have read and approved the final manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fvets.2021.764135/full#supplementary-material

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Estrogen Regulates Glucose Metabolism in Cattle Neutrophils Through Autophagy

*Xinbo Wang†, Yuming Zhang†, Yansong Li, Mingyu Tang, Qinghua Deng, Jingdong Mao and Liyin Du**

Clinical Veterinary Laboratory, College of Animal Science and Technology, Inner Mongolia MINZU University, Tongliao, China

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***Correspondence:**
Liyin Du
duliyin2016@163.com

†These authors have contributed equally to this work

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Hypoglycemia resulting from a negative energy balance (NEB) in periparturient cattle is the major reason for a reduced glycogen content in polymorphonuclear neutrophils (PMNs). The lack of glycogen induces PMNs dysfunction and is responsible for the high incidence of perinatal diseases. The perinatal period is accompanied by dramatic changes in sex hormones levels of which estrogen (17β-estradiol, E2) has been shown to be closely associated with PMNs function. However, the precise regulatory mechanism of E2 on glucose metabolism in cattle PMNs has not been elucidated. Cattle PMNs were cultured in RPMI 1640 with 2.5 (LG), 5.5 (NG) and 25 (HG) mM glucose and E2 at 20 (EL), 200 (EM) and 450 (EH) pg/mL. We found that E2 maintained PMNs viability in different glucose conditions, and promoted glycogen synthesis by inhibiting PFK1, G6PDH and GSK-3β activity in LG while enhancing PFK1 and G6PDH activity and inhibiting GSK-3β activity in HG. E2 increased the ATP content in LG but decreased it in HG. This indicated that the E2-induced increase/decrease of ATP content may be independent of glycolysis and the pentose phosphate pathway (PPP). Further analysis showed that E2 promoted the activity of hexokinase (HK) and GLUT1, GLUT4 and SGLT1 expression in LG, while inhibiting GLUT1, GLUT4 and SGLT1 expression in HG. Finally, we found that E2 increased LC3, ATG5 and Beclin1 expression, inhibited p62 expression, promoting AMPK-dependent autophagy in LG, but with the opposite effect in HG. Moreover, E2 increased the Bcl-2/Bax ratio and decreased the apoptosis rate of PMNs in LG but had the opposite effect in HG. These results showed that E2 could promote AMPK-dependent autophagy and inhibit apoptosis in response to glucose-deficient environments. This study elucidated the detailed mechanism by which E2 promotes glycogen storage through enhancing glucose uptake and retarding glycolysis and the PPP in LG. Autophagy is essential for providing ATP to maintain the survival and immune potential of PMNs. These results provided significant evidence for further understanding the effects of E2 on PMNs immune potential during the hypoglycemia accompanying perinatal NEB in cattle.

Keywords: estrogen, glucose metabolism, autophagy, ATP, polymorphonuclear neutrophils, cattle

INTRODUCTION

Negative energy balance (NEB) during the perinatal period in cattle increases the incidence of mammary gland and uterine infectious diseases, such as mastitis, uteritis, retained fetal membranes (RFM), and endometritis. These diseases are associated with polymorphonuclear neutrophils (PMNs) dysfunction induced by the dramatic changes in steroid hormone levels. Studies have found that changes in estrogen (17β-estradiol, E2), a steroid hormone, may be responsible for the reduced immune response of PMNs before and after parturition (1). E2 in periparturient cows is known to increase in the 2 weeks immediately before parturition, rising from a basal level of 20 pg/mL to a peak of 450 pg/mL, then rapidly declining to the basal level (2). Many studies have confirmed that abnormal E2 levels may lead to perinatal diseases. For example, the level of E2 in cows that suffered from RFM was higher than that of normal cows 6 days before parturition (3, 4). Cows suffering from subclinical mastitis showed low circulating E2 levels (5). These results suggest that normal levels of E2 could relieve immunosuppression in periparturient cattle.

Previous studies have shown that ATP in PMNs is mainly produced by glucose metabolism, and elevated ATP levels are conducive to normal PMNs function during parturition (6). Regarding glucose metabolism in PMNs, glucose is first phosphorylated by hexokinase (HK) to produce glucose 6-phosphate (G6P), after which G6P is used for ATP and NADPH production via glycolysis and the pentose phosphate pathway (PPP) and glycogen synthesis (7). Hypoglycemia after parturition affects the maintenance of optimal intracellular glycogen levels and PMNs function, especially in cows suffering from uterine or mammary disease as described above. E2 has been shown to promote glycogen synthesis in various tissues and cells by regulating glycogen synthase kinase-3β (GSK-3β), for example, in the uterus (8), astrocytes (9), and neurons (10). However, to the best of our knowledge, there is no research on E2 regulation of glycogen synthesis in cattle PMNs. Reports have shown that glycolysis is the major pathway for ATP generation in PMNs, while the PPP is involved in NADPH generation (11). Both these pathways, together with the glycogen synthesis pathway, play important roles in glucose metabolism (12). As one of the most important regulatory enzymes in glycolysis, phosphofructosekinase-1 (PFK1) catalyzes the conversion of fructose-6-phosphate to fructose-1,6-diphosphate in response to cellular energy requirements, while glucose-6-phosphate dehydrogenase (G6PDH), the key enzyme in the PPP, fuels NADPH to produce superoxide. Numerous studies have shown that E2 plays an important regulatory role in glycolysis, the PPP, and other pathways of glucose metabolism in various types of cells, including MCF-7 breast cancer (13), uterine (14), and HeLa cervical cancer cells (15). Unfortunately, the specific mechanism of E2 action on glucose metabolism in PMNs is still unclear. It is, thus, worthwhile to improve our understanding of the role of glucose deficiency and E2 level on the immune potential of PMNs.

It is well-known that low extracellular glucose levels result in a deficiency in glucose uptake and utilization, and the glucose transport system of PMNs is responsible for the uptake of circulating glucose (16). To date, two glucose transporter families have been identified, including the main GLUT superfamily (GLUTs) and the sodium-glucose co-transporter SGLT family (SGLTs) (17). As the members of the GLUTs, the expression of GLUT1 and GLUT4 varies with PMNs biological conditions with glucose mainly transported across cell membranes by GLUT1 under physiological conditions (18). Meanwhile, SGLTs transport glucose through a secondary active transport mechanism, which promotes glucose uptake by using the sodium concentration gradient established by the Na^+/K^+-ATPase pump (19). As a member of SGLTs, the SGLT1 is mainly expressed in the kidney, heart and trachea. SGLT1 expression and its relationship to GLUT1/4 in cattle PMNs under changed glucose environments is completely unknown.

AMP-activated protein kinase (AMPK) senses available energy in cells by binding directly to ATP. Activated AMPK increases the translocation of glucose transporters and promotes ATP preservation and production. Once ATP production pathways such as glycolysis, fatty acid oxidation (FAO) and oxidative phosphorylation (OXPHOS) are activated, pathways involving ATP consumption, such as protein synthesis, fatty acid synthesis, gluconeogenesis and glycogen synthesis pathways, are reduced. However, reports on the role of AMPK in glucose metabolism are contradictory. Some studies have shown that activated AMPK phosphorylates key proteins of multiple pathways such as glycolysis, leading to enhanced catabolism and reduced anabolism (20, 21). Other studies have shown that AMPK activation is associated with glycogen accumulation rather than glycogen consumption (22, 23). Therefore, this study aimed to clarify the role of AMPK in the glucose metabolism of cattle PMNs.

The Bcl-2 superfamily both promotes and inhibits apoptosis, and the balance pro-apoptotic and anti-apoptotic proteins, such as Bax and Bcl-2, respectively, is critical for determining the survival time of mature PMNs. Previous study have found that the spontaneous apoptosis of human PMNs in the absence of sufficient nutrients could be inhibited by increased glucose *in vitro* (24). As is well-known, autophagy can supplement anabolic substrates and energy under low energy conditions by degrading internal cellular components (25) and autophagy markers including ATG5, Beclin1, LC3 and p62 play key roles in autophagy initiation in various cells (26). Studies have shown that E2 can promote autophagy, delay senescence (27) and inhibit apoptosis (28). Although increasing evidence shows that E2 can regulate cellular glucose metabolism, there are few studies on the mechanism of E2 regulation of PMNs autophagy.

The effects of E2 on the immune potential of cattle PMNs and its association with glucose levels have not been studied. Here, we investigated the effect and mechanism of E2 on glucose metabolism through regulation glucose uptake and utilization, to determine whether E2 enhances PMNs immune potential by activating autophagy under low-glucose conditions. This study provided valuable new perspectives on how E2 controls the immune potential of PMNs in cattle suffering from perinatal NEB.

MATERIALS AND METHODS

Animals

All experiments were conducted in accordance with relevant guidelines and regulations. The current study was conducted at the Inner Mongolia University for Nationalities in Tongliao, China. Jugular venipuncture blood samples were collected from the ovariectomized Chinese Simmental cattle aged about 2 years.

Isolation and Culture of PMNs

The blood was collected by jugular vein puncture in cattle into 50 mL centrifuge tubes containing 0.1 mL heparin (Gentihold) as an anticoagulant. Heparinized blood was diluted with equal amounts of 1 × PBS, placed on the Percoll (GE Healthcare) separation solution, and centrifuged at 800 × g for 15 min. After removal of the plasma, red blood cell and PMNs were collected, washed once with PBS, then the red blood cell lysates were added and centrifuged at 800 × g for 8 min. PMNs were washed once again with 1 × PBS and once with RPMI 1,640 medium (Procell), and then were resuspended in RPMI 1,640 medium (2.0×10^6 cells/mL). The PMNs were incubated in RPMI 1,640 containing 10% fetal cattle serum (Hyclone, Logan, UT, USA) at 37°C and 5% CO_2 for 45 min. After 45 min, the PMNs were cultured in fresh medium.

PMNs were incubated with different concentrations of 17β-estradiol (Sigma) and glucose (Sigma) for specified times. The concentrations of E2 were 20 pg/mL (EL), 200 pg/mL (EM) and 450 pg/mL (EH). The glucose concentrations were 2.5 mM (LG), 5.5 mM (NG) and 25 mM (HG). PMNs were incubated with EH for 6 h under LG, NG and HG conditions. The PMNs were then collected for subsequent tests.

Cell Counting Kit-8 Assay

The viability of PMNs cells was determined with a Cell Counting Kit-8 detection kit (CCK-8; Biosharp, China) in accordance with the manufacturer's protocol. Briefly, the cells were seeded into 96-well plates at the density of 5×10^3 cells per well. At the indicated time point, 10 μL of CCK-8 solution was added, and PMNs were incubated at 37°C in a 5% CO_2 incubator for 0, 2, 4, 6, 8, and 12 h at different concentrations of E2 and glucose. The absorbance was measured at 450 nm under an automatic microplate reader (Multiskan Spectrum; Thermo Scientific, USA).

Biochemical Analyses

Biochemical analysis was used to detect activities of different enzymes in PMNs cultured with different concentrations of E2 and glucose for 6 h. All biochemical tests, HK and G6PDH activity, and ATP and glycogen content, were performed using commercial test kits (Solarbio, Beijing, China) at 37°C in an automatic microplate reader (Multiskan Spectrum; Thermo Scientific). Biochemical analyses were conducted in strict accordance with the instructions of the kits.

ELISA Analysis

PMNs cultured with different concentrations of E2 and glucose for 6 h were collected and the activity of PFK1 and GSK-3β in cells was detected by ELISA kit (SolarBio). ELISA analysis was conducted in strict accordance with the instructions of the kit.

Western Blotting Analysis

The PMNs were treated for 6 h with 2.5, 5.5 and 25 mM glucose and EH. Total protein was extracted from PMNs with lysis buffer (Solarbio). The protein concentration was quantified using a BCA protein assay kit (Applygen). Then, the protein samples were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto polyvinylidene fluoride (PVDF) membranes (Immobilon). After blocking with 5% BSA for 2 h, the membranes were blotted with 1:700 diluted primary antibodies against GLUT1 (Abcam, MA, USA), GLUT4 (Abcam), SGLT1 (Cell Signaling Technology, MA, USA), Beclin1 (Abcam), ATG5 (Abcam), p62 (Abcam), LC3 (Cell Signaling Technology), β-actin (Absin), AMPK (Abcam), p-AMPK (PL Laboratories, USA), Bax (Abcam), or Bcl-2 (Abcam) at 4°C overnight. The membrane was washed with Tris-buffered saline containing 0.1% Tween-20 (TBST), and the secondary antibodies (Cell Signaling Technology) conjugated to horseradish peroxidase were incubated for 1 h at room temperature. The bands were visualized using the enhanced chemiluminescence (ECL) system and the gray densities were quantified with ImageJ software.

Flow Cytometry

The apoptotic rate was measured by flow cytometry using an Annexin V-FITC/PI apoptosis assay kit (Beyotime, Shanghai, China). After PMNs culture for 6 h, the cells were resuspended in 500 mL binding buffer containing 5 mL Annexin V FITC and 10 mL PI, and incubated at room temperature in the dark for 20 min. The presence of apoptotic cells was analyzed by flow cytometry on a Beckman flow cytometer (CA, USA) within no more than 1 h.

Statistical Analysis

The gray values of the protein electrophoresis bands were analyzed by ImageJ software (National Institutes of Health, Bethesda, MD, USA). The results are presented as the mean ± standard error of the mean and analyzed using SPSS 19.0 software (IBM Corp, Armonk, NY, USA). GraphPad Prism 8.0 was used for graphical analysis. Analysis of variance was performed to evaluate the differences among the groups while the *t*-test was used for between-group analysis. A *P*-value lower than 0.05 was considered statistically significant and a *P*-value lower than 0.01 was considered highly significant ($^*P < 0.05$, $^{**}P < 0.01$).

RESULTS

E2 Enhances PMNs Viability

To investigate the effect of E2 on PMNs viability, the CCK-8 viability assay was used. The results showed that compared with NG, PMNs viability did not change significantly at 0–2 h in LG and HG; PMNs viability decreased significantly in LG group and increased significantly at 4–6 h in HG; PMNs viability decreased significantly at 8–12 h in both LG and HG (**Figure 1A**). E2, including low E2 (EL), moderate E2 (EM) and high E2

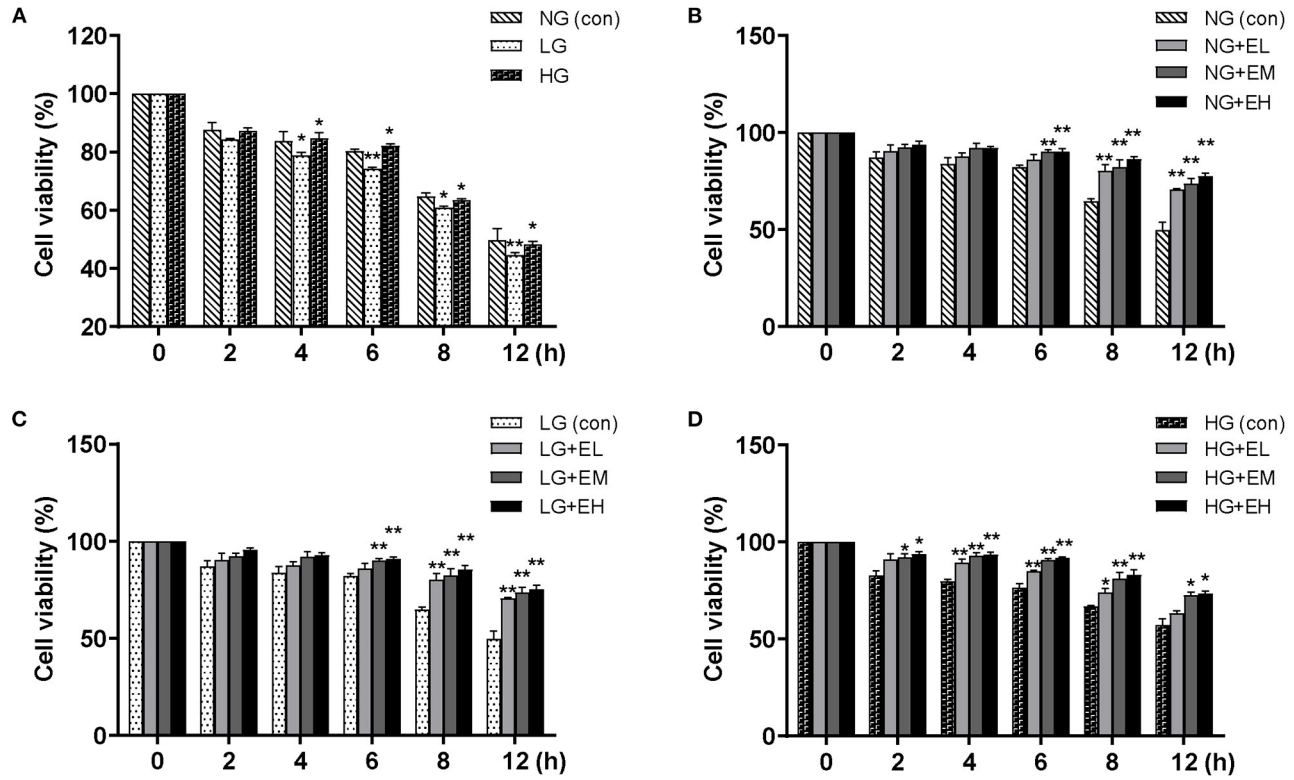

FIGURE 1 | E2 enhances PMNs viability. PMNs were treated with NG (5.5 mM), LG (2.5 mM), HG (25 mM), NG+EL (20 pg/mL), NG+EM (200 pg/mL), NG+EH (450 pg/mL), LG+EL, LG+EM, LG+EH, HG+EL, HG+EM and HG+EH, for 0, 2, 4, 6, 8, and 12 h. **(A–D)** PMNs viability detected by CCK-8. The results are shown as the mean ± SD ($n = 3$). The *t*-test was used to analyze differences. $^{*}P < 0.05$, $^{**}P < 0.01$. The asterisk indicates a significant difference between the treatment group and the control group (con).

(EH) doses, significantly increased PMNs viability, especially enhancing viability after 6 h at low glucose (LG), normal glucose (NG) levels and high glucose (HG) levels (**Figures 1B–D**). These data indicated that E2 influences and maintains the viability of PMNs *in vitro*.

E2 Promotes PMNs Glycogen Synthesis by Inhibiting GSK-3β Activity

To understand the glucose metabolism of PMNs in different glucose conditions and the possible regulatory role of different concentrations of E2, we first measured the glycogen content of the cells by biochemical methods and GSK-3β activity by ELISA. The results showed that the glycogen content was significantly increased, whereas the GSK-3β activity decreased in a concentration-dependent manner with the glucose levels (**Figures 2A,E**). E2 significantly increased the glycogen content, and this increase was related to the E2 concentration in LG, NG and HG in a dose-dependent manner (**Figures 2B–D**) while GSK-3β activity decreased significantly with increasing E2 concentration at LG, NG and HG (**Figures 2F–H**). These results suggested that EH strongly promotes glycogen synthesis through the inhibition of GSK-3β activity.

E2 Regulates the Activity of PFK1 and G6PDH and Maintains ATP Homeostasis in PMNs

To determine the action of E2 on increased glycogen content in PMNs, we focused on glycolysis and the PPP in glucose metabolism pathways which provide ATP to meet the requirements of glycogen synthesis and energy expenditure in PMNs. The results showed that PFK1 activity was significantly increased in a glucose-dependent manner (**Figure 3A**). Under LG and NG conditions, EL, EM and EH significantly decreased PFK1 activity in a time-dependent manner, while under HG conditions, only EH significantly increased PFK1 activity (**Figures 3B–D**). The activity of G6PDH decreased in a dose-dependent manner with increasing glucose concentration (**Figure 3E**), specifically, decreasing with increasing E2 concentration at both NG and LG (**Figures 3F,G**) while showing the opposite effect at HG (**Figure 3H**). The results showed that EH was able to promote or inhibit PFK1 and G6PDH, the key catabolic enzymes of cellular glucose in PMNs under different glucose conditions. In addition, the results showed that the ATP content increased significantly in a glucose-dependent manner (**Figure 3I**), increasing with increased E2 concentrations at LG and NG (**Figures 3J,K**), while decreasing in response to E2 at

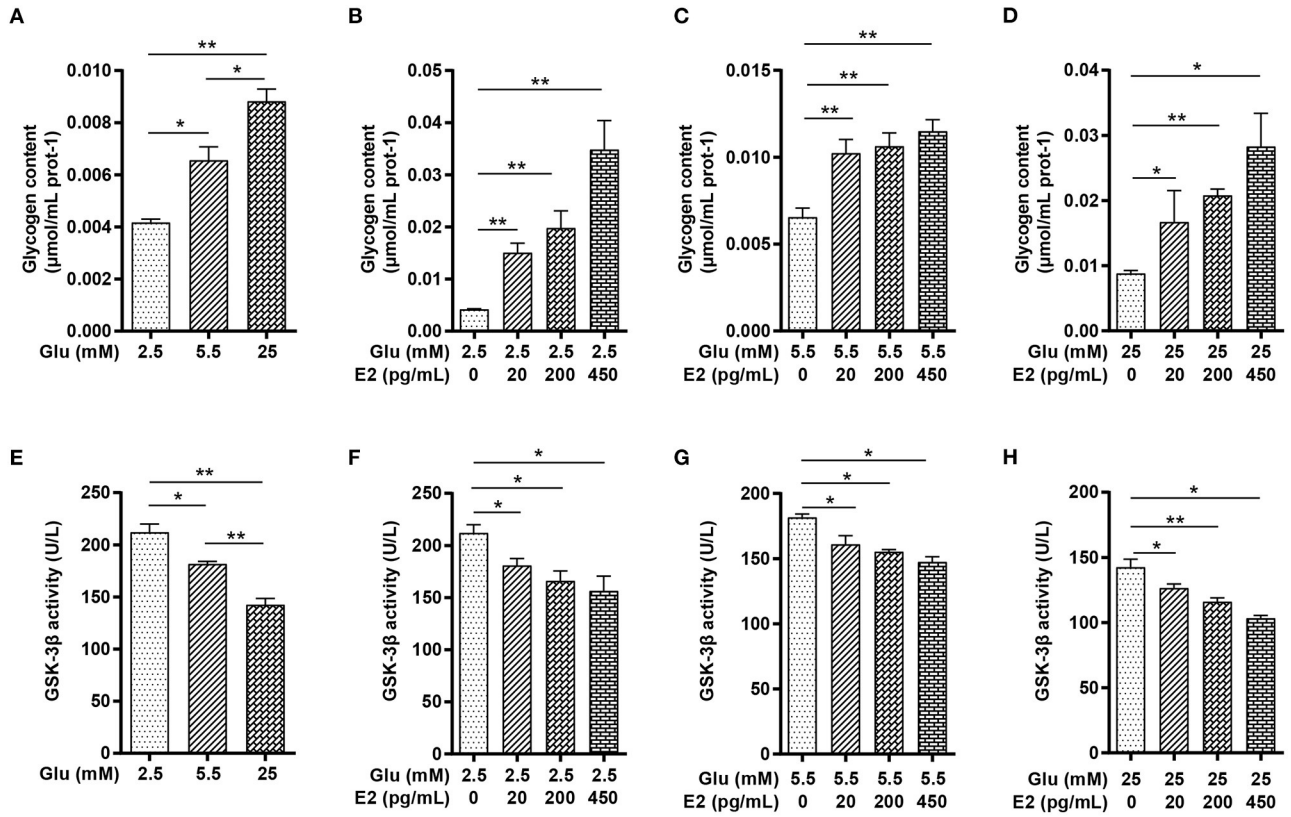

FIGURE 2 | E2 promotes PMNs glycogen synthesis by inhibiting GSK-3β activity. PMNs was treated with LG, NG, HG, LG+EL, LG+EM, LG+EH, NG+EL, NG+EM, NG+EH, HG+EL, HG+EM and HG+EH for 6 h. **(A–D)** Glycogen content was determined by the biochemical method. **(E–H)** GSK-3β activity was determined by ELISA. The results are shown as the mean ± SD ($n = 3$). Differences were analyzed using the t-test. $^{*}P < 0.05$, $^{**}P < 0.01$. The significant difference between the two groups is indicated by a line and an asterisk.

HG and with the lowest level at EH (**Figure 3L**). These results indicated that EH had significant effects on cellular glucose catabolism by regulation of the activity of PFK1 and G6PDH, and on ATP production. Therefore, the EH concentration was used for the following experiments.

E2 Regulates Glucose Uptake and Utilization by Regulating Glucose Transporters Expression and HK Activity

HK is a key enzyme of glucose catabolism: once glucose has been taken up by transporters from the extracellular environment, HK transforms the absorbed glucose into G6P to provide substrates for glycogen synthesis, glycolysis or the PPP. To identify the specific role of E2 on glucose uptake and utilization, we analyzed the expression of GLUT1, GLUT4 and SGLT1 by WB and HK activity by biochemical measurement. The results showed that HK activity increased in response to glucose in a dose-dependent manner (**Figure 4A**). EL and EM did not increase HK activity while EH significantly enhanced HK activity at LG (**Figure 4B**). Although E2 had no significant effect on HK activity at NG (**Figure 4C**), HK activity was decreased in an E2 concentration-dependent manner at HG (**Figure 4D**). The WB results showed that compared with NG, the expression of GLUT1 and SGLT1 were significantly increased and GLUT4 expression was significantly decreased at LG and HG levels (**Figures 4E–H**). E2 thus promoted the expression of GLUT1, GLUT4 and SGLT1 at LG while, in contrast, inhibiting expression at HG. At NG levels, E2 promoted the expression of both GLUT1 and SGLT1 while inhibiting GLUT4 expression. These results suggested that the regulation of glucose uptake and utilization by E2 depends on up-regulating or down-regulating the expression of transporters and HK activity, and that this is a crucial mechanism by which PMNs handle energy stress.

E2 Regulates AMPK-Dependent Autophagy in PMNs

To determine whether the variation in ATP content under different glucose conditions is the result of pathways other than glycolysis and the PPP, we further investigated the effect of E2 on the autophagy pathway of AMPK and the expression of autophagy-related proteins LC3, ATG5, Beclin1 and p62 by WB. The results indicated that the p-AMPK/AMPK ratio at both LG and HG was significantly higher than at NG, and was significantly higher at HG than that at LG (**Figures 5A,B**). E2 significantly

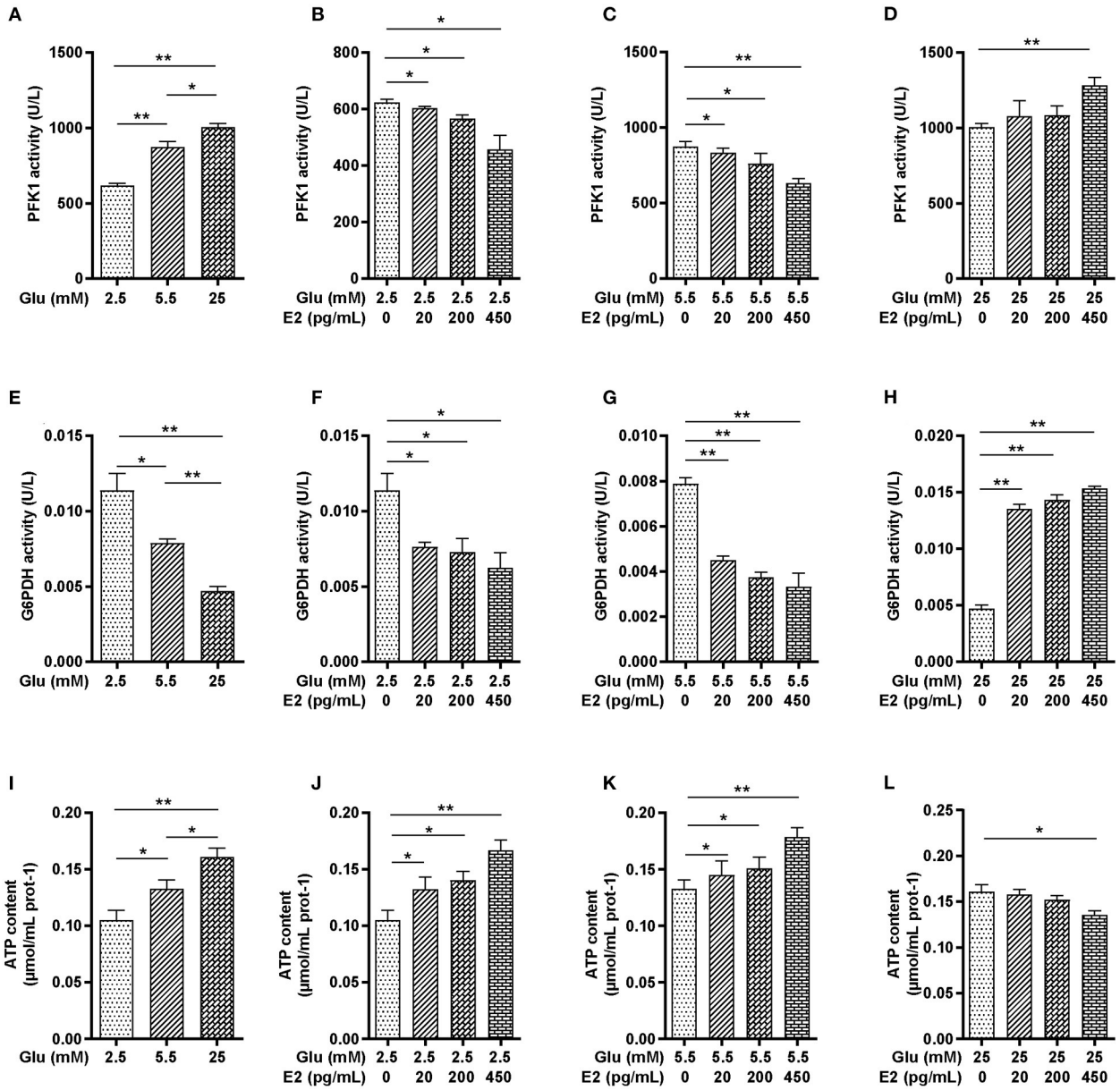

FIGURE 3 | E2 regulates the activity of PFK1 and G6PDH and maintains ATP homeostasis in PMNs. PMNs were treated with LG, NG, HG, LG+EL, LG+EM, LG+EH, NG+EL, NG+EM, NG+EH, HG+EL, HG+EM and HG+EH for 6 h. **(A–D)** PFK1 activity was determined by ELISA. **(E–H)** G6PDH activity was determined by biochemical methods. **(I–L)** ATP contents were determined by biochemical methods. The results are shown as the mean ± SD ($n = 3$). The significance of the difference was analyzed by the t- test. $^{*}P < 0.05$, $^{**}P < 0.01$.

increased the level of AMPK phosphorylation at LG and NG but decreased it at HG. The expression of LC3, ATG5 and Beclin1 was increased while that of p62 was reduced at LG and HG rather than at NG, especially this expression was most significant at HG (**Figures 5A,C–F**). E2 significantly increased the expression of LC3, ATG5 and Beclin1, and significantly decreased p62 at LG and NG, but showed the opposite results at HG. These results suggested that E2 can maintain the optimal concentration of ATP by regulating autophagy according to glucose environment and intracellular ATP level, which may provide a material guarantee for maintaining cell survival and the development of immune potential in PMNs.

E2 Inhibits PMNs Apoptosis

Low ATP levels in apoptotic PMNs indicate the importance of the relationship between spontaneous apoptosis and autophagy for cell survival. We, therefore, verified the effect of E2 on PMNs apoptosis under different glucose conditions by WB and flow cytometry. The results showed that at LG, there was a significantly higher Bax expression, lower Bcl-2 expression and

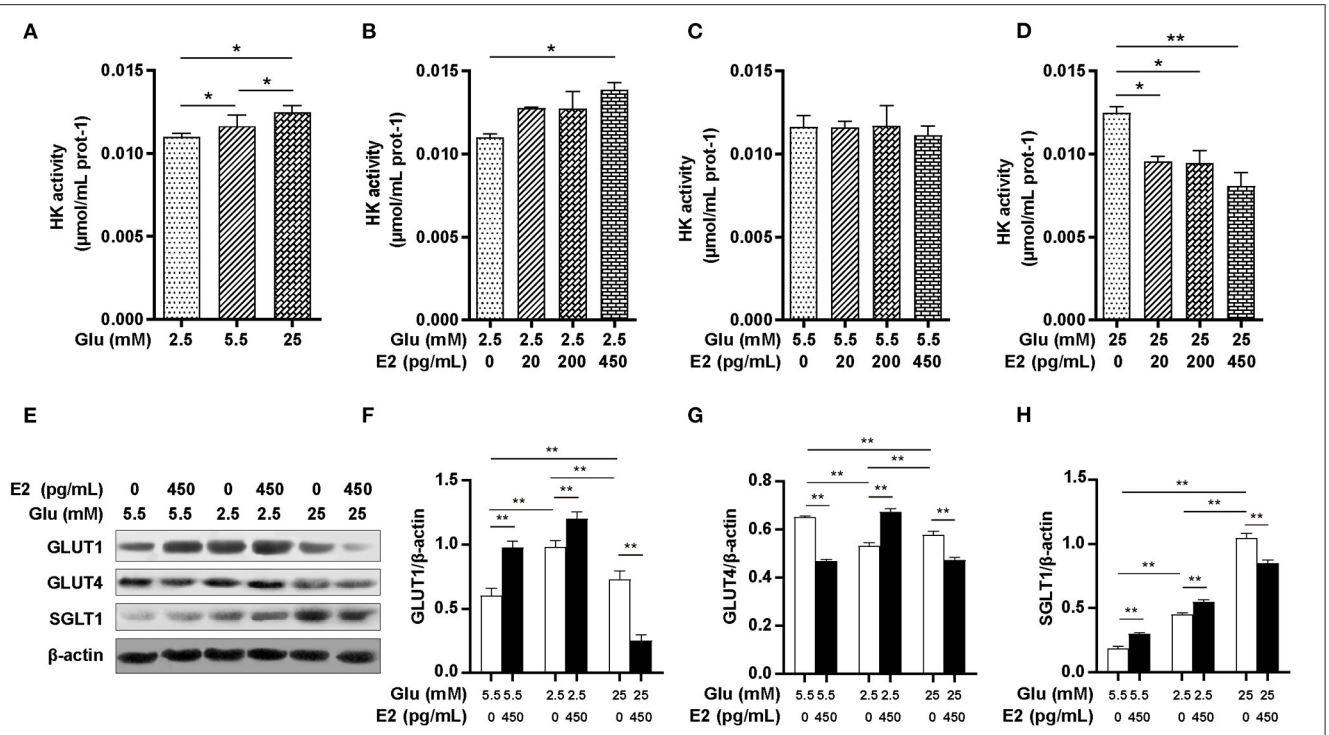

FIGURE 4 | E2 regulates glucose uptake and utilization by regulating the activities of HK and glucose transporters. PMNs were treated with LG, NG, HG, LG+EL, LG+EM, LG+EH, NG+EL, NG+EM, NG+EH, HG+EL, HG+EM and HG+EH for 6 h. **(A–D)** HK activity was measured by the biochemical method. **(E–H)** The expression of GLUT1, GLUT4 and SGLT1 were detected by WB, and β-actin was used as an internal control. The results are shown as the mean ± SD ($n = 3$). The significance of the difference was analyzed using the *t*-test. $^{*}P < 0.05$, $^{**}P < 0.01$.

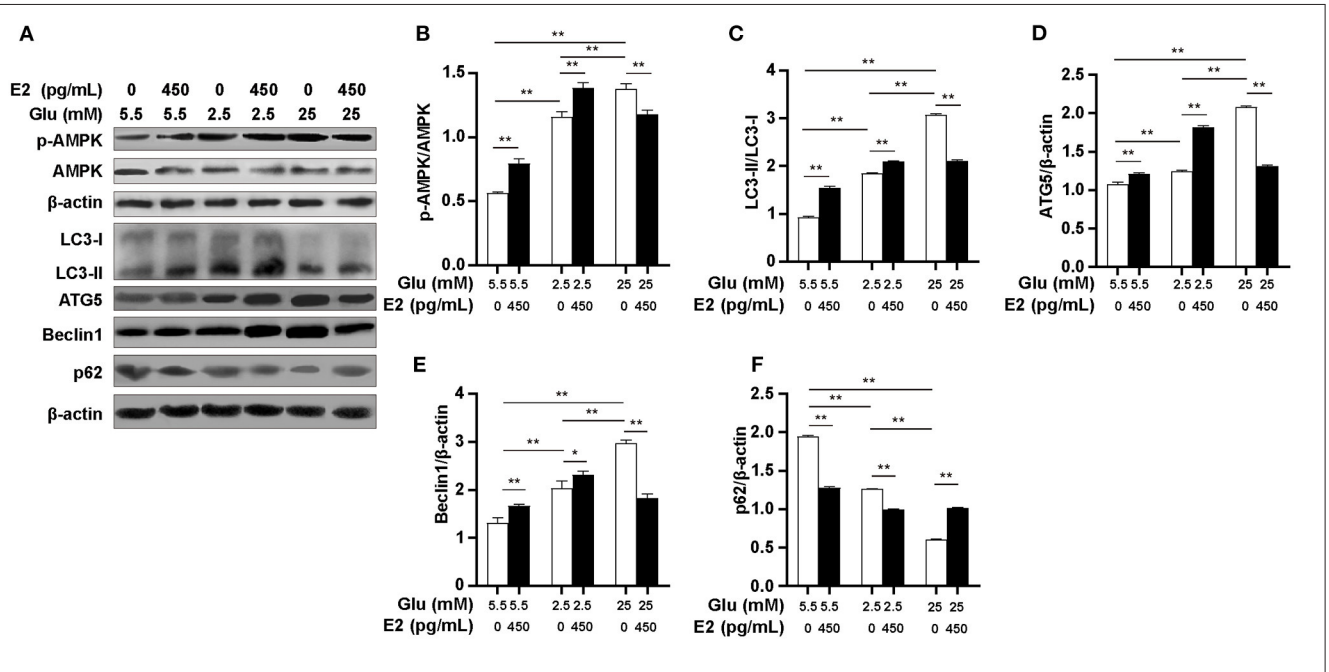

FIGURE 5 | E2 regulates AMPK-dependent autophagy in PMNs. PMNs were treated with NG, NG+EH, LG, LG+EH, HG and HG+EH for 6 h. **(A–F)** Expressions of LC3, ATG5, Beclin1, p62, p-AMPK and AMPK were analyzed by WB with β-actin used as an internal control. The results are shown as the mean ± SD ($n = 3$). Differences were analyzed using the *t*-test.$^{*}P < 0.05$, $^{**}P < 0.01$.

Bcl-2/Bax ratio (**Figures 6A–D**) and an increased apoptosis rate compared to NG (**Figure 6E**), but there was an opposite effect at HG. However, E2 significantly increased the Bcl-2/Bax ratio and decreased the apoptosis rate at LG and HG. Our results suggested that E2 can protect PMNs by inhibiting apoptosis resulting from the environmental stress of lack or excess of glucose. In other words, the inhibition of apoptosis by E2 requires the change of autophagy level to maintain cell survival, and the occurrence of autophagy and apoptosis is based on the changes in glucose intake and metabolism.

DISCUSSION

We found that E2 promotes glycogen storage by promoting glycogen synthesis and maintains ATP homeostasis by enhancing glucose uptake and regulating autophagy in the context of changing extracellular glucose levels. These results suggested that E2 exerts a significant effect on both glucose uptake and utilization and, in particular, plays an important role in sustaining cell viability and promoting the glycogen storage and ATP content in situations of glucose deficiency in cattle PMNs *in vitro*. Therefore, E2 may be a key factor in maintaining the viability and enhancing the immune potential of PMNs for fulfilling immune function in periparturient cattle suffering from NEB.

Vazquez-Anon et al. (29) observed that hypoglycemia in periparturient cattle affects chemotaxis, phagocytosis, and killing capacity in PMNs due to reduced glycogen storage. Similarly, hyperglycemia during calving also impairs PMNs function and increases the risk of postpartum infection (30). Our results showed that PMNs viability was significantly affected by both low and high glucose, and the higher glucose condition significantly increased the PMNs glycogen content. Galvao et al. (31) found that treatment with glucose narrowed the difference in PMNs viability between cows suffering from uteritis and healthy cows,

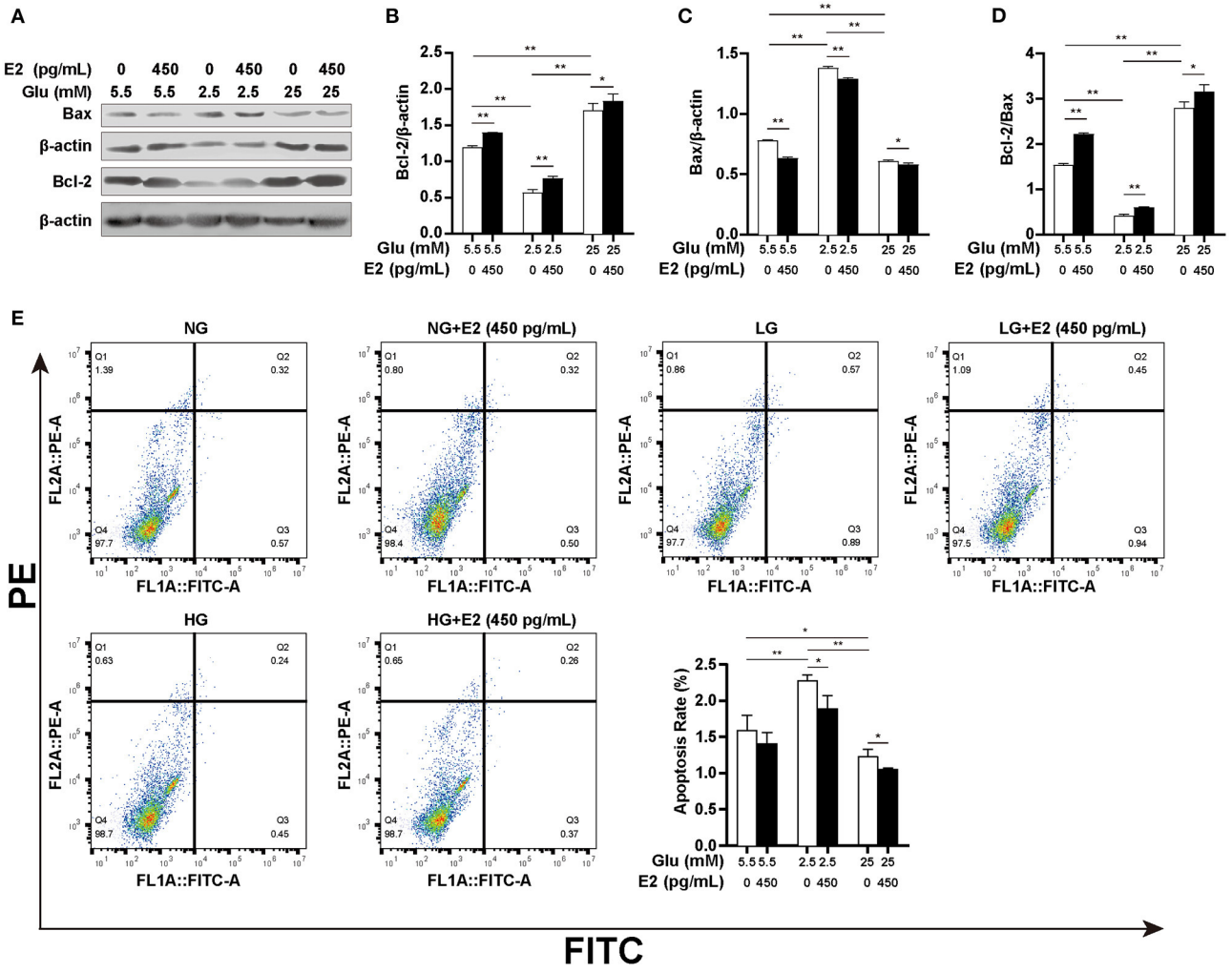

FIGURE 6 | E2 inhibits PMNs apoptosis. PMNs were treated with NG, NG+EH, LG, LG+EH, HG and HG+EH for 6 h. **(A–D)** The expression of the apoptosis-related proteins Bax and Bcl-2 and the Bcl-2/Bax ratio were analyzed by WB. **(E)** Apoptosis rates in PMNs were assayed by flow cytometry. The results are shown as the mean ± SD ($n = 3$). Differences were analyzed using the *t*-test. $^{*}P < 0.05$, $^{**}P < 0.01$.

suggesting that hypoglycemia during the perinatal period is highly correlated with PMNs dysfunction and leads to disease susceptibility. It is well known that the E2 concentrations in perinatal cattle typically change from basal to peak and back to basal again. Our findings showed that different E2 concentrations significantly enhanced the viability of PMNs in low or high glucose environments *in vitro*, and we speculate that E2 exerts an active action on maintaining PMNs viability by regulating cellular glucose metabolism.

To further understand the E2 regulatory mechanism on PMNs glucose metabolism, we first evaluated the effect of E2 on PMNs glycogen synthesis. As the main energy source in PMNs, the glycogen content mostly depends on extracellular glucose uptake, and glucose deficiency leads to glycogen reduction in PMNs (31). Although previous studies have confirmed that E2 can promote utero glycogen synthesis in rats and rabbits (8, 32), the effect of E2 on glycogen synthesis in PMNs has been less studied. Our study showed that E2 promotes glycogen synthesis in cattle PMNs under different glucose concentrations *in vitro*, therefore, may benefit PMNs function in periparturient cattle suffering from NEB. ERα is the specific receptor for E2 and is a substrate of GSK-3β. E2 inhibits GSK-3β activity depending on the detachment of ERα from the ERα/GSK-3β complex (33). Reports have shown that E2 activates uterine epithelial cell proliferation by inhibiting GSK-3β-induced PI3K pathway activation (34). Our results showed that glycogen content depends on increased glucose concentration, and that E2 enhanced glycogen synthesis and inhibited GSK-3β activity in PMNs. Recent studies have shown that in mouse PMNs, the glycogen cycle can be used to produce energy at inflammatory sites where nutrients are limited (35). This suggested that moderate and well-timed amounts of E2 may contribute to the maintenance of the immune potential of PMNs in periparturient cattle by increasing the glycogen storage.

In terms of the glucose absorbed from the environment in PMNs, one part is stored in the form of glycogen while the other is competitively utilized between the PFK1-mediated glycolysis pathway and the G6PDH-mediated PPP. In mouse PMNs incubated with 25 mM glucose, there was a 50% dose-dependent reduction in G6PDH and oxygen production (36), and G6PDH deficiency in PMNs from diabetic mice resulted in reduced germicidal capacity and peroxide production (37). Therefore, we examined the activities of PFK1 and G6PDH in cattle PMNs and found that G6PDH activity decreased in response to increased glucose concentration. Addition of E2 to low- or normal-glucose medium attenuated G6PDH activity while E2 addition to high-glucose medium enhanced G6PDH activity, indicating that the impact of E2 on the PPP is involved in the extracellular glucose level. Newsholme et al. (38) found that the addition of glucose increased enzyme activity in glycolysis and ATP production in PMNs. Similarly, this study found that increased extracellular glucose enhanced PFK1 activity and ATP production. However, E2 significantly inhibited PFK1 activity and increased ATP content under low- or normal-glucose conditions, and enhanced PFK1 activity, and decreased ATP content under high-glucose conditions. These results raise an interesting question: E2 has been found to promote glycogen synthesis by inhibition of PFK1, G6PDH, and GSK-3β activity under low- or normal-glucose conditions, which ought to reduce the ATP content, however, our results showed an increase in ATP content. To answer this question, we next evaluated HK activity, glucose transporter expression, and autophagy levels in cattle PMNs.

Evidence has suggested that glucose uptake by cells is the result of functional coupling between GLUTs and HK (39). GLUT1 is expressed and up-regulated in a glucose-rich environment and mediates extracellular glucose uptake in mouse (40) and human (41) PMNs, and GLUT1, but not GLUT4, is regarded as the key link between numerous factors regulating glucose uptake in PMNs. However, we have not seen the report regarding the E2-mediated GLUT1 in PMNs, although E2 could up-regulate GLUT1 expression in breast cancer cells (42). Our study demonstrated that GLUT1, GLUT4, and SGLT1 protein are expressed in cattle PMNs. Low- and high-glucose promoted the expression of GLUT1 and SGLT1 and inhibited GLUT4 expression. We observed that the enhanced glucose uptake depends mainly on GLUT1 and SGLT1 in low-glucose environments, and which only relies on SGLT1 in the high-glucose environment. E2 enhanced the expression of GLUT1/4 and SGLT1 at low glucose levels, and inhibited GLUT4 expression at normal and high glucose levels, whereas E2 promoted SGLT1 expression at low- and normal-glucose levels and inhibited SGLT1 at high glucose levels. This demonstrates that E2 enhanced glucose uptake by promoting the expression of SGLT1 and GLUT1/4 in low glucose environments and preventing excessive glucose uptake by inhibiting SGLT1 and GLUT1/4 in high glucose environments. Thus, our results obviously defined that the promoting or inhibiting glucose uptake by E2 depends on glucose transporters function relating with the extracellular glucose level. Meanwhile, we found that HK activity was positively correlated with the level of environmental glucose and that HK activity was promoted by E2 under low glucose conditions while remaining unchanged under normal glucose conditions and being inhibited under high glucose conditions. This further provided the direct evidence for revealing glycogen increase in the perspective of intracellular glucose metabolism. Clearly, E2 enhanced the PMNs glycogen storage by upregulating glucose uptake, which lies in HK activity and the level of GLUT1/4 and SGLT1 expression in different glucose environments. Considering the process of glycogen synthesis, by its nature, needs to consume a large amount of ATP, here raises an interesting question of where the increased ATP come from when the activity of PFK1, G6PDH, and GSK-3β are inhibited by E2 in low- or normal-glucose environments in this study. Previous studies showed that activated AMPK enhance the plasma membrane localization of GLUT1 and GLUT4 in skeletal muscle (43) and leads to SGLT1 upregulation and glucose uptake promotion in cardiomyocytes (44). These finding remind us to evaluate AMPK activation may help to uncover the underlying relevance of E2 to cellular ATP content.

We observed that activated AMPK is synchronized with the increased glucose uptake, E2 promoted AMPK phosphorylation in the low- and normal-glucose environment, but inhibited AMPK activation under high-glucose conditions. This indicates that E2 can regulate ATP levels. Some studies have found that

inhibiting glycolysis-dependent ATP production could activate AMPK (45) and activation of AMPK could increase the ATP level in ovarian cancer cells (46). Correspondingly, our results showed that E2 increased the ATP content by inhibiting PFK1 and activating AMPK at low and normal glucose levels, and yet E2 reduced the ATP content by enhancing PFK1 activity and inhibiting AMPK at high glucose levels. Therefore, it is reasonable to speculate that the change in ATP level resulting from addition of E2 at different glucose levels may depend on AMPK activity, and the increased ATP may be derived from an AMPK-dependent pathway, such as the autophagy pathway. We, therefore, next investigated autophagy-related proteins to answer this question.

Activation of autophagy at low glucose levels helps to maintain cellular energy homeostasis, whereas several identified signaling pathways also activate autophagy at high glucose levels (47). Ma et al. found that high glucose (20 mM) induced podocyte autophagy and damage by enhancing the expression of LC3 and Beclin1 proteins (48). Similarly, our study showed that autophagy was promoted in PMNs under low- and high-glucose conditions. The evidence showed that E2 could protect the vascular endothelium by promoting autophagy (49), but E2 protects cardiomyocytes from LPS damage by inhibiting autophagy (50). The controversial results may lies in the different conditions or cells. Our results suggested that E2 enhanced autophagy in low- or normal-glucose conditions and inhibited autophagy under high glucose conditions by regulating AMPK activity in PMNs *in vitro*. It may indicate that E2 promotes autophagy during energy deficiency or prevent damage to the cells by inhibiting autophagy in nutrient-rich environments. Therefore, the glucose environment is likely to be an essential prerequisite for E2-regulated autophagy for cattle PMNs. As known that glycolysis is the main pathway of ATP production in PMNs, whereas in the case of limited glucose supply, PMNs also can obtain the required energy through fatty acid oxidation (36). In human PMNs, autophagy provides sufficient free fatty acids through the decomposition of lipid droplets and supports the FAO-OXPHOS pathway to produce more ATP (51). This verifies our results from another aspect, that is, E2 may promote lipophagy to maintain a steady-state ATP content in cattle PMNs under deficient glucose conditions.

Both apoptosis and autophagy are necessary for PMNs survival, and the ATP content is lower in apoptotic PMNs (52), while E2 can protect mouse pancreatic β cells from apoptosis through the ERα mechanism (53). To further understand how E2 protects PMNs from spontaneous apoptosis, we analyzed the expression of autophagy- and apoptosis-related proteins Bcl-2 and Bax and the apoptosis rate. The results showed that low glucose promoted apoptosis and high glucose inhibited apoptosis. This indicated that autophagy is not sufficient to counteract spontaneous apoptosis under low-glucose conditions. The lower apoptosis rate in high-glucose, not in low-glucose conditions, could be attributed to enough ATP production resulting from increased autophagy and glycolysis being sufficient to inhibit spontaneous apoptosis. Moreover, our study also found that E2 inhibited apoptosis accompanied by inhibition of GSK-3β activity in PMNs under different glucose conditions, suggesting that E2-induced GSK-3β inactivation is the initial point of regulating glucose metabolism and the root cause of triggering or inhibiting lipophagy.

CONCLUSION

In conclusion, we demonstrated that E2 promotes glycogen synthesis and increases the ATP content of cattle PMNs by enhancing the activity of HK, expression of GLUT1/4 and SGLT1, and the level of autophagy, as well as by inhibiting the activity of PFK1, G6PDH and GSK-3β under conditions of glucose restriction. This finding suggested that the molecular mechanisms by which E2 controls cellular energy levels is essential for protecting the cells from apoptosis and reinforcing the immune potential of PMNs. More research is required to further elucidate the mechanism by which E2 regulates glucose metabolism. This study provided a meaningful understanding of the effects of E2 on PMNs function in periparturient cattle suffering from NEB.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Materials**, further inquiries can be directed to the corresponding authors.

ETHICS STATEMENT

The animal study was reviewed and approved by Inner Mongolia Nationalities University Animal Care and Use Committee (no. SCXK-2020-0002).

AUTHOR CONTRIBUTIONS

XW and YZ performed the experiments and wrote the manuscript. YL and MT collected and analyzed the data. QD and JM revised the manuscript. LD revised the manuscript and supervised the entire project. All authors read and approved the final manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fvets.2021.773514/full#supplementary-material

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GLOSSARY

NEB, Negative energy balance; PMNs, Polymorphonuclear neutrophils; E2, 17β-estradiol, estrogen; GLU, Glucose; NG, Normal concentration of glucose; LG, Low concentration of glucose; HG, High concentration of glucose; EL, Low concentration of estrogen; EM, Medium concentration of estrogen; EH, High concentration of estrogen; CCK-8, Cell counting kit-8; HK, Hexokinase; G6P, glucose 6-phosphate; PFK1, Phosphofructokinase-1; PPP, Pentose phosphate pathway; G6PDH, Glucose-6-phosphate dehydrogenase; GSK-3β, Glycogen synthase kinase-3β; ATP, Adenosine triphosphate; SGLT, Sodium-glucose co-transporter; GLUTs, Glucose transporters system; AMPK, AMP-activated protein kinase; ATG5, Autophagy-related gene 5; LC3, light chain 3; p62, Sequestosome-1; Bcl-2, B-cell lymphoma-2; Bax, Bcl-2-associated x; WB, Western blotting.

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Plasma Proteomics Characteristics of Subclinical Vitamin E Deficiency of Dairy Cows During Early Lactation

Weidong Qian, Hongyi Yu, Cuiyu Zhang, Hongyou Zhang, Shixin Fu and Cheng Xia**

Heilongjiang Provincial Key Laboratory of Prevention and Control of Bovine Diseases, College of Animal Science and Veterinary Medicine, Heilongjiang Bayi Agricultural University, Daqing, China

Vitamin E (VE) is an essential fat-soluble nutrient for dairy cows. Vitamin E deficiency leads to immune suppression and oxidative stress and increases the susceptibility of cows to reproductive disorders in the early post-partum period. However, studies on plasma proteomics of VE deficiency have not been reported so far. Therefore, the purpose of this study was to understand the changes of blood protein profile in cows with subclinical VE deficiency in the early post-partum period. In this study, plasma protein levels of 14 healthy cows (>4 μg/ml α-tocopherol) and 13 subclinical VE-deficient cows (2–3 μg/ml α-tocopherol) were analyzed by tandem mass tag (TMT). The results showed that there were 26 differentially expressed proteins (DEPs) in the plasma of cows with subclinical VE deficiency compared with healthy controls. Twenty-one kinds of proteins were downregulated, and five kinds were upregulated, among which eight proteins in protein–protein interactions (PPI) network had direct interaction. These proteins are mainly involved in the MAPK signaling pathway, pantothenic acid and coenzyme A (CoA) biosynthesis, PPAR signaling pathway, and glycosylphosphatidylinositol (GPI)-anchor biosynthesis. The top four DEPs in PPI (APOC3, APOC4, SAA4, PHLD) and one important protein (VNN1) by literature review were further verified by ELISA and Western blot. The expression levels of APOC3, VNN1, and SAA4 were significantly lower than those of healthy controls by ELISA. VNN1 was significantly lower than those of healthy controls by Western blot. VNN1 is closely related to dairy cow subclinical VE deficiency and can be a potential biomarker. It lays a foundation for further research on the lack of pathological mechanism and antioxidative stress of VE.

Keywords: subclinical VE deficiency, TMT, differentially expressed proteins, biomarkers, plasma

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***Correspondence:**
Shixin Fu
fushixin@163.com
Cheng Xia
xcwlxyf2014@163.com

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INTRODUCTION

The development of the dairy industry is closely related to the regulation of vitamin nutrition, which is the key problem in dairy cows' healthy breeding (1). Vitamin E (VE) is one of the most important components of cellular antioxidant systems and involved in maintaining the oxidative stability (2). The main function of VE is to protect lipid peroxidation, scavenge free radicals *in vivo*, so as to maintain the integrity of cell membrane function (3). Dietary vitamin additives, forage, and legume silage are the main sources of VE for dairy cows (4, 5). A large number of studies on dairy cows show that the plasma VE concentration decreased gradually before and after delivery and reached the minimum value before and after calving (6–8).

Although with the continuous progress of dairy cows' feeding and management, serious VE deficiency in dairy cows rarely occurs. However, during the transition period, in order to enhance the antioxidant capacity of cow cells, excessive consumption of vitamin E will be caused to achieve the purpose of scavenging the oxidative free radicals of excess cells (6, 9). This process makes early post-partum cows prone to subclinical VE deficiency and systemic oxidative stress. Plasma concentrations of α-tocopherol in cows at 2–3 μg/ml can be identified as subclinical VE deficiency (10–12). Vitamin E deficiency and oxidative stress are important causes of perinatal dairy cows' susceptibility to productive diseases (13). Studies have found that VE deficiency can increase the risk of diseases such as retention of placenta, hysteritis, and mastitis in cows (13, 14). Because there were no practical early monitoring methods to measure the level of VE, subclinical VE deficiency is difficult to be found in due time, which brings serious economic losses to the dairy industry. Therefore, the search for new characteristic biomarkers of subclinical VE deficiency is a key technical problem to be solved in the early monitoring and rapid diagnosis of subclinical VE deficiency for high-yield dairy cows.

Mass spectrometry (MS) has become the preferred method for large-scale protein identification and characterization due to its sensitivity and specificity (15, 16). The MS analysis has been found to reveal changes in protein expression. These proteins can be identified as intermediate biomarkers of early disease effects (17). Tandem mass tag (TMT) is a kind of chemical label used for molecular recognition and quantification based on MS. The TMT has been established as an effective method for proteome quantification (18). The body fluid often detected in the clinic is blood, which is easy to obtain and contains abundant biological information of physiological and pathological processes (19). In this study, proteomics techniques were used to identify the differentially expressed proteins (DEPs) in the plasma between subclinical VE deficiency and healthy cows in the early post-partum period. To the best of our knowledge, no data have been published on plasma proteins in early lactation with subclinical VE deficiency in dairy cows. Thus, this is a comprehensive study to explore the potential biological significance of DEPs between subclinical VE deficiency and healthy cows, providing valuable insights into subclinical VE deficiency plasma proteins that may be applied for developing diagnostic markers in subclinical VE deficiency.

MATERIALS AND METHODS

Animals and Experimental Design

All animals involved in this study were cared for according to the principles of Heilongjiang Bayi Agricultural University Animal Care and Use Committee (Daqing, China). The experiment was conducted at a large intensive cattle farm in Heilongjiang Province (Suihua, China). All Holstein cows were fed the same total mixed ratio diets (in accordance with NRC 2001 standard) with similar age, parity, body condition score, and milk yield. The cows were fed a total mixed ratio diet during early lactation, which mainly consisted of 39.58% of corn, 19.61% of corn silage, 26.99% of *Leymus chinensis*, 8.48% of soybean meal, and 4.21% of concentrated feed at the early stage of lactation (ingredient, % of DM). The basal diet was formulated to meet the nutrient requirements according to the Feeding Standards of Dairy Cattle in China. Detailed feed composition is shown in **Supplementary Table 1**.

According to the concentration of α-tocopherol in plasma, the subclinical VE deficiency group (2–3 μg/ml α-tocopherol) and the healthy control group (>4 μg/ml α-tocopherol) were determined (10–12). Finally, after excluding all other cases of perinatal disease, 67 cows were selected as test animals. Thirteen cows were used as the subclinical VE deficiency group (QF), and 14 cows were used as the healthy control group (BQF) for proteomics study. In addition, 40 cows were used for ELISA verification of the screened and identified differential proteins.

Blood Sample Collection

For this study, blood samples were obtained from the coccygeal veins of 80 transition dairy cows from 0 to 30 days after calving. The blood samples of each cow were collected on an empty stomach in the morning. Plasma was obtained by centrifugation of blood collected in a 10 ml lithium-heparin tube. After centrifugation at 4°C for 10 min (3,000 rpm), the supernatant was collected for secondary centrifugation by high speed (12,000 rpm) for 5 min. The supernatant was placed in a 1.5 ml centrifuge tube and cryopreserved at −80°C until analyzed. All samples used for repetitive analysis were frozen in aliquots, and only vials needed for each assay run were used, to avoid the repetitive thawing and freezing effect.

Plasma Sample Processing and TMT Labeling

To determine the biomarkers of subclinical VE deficiency, 27 plasma samples were analyzed by protein quality inspection, trypsin digestion, and TMT differential labeling. Total protein concentration determination was assessed using a Bradford method (20) (Enzyme labeled instrument, Thermo: Multiskan MK3, USA). Firstly, the sample was diluted with lysis buffer to make its final concentration fall within the range of standard curve. The diluted sample and standard sample (bovine serum albumin, BSA: Sigma-Aldrich, A2058, AUS; BSA was dissolved into a series of standard protein by lysis buffer) were diluted with 5 and 250 μl protein quantitative dye, respectively, and the light absorption value of standard substance and sample at 595 nm was determined by enzyme label instrument. The standard curve was drawn, and the sample concentration was calculated. Then, the protein concentration of each sample was calculated according to the curve formula, and the protein concentration of each sample could meet the requirements of the next experiment.

After protein quantification, 100 μg of protein per sample solution was put into a centrifuge tube, and the final volume was 100 μl with Dissolution Buffer (Thermo Scientific, PN: 1861436). Then, 25 μl of 100 mM reducing reagent (Thermo Scientific, PN:1861438) was added and incubated at 55°C for 1 h, and 5 μl of 375 mM iodoacetamide solution (Thermo Scientific, PN: 1861445) was added and incubated for 30 min in a dark room. The processed samples were transferred to a 10 kDa ultrafiltration tube (Sartorius, PN: VN01H02), and 200 μl of 100 mM dissolution buffer was added, centrifuged at 12,000 *g* for 20 min, discarded the solution at the bottom of the collecting

tube, and repeated four times (pH value should be measured at 8.0). Trypsin (Thermo Scientific, PN: 1862748) was added to the sample (2.5 μl/per sample, 37°C for 14 h). On the next day, the samples were washed with ultrapure water three times, and the bottom of the enrichment tube was lyophilized and then redissolved with 100 mM dissolution buffer.

TandemMassTag™ (TMT™) technology is an *in vitro* peptide labeling technology developed by Thermo Scientific. In this experiment, 27 serum samples were divided into three groups by labeling with 10 isotopes. The amino groups of peptides were specifically labeled and then analyzed by tandem MS. The relative protein content of 10 different samples in each group could be compared simultaneously. After thawing at room temperature, the TMT reagent (10 standard TMT Kit, Thermo Scientific, PN: 90111) was opened, and 0.8 mg of TMT reagent and 41 μl of absolute ethanol were added into each tube and vibrated for 5 min. Then, 100 μl of enzyme digested sample was added (100 μl/sample) and reacted for 1 h at room temperature. Next, 8 μl of 5% of quenching reagent (Thermo Scientific, PN: 1861439) was added and incubated for 15 min to terminate the reaction. After the labeled samples were mixed, vortex oscillation was performed and centrifuged to the bottom of the tube. The sample after vacuum freeze centrifugation is frozen and stored for use (Vacuum freeze dryer, Thermo: SPD2010-230).

Pre-separation of Enzymatic Peptides and LC–MS/MS Analysis

Rigoll-3000 high performance liquid chromatography system was used to separate the samples at high pH (Beijing Puyuan Jingdian Technology Co., Ltd). The experimental methods in this part refer to the proteomic studies published by Zhao et al. (21). The mixed labeled samples were dissolved in 100 μl mobile phase A (98% ddH_2O, 2% acetonitrile, pH 10) and centrifuged at 14,000*g* for 20 min, and the supernatant was taken for use. Firstly, the system condition was detected, and 400 μl BSA was used for separation (column temperature 45°C, detection wavelength 214 nm). Then, 100 μl of the prepared sample was separated in mobile phase B (98% acetonitrile, 2% ddH_2O, pH 10) with a linear gradient of 5–95% over 72 min at the flow rate of 0.7 ml/min, in detail **Supplementary Table 2**.

Each fraction was injected for nanoLC–MS/MS analysis (high performance liquid chromatography: Thermo Scientific EASY-nLC 1000 System, Nano HPLC; MS system: Thermo, Orbitrap Fusion Lumos). The components obtained by reverse phase separation at high pH were redissolved with 20 μl of 2% methanol and 0.1% formic acid (centrifuged at 12,000 rpm for 10 min), 10 μl of supernatant was aspirated, and the sample was loaded by sandwich method (loading pump flow rate to 350 nl/min for 15 min) and mobile phase B (100% acetonitrile, 0.1% formic acid) with a linear gradient of 6–95% over 75 min at the flow rate of 600 nl/min, in detail **Supplementary Table 3**.

Protein Identification and Quantification

The obtained data were processed by UniProt_Bovin (2019.07.16 Download) database for MS. Maxquant, the commercial software supporting Thermo Company, was used to process the original MS file to obtain the quantitative value of the sample. The detailed parameters are retrieved in **Supplementary Table 4**.

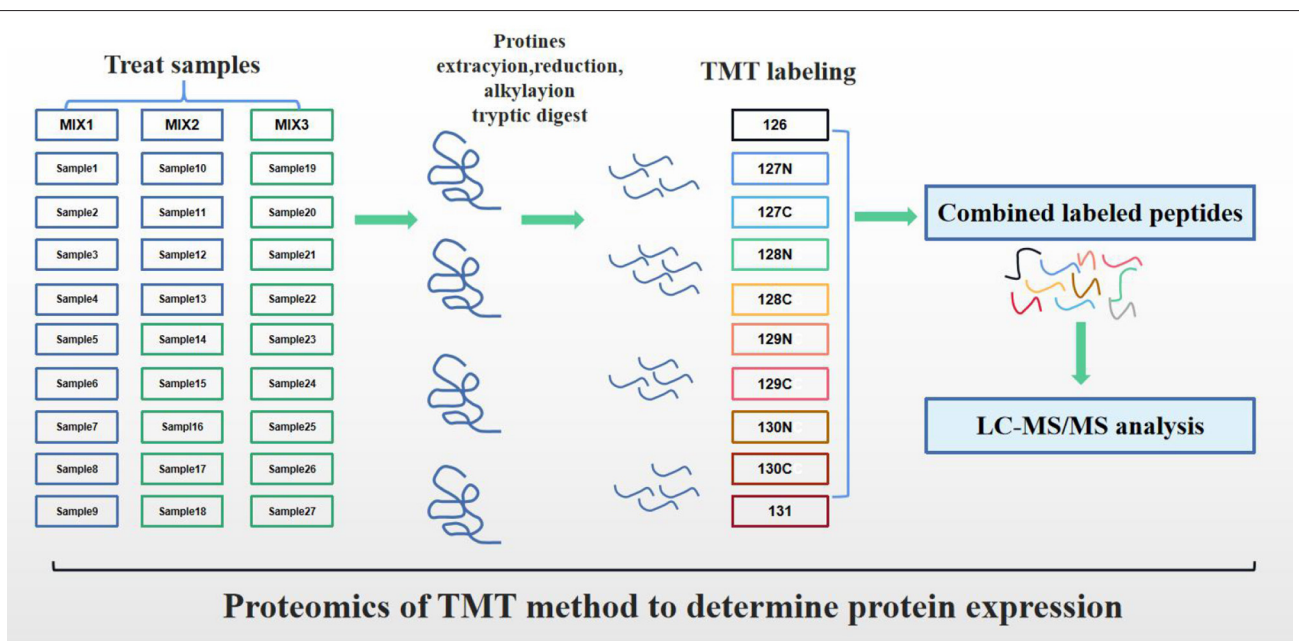

FIGURE 1 | Experimental workflow for proteome analysis.

Bioinformatics Analysis of DEPs

Clusters of Orthologous Groups (COG) analysis is realized by Blast to kyva sequence, and then statistical analysis is carried out to draw the corresponding graph. Gene Ontology (GO) is a standard vocabulary describing the function, location, and activity of genes. It has a tree structure and is the most widely used ontology in molecular biology. KEGG is a Kyoto Encyclopedia of Genes and Genomes (http://www.genome.jp/kegg).

Enzyme-Linked Immunosorbent Assay

According to the α-tocopherol concentration in plasma, 40 cows were divided into the subclinical VE deficiency group and healthy control group (the same as the Animals and Experimental Design section). Plasma levels of apolipoprotein C3 (APOC3), apolipoprotein A4 (APOC4), serum amyloid protein A4 (SAA4), phosphatidylinositol-glycan-specific phospholipase D (PHLD), and pantetheinase-1 (VNN1) were determined by enzyme-linked immunosorbent assays according to the manufacturer's instructions (ELISA kits, Shanghai Sinovac Biotechnology Co., Ltd., China). Optical density was measured at 450 nm using a microplate reader (Thermo Multiskan FC microplate reader).

Western Blotting Analysis

Lysates from plasma samples from normal or subclinical VE deficiency were separated on a 10% SDS-PAGE gel, and the proteins were then transferred to a nitrocellulose membrane. The membrane was blocked in TBST containing 5% non-fat milk powder for 1 h and then incubated overnight with primary antibodies against VNN1 protein; the primary antibody used was anti-VNN-1 (dilution 1:1,000, rabbit, LSBio, USA, LS-C374585) and washed three times with TBST (5 min each), and then the membrane was incubated for 1 h at room temperature with horseradish peroxidase conjugated rabbit IgG. Antibody binding was detected using enhanced chemiluminescence ECL Plus Western blotting detection reagents (GE).

TABLE 1 | Proteins showing significant differences in abundance between plasma from cows with subclinical vitamin E deficiency and healthy control.

ID[a]	Gene name	Protein name[b]	FDR- corrected *p*-value	Fold_change[c]	Change[d]
C4T8B4_BOVIN	CRP	Pentraxin	0.000055	−2.030082929	↓
G3X6K8_BOVIN	HP	Haptoglobin	0.046888	−1.826079462	↓
APOC3_BOVIN	APOC3	Apolipoprotein C-III	0.000048	−1.418673822	↓
Q1RMN9_BOVIN	–	C4b-binding protein alpha-like	0.001169	−0.912143442	↓
APOC4_BOVIN	APOC4	Apolipoprotein C-IV	0.000173	−0.808904848	↓
VNN1_BOVIN	VNN1	Pantetheinase	0.001734	−0.743950694	↓
F1MJK3_BOVIN	–	Uncharacterized protein	0.000734	−0.711301256	↓
F1N0H3_BOVIN	CA2	Carbonic anhydrase 2	0.002981	−0.698077617	↓
SAA4_BOVIN	SAA4	Serum amyloid A-4 protein	0.007418	−0.683821812	↓
Q32PA1_BOVIN	CD59	CD59 molecule	0.000926	−0.522529897	↓
Q2KIW1_BOVIN	PON1	Paraoxonase 1	0.00612	−0.476851592	↓
F1MRD0_BOVIN	ACTB	Actin, cytoplasmic 1	0.006124	−0.463760059	↓
LBP_BOVIN	LBP	Lipopolysaccharide-binding protein	0.037565	−0.453306513	↓
PHLD_BOVIN	GPLD1	Phosphatidylinositol-glycan-specific phospholipase D	0.000006	−0.447579644	↓
E1B805_BOVIN	–	Uncharacterized protein	0.007075	−0.422350268	↓
A0A3B0IZF8_BOVIN	C1QC	Adiponectin B	0.035116	−0.403812868	↓
A0A3Q1LU84_BOVIN		Uncharacterized protein	0.01683	−0.400493668	↓
A0A3Q1LL04_BOVIN	YIPF1	Protein YIPF	0.017006	−0.387877785	↓
A0A3Q1LS74_BOVIN	CFH	Complement factor H	0.029566	−0.305647518	↓
F6PSK5_BOVIN	IL1RAP	Interleukin 1 receptor accessory protein	0.003925	−0.285444103	↓
F1MMP5_BOVIN	ITIH1	Inter-alpha-trypsin inhibitor heavy chain H1	0.030626	−0.27646765	↓
FA10_BOVIN	F10	Coagulation factor X	0.036694	0.280035783	↑
HABP2_BOVIN	HABP2	Hyaluronan-binding protein 2	0.002546	0.306909645	↑
COMP_BOVIN	COMP	Cartilage oligomeric matrix protein	0.029325	0.350653389	↑
REG1_BOVIN	–	Regakine-1	0.01005	0.546086942	↑
CD14_BOVIN	CD14	Monocyte differentiation antigen CD14	0.000123	0.756306843	↑

[a] *ID from NCBI protein database for BOVIN.*

[b] *Displays the protein name of the comment in the Fasta header column.*

[c] *Fold changes calculated as: –log2 (mean disease/mean control), mean value of peak area obtained from the QF group/mean value of peak area obtained from the BQF group. Choose p-value 0.05 and fold change 1.2 times as significant difference (21).*

[d] *"↑", upregulated plasma proteins; "↓", downregulated plasma proteins.*

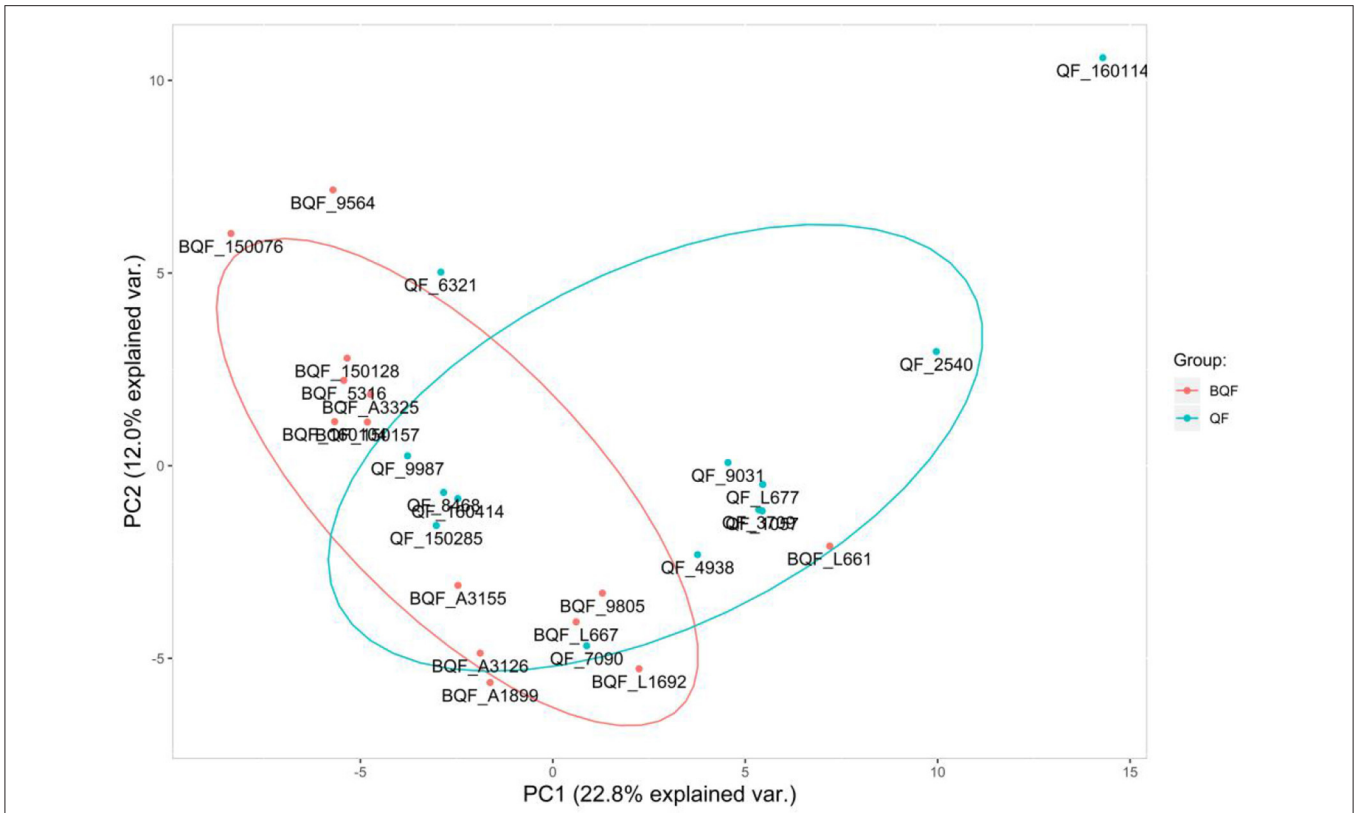

FIGURE 2 | Principal component analysis. The abscissa is the PC1 result, and the ordinate is the PC2 result. Red represents the healthy controls (BQF group), and blue represents the subclinical VE deficiency cows (QF group). It can be seen that there is a certain separation trend between the two groups of samples.

Statistical Analysis

The basic information analyses were performed using SPSS statistical software (V18.0), which was considered significant when the *p*-value was below 0.05. MS data analyses used the Uniprot_BOVIN (2019.07.16 download) database. The original MS of TMT file is processed by Maxquant, a commercial software of Thermo Company. When the *p*-value is 0.05 and fold change 1.2 times, the difference protein is determined to have significant difference (21).

RESULTS

Proteomics of TMT Method to Determine Protein Expression

The experimental workflow of proteomic analysis is shown in **Figure 1**. The clinical characteristics of the subclinical VE deficiency group (QF) and normal control group (BQF) samples for proteomic analysis are shown in **Supplementary Figure 1**. According to the Uniprot_BOVIN database, 3,614 peptides (**Supplementary Table 5**) and 270 proteins (**Supplementary Table 6**) were identified in the protein qualitative results, which revealed 26 DEPs. The specific information concerning the DEPs is shown in **Table 1**. Compared with the healthy control group, the DEPs in the plasma of the subclinical VE deficiency group were 21 downregulated proteins and 5 upregulated proteins (**Figures 2**, **3**).

Functional Annotation and Analysis

Clusters of Orthologous Groups analysis was based on the homologous classification of gene products based on the COG database. The analysis identified protein ortholog classifications *via* the COG database, allowing us to predict the possible functions of these proteins and potentially uncover further functional classifications. The highest protein functional classifications were defense mechanisms with 28 proteins, general function prediction with 20 proteins, post-translational modification, protein turnover, and chaperones with 15 proteins, amino acid transport and metabolism with 10 proteins, and signal transduction mechanisms with 15 proteins (**Figure 4**).

To understand the cellular and molecular functions (MF) of subclinical VE deficiency-related proteins, GO analysis of these related proteins was performed based on biological processes (BP), cellular components (CC), and MF. In this analysis, the protein number was used to assess the importance of subclinical VE deficiency-related proteins in regulating cellular and molecular functions (**Figure 5**). Based on BP, these subclinical VE deficiency-related proteins were mainly involved in innate immune response, triglyceride homeostasis, negative regulation of triglyceride, G protein-coupled receptor signaling,

inflammatory response, and phosphatidylcholine metabolic process. The higher enrichment of CC was the plasma membrane and high-density lipoprotein (HDL) particle, and the functions of higher MF enrichment were calcium ion binding and phospholipid binding.

For KEGG signaling pathway analysis, these subclinical VE deficiency-related proteins are mainly involved in the MAPK signaling pathway, and these subclinical VE deficiency downregulated differential-related proteins are mainly involved in pantothenate and CoA biosynthesis, PPAR signaling pathway, and glycosylphosphatidylinositol (GPI)-anchor biosynthesis (**Figure 6**).

Interaction Network Between Proteins

In this study, 26 differential proteins were obtained through high-throughput screening. Based on the STRING PPI (protein–protein interaction) database and Cytoscape tools, we established a PPI network and found that 10 of these proteins have direct interactions. In the network, the number of proteins that directly interact with a certain protein A is called the connection degree of protein A. Generally speaking, the greater the connection degree of a protein, the greater the disturbance to the entire system when the protein changes; this protein may be the key to maintaining the balance and stability of the system. The Cytoscape was used as a tool to set the size of the node to reflect the degree of the node. A larger node indicates a higher degree of the node. The top four proteins were selected in the degree of the node in this network as candidate proteins for subsequent experimental verification, namely, APOC3, APOC4, SAA4, and PHLD (**Figure 7**).

Reduced Plasma Levels of VNN1, SAA4, APOC3, APOC4, and PHLD in Cows With Subclinical VE Deficiency

To validate differentially expressed candidate proteins between the subclinical VE deficiency group and the healthy control group, 40 plasma samples were verified (20 subclinical VE deficiency and 20 healthy plasma) by ELISA. The top four DEPs in PPI (APOC3, APOC4, SAA4, PHLD) and one important protein (VNN1) by literature review were further verified by ELISA. The results showed that VNN1, SAA4, and APOC4 were significantly downregulated in subclinical VE deficiency plasma samples ($p < 0.05$; **Figure 8**), while the expression levels of PHLD and APOC3, protein were not significantly changed ($p > 0.05$).

Confirmation of Differentially Regulated Proteins by Western Blot

Four samples (the subclinical VE deficiency group and the healthy control group) were selected from the collected plasma samples, and Western blot analysis was performed on one important protein, VNN-1 to verify the results of LC–MS/MS. **Figure 9** shows a Western blot image that can quantify proteins. The results of Western blot provide reliable evidence for TMT proteomics.

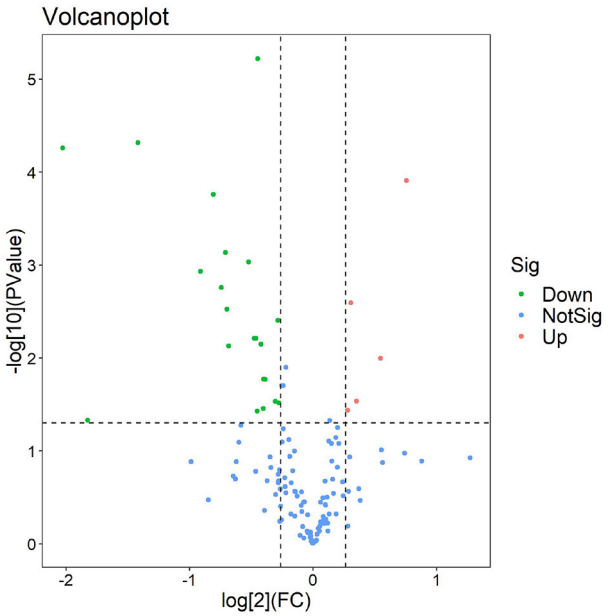

FIGURE 3 | Volcano plot of subclinical vitamin E deficiency vs. healthy control. The volcanic map was drawn using two factors, the fold change (Log2) between the two groups of samples and the *p*-value (–Log10) obtained by the *t*-test, to show the significant difference in the data of the two groups of samples. Red and green dots in the figure are proteins that are significantly differently expressed (1.2 times of fold change and 0.05 of *p*-value). Green dots are downregulated proteins, red dots are upregulated proteins, and blue dots are proteins that have no significant difference.

DISCUSSION

The aim of this study was to report a comprehensive analysis of DEPs in the plasma of early subclinical VE deficiency and healthy cows using TMT-labeled quantitative proteomics. Twenty-six plasma proteins were changed in the subclinical VE deficiency group, of which 21 proteins were downregulated, and 5 proteins were upregulated. This is a comprehensive study to explore the potential biological significance of DEPs between subclinical VE deficiency and healthy cows, providing valuable insights into subclinical VE deficiency plasma proteins that may be applied for developing diagnostic markers in subclinical VE deficiency.

Those DEPs are mainly involved in innate immune response, triglyceride homeostasis, and negative triglyceride regulation according to GO analysis. The changes of regakine-1, coagulation factor X (F10), CD59, haptoglobin (HP), lipopolysaccharide-binding protein (LBP), and serum amyloid A-4 protein (SAA4) involved in congenital immune response are noteworthy in the DEPs. Synergistic effects of regakine-1 with other neutrophil chemokines suggest that it also enhances inflammatory responses to infection (22). F10 regulates inflammatory signaling by inducing the expression of interleukin (IL)-6, IL-8, monocyte chemotactic protein-1, and intracellular adhesion molecules (23, 24). CD59 is a key regulator of the complement system, which inhibits the formation of the MAC terminal pathway by binding to C9 or C5B-8 to prevent complement attack (25).

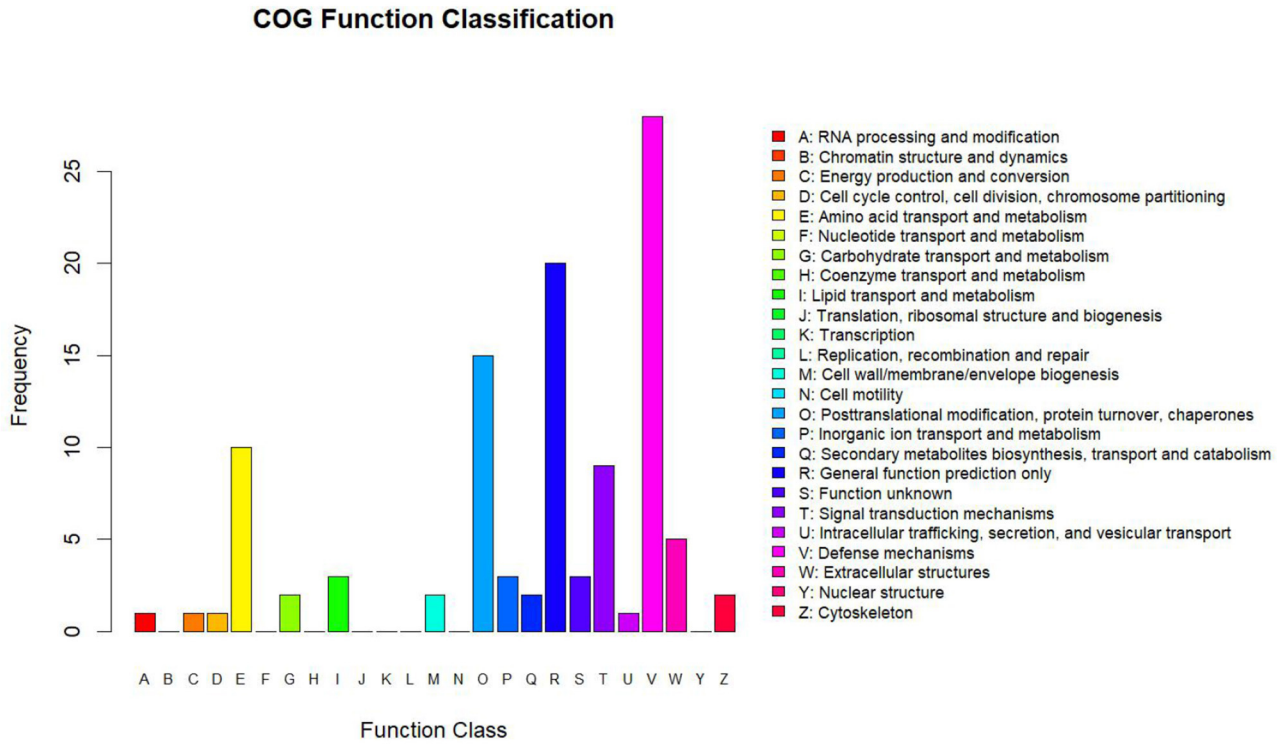

FIGURE 4 | Statistics of COG function classification. The abscissa shows the COG function classification, and the ordinate shows the number of proteins by functional classification.

Haptoglobin is an acute phase protein released by hepatocytes in response to the production of pro-inflammatory cytokines in an inflammatory state (26). Lipopolysaccharide-binding protein is an acute phase protein synthesized in the liver that is involved in the host response to both Gram-negative and Gram-positive pathogens (27); it also promotes the presentation of lipopolysaccharide LPS to CD14 and induces the release of pro-inflammatory cytokines (28). Serum amyloid A-4 protein is a secondary apolipoprotein on HDL in plasma (29). Studies have shown that the concentration of SAA4 decreases when the body is under inflammation (30, 31). The expression of regakine-1 and F10 was upregulated. It indicates that subclinical VE deficiency may aggravate the inflammatory response of the body and increase the risk of infection with other diseases. The expression of CD59, CRP, HP, LBP, and SAA4 was downregulated in the subclinical deficiency group, suggesting that the subclinical deficiency of VE may impair the innate immune response of the body, leading to inflammation and immunosuppression. However, its molecular mechanism needs to be further studied.

The involvement of hyaluronan-binding protein 2 (HABP2), apolipoprotein C4 (APOC4), paraoxonase 1 (PON1), complement factor H (CFH), inter-alpha-trypsin inhibitor heavy chain H1 (ITIH1), and APOC3 in triglyceride homeostasis and negative triglyceride regulation is significant in DEPs. Hyaluronan-binding protein 2 is a calcium-dependent serine protease that provides structural and functional integrity for cells and plays an important role in blood coagulation and fibrinolysis (32). Apolipoprotein C-IV expression is regulated by the oxisome proliferation-activated receptor complex and is associated with hepatic steatosis (33). Paraoxonase 1 is a mammalian antioxidant/anti-inflammatory enzyme synthesized in the liver and secreted into the blood (34). Complement factor H is a major MDA binding protein that can induce the pro-inflammatory effects of MDA (35). Inter-alpha-trypsin inhibitor heavy chain H1 is one of the heavy chains of serine protease inhibitors that carry hyaluronic acid in plasma and play a role in inflammation and carcinogenesis (36). APOC3 is recognized as one of the most important regulators of plasma triglyceride (37). APOC3 of bovine is a low molecular weight protein mainly synthesized by the liver and mainly distributed in HDL (38, 39). Under normal conditions, APOC3 concentration in bovine plasma was the lowest in the non-lactation period and gradually increased in the early lactation stage. In the early lactation period, the plasma APOC3 concentration of cows with fatty liver and ketosis was lower than that of healthy cows (40, 41). In the subclinical VE deficiency group, HABP2 was upregulated, while CFH, ITIH1, and APOC3 were downregulated, indicating that subclinical VE deficiency can aggravate lipid peroxidation, cause oxidative stress induced by inflammatory response, and may cause hyaluronic acid synthesis and transport disorders at the cellular level, thus affecting extracellular matrix synthesis and changing cell structural integrity (42). Paraoxonase 1 is involved in the hydrolysis of lipid hydroperoxides and phospholipids

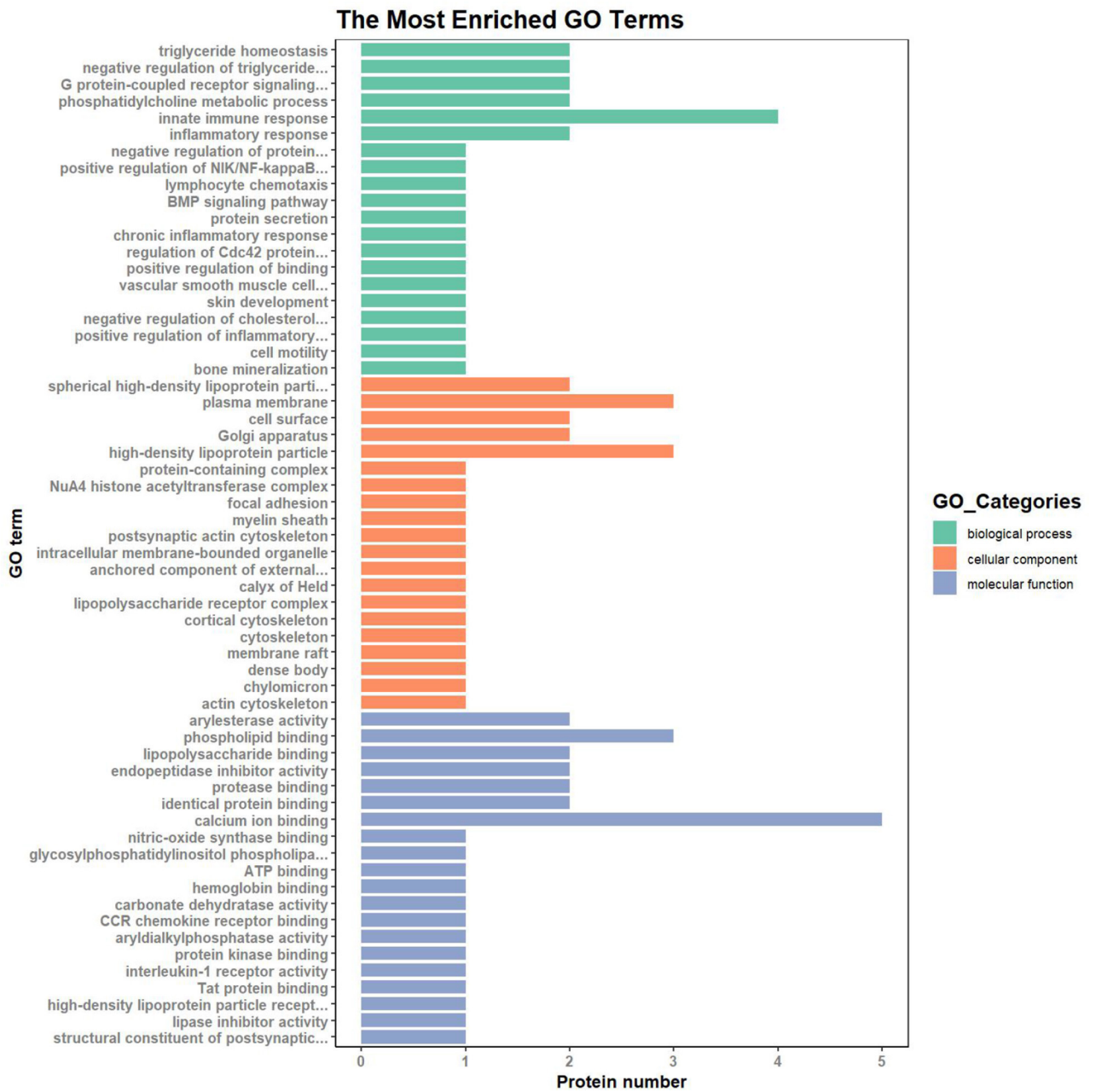

FIGURE 5 | GO (Gene Ontology) analysis of subclinical VE deficiency-related proteins. Green represents biological process, orange represents cellular process, and blue represents molecular process. Classification of 26 DEPs based on biological process, molecular function, and subcellular localization. The abscissa represents the number of differential proteins in each functional category. Based on BP, these subclinical VE deficiency-related proteins were mainly involved in innate immune response and triglyceride homeostasis. The higher enrichment of CC was the plasma membrane and high-density lipoprotein particle, and the functions of higher MF enrichment were calcium ion binding and phospholipid binding.

produced during oxidative stress (43), which may be the main reason for the downregulation of VE in subclinical deficiency.

The main typical pathways were identified between subclinical VE deficient and control cows by KEGG. For KEGG signaling pathway analysis, these subclinical VE deficiency-related proteins are mainly involved in the MAPK signaling pathway, and these subclinical VE deficiency downregulated differential-related proteins are mainly involved in pantothenate and CoA biosynthesis, PPAR signaling pathway, GPI-anchor biosynthesis. Mitogen-activated protein kinases (MAPKs) are a kind of protein kinases that regulate cell proliferation, differentiation, apoptosis, and migration (44). Mitogen-activated protein kinase activation

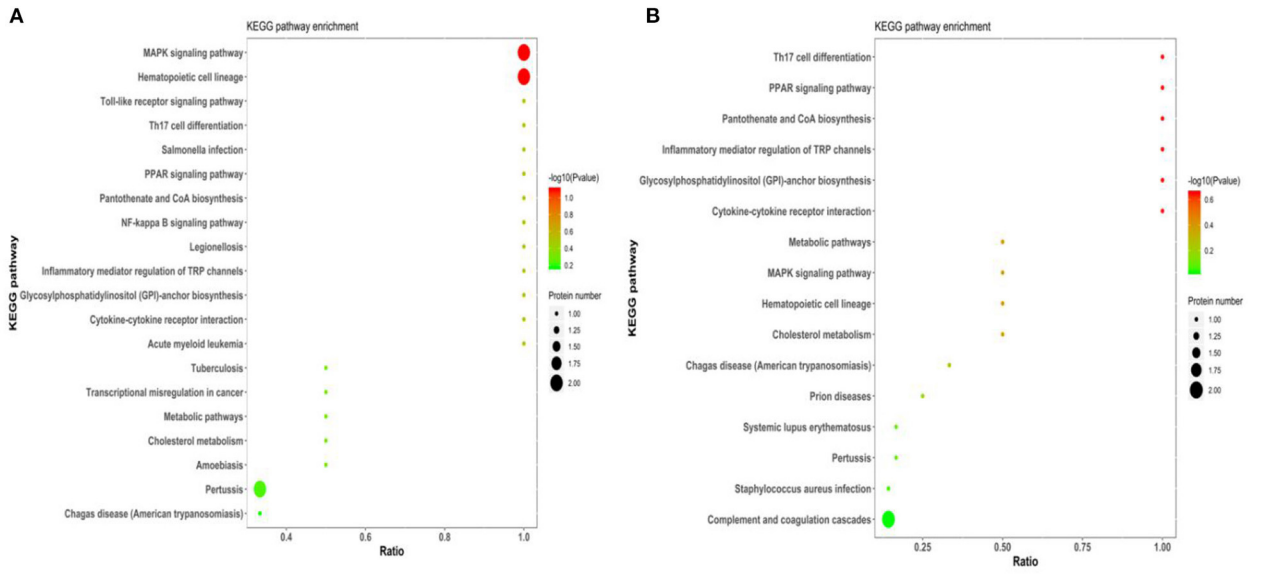

FIGURE 6 | KEGG pathway of subclinical VE deficiency-related proteins enrichment results. Abscissa is ratio, ordinate is each KEGG pathway entry, color represents enrichment [–log10 (*p*-value)], and circle size represents protein number. **(A)** The enrichment results of all differential proteins. **(B)** The enrichment results of downregulated differential proteins.

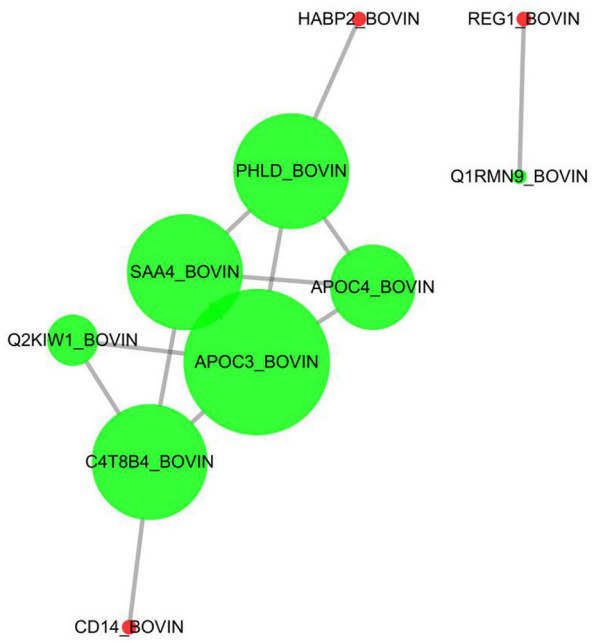

FIGURE 7 | Protein–protein directive interaction network. Ten among 26 DEPs were predicted to have directive protein–protein interactions. The interactions were based on "evidence" mode and of medium confidence. Nodes represent proteins, and edges represent protein–protein interactions. Degree determines the node size, and combined_score determines the edge size.

interacts with protein lipase to change cell behavior rapidly in response to environmental changes (45). The upregulated protein monocyte differentiation antigen CD14 (CD14) and downregulated protein interleukin 1 receptor accessory protein (IL-1RAP) were related to the MAPK signaling pathway. CD14 is a pattern recognition receptor (PRR) of the innate immune system. After recognizing pathogen associated molecular pattern (PAMP), CD14 transmits signals to cells to activate transcription factors and initiate inflammatory reaction (46). Interleukin 1 receptor accessory protein is a kind of auxiliary protein IL-1 signaling pathways, involved in the IL-1 functional receptors (IL-1R I) that both belong to the immunoglobulin superfamily member (47). Interleukin 1 receptor accessory protein increases the supply of cysteine through uptake and biogeneration and controls cysteine metabolism to participate in regulating oxidative stress (48). The downregulation of plasma IL1RAP in dairy cows with subclinical VE deficiency may be due to its involvement in the regulation of cysteine metabolism. Cysteine metabolism is a key substrate and determinant of antioxidant glutathione (GSH) synthesis (48). Plasma CD14 protein expression was up-regulated and IL-1RAP protein expression was down-regulated, suggesting that stress and inflammation were more serious in cows with subclinical VE deficiency.

Glycosylphosphatidylinositol-anchored modification is one of the most common post-translational modifications of eukaryotic cell membrane proteins (49, 50). Glycosylphosphatidylinositol-anchored biosynthesis pathway related protein phosphatidylinositol glycosyl specific phospholipase D (GPLD) was downregulated in post-partum VE-deficient dairy cows. Glycosylated phosphatidylinositol specific phospholipase D (GPI-PLD) in plasma can specifically act on the GPI-anchored substrate in the presence of detergents, thus releasing anchored proteins and phospholipid acids (51). Liver is an important source of plasma GPI-PLD in both human

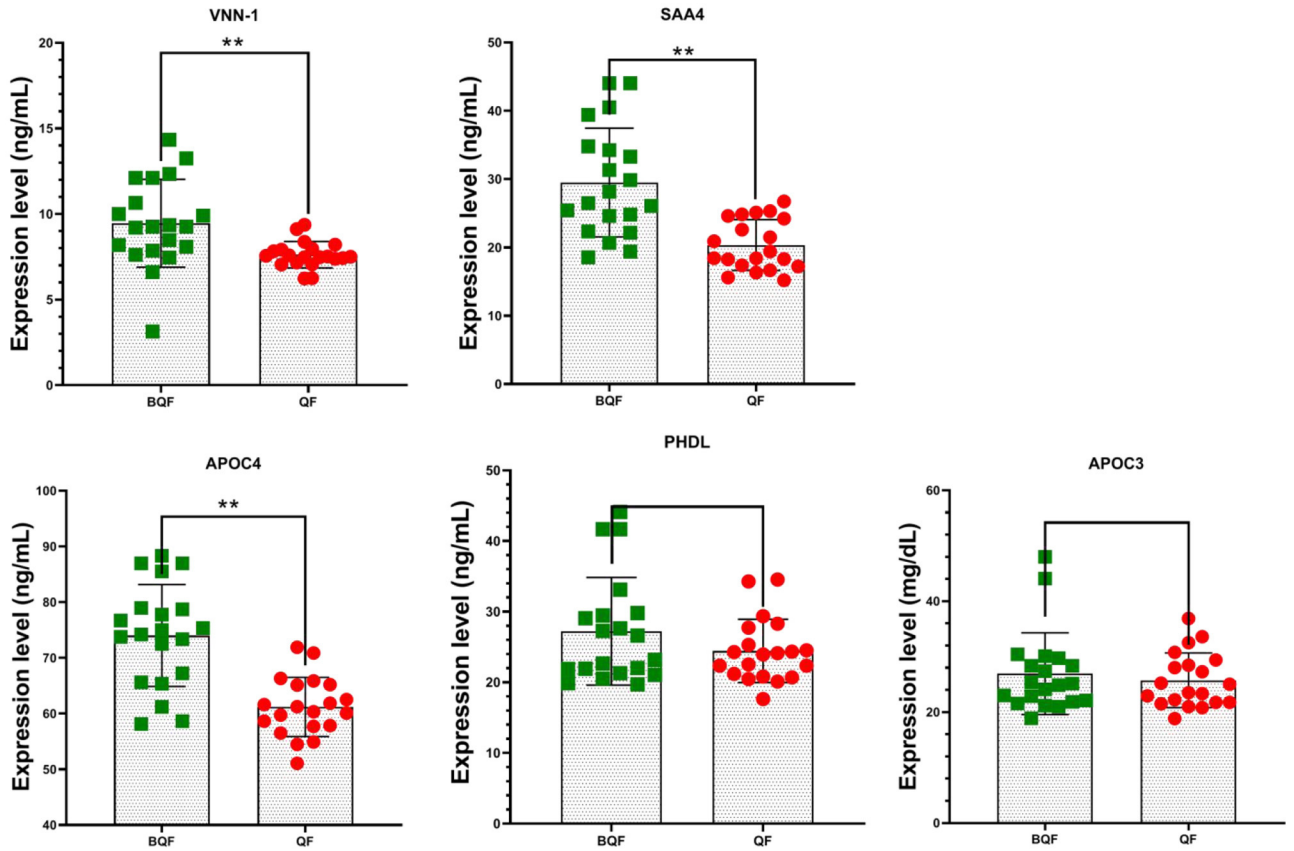

FIGURE 8 | Five plasma protein levels in subclinical VE deficiency cows (QF) and healthy controls (BQF) were selected for verification using enzyme-linked immunosorbent assay in subclinical VE deficiency cows ($n = 20$) and healthy controls ($n = 20$). Plasma levels of VNN-1, SAA4, and APOC4 were significantly lower in subclinical VE deficiency cows than those of the control group (*$p < 0.05$, **$p < 0.01$), while no significant differences were detected in the expression of APOC3 and PHDL ($p > 0.05$).

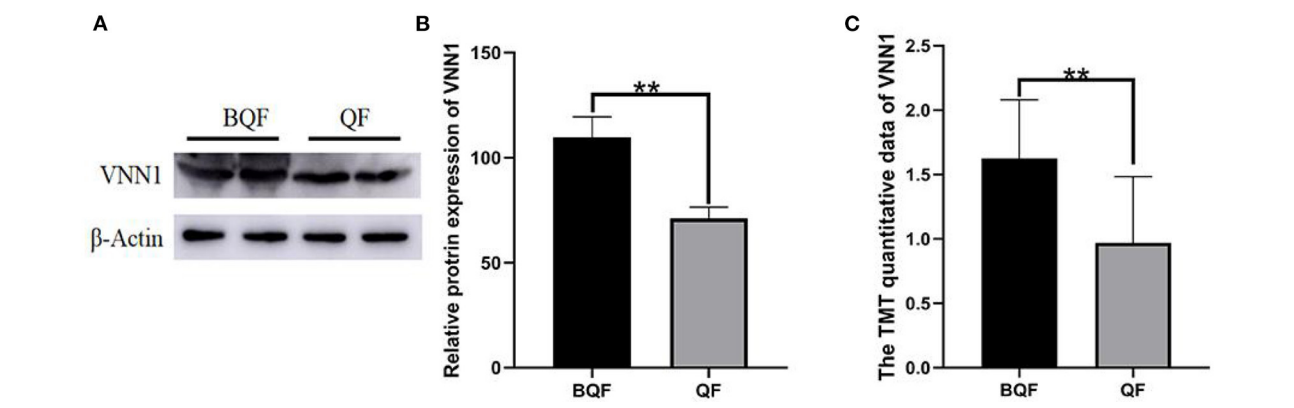

FIGURE 9 | Validation of VNN-1 by immunoblot analysis. **(A)** Representative immunoblotting of DEPs between subclinical VE deficiency cows (QF) and controls (BQF). **(B)** Quantification of relative gray values of VNN-1 compared with actin (**$p < 0.01$). **(C)** The TMT quantitative data of VNN1 between QF group and BQF (**$p < 0.01$).

and bovine (52, 53). The lysosomes of hepatocytes are rich in GPI-PLD, which plays an important role in the degradation of GPI and GPI-anchored proteins in hepatocytes. It is speculated that GPI-PLD in hepatocytes may enter plasma with HDL secreted by hepatocytes (54). Therefore, liver diseases may affect the activity of GPI-PLD in plasma. Downregulation of plasma GPLD1 in dairy cows with subclinical VE deficiency indicated that oxidative stress aggravates abnormal liver metabolism and

abnormal degradation of GPI and GPI-anchored protein of dairy cows with subclinical VE deficiency.

Pantothenic acid (PA) and its salts, as a component of coenzyme A (CoA) or acyl carrier protein (ACP), play an important role in many metabolic reactions (55). Coenzyme A-bound PA is involved in the energy release of carbohydrates, fatty acids, and amino acids. The PA binding to ACP is related to the synthesis of fatty acids (56). In the post-partum subclinical VE deficiency group, PA and CoA biosynthesis pathway-related protein pantothenyl thioglycolaminase (VNN1) were downregulated. VNN1 is a kind of oxidative stress sensor rich in the liver, which is a GPI-anchored pantothenase. It is involved in the regulation of multiple metabolic pathways and is highly expressed in the liver, intestine, and kidney (57, 58). Its pantothenase activity hydrolyzes PA into PA (vitamin B5) and cysteamine (59). Some studies have shown that VNN1 deficiency can increase liver GSH levels (60, 61). In this study, VNN1 was downregulated in subclinical VE-deficient dairy cows after middle production, which may be due to the increase of liver GSH level to resist oxidative stress.

Subclinical VE deficiency in cows in the early post-partum period can aggravate oxidative stress and inflammation and aggravate abnormal lipid metabolism in cows (11, 62, 63). Vitamin E is a major protective agent for circulation and intracellular lipid peroxidation, which can reduce the level of cellular oxidative stress and improve the functional environment of intracellular signaling pathways. It has anti-inflammatory and antioxidant effects (64). The five candidate proteins (APOC3, APOC4, SAA4, PHLD, and VNN1) were identified by interaction network analysis and literature review. The DEPs were further verified by enzyme-linked immunosorbent assay and Western blot. APOC3, VNN1, and SAA4 all had significantly lower expression levels than the healthy control group. APOC3 plays a role in PPARα metabolism by controlling lipolysis of PPARα ligands (65). VNN1 is an important target gene of PPARα, which participates in regulating its activity (66–68). VNN1 is involved in oxidative stress and inflammation by regulating the synthesis of cysteamine and GSH (69, 70). Under physiological conditions, SAA4 accounts for more than 90% of the total SAA (29). However, its concentration did not increase in the inflammatory state, but showed a downward trend (30, 31). As a new molecule of concern, SAA4 may be one of the diagnostic markers of post-partum cow subclinical VE deficiency. More research is needed to explore the regulatory mechanisms of APOC3, VNN1, and SAA4 proteins against the dairy cow subclinical VE deficiency and how the three proteins interact.

CONCLUSIONS

In this study, proteomic TMT methods were used to reveal the subclinical deficiency of cow VE in the early post-partum period and changes in plasma protein abundance in healthy control. Subclinical VE-deficient cows aggravate oxidative stress, abnormal lipid metabolism, and immunosuppression. The top canonical pathways and biological functions identified by KEGG and GO indicate this. Based on the different abundance of proteins in these pathways, fat mobilization, ROS production, and inflammatory immune response of subclinical VE-deficient dairy cows increased, which would cause the body to be susceptible to infection. These changes of oxidative stress and inflammation-related proteins may be related to early lactation diseases and slow recovery of reproductive performance. These findings contribute to further research to better understand the molecular mechanism of protein changes that may promote inflammation and oxidative stress.

DATA AVAILABILITY STATEMENT

The data presented in the study are deposited in the IPROX repository, ProteomeXchange ID: PXD026856. The link is https://www.iprox.cn/page/PSV023.html;?url=1624347711767BLKj, view password is jWOm.

ETHICS STATEMENT

The animal study was reviewed and approved by Heilongjiang Bayi Agricultural University Animal Care and Use Committee.

AUTHOR CONTRIBUTIONS

WQ, SF, and CX conceived the study, interpreted the data, and wrote the manuscript. WQ, HY, CZ, XS, HZ, SF, and CX carried out experiments and data analysis. All authors approved the final version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fvets.2021.723898/full#supplementary-material

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Peripartal Rumen-Protected L-Carnitine Manipulates the Productive and Blood Metabolic Responses in High-Producing Holstein Dairy Cows

Mohsen Danesh Mesgaran[1]*, *Hassan Kargar*[1], *Sadjad Danesh Mesgaran*[2] and *Ali Javadmanesh*[1,3]

[1] Department of Animal Science, Faculty of Agriculture, Ferdowsi University of Mashhad, Mashhad, Iran, [2] Kaesler Nutrition GmbH, Cuxhaven, Germany, [3] Stem Cell Biology and Regenerative Medicine Research Group, Research Institute of Biotechnology, Ferdowsi University of Mashhad, Mashhad, Iran

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***Correspondence:**
Mohsen Danesh Mesgaran
danesh@um.ac.ir

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This study aimed to monitor the effect of including rumen-protected L-carnitine (Carneon 20 Rumin-Pro, Kaesler Nutrition GmbH, Cuxhaven, Germany) in the transition diet on the productive and metabolic responses of multiparous high-producing Holstein dairy cows. Thirty-two multiparous cows were allocated in a completely randomized design to receive the same diet plus 60 g fat prill containing 85% palmitic acid (control, $n = 16$) or 100 g rumen-protected L-carnitine (RLC, $n = 16$); at 28 days before expected calving until 28 days in milk (DIM). Fat prill was included in the control diet to balance the palmitic acid content of both experimental diets. Milk production over the 28 DIM for the control and RLC groups was 46.5 and 47.7 kg, respectively. Milk fat content tended to increase upon rumen-protected L-carnitine inclusion ($p = 0.1$). Cows fed rumen-protected L-carnitine had higher fat- and energy-corrected milk compared with the control group. Pre- and post-partum administration of L-carnitine decreased both high- and low-density lipoprotein concentrations in peripheral blood of post-partum cows. The results of this study indicated that the concentration of triglycerides and beta-hydroxybutyrate was not significantly different between the groups, whereas the blood non-esterified fatty acid concentration was markedly decreased in cows supplemented with L-carnitine. Animals in the RLC group had a significant ($p < 0.05$) lower blood haptoglobin concentration at 7 and 14 DIM than the control. Animals in the RLC group had a lower concentration of blood enzymes than those of the control group. The mRNA abundance of Toll-like receptors 4, cluster of differentiation 14, and myeloid differential protein 2 did not significantly change upon the supplementation of L-carnitine in the transition diet. In summary, the dietary inclusion of RLC improved dairy cow's performance during the early lactation period. Greater production, at least in part, is driven by improved energy utilization efficiency and enhanced metabolic status in animals during the periparturient period.

Keywords: L-carnitine, cow, periparturient period, milk, metabolism

INTRODUCTION

In dairy cows, the transition from gestation to lactation is challenged by energy requirement for milk production and secretion, inadequate feed intake, and metabolic disorders (1, 2). Therefore, this period is critical for determining the productive responses, metabolic health (3), and profitability of the dairy cows (4). As parturition approaches, concentrations of various hormones and metabolites begin to alter in order to support the milk yield (2). This would eventually lead to higher milk production while there is a lag in the dry matter intake (DMI) to provide nutrient demands of the animals post-partum. This phenomenon triggers the animals to mobilize the body fat reservoirs, which enters them in a state of negative energy balance and could last for a long period (i.e., several months) in various cases (5). A severe negative lag between DMI and milk yield is a risk factor for metabolic imbalancing as well as infectious and reproductive disorders (6). Besides, metabolic imbalancing initiates a cluster of risk factors in dairy cows which leads to an increased susceptibility to certain health disorders (2, 7). For example, fresh cows, which cannot meet energy demands through DMI, are associated with higher blood biomarkers of fat mobilization, such as non-esterified fatty acids (NEFA) (7, 8). Excessive fat mobilization may result in overproduction of the ketones, e.g., beta-hydroxybutyrate (BHB) (9, 10). This can be further elaborated by a higher degree of mobilization of energy reserves due to the severe negative energy balance and lower rumen fill index because of decreased DMI. Hence, the body condition score (BCS) may be considered as an indicator for the mobilization of adipose tissue. In particular, the post-partum decrease in BCS is associated with metabolic imbalancing and infectious disorders (11). Cows with a metabolic challenge have a more pronounced decrease in BCS from days 14 to 35 after calving, indicative of a higher degree of body fat mobilization.

Previous works have clearly pinpointed the state of immune dysregulation in dairy cows during the transition period (12, 13). The presence of any metabolic imbalancing resulted from aberrant nutrient metabolism, causing metabolic stress and inflammatory responses in the early lactating cows (1). Higher concentrations of NEFA and BHB may influence early lactation disease and alter immune competence (14, 15). Pathological levels of both NEFA and BHB have been negatively associated with polymorphonuclear leukocytes and peripheral blood mononuclear cell functionality (16–18). In particular, increasing levels of NEFA during the periparturient period manipulate the inflammatory response of dairy cows *via* its impact on Toll-like receptors (TLR) and their signaling pathways (19). TLR are among the pathogen recognition receptors within the immune system. Toll-like receptors 4 (TLR4) are able to recognize lipopolysaccharides (LPS), i.e., endotoxins of gram-negative bacteria, which are located in various cell types (20). Cluster of differentiation 14 (CD14) are accessory proteins, which facilitate the interaction between LPS and TLR4. Subsequently, CD14 transfers LPS to myeloid differential protein 2 (MD2), a protein complexed with TLR4 on the cell surface, which initiates the myeloid differentiation factor 88 pathway and in turn transcription of the inflammatory cytokines.

Carnitine is a water-soluble quaternary amine, which influences the function of all living cells. In dairy cows, L-carnitine is a necessary molecule for the normal activity of the tissues encompassing mitochondria. This molecule is involved in the shuttle of activated long-chain fatty acids from cytosol to the mitochondria. Besides, this molecule has a potential to influence, although indirectly, the rate of energy production from glucose (21, 22). In addition, previous experiments in monogastrics have demonstrated that L-carnitine has an antioxidative potential, which is quite vital for scavenging the excess reactive oxygen species and maintaining the health of livestock (23). L-Carnitine has been shown to modulate the liver inflammation as well as circulating pro-inflammatory markers *via* specific signaling pathways (24). However, in a recent study in mid-lactating dairy cows challenged with LPS, authors were not able to reveal significant effects of L-carnitine supplementation before and 2 weeks after the challenge (25). The aforementioned functions indicate that L-carnitine may play a unique function in the transition period. Therefore, the present work aimed to investigate the influence of dietary inclusion of L-carnitine, through a rumen-protected L-carnitine (Carneon 20 Rumin-Pro containing 20% L-carnitine, Kaesler Nutrition GmbH, Cuxhaven, Germany) product, during the transition period on (a) milk yield, chemical composition, and fatty acid profile; (b) plasma concentrations of glucose, urea, albumin, cholesterol, high-density lipoprotein (HDL), low-density lipoprotein (LDL), triglyceride, haptoglobin, calcium, blood enzymes, i.e., alanine aminotransferase (SGPT) and aspartate aminotransferase (SGOT), NEFA, and BHB; (c) mRNA expression of CD14, TLR4, and MD2; and (d) distinct animal behavioral indices such as rumen fill index, manure score, rumination activity, and BCS. Determining the aforementioned parameters would enable to observe the impact of rumen-protected L-carnitine on the productivity of the animal as well as their inflammatory status at the molecular level.

MATERIALS AND METHODS

Animals, Feeding, and Management

Thirty-two multiparous Holstein cows (average 305-day milk yield of 12,000 kg in the previous lactation) were paired by expected calving date and randomly assigned to receive a similar basal diet plus 60 g fat prill containing 85% palmitic acid (control group, $n = 16$) or 100 g of the rumen-protected L-carnitine (RLC group, $n = 16$), beginning at 28 days before the expected calving through 28 days in milk (DIM). The rumen-protected L-carnitine top-dressed on the basal diet was Carneon 20 Rumin-Pro (Kaesler Nutrition GmbH, Cuxhaven, Germany), which is a commercial source of L-carnitine (20% L-carnitine coated with rumen bypass fat rich in palmitic acid). As described previously (21), the specific inclusion rate of a protected fat, i.e., fat prill, was included in the control group in order to balance the coated fat of the protected L-carnitine source. Hence, the experimental diets were either without or with extra supplemented L-carnitine through the rumen-protected L-carnitine source. The specific inclusion rate of protected L-carnitine source was decided based on the previous research observing the impact of L-carnitine

around parturition and during the high lactation period (21, 26). During pre- and post-partum, the cows were housed in two separated free stall barns, while they had free access to feed and water in a commercial dairy farm with 820 milking cows. Diets were fed as a total mixed ration (TMR) two times per day at 0730 to 1830 h in amounts that ensured *ad libitum* consumption and ∼4–8% feed refusals. The ingredients and chemical composition of the pre- and post-partum diets are presented in **Table 1**. Daily samples of TMR and feed refusal and weekly samples of the diet ingredients were collected, dried in a forced air oven for 72 h at 55°C, and ground using a Wiley mill to pass a 1-mm screen, then analyzed for DM and the chemical composition (27). Dry matter was determined after 24 h at 95°C (ISO 6496). Ash was determined after 3 h at 550°C (ISO 5984). Nitrogen was assessed using the Kjeldahl method (Kjeltec 2300 Autoanalyzer, Foss Tecator AB, Hoganas, Sweden) with crude protein (CP) as N × 6.25. Starch content was evaluated by an anthrone/sulfuric acid method using glucose as standard and estimated as 0.9 × glucose content after liberating the starch by heating in a boiling water bath in the presence of 2 N HCl (28). For NDF and ADF, the method of Goering and Van Soest (29) was used.

Cows were milked three times daily at ∼0400, 1200, and 2000 h. The incidence of health problems was accurately recorded for each cow throughout the experiment.

TABLE 1 | Ingredient, chemical composition, and calculated energy content of pre-partum (from −28 to parturition) and post-partum (from 1 to 28 DIM).

Items	Pre-partum	Post-partum
Ingredient, % of DM		
Corn silage	35.0	17.6
Alfalfa hay	17.2	21.8
Wheat straw	4.8	0.6
Corn grain	8.6	12.3
Barley grain	7.9	14.1
Wheat grain	7.3	-
Sugar beet pulp	1.2	2.6
Extuded soybean meal	4.8	10.9
Wheat bran	1.3	6.3
Cottonseed whole	2.6	3.9
DDGS	1.3	2.7
Rape seed meal	1.3	3.2
Supplement[a,b]	6.6	3.9
Metabolizable energy (MJ/kg DM)	10.3	11.2
Chemical composition (% DM) and		
CP	11.8	15.8
NDF	39.5	36.3
ADF	21.9	18.0
ASH	7.8	7.3
Starch + soluble sugar	25.3	27.3
EE	3.1	4.0

[a] *Pre-partum: contained 400 g anionic salts (www.javanehkhorasan.com), 200 g VitalG (rumen protected glucose, www.groupsana.comena), 100 g OptiMate (essential omega-3 from salmon oil, rumen protected with vitamins, www.agritech.ie), 15 g encapsulated choline chloride (www.Kemin.com), 25 g Lutrell® Pure [conjugated linoleic acid (CLA), BASF], 60 g vitamin D3 (5,000,000 iu/kg), 80 g vitamin E and Se (11,000 iu and 300 mg/kg, respectively), 200 g mineral/vitamin premix/kg (vitamins including A: 1,500,000 iu, D3: 400,000 IU, E:3,000 IU, biotin: 120 mg; minerals including Ca, P, Mg Na, K, Mn Zn, Cu, Se, I, Fe, Co, and S with the quantity of 44, 20, 2.3, 20, 1.6, 3.4, 6, 5, 0.14, 0.25, 4 0.043, and 17.6 g, respectively, www.javanehkhorasan.com), and 15 g toxin bonder (Toxytrap, Iran).*

[b] *Post-partum: contained 160 g sodium bicarbonate (Petro Tarh, Iran), 60 g di-calcium phosphate (www.javanehkhorasan.com), 200 g VitalG (Rumen protected glucose, www.groupsana.comena), 150 g Optimate (essential omega-3 from salmon oil, rumen protected with vitamins, www.agritech.ie), 30 g encapsulated choline chloride (www.Kemin.com), 25 g Lutrell® Pure [conjugated linoleic acid (CLA), BASF], 60 g vitamin D3 (5,000,000 iu/kg), 80 g vitamin E and Se (11,000 iu and 300 mg/kg, respectively), 200 g mineral/vitamin premix/kg (vitamins including A: 1,500,000 iu, D3: 400,000 IU, E:3,000 IU, biotin: 120 mg; minerals including Ca, P, Mg Na, K, Mn Zn, Cu, Se, I, Fe, Co, and S with the quantity of 44, 20, 2.3, 20, 1.6, 3.4, 6, 5, 0.14, 0.25, 40.043, and 17.6 g, respectively, www.javanehkhorasan.com), and 15 g Toxin bonder (Toxytrap, Iran).*

Sample Collection and Processing

Feed refusals of each group were measured daily, and feed intake for each group was determined by difference assuming a different DM content of feed offered and the ort. Milk yield was recorded daily but reported from 3 days after calving. Weekly milk samples, for 4 weeks after the calving, from individual cows were obtained at 3 consecutive milking, preserved with 2-bromo-2-nitropropane-1,3-diol, and analyzed for protein, fat, lactose, milk urea nitrogen (MUN), somatic cell scores (SCC), solid non-fat (SNF), and total solid content using Fourier-transform infrared spectroscopy (FT-IR; CombiScope FTIR 600 HP, Delta Instruments, Drachten, The Netherlands) in a commercial laboratory (Sazan Rojan Alvand Co., Alborz, Iran). In addition, the milk fat samples obtained from cows at 21 DIM were analyzed for fatty acid composition. This particular sampling day for milk fatty acid composition analysis was chosen as feed intake in animals around 21–28 days in lactation would be higher (30); hence, the milk fatty composition would be more heavily relied from the diet rather than mobilized fat depot (31). For this, fatty acid methyl esters were prepared by transmethylation and were then quantified by using a gas chromatograph (Shimadzu GC-2010, Kyoto, Japan) equipped with a flame-ionization detector and a CP-7489 fused-silica capillary column (100 m × 0.25 mm i.d. with 0.2-μm film thickness; Varian, Walnut Creek, CA, USA). The initial oven temperature (50°C) was held for 1 min then ramped at 5°C/min to 160°C, where it was held for 42 min and then ramped at 5°C/min to 190°C and held for 22 min. Inlet and detector temperatures were maintained at 250°C, and the split ratio was 100:1. The hydrogen carrier gas flow rate through the column was 1 ml/min. The hydrogen flow to the detector was 30 ml/min, airflow was 400 ml/min, and the nitrogen make-up gas flow was 25 ml/min. Peaks in the chromatogram were identified and quantified using pure methyl ester standards.

Fat-corrected milk standardized to 4% fat was calculated using the equation of Gaines (32), FCM = [0.4 × milk yield (kg)] + [15 × milk fat (kg)], and ECM was calculated as presented by Muñoz et al. (33).

Bleeding was conducted from 10 cows per each group at 0800 h *via* puncture of the coccygeal vessels on days −14, −7, +7, +14, and +21 relative to calving as proposed by Greenfield et al. (34). The aforementioned sampling dates were

chosen, as previous studies have shown extensive metabolic changes from 2 weeks pre- until 2 weeks post-partum, which could be associated with overall health alteration in dairy cows and higher culling rates (35). Samples on day 21 after calving was also taken to ensure a better depiction of dynamics of the selected metabolic and health parameters. The samples were kept at room temperature, and the serum was separated within 0.5 h, then stored frozen at −20°C until analyses for glucose (GOD-PAP, https://parsazmun.de/GLUCOSE/), triglycerides (GPO-POD, www.Bionik.web.com), NEFA (colorimetric method, Randox, County Antrim, UK), BHB (kinetic enzymatic method, Randox, County Antrim, UK), urea (http://paadco.co), cholesterol (CHOD_POG, http://paadco.co), HDL (direct enzymatic colorimetric method, http://paadco.co), LDL (direct enzymatic colorimetric method, http://paadco.co), SGOT (kinetic UV method based on IFCC recommendations, http://paadco.co), SGPT (kinetic UV method based on IFCC recommendations, http://paadco.co), calcium (Arsenazo III Colorimetric method, http://paadco.co), albumin (BGC method, https://parsazmun.de), and haptoglobin (an immunoturbidimetric assay).

RNA Isolation, Reverse Transcription, and Quantitative Real-Time PCR

Samples of blood were obtained at 0800 h *via* puncture of the coccygeal vessels on −7, +7, and +14 days related to calving. These sampling points were chosen, as previous studies revealed that unresolved subacute inflammations as early as 7 days post-calving would damage the productivity of dairy cows in the subsequent lactation (36). These samples were immediately frozen at −80°C and used for the analysis of mRNA expression. Total RNA was extracted by the AccuZol™ Total RNA Extraction Solution (Bioneer, Daejeon, South Korea) according to the manufacturer's instruction. The purity and integrity of RNA were assessed using the Epoch microplate spectrophotometer (BioTek, Winooski, USA) and agarose gel electrophoresis, respectively. One μg of RNA was treated with DNase and reverse transcribed to cDNA using AccuPower® RT PreMix (Bioneer, Daejeon, South Korea) according to the supplier's instruction.

The cDNA was then subjected to real-time quantitative PCR (qPCR) for amplification. Oligonucleotide primers specific for studied and reference genes were used (**Table 2**). All qPCR reaction conditions were in compliance with MIQE [minimum information for publication of qPCR experiments, (39)]. Quantitative PCR was performed in duplicate, using the RealQ Plus 2X master mix (Ampliqon, Odense, Denmark) in a LightCycler® 96 instrument (Life Technologies Roche Life Science, Basel, Switzerland). Amplification was performed in 0.1-ml 8-strip tubes (Gunster Biotech, Viluppuram, Taiwan) as the reaction mixture containing 2 μl of cDNA, 5 pmol of each primer, and 10 μl of 2× master mix in a total volume of 20 μl. The following PCR program was used: the initial step of 95°C for 10 min and the amplification step of 40 cycles which started with 15 s at 95°C followed by 30 s at 60°C and 20 s at 72°C. This program was followed by analyzing the melting curve performed with linear heating from 60 to 90°C. Reaction efficiency was calculated based on the slope of the standard curve (equation: efficiency = (10 (−1/slope) − 1) × 100). Correlation coefficients ($R^2 \geq 0.99$) were considered. The relative copy number of CD14, TLR4, and MD2 transcripts was normalized to the geometric means of both RPS9 and GAPDH reference genes.

TABLE 2 | Species-specific primers for the quantification of selected as well as reference genes using a real-time qPCR assay.

Name	Sequence	References	Product size (bp)
TLR4-F[a]	CCTTGCGTACAGGTTGTTCC	(37)	129
TLR4-R[b]	GCCTAAATGTCTCAGGTAGTTAAAGC		
CD14-F	CACCACATTGCACACCTGTT	(37)	124
CD14-R	CACCACATTGCACACCTGTT		
MD2-F	GGAGAATCGTTGGGTCTGCT	(37)	92
MD2-R	GCTCAGAACGTATTGAAACAGGA		
GAPDH-F	TCATTGAAGCCTTCACTACATGGTCT	(37)	147
GAPDH-R	TGATGTTGGCAGGATCTCG		
RPS9-F	TAGGCGCAGACGGGCAAACA	(38)	136
RPS9-R	CCCATACTCGCCGATCAGCTTCA		

[a] *F: forward primer.*
[b] *R: reverse primer.*

Animal Behavior

The weekly animal behavior including the time spent ruminating was recorded every 10 min per 24 h and calculated by multiplying the total number of observed activities in each duration (40). The body condition score, manure score, and rumen fill score were assessed weekly from 21 days before the expected parturition to 4 weeks after calving. The body condition score was recorded by the same operator using a 1–5 scale with 0.1 intervals as proposed by Ferguson et al. (41) and Roche et al. (11). Both the manure score (42) and rumen fill score (43) were assessed 6 times per day which started 2 h before the morning feeding by the same operator using a 1–5 scale.

Statistical Analysis

Data obtained weekly were statistically analyzed using the Proc Mixed procedure of SAS (44) for a completely randomized design with repeated measures. The model included the effects of group, day relative to calving, and the interaction between group and day. Days relative to calving was used as a repeated measurement, with cow within experimental groups as the subject. Daily DMI of the groups was analyzed as previously described (45); however, the interaction between group and day was taken out from the model. A gamma-type function model (Y = a*EXP – cd) was generated to describe the relation between daily milk production (Y) and time (d), while (c) is the slope. Data of milk fatty acid composition were analyzed using a completely randomized design. Data of gene expression were analyzed in JMP® 4.0 (SAS Institute, Cary, NC, USA) using the analysis of variance method (ANOVA) by least-square fit. Differences were considered significant at $p < 0.05$, whereas tendency was determined at $0.05 < p < 0.1$. Data are expressed as the mean ± SEM.

TABLE 3 | Milk production and composition of lactating Holstein dairy cows fed a post-partum diet plus fat prill containing 85% palmitic acid (control group) or Carneon 20 Rumin-pro (RLC group).

Items	Experimental groups								SEM	*p-value*		
	Control				RLC					Group	Time	Group*time
	+7 days	+14 days	+21 days	+28 days	+7 days	+14 days	+21 days	+28 days				
DMI (kg)	15.9	20.2	24.1	26.9	16.1	20.9	24.4	27.2	1.71	ns	*	-
Milk yield (kg)	36.2	44.7	50.1	52.3	36.0	45.0	52.1	54.5	1.01	ns	*	ns
Fat (%)	4.16	3.28	3.07	3.26	4.12	3.59	3.90	3.65	0.22	0.10	*	ns
Protein (%)	3.80	3.20	3.10	3.00	3.80	3.20	3.10	3.00	0.06	ns	*	ns
Lactose (%)	4.10	4.50	4.50	4.50	4.20	4.40	4.50	4.50	0.03	ns	ns	ns
Solids (%)	13.2	12.1	11.8	11.9	13.1	12.3	12.5	12.2	0.24	*	*	ns
SNF (%)	9.00	8.80	8.70	8.60	9.00	8.70	8.60	8.50	0.08	*	*	ns
4% FCM (kg)	37.1	39.8	43.1	46.5	36.7	42.2	51.4	51.6	0.91	*	*	ns
ECM (kg)	41.3	43.9	47.5	50.5	40.9	46.0	55.0	55.3	1.05	*	*	ns
Fat (g/day)	1.51	1.47	1.54	1.70	1.48	1.62	2.03	1.99	0.05	*	*	ns
Protein (g/day)	1.38	1.43	1.55	1.57	1.37	1.44	1.62	1.63	0.01	*	*	ns
MUN (g/dL)	12.1	14.0	14.0	13.5	13.3	13.5	12.8	13.2	0.43	ns	ns	ns
SCC (×1,000)	305	397	117	146	85.3	108	113	137	42.0	*	*	ns

*d: day relative to calving; *p < 0.05; ns: p > 0.05; when the difference between means is >2 times the SEM, it is considered as significant (p < 0.05).*

RESULTS

Milk Production and Composition

Dry matter intake and the productive responses of the cows within the experimental groups are depicted in **Table 3**. There was no significant difference in feed intake between groups during this study (21.77 vs. 22.15 kg for the control and RLC group, respectively). The mean milk production over the 28 DIM for the control and LC group was 46.5 and 47.7 kg, respectively. The mathematical model indicated that the rate of increase in the milk production of the animals in the RLC group, compared with the control, was maximum after 10–15 days post-calving (**Figure 1**). The milk component content and production of the cows over the 4-week study period are presented in **Table 3**. Overall, milk fat content showed a tendency to increase by 10% upon rumen-protected L-carnitine supplementation in the transition diets ($p = 0.1$). There was no group or group-by-day interaction effect on the content of milk protein or lactose concentration. However, there was a significant effect of group and day ($p < 0.05$) on milk protein yield (1.48 vs. 1.51 g/day for the control and RLC group, respectively). Both 4% fat-corrected milk and energy-corrected milk were significantly ($p < 0.05$) influenced by the experimental group and DIM. Cows in the RLC group had roughly 4 kg higher fat- and energy-corrected milk compared with those of the control group. Milk urea-N content did not show any significant differences between the experimental groups. Milk SCC clearly decreased ($p < 0.05$) in the cows fed the transition diet supplemented with the rumen-protected L-carnitine.

Table 4 depicts the effect of the experimental groups on milk fatty acid profiles. In the current study, cows in both groups had generally similar milk fatty acid concentrations. Nevertheless, cows in the RLC group had significant lower concentrations of C14:1 cis-9, C15:0, C16:1 cis-9, and C18:2 and higher C18:1 trans-9, trans-11 content in comparison with the control ($p < 0.05$).

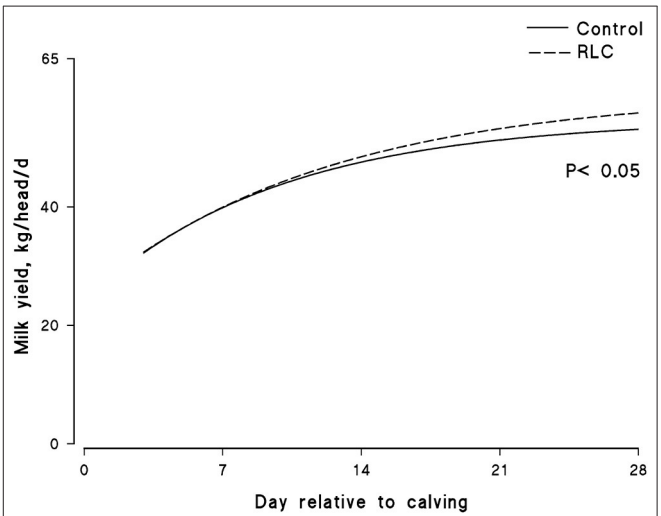

FIGURE 1 | Effects of supplementing multiparous Holstein cows during the transition period with rumen-protected L-carnitine (Carneon 20 Rumin-pro, Kaesler Nutrition GmbH, Cuxhaven, Germany) on lactation curve of early-lactating Holstein dairy cows. A gamma-type function model (Y = a*EXP –cd) was generated in order to describe the relation between daily milk production (Y) and time (d), while (c) is the slope in Holstein dairy cows during the transition period. The group fed rumen-protected L-carnitine depicted as RLC.

Blood Metabolites

Pre- and post-partum blood serum metabolites are presented in **Table 5**. Results of the current study showed that the effect of

TABLE 4 | Milk fatty acid profile of lactating Holstein dairy fed a post-partum diet plus fat prill containing 85% palmitic acid (control group) or plus Carneon 20 Rumin-pro (RLC group).

Fatty acids	Experimental groups		SEM	*p*-value
	Control	RLC		
C4:0	6.62	7.55	1.016	ns
C6:0	3.52	4.40	0.642	ns
C8:0	1.83	2.18	0.327	ns
C10:0	3.07	3.33	0.484	ns
C12:0	2.75	2.53	0.273	ns
C14:0	7.70	7.07	0.384	ns
C14:1 *cis-9*	1.22	1.07	0.076	*
C15:0	0.85	0.70	0.043	*
15:1	0.20	0.26	0.027	ns
C16:0	23.64	23.75	0.685	ns
C16:1 *cis-9*	2.68	2.35	0.122	*
C17:0	0.65	0.66	0.031	ns
C17:1	0.32	0.33	0.027	ns
C18:0	12.38	12.09	0.637	ns
C18:1 *trans-9, trans-11*	0.94	1.40	0.133	*
C18:1 *cis-9*	24.92	24.67	1.324	ns
C18:2	0.90	0.60	0.138	*
C18:2 *cis-9*	4.38	3.59	0.487	ns
C20:0	0.70	0.73	0.151	ns
C18:3n-3	0.45	0.48	0.059	ns
C20: >1; n_3,n_6	0.31	0.24	0.042	ns
Saturated fatty acids	63.70	65.01	1.878	ns
Monounsaturated fatty acids	30.28	30.08	1.565	ns
Polyunsaturated fatty acids	6.02	4.90	0.542	ns
C18:2 cis-9/C15	27.34	26.01	2.21	ns

*$p < 0.05$; ns: $p > 0.05$.

the experimental group on cholesterol (mg/dL), HDL (mg/dL), LDL (mg/dL), haptoglobin (mg/dL), SGPT (U/l), SGOT (U/l), and NEFA (mmol/l) was significant ($p < 0.05$). Animals in the RLC group had a lower blood cholesterol concentration compared with those of the control group (99.4 and 82.6 mg/dl for the control and RLC groups, respectively). Pre- and post-partum dietary inclusion of the rumen-protected L-carnitine source decreased both LDL and HDL concentrations in peripheral blood of post-partum cows in the present study. The concentrations of cholesterol, HDL, and LDL decreased at 7 days before calving and eventually increased from 2 weeks after calving. In the present experiment, rumen-protected L-carnitine supplementation hardly influenced the albumin concentration during the transition period ($p > 0.05$). The serum albumin level in cows of both experimental groups increased by 8% after calving, whereas the urea concentration in the animals hardly changed compared with the pre-partum period. Results from this work showed that cows in the RLC group had significantly ($p < 0.05$) lower blood haptoglobin concentrations at 7 and 14 days after calving than the control group (22.8 and 11.1 mg/dl for the control and RLC groups, respectively). Both serum glucose and calcium concentrations declined as parturition approached and started to increase from 21 days post-calving. There was no significant effect ($p > 0.05$) of the experimental group or group and day interactions on blood calcium or glucose concentration. Data regarding the concentration of blood triglycerides indicated that there were no significant differences between the groups. The blood NEFA concentration was evidently decreased in cows supplemented with rumen-protected L-carnitine during the periparturient period ($p < 0.05$). Animals in the control group had a 20% higher NEFA concentration compared with those levels in the RLC group. Nevertheless, dietary rumen-protected L-carnitine hardly changed the BHB level in animals in comparison with control ($p > 0.05$). Generally, both blood NEFA and BHB concentrations were increased before calving (50 and 30% for NEFA and BHB, respectively) and decreased from 14 DIM onward. Circulating concentrations of both blood enzymes, i.e., SGPT and SGOT, increased from 1 week before calving. Moreover, animals in the RLC group had lower concentrations of both enzymes than the control group ($p < 0.05$).

mRNA Expression

Effects of the dietary inclusion of rumen-protected L-carnitine on mRNA abundance of CD14, TLR4, and MD2 of early lactating Holstein dairy cows is shown in **Figures 2A–C**, respectively. Results of the current work indicated that supplementing the transition diet with rumen-protected L-carnitine hardly showed any significant impact on the mRNA abundance of TLR4, CD14, and MD2 ($p > 0.05$).

Animal Behavior

The experimental group effect on BCS of the animals throughout the study is presented in **Figure 3**. L-Carnitine supplementation did not evidently affect the BCS of the animals. The initial BCSs of animals in both groups were similar; however, it decreased with the increase in days post-calving. The data of manure score and rumen fill score are shown in **Figures 4A,B**, respectively. The manure score was unaffected by the experimental groups. The rumen fill score was significantly ($p < 0.05$) influenced by the experimental group and days relative to calving. Animals in the RLC group had a higher rumen fill score in comparison with the control. The rumination activity for each experimental group is presented in **Figure 5**. There was a significant difference ($p < 0.05$) among the cows allocated in the experimental groups, in which animals in the RLC group demonstrated 11% higher rumination time compared with their counterparts in the control group.

DISCUSSION

Results from the present experiment underline the ability of L-carnitine, in a protected form, to support the production responses, enhancing the liver metabolism and modulating the health biomarkers of high-producing dairy cows during the periparturient period. This study was able to show significant changes in particular cow behavior indices, which can be used for identifying cows with risk of illness (46). Taken together, data generated from this experiment

TABLE 5 | Concentration of blood metabolites in Holstein dairy cows during the transition period fed diets plus fat prill (control group) or Carneon 20 Rumin-Pro (RLC group).

Items	Experimental groups										SEM	*p*-value		
	Control					RLC						Group	Time	Group*Time
	−14 days	−7 days	+7 days	+14 days	+21 days	−14 days	−7 days	+7 days	+14 days	+21 days				
Glucose (mg/dL)	56.8	52.1	48.8	42.2	50.7	54.1	49.4	47.2	45.7	49.2	0.77	ns	*	ns
Urea (mg/dL)	38.0	35.3	40.5	39.8	43.2	39.0	32.0	40.5	40.2	39.7	0.62	ns	ns	ns
Albumin (g/dL)	3.94	3.95	3.92	4.00	4.14	3.80	3.81	3.82	3.82	3.99	0.04	ns	*	ns
Cholesterol (mg/dL)	104	102	77.6	89.9	125	92.3	79.0	65.8	76.6	99.6	2.40	*	*	ns
HDL (mg/dL)	81.8	82.4	68.7	73.7	90.6	74.5	67.1	57.5	61.0	78.5	1.57	*	*	ns
LDL (mg/dL)	12.6	9.60	7.60	11.5	15.9	9.80	8.30	5.50	8.60	12.7	0.50	*	*	ns
Triglyceride (mg/dL)	25.1	27.9	12.0	10.4	12.3	27.2	27.0	12.7	10.2	8.2	1.11	ns	*	ns
Haptoglobin (mg/dL)	-	-	19.5	26.1	-	-	-	10.9	11.3	-	2.10	*	ns	ns
Calcium (mg/dL)	8.75	8.69	7.98	8.67	9.27	8.56	8.43	7.90	8.49	9.11	0.15	ns	*	ns
SGPT (U/l)	18.5	16.8	16.6	17.1	20.1	14.9	13.4	12.5	14.9	18.5	0.56	*	*	ns
SGOT (U/L)	75.3	80.5	103	103	107	79.2	72.9	90.6	99.8	105	2.50	*	*	ns
NEFA (mmol/L)	-	0.43	1.15	1.05	0.75	-	0.33	0.83	0.89	0.77	0.05	*	*	ns
BHBA (mmol/L)	-	0.53	0.67	1.01	0.65	-	0.53	0.77	0.85	0.75	0.04	ns	*	ns

*d: day relative to calving; *p < 0.05; ns: p > 0.05; when the difference between means is >2 times the SEM., it is considered as significant (p < 0.05).*

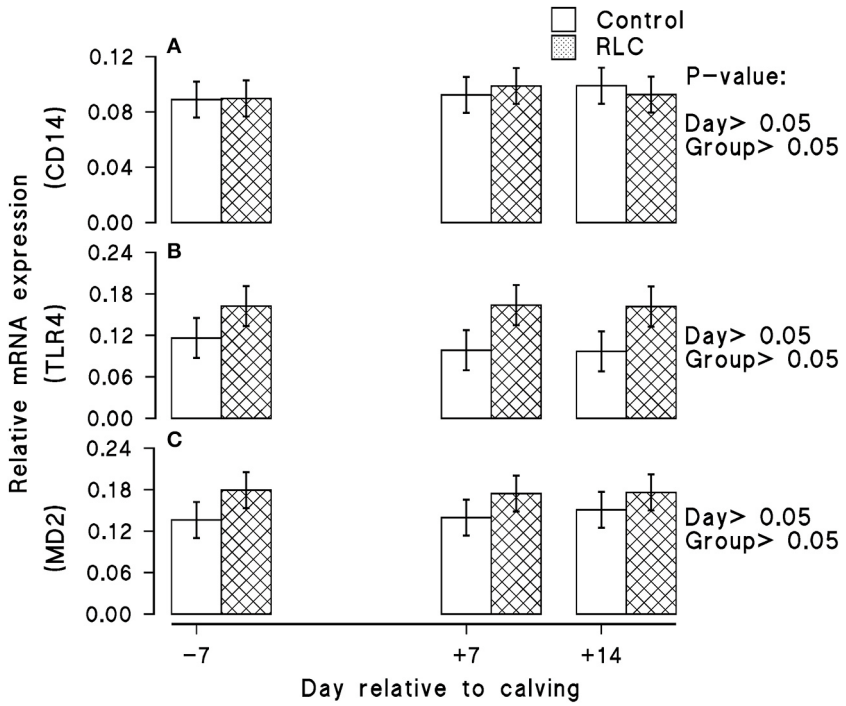

FIGURE 2 | Effects of supplementing multiparous Holstein cows during the transition period with rumen-protected L-carnitine (RLC; Carneon 20 Rumin-Pro, Kaesler Nutrition GmbH, Cuxhaven, Germany) on the relative mRNA expression of CD14 **(A)**, TLR4 **(B)**, and MD2 **(C)** genes. Values are means, with standard errors of means ($n = 10$/group) represented by vertical bars. The group fed rumen-protected L-carnitine depicted as RLC.

could help develop more detailed feeding management, i.e., including particular feed additives, to ensure dairy cattle to overcome the transition from parturition to lactation with less noticeable damage to their entire lactation performance and longevity.

Productive Performance of the Animals

Our results showed that the animals in the RLC group had better productive responses. It has been demonstrated that energy intake during early lactation is insufficient to meet the animal needs for milk synthesis. Indeed, cows very often

enter into a negative energy balance where their successful lactation would be effectively disturbed (47). Therefore, dairy cattle during the transition from gestation to lactation requires substantial nutrients to shift their situation (48). Previous studies claimed that increasing the energy availability of the transition diet through L-carnitine supplementation may have some benefits (26, 49), allowing the animals to adapt and decrease fatty acid mobilization from adipose tissue, eventually being less prone to lipid-related metabolic disorders (50). Hence, following parturition, this resulted in cows showing a better milk production and composition responses as those observed in the present work. Moreover, the findings of the current study suggest that upon dietary inclusion of an L-carnitine source, resisting ruminal degradation, to both pre- and post-partum diets, cows produce more milk components. It seems that a more favorable metabolic situation by including rumen-protected L-carnitine in the transition diet may decrease the negative physiological situation of this period (48). Therefore, effects of the rumen-protected L-carnitine supplementation to the transition diets cause an increase to the uptake of energy and nutrients for milk yield components. The outcome from our investigation indicated an obvious lower milk SCC in dairy cattle fed with L-carnitine. These results are not in line with previous studies, in which dietary supplementation of L-carnitine did not show any significant changes in SCC parameter (26). Scholz et al. (51) merely observed numerical lower SCC in dairy cows fed rumen-protected L-carnitine in comparison with control. Regular SCC observation has been globally recognized as an optimal index for measuring inter-mammary infection and milk quality (52). This parameter along with udder health monitoring programs has been quite advantageous on individual cows as well as the entire herd (53). Current data indicated that cows receiving rumen-protected L-carnitine, i.e., increasing the post-ruminal L-carnitine availability, could be less prone to develop mammary disorders in early lactation period. This is quite important for the productivity and longevity of the animals in the subsequent lactation.

Data from our experiment indicated that dietary supplementation with rumen-protected L-carnitine was able to merely regulate few milk fatty acid concentrations during the early lactation period. In dairy cows, the short- and medium-chain fatty acids (C4:0–C14:0) are synthesized in the mammary gland. Overall, short- and medium-chain fatty acids were numerically higher in animals in the RLC group. The only difference between the C14:1 cis-9 concentrations was significant. Buitenhuis et al. (54) evaluated the effect of microbiome on milk fatty acid composition and reported the heritability and microbiability for each trait. They showed that, in general, the heritability was relatively high for all milk fatty acids [ranging from 0.69 (C14:1 cis-9) to 0.11 (C18:1 trans-11; C18:1 cis-9)]. Therefore, the difference in C14:1 cis-9 obtained in the present study might be explained by the different rumen microbiomes. During the negative energy balance period, by increasing the energy demand for milk production, body fat is mobilized and transported as NEFA to several organs, particularly to the liver (15, 55). Excessive amounts of NEFA [particularly rich in long-chain fatty acids, e.g., C18:1 cis-9 and C18:0 (56)], which is released during body fat mobilization, have a potential to transfer to the milk, resulting in their elevated concentrations in the milk fat. It has been proposed

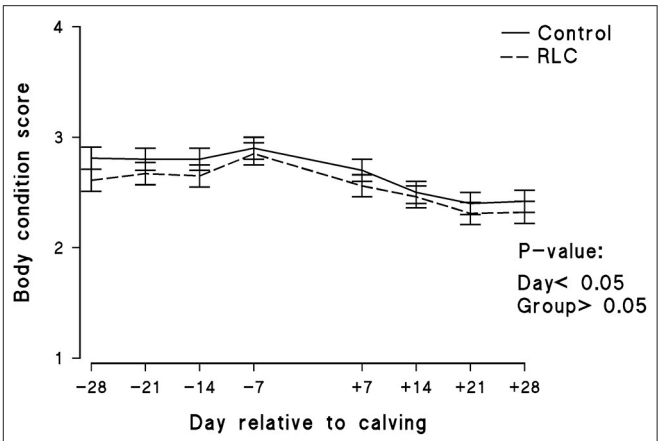

FIGURE 3 | Effects of supplementing multiparous Holstein cows during the transition period with rumen-protected L-carnitine (Carneon 20 Rumin-Pro, Kaesler Nutrition GmbH, Cuxhaven, Germany) on the body condition score of the animals. Values are means, with standard errors of means represented ($n = 16$/group) by vertical bars. The group fed rumen-protected L-carnitine depicted as RLC.

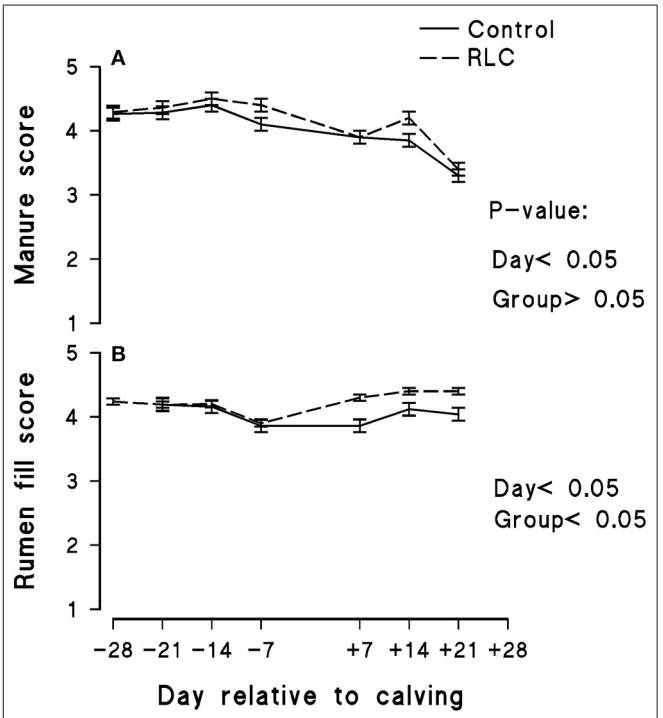

FIGURE 4 | Effects of supplementing multiparous Holstein cows during the transition period with rumen-protected L-carnitine (Carneon 20 Rumin-Pro, Kaesler Nutrition GmbH, Cuxhaven, Germany) on manure score **(A)** and rumen fill score **(B)**. Values are means, with standard errors of means ($n = 16$/group) represented by vertical bars. The group fed rumen-protected L-carnitine depicted as RLC.

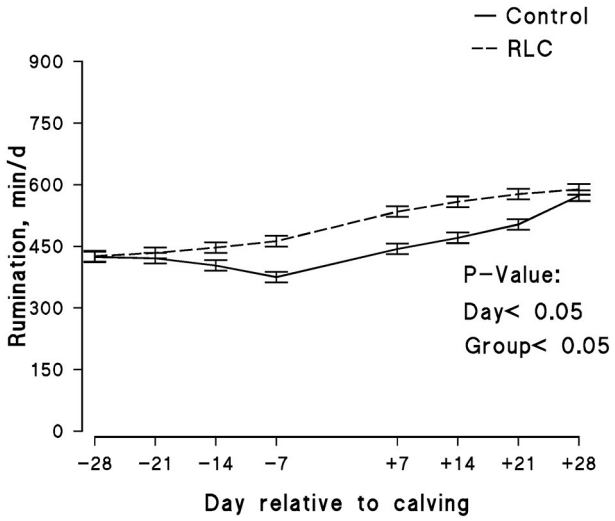

FIGURE 5 | Effects of supplementing multiparous Holstein cows during the transition period with rumen-protected L-carnitine (Carneon 20 Rumin-Pro, Kaesler Nutrition GmbH, Cuxhaven, Germany) on rumination activity. Values are means, with standard errors of means ($n = 16$/group) represented by vertical bars. The group fed rumen-protected L-carnitine depicted as RLC.

that these fatty acids in milk fat were identified as valuable early warning biomarkers for health status during transition period (57). Jorjong et al. (58) assessed the potential of milk fatty acids as biomarkers to predict the health status of the early-lactating dairy cows. The authors claimed that the milk fat C18:1 cis-9/C15:0 ratio may be a useful factor for the diagnosis of hyperketonemia in early-lactating dairy cows. Therefore, based on the data reported by Jorjong et al. (58), the ratio of C18:1 cis-9/C15 between 34 and 45 seems to be a valuable threshold in the early-lactating dairy cows. In the current study, the ratio of C18:1 cis-9/C15:0 for each experimental group was below 40 (**Table 4**). This shows that the animals enrolled in the current study were not generally in a critical health status.

In the current study, we used similar fat concentrations (with the same sources) in the experimental diets. We even used very similar concentrations of palmitic acid in the diet. Dietary fat enhances the supply of fatty acids to the mammary gland, which results in a lower proportion of *de novo* synthesized, saturated short- and medium-chain fatty acids in milk fat and a higher proportion of long-chain fatty acids (59). On the other hand, any differences in the supply of fermentable carbohydrate lead to altered production of acetate in the rumen, as a precursor of mammary *de novo* fatty acid production. Therefore, it has been proposed that fatty acids with <16 C originated from *de novo* synthesis and those >16 C were preformed fatty acids taken up by the mammary gland, and 16:0 and 16:1 fatty acids come from both *de novo* and preformed sources (59, 60). However, Dewhurst et al. (61) showed that actual milk yields of C15:0 and C17:0 exceeded the duodenal flow of these fatty acids. They suggested that there is a possibility of some *de novo* synthesis within animal tissue or transfer of these fatty acids (mobilized from adipose tissue) to the mammary gland. All of the above information clearly stated that when we used similar dietary carbohydrates (i.e., similar fermentation pattern) and fat supplements, as well as when observing similar BCS (i.e., same fat mobilization), it should be expected to observe relatively similar milk fatty acid profiles between the experimental groups.

Metabolic and Health State of the Animals

Data from the current study revealed that dietary inclusion of L-carnitine resulted in lower circulating concentrations of cholesterol, HDL, and LDL. Typically, the cholesterol concentration in dairy cows declines close to parturition and starts to gradually increase post-parturition (62), following the pattern of changes in animals' feed intake during this period (63). Previous studies have pinpointed higher risk of post-partum diseases associated with higher cholesterol concentrations pre-partum (62). The total cholesterol level in the blood has been also attributed to the changes in serum lipoprotein concentrations during lactation (64). Therefore, lower concentrations of LDL and HDL in dairy cows in the RLC group were expected. Stefanska et al. (37) reported higher concentrations of HDL in non-healthy cows compared with the healthy group (78.16 vs. 68.32 mg/dl). They proposed that higher concentrations of blood HDL in non-healthy cows may be a protective mechanism against endotoxemia. Our data regarding the blood HDL concentration confirmed those findings. More recently, Jukema et al. (65) indicated that disorders in lipid metabolism initiate an inflammatory and immune-mediated response, in which the concentration of blood LDL was increased. Hence, they suggested that blood LDL cholesterol has strong potential to induce inflammation in animals. The lower blood LDL concentration in cows fed the rumen-protected L-carnitine may explain a much better health status in these animals as observed in the current experiment.

In the present work, we have determined the circulating concentrations of two acute phase proteins (APP), i.e., albumin and haptoglobin. The liver is vital for an optimal immune response as it will redirect the priority from metabolism to defense during an incidence of inflammation in animals. This change is known as APP response, which depicts the reduced synthesis of necessary proteins for normal liver metabolism, e.g., albumin, retinol-binding protein, paraoxonase, and increased synthesis of proteins, which are involved in immune and detoxification response (66). Positive APP play an important role in pathogen elimination, removal of toxic substances, and maintenance of a balanced inflammatory response (67). Haptoglobins are among the positive APP, in which their blood levels increase as a result of an inflammation stage in animals (68). Stefanska et al. (37) reported a lower blood concentration of haptoglobin in healthy vs. acidotic cows (470.19 vs. 516.85 ng/ml). A previous experiment revealed that elevated concentrations of blood haptoglobin from 2 to 8 days post-partum were associated with enhanced innate immune responses (69). These findings provide evidence that the blood haptoglobin concentration is associated with both systemic inflammatory responses and liver inflammation. Post-partum blood concentrations of haptoglobin >1.1 g/l were associated with a 947-kg decrease in 305-day mature equivalent milk yield

(70). Inflammatory response during the periparturient period has been characterized with an increase in the production of positive APP and a concomitant decrease in the production of negative APP, e.g., albumin (71). The lower serum haptoglobin concentration in cows in the RLC group may indicate a better health status of the animals. This in part could be the reason that animals in this group produce higher fat- and energy-corrected milk during 4 weeks after calving. Nevertheless, we did not observe any significant difference in the serum albumin level between the groups. Others also did not find a clear difference in blood albumin concentration of animals treated with anti-inflammatory drugs from −7 until +35 days relative to calving, while haptoglobin levels were clearly decreased upon treatment (72).

Although the blood concentrations of triglyceride and BHB did not significantly differ between the groups, dietary L-carnitine supplementation showed an evident effect to decrease the circulatory NEFA concentration. In dairy cows when there is an energy deficiency, a mobilization of body fat reserves occurs and, thus, the concentration of NEFA increases in blood (21). On the other hand, an efficient utilization of NEFA depends on an adequate L-carnitine availability for fatty acid transfer into the mitochondrial matrix as the site of their oxidation (73). Insufficient L-carnitine availability at times of an increased energy requirement, such as early lactating status, could alter the liver metabolism of lipids. Carlson et al. (74) demonstrated that carnitine modulates nutrient metabolism in dairy cows. They indicated that carnitine supplementation of 50 and 100 g/day had more potent effects on lipid metabolism as a result of enhanced capacity for hepatic long-chain fatty acid β-oxidation. In their experiment, the marked increases in hepatic carnitine concentrations confirm that exogenous carnitine is readily taken up by the liver. This idea has also been previously demonstrated in mid-lactation dairy cows (73). Therefore, an insufficient L-carnitine supply to the liver was proposed as a limiting factor for fatty acid metabolism (75). Besides, it has been shown in dairy cows that many LPS-induced metabolic challenges are related to the energy metabolism, in which L-carnitine is involved (76). The authors showed that an intravenous LPS injection followed by an increase in blood tumor necrosis factor-α was accompanied with a rise in blood NEFA concentration.

Metabolic alterations in the liver during the transition period are one of the key points in dairy cow performance (21). As seen in our results, an evident decline of the liver enzymes during the periparturient period in rumen-protected L-carnitine-supplemented animals seems to improve their productive responses in the subsequent lactation. Recently, a number of blood metabolites were used to monitor clinical or subclinical signs of metabolic disorders in high-yielding cows around parturition (1). The blood elevation of SGPT and SGOT may indicate an accumulation of NEFA transported from blood to hepatocytes (77). Higher concentrations of blood SGOT can also imply damages in organs other than the liver, as SGOT exists in the muscle, kidney, intestine, and brain. West (78) proposed that a rise in blood SGOT shortly after calving might indicate a muscle damage in the animals. Therefore, metabolic alterations in liver function may be an important point in early lactating cows. Olagaray et al. (49) reported that fatty liver is a metabolic disease that occurs during the first few weeks of lactation and affects up to 50% of dairy cows. Higher incidence of fatty liver accompanies with a decline in the concentration of free carnitine. Dietary administration of L-carnitine during the transition period was effective at increasing hepatic carnitine concentrations, with a subsequent decrease in total liver lipid content (79). This will in turn help the liver to reduce the hepatocyte damages, moderate its metabolic function, and enhance the health status and productive performance of the animal.

mRNA Abundance of TLR4, CD14, and MD2

Results from the current work indicated that the expression of genes involved in bacterial LPS recognition was not evidently regulated upon L-carnitine supplementation. During states of inflammation and inflammatory disease, the expression of TLR4 and associated signaling proteins could increase and facilitate receptor-mediated endocytosis of LPS (80, 81). Results of the current study are not in agreement with previous works, where higher transcription levels of TLR4 and CD14 were noticed in early-lactating dairy cow with puerperal diseases (82, 83). Interaction of TLR4/CD14/MD-2 with the bacterial endotoxin triggers the expression of inflammatory biomarkers such as cytokines, antimicrobial peptides, and chemokines. A plausible reason for the current outcome would be that animals during the course of this experiment were not suffering from clinical inflammatory diseases associated with the periparturient period.

Behavioral Parameters of the Animals

We did not observe any evident differences of manure scores, yet the rumen fill scores were significantly affected by the addition of L-carnitine (**Figures 4A,B**). The rumen fill score is related to the DMI, especially the proportion of fiber in the diet (43). Kawashima et al. (84) showed that the rumen fill score is also associated with energy status in dairy cows. They concluded that the rumen fill score during the transition period might indicate the real-time feed intake based on its correlation with serum total cholesterol levels. Consequently, it might be used as a practical indicator to describe the metabolic health status in dairy cows. In the present study, all cows had very good rumen fill scores during the pre- and post-partum periods. Nevertheless, addition of rumen-protected L-carnitine in transition diets was able to enhance this parameter in the animals.

It has been previously reported that early-lactation cows would produce much more milk per unit of dry matter consumed and per minute of rumination resultant from that feed consumed (85). In ruminants, rumination is a natural behavior and mostly influenced by the physically effective fiber (86), which would increase the surface area of the feed particle. In addition, rumination stimulates saliva production to help buffer the rumen and facilitates the passage of dry matter from the rumen to the intestine. Therefore, as the passage rate increases, DMI increases in dairy cows (85), eventually improving the productive responses of the animals. The time that cows spend chewing might be a valuable management tool for detecting health problems and optimizing the herd heath status (40), as monitoring rumination time is easier than monitoring

feed intake. Kaufman et al. (85) monitored the relationship between health status of dairy cows with rumination time, milk yield, milk fat, and protein content. They concluded that rumination time was positively associated with health status and milk yield in early-lactation dairy cows across all parities. It was concluded that healthy animals had better productive responses compared with unhealthy cows across all lactations. Besides, early-lactating cows are more challenging in the view of metabolic stress and are more susceptible to health disorders that cause significant production losses. Stangaferro et al. (87) evaluate the rumination time to score the health of cows from 21 days before expected calving until 80 days post-partum. The rumination time of cows was lower in clinical diagnosis, depending on the disorder, compared with the healthy cows. Taken together, higher rumination time in cows of the RLC group in the present work would be an indicator of the better health situation of the animals.

CONCLUSION

Altogether, results of the present experiment provide evidence that dietary inclusion of rumen-protected L-carnitine during the transition period could improve the productivity of high-producing dairy cows early post-partum. This can be to some extent explained by the ability of L-carnitine to modulate metabolic indicators as indexed in the energy metabolism and liver functionality. This experiment proves that eating and ruminating of transition dairy cows will be positively affected by the dietary inclusion of L-carnitine. Although the overall health of the animals enrolled in the current study was relatively good, addition of L-carnitine seems to even enhance the health status of the animals. However, further research is warranted to gain a deeper understanding on the impact of pre- and post-partum dietary L-carnitine on various pro- and anti-inflammatory as well as oxidative status biomarkers in dairy cows. Another compelling idea would be to investigate the *in-utero* effect of feeding L-carnitine during the periparturient period, thereby determining the potent impact of this natural molecule on the offspring performance and health.

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 author.

ETHICS STATEMENT

The animal study was reviewed and approved by Institutional Animal Care Committee, Ferdowsi University of Mashhad.

AUTHOR CONTRIBUTIONS

MDM, HK, and SDM conceived and designed the study and wrote the paper with a critical review by all authors. HK conducted the experiment. AJ conducted the qPCR analysis. All authors reviewed and approved the final manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fvets.2021.769837/full#supplementary-material

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Resveratrol Ameliorates Intestinal Damage Challenged With Deoxynivalenol Through Mitophagy *in vitro* and *in vivo*

Yujian Huang[1,2†], *Changbing Zheng*[1†], *Bo Song*[1], *Li Wang*[1], *Hao Xiao*[1*] and *Zongyong Jiang*[1]

[1] State Key Laboratory of Livestock and Poultry Breeding, Ministry of Agriculture Key Laboratory of Animal Nutrition and Feed Science in South China, Guangdong Public Laboratory of Animal Breeding and Nutrition, Guangdong Provincial Key Laboratory of Animal Breeding and Nutrition, Maoming Branch, Guangdong Laboratory for Lingnan Modern Agriculture, Institute of Animal Science, Guangdong Academy of Agricultural Sciences, Guangzhou, China, [2] Guangdong Provincial Key Laboratory of Animal Nutrition Control, Institute of Subtropical Animal Nutrition and Feed, College of Animal Science, South China Agricultural University, Guangzhou, China

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Tarique Hussain,
Nuclear Institute for Agriculture and Biology, Pakistan

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Elsayed Metwally,
Suez Canal University, Egypt
Muhammad Saleem Kalhoro,
Sindh Agriculture University, Pakistan

***Correspondence:**
Hao Xiao
xiaohao@gdaas.cn

[†] These authors have contributed equally to this work

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Deoxynivalenol (DON) reduces growth performance and damage intestinal function, and resveratrol (RES) has positive effects on growth performance and intestinal function. The purpose of this study was to investigate the protective mechanism of RES *in vitro* and *vivo* challenged with DON. The results showed that dietary supplementation with DON significantly increase the mRNA expression levels of mitophagy- related genes, and protein level for PINK1, Parkin, Beclin-1, Lamp, Atg5, Map1lc, Bnip3, Fundc1, Bcl2l1 and SQSTMS1 ($P < 0.05$), while supplementation with both RES and DON decreased those indexes in the ileum. Besides DON significantly decreased protein level for Pyruvate Dehydrogenase, Cytochrome c, MFN1, OPA1, and PHB1 ($P < 0.05$), while supplementation with both RES and DON increased protein level for PHB1, SDHA, and VDAC in the ileum. Moreover, *in vitro*, we found that DON significantly decreased mitochondrial respiration ($P < 0.05$), while RES + DON increased the rate of spare respiratory capacity. Also, DON significantly decreased total NAD and ATP ($P < 0.05$), while RES + DON increased the total NAD and ATP. These results indicate that RES may ameliorates the intestinal damage challenged with deoxynivalenol through mitophagy in weaning piglets.

Keywords: piglets, resveratrol, deoxynivalenol, mitophagy, intestinal function

INTRODUCTION

Deoxynivalenol (DON) or vomiting toxin, is the most common trichothecenes toxin produced by Fusarium, which mainly contaminates cereal crops (1). DON could reduce animal feed intake, vomiting, fever, diarrhea, and anorexia, even death. However, different animals have different sensitivity to DON. Pigs are the most sensitive animals among monogastric animals and ruminants (2–5). Recent study has shown that DON could reduce the growth performance of pigs, affect immune system, antioxidant system, cell signal transduction, gene expression and protein synthesis of livestock (6). Our previous studies have found that DON decreased the growth performance

weaned piglets, destroyed intestinal function and structural integrity, weakened antioxidant capacity and protein synthesis levels of weaning piglets (2, 3). The mechanism by which DON exerts its toxicological effects is through binding to the phthaloyltransferase on the 60 s subunit of eukaryotic ribosomes, causing MAPK phosphorylation, inducing inflammatory process in the organism, and leading to lipid peroxidation damage in cell membranes, which in turn inhibition of protein and genetic material synthesis. However, the traditional chemical detoxification of DON pollution is unable to meet the low-energy, high-efficiency, and green environmental protection in modern farming. In recent years, the control of piglet stress damage through nutrition has received ever greater attention. Therefore, it is one of the hot spots in animal nutrition research to effectively mitigate DON damage to piglets through nutritional regulation to improve piglet growth performance.

Studies have shown that the addition of arginine, glutamic acid and antibacterial peptides to feed can effectively mitigate the toxic effect of DON on the intestinal damage in weaned piglets (4). RES is a bioactive material, naturally occurring polyphenolic plant antitoxin with anti-inflammatory, anti-aging, anti-cancer and cardioprotective properties. Found mainly in wine, blueberries, peanuts and other nuts, it is a potential additive to mitigate the toxic effects of DON (7). RES was shown to improve mitochondrial respiratory metabolism and lipid oxidation through Sirt1, and to target mitochondria to ameliorate stress damage (8). Mitochondria are the main target organelles for oxidative damage. Excessive ROS produced by damaged mitochondria will activate proteins such as p53 and Caspase to initiate apoptosis. Effective identification and removal of damaged mitochondria from the cells is therefore essential to ameliorate stress damage. Study shows RES attenuates the oxidative damage via mitochondrial autophagy in Parkinson's patients (9), and mitigate mitochondrial damage, and improve the intestinal function in diquat-challenge piglets (10). Thus, RES could mitigate organismal damage through mitochondrial autophagy, however, its mitigating effect on DON-induced intestinal damage are not well understood.

Therefore, in this study, by using DON-induced model *in vitro* and *vivo*, we want to know the protective regulatory role of resveratrol on intestinal function challenged with DON. This study provides a theoretical basis for the nutritional regulation of early weaned piglets.

MATERIALS AND METHODS

Animals and Diets

All animal procedures used in the present study were approved by the Animal Care and Use Committee of Guangdong Academy of Agricultural Sciences and followed the Guidelines for the Care and Use of Animals for Research and Teaching. A total of 64 weaned piglets [Duroc × (Landrace × Yorkshire), 21 days old, barrow] with an initial weaning weight of 6.97 ± 0.10 kg were randomly allocated to four dietary treatments. The piglets fed a basal diet were considered the control group (CON), and the other groups were fed the basal diet supplemented with 300 mg RES/kg diet (RES), 3.8 mg DON/kg diet (DON) or 3.8 mg DON plus 300 mg RES per kg diet (DON+RES group) for a 28-days feeding trial. RES (> 99.0%) was obtained commercially from Shaanxi Ciyuan Biotechnology Co., Ltd. (Xian, China). Each treatment consisted of eight replicate pens, with two piglets per pen (n = 16 piglets per treatment). The basal diet was formulated to meet the nutrient recommendations of the National Research Council (NRC) 2012.

Sample Collection and Processing

At the end of the experiment, eight piglets from each group were anesthetized and bled, the abdominal cavity was quickly dissected and the viscera removed. The intestinal segments were ligated and 1 cm sections of intestine were taken from the middle of the whole jejunum and ileum respectively, washed with pre-cooled PBS, collected in centrifuge tubes and snap frozen in liquid nitrogen, then stored at −80°C for further study.

Cell Culture and Treatment

The cell culture refers to our previous study (11). High-glucose (25 mM) Dulbecco's modified Eagle's (DMEM-H), fetal bovine serum (FBS), and antibiotics were procured from Invitrogen (Grand Island, NY, USA). Plastic culture plates were manufactured by Corning Inc. (Corning, NY, USA). Unless indicated, all other chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA). IPEC-J2 cells were seeded and cultured with DMEM-H medium containing 10% FBS, 5mM l-glutamine, 100 U/mL penicillin, and 100 μg/ml streptomycin at 37°C in a 5% CO_2 incubator. After an overnight incubation, the cells were changed to culture in 15 μmol/L RES for 24 h and then exposed to 0.5 μmol/L DON for another 24 h. Cells were treated or collected for the analysis of extracellular flux, and GC-MS.

Real-Time PCR

The protocol of total RNA extraction, quantification, cDNA synthesis and real-time PCR was adapted from the method of (12). Briefly, total RNA was isolated from intestinal samples by using the Trizol method. Real time PCR was carried out by using forward and reverse primers (**Supplementary Table 1**) to amplify the target genes. For quantification, amplification efficiencies curves were constructed from serial 1:2 dilutions, and the $2^{-\Delta\Delta CT}$ method was used to calculate the mRNA expression of the target genes relative to housekeeping gene (β-actin).

Western Blotting Analysis

Frozen intestinal samples were collected as described by Tan et al. (13). Protein concentrations of tissue homogenates were measured by using the BCA method and bovine serum albumin as standard. All samples were adjusted to an equal concentration (50 μg protein). The western blotting was conducted based on previous description. The primary antibodies are LC-3B (1: 1,000; Cell Signaling Technology), P62 (1: 1,000; Cell Signaling Technology), Parkin (1: 1,000;

Cell Signaling Technology), BNIP3/Nix (1: 1,000; Cell Signaling Technology), BNIP3 (1: 1,000; Cell Signaling Technology), Pyruvate Dehydrogen (1: 1,000; Cell Signaling Technology), COX IV(1: 1,000; Cell Signaling Technology), Cytochrome c(1: 1,000; Cell Signaling Technology), HSP 60(1: 1,000; Cell Signaling Technology), Mitofusin 1(1: 1,000; Cell Signaling Technology), Mitofusin 2(1: 1,000; Cell Signaling Technology), OPA1(1: 1,000; Cell Signaling Technology), PHB1(1: 1,000; Cell Signaling Technology), SDHA(1: 1,000; Cell Signaling Technology), SOD1(1: 1,000; Cell Signaling Technology), TOM20(1: 1,000; Cell Signaling Technology), VDAC(1: 1,000;

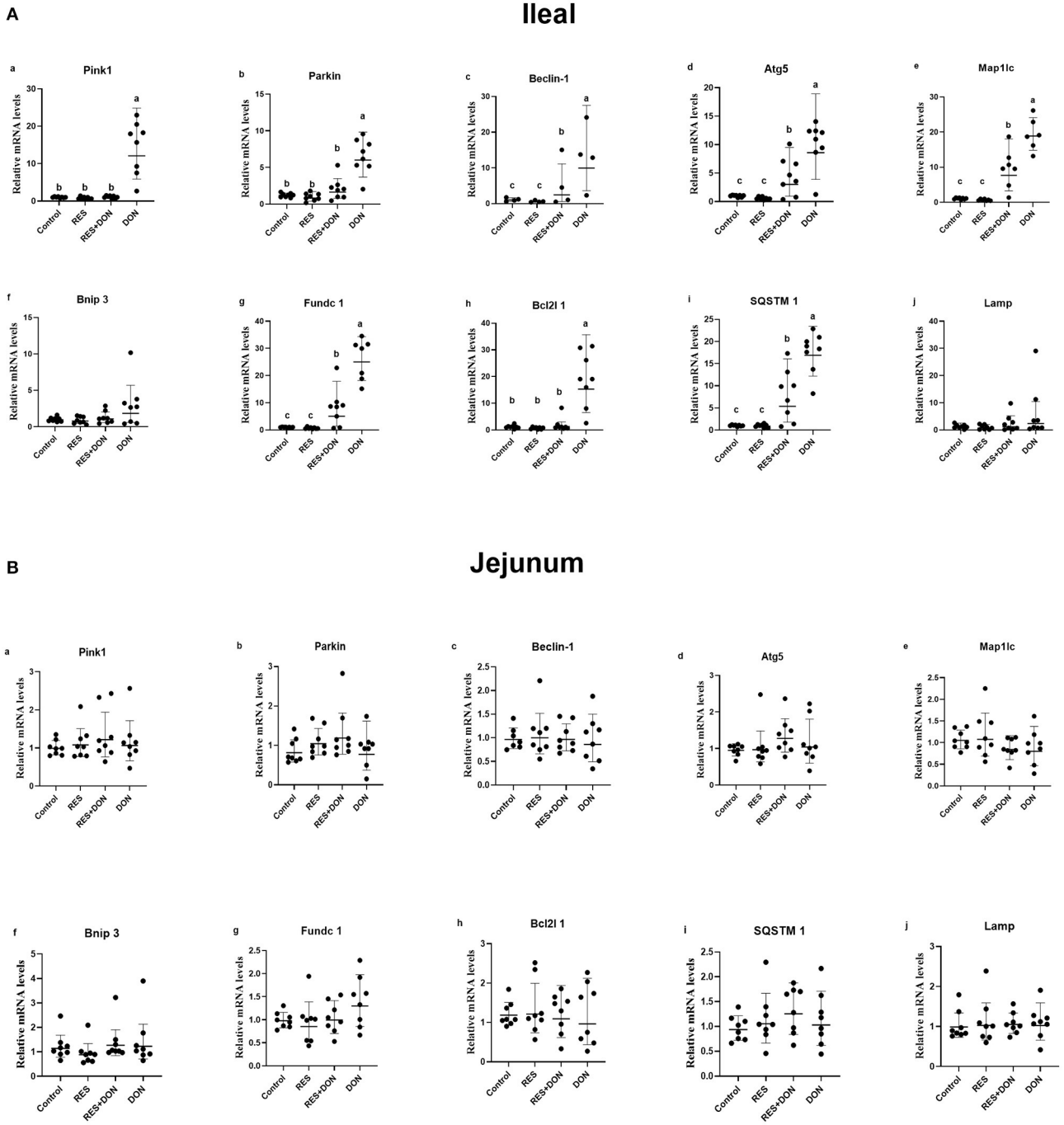

FIGURE 1 | Dietary supplementation with RES alleviated the negative effects on mRNA expression levels of mitophagy-related genes challenged with DON. Data were expressed as means ± SEM of at least three independent experiments. **(A,B)** Values with different letters are significantly different ($P < 0.05$).

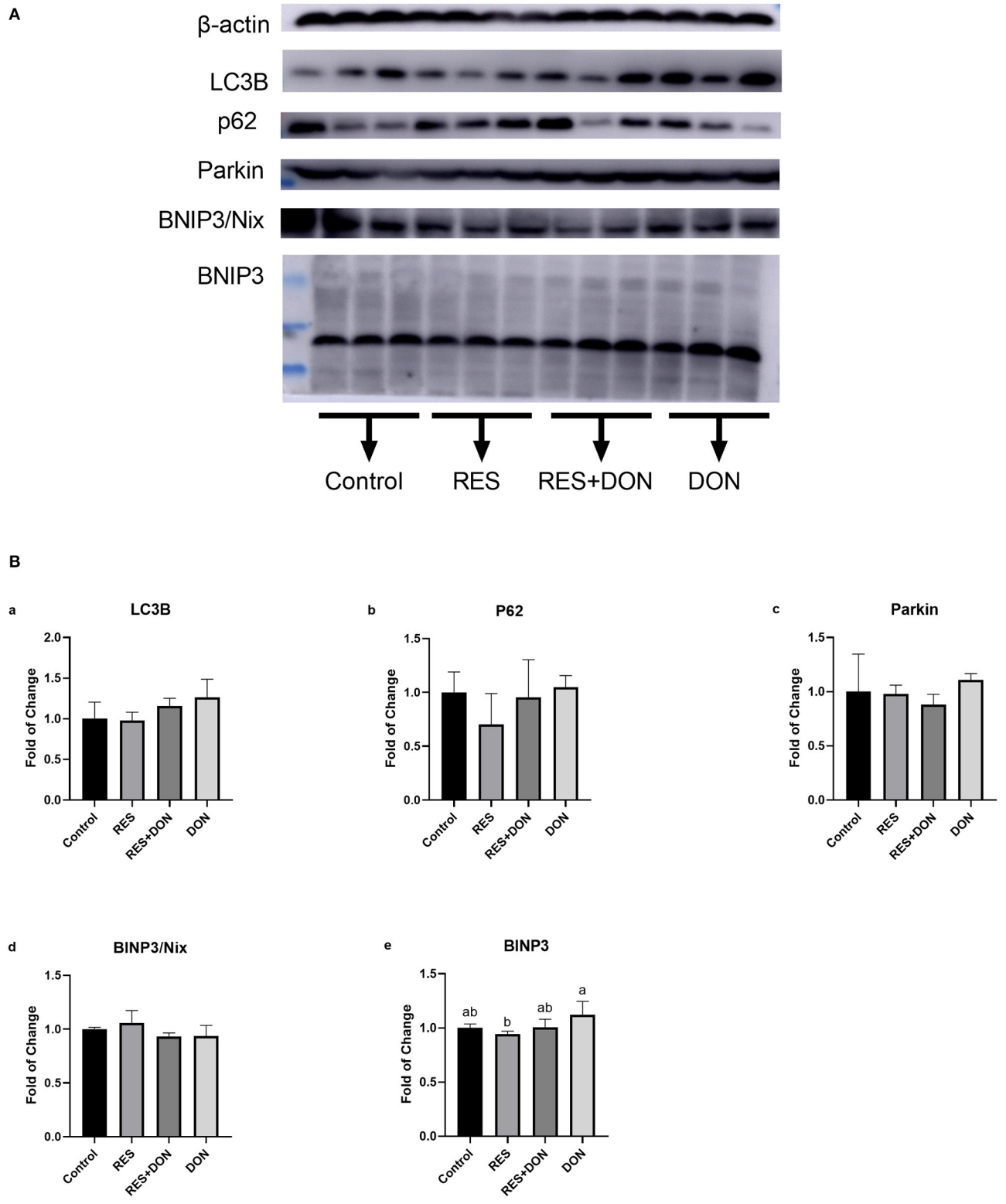

FIGURE 2 | Dietary supplementation with RES increases the expression of mitophagy-related genes. Cells were treated with 0 (NC) or 0.5 μmol/L DON and 0 or 15 μM RES, respectively. Data were expressed as means ± SEM of at least three independent experiments. **(A,B)** Values with different letters are significantly different ($P < 0.05$).

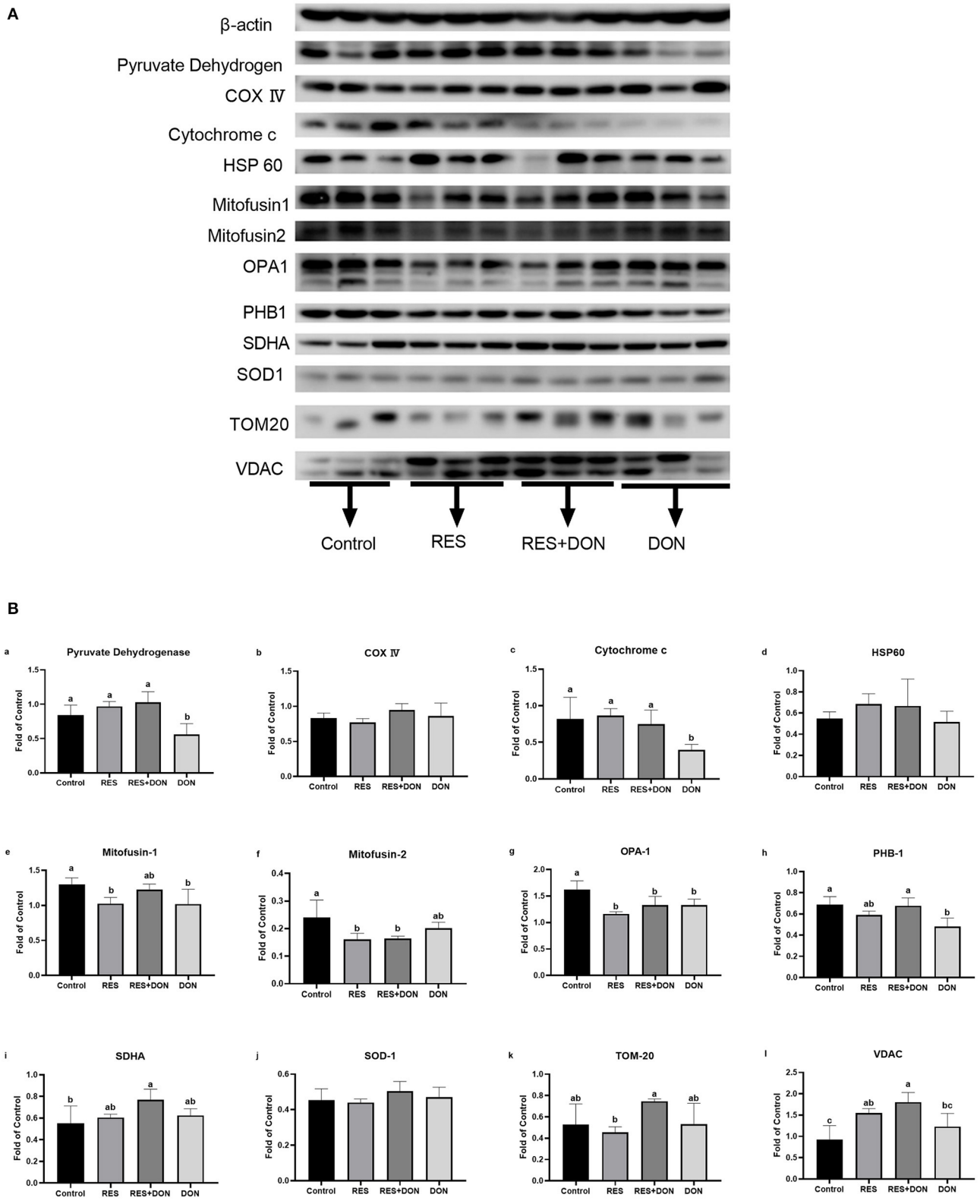

FIGURE 3 | Dietary supplementation with RES affects the expression of mitochondrial related genes. Cells were treated with 0 (NC) or 0.5 μmol/L DON and 0 or 15 μM RES, respectively. Data were expressed as means ± SEM of at least three independent experiments. **(A,B)** Values with different letters are significantly different ($P < 0.05$).

Cell Signaling Technology) or β-actin(1: 1,000; Cell Signaling Technology). All protein measurements were normalized to β-actin.

Extracellular Flux Assays

The XF-24 Extracellular Flux Analyzer and Cell Mito Stress Test Kit from Seahorse Biosciences were used to examine the effects of addition of different treated with 0 (NC) or 0.5 μmol/L or 1 μmol/L DON and 0 or 15 μM RES, respectively on mitochondrial respiration in IPEC-J2 cells. Cells in four replicates per group. Owing to the effects of DON on IPEC-J2 cell proliferation, total cellular protein was determined and used to normalize mitochondrial respiration rates.

Gene Knockout With CRISPR-Cas9

Thanks to Yulong Yin lab for providing ATG5 plasmid (13). Guide RNAs were designed using the online CRISPR design tool (http://crispr.mit.edu/) and then cloned into the BbsI-digested plasmids (pSpCas9n) containing the entire guide RNA scaffold. The genomic region flanking the ATG5 target site was amplified using polymerase chain reaction (PCR). The products underwent a reannealing process to facilitate heteroduplex formation. After re-annealing, the products were treated with T7 Endonuclease I (NEB) following the manufacturer's recommended protocol. Then used lentiviral transfection, the viral solution was added to the cell culture medium and co-incubated with the cells.

Statistical Analysis

Results are expressed as Mean ± SEM. The statistical analysis was performed by one-way ANOVA using SPSS 17.0 (SPSS Inc., Chicago, IL, USA). Probability values < 0.05 and <0.01 were considered statistically significant. *P*-values were calculated using a two-tailed paired Student's *t*-test.

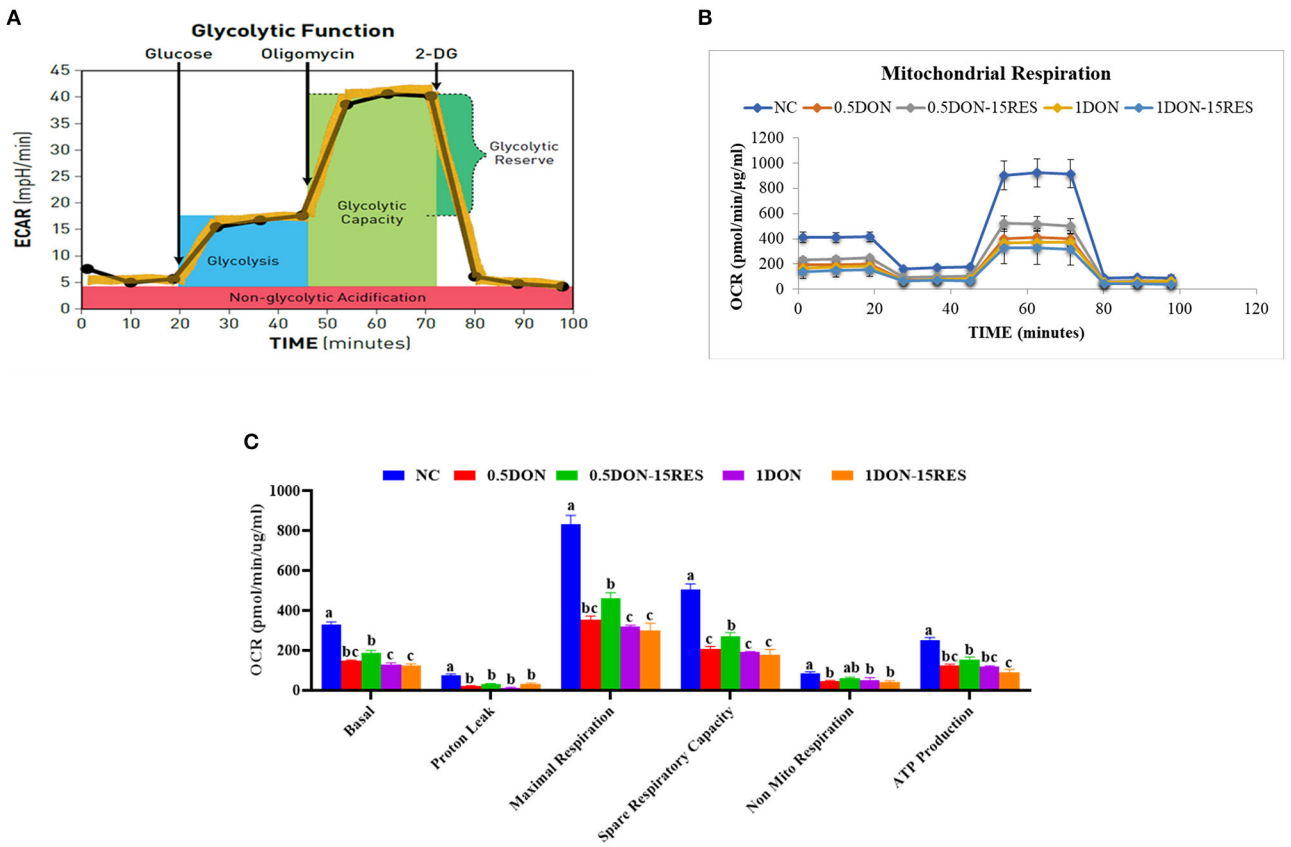

FIGURE 4 | RES improved the negative effect on mitochondrial respiration by DON *in vitro*. **(A)** Schematic and **(B)** oxygen consumption rate (OCR) assessed by extracellular flux analysis. OCR was measured under basal conditions followed by the sequential addition of oligomycin (0.5 μM), FCCP (1 μM), rotenone (1 μM), or antimycin A (1 μM). Each data point represents an OCR measurement. **(C)** Individual parameters for basal respiration, proton leak, maximal respiration, spare respiratory capacity, nonmitochondrial respiration, and ATP production were determined. Cells were treated with 0 (NC) or 0.5μmol/L or 1μmol/L DON and 0 or 15μM RES, respectively. Data were expressed as means ± SEM of at least three independent experiments. **(A–C)** Values with different letters are significantly different ($P < 0.05$).

RESULTS

Dietary Supplementation With RES Alleviated the Negative Effects on mRNA Expression Levels of Mitophagy-Related Genes Challenged With DON

To determine the molecular mechanism of RES on DON-fed piglets, we analyzed the mRNA expressions of mitophagy-related genes in the ileum and jejunum of weaning piglets (**Figure 1**). Dietary supplementation with DON increased ($P < 0.05$) the mRNA expressions of PINK1, Parkin, Beclin-1, Lamp, Atg5, Map1lc, Bnip3, Fundc1, Bcl2l1 and SQSTM1 in the ileum, while there were no differences ($P > 0.05$) in those indexes among the Control, RES, and RES+ DON treatments in the ileum. However, there were no differences ($P > 0.05$) in the mRNA expressions of PINK1, Parkin, Beclin-1, Lamp, Atg5, Map1lc, Bnip3, Fundc1, Bcl2l1 and SQSTM1 expressions among the four treatments in the jejunum.

Dietary Supplementation With RES Increases the Expression of Mitophagy-Related Genes

Since we know that there are significant differences in mitophagy-related genes in the ileum, and then we analyzed the protein expressions of autophagy genes in ileum. The relative expression levels of LC3B, p62, Parkin, Binp3/Nix, and BINP3 are shown in **Figure 2**. Dietary supplementation with 3.8 mg DON/kg diet (DON) increased ($P < 0.05$) protein levels for BNIP3, while there were no differences ($P > 0.05$) in those indexes among the Control, RES, and RES+ DON treatments in the ileum.

Dietary Supplementation With RES Affects the Expression of Mitochondrial Related Genes

In order to know whether the mitochondrial is related to this study, we analyzed the protein expressions of mitochondrial related genes in ileum. The relative expression levels of C54G1, COX IV, Cyt c, HSP60, Mitofusin1, Mitofusin2, OPA1, PHB1, SDHA, SOD1, TOM20, and VDAC are shown in **Figure 3**. Dietary supplementation with 3.8 mg DON/kg diet (DON) decreased ($P < 0.05$) protein levels for Pyruvate Dehydrogenase, Cytochrome c, MFN1, OPA1, and PHB1 ($P < 0.05$), while supplementation with 300 mg RES increased ($P < 0.05$) protein levels for PHB1, SDHA, and VDAC. Supplementation with RES decreased ($P < 0.05$) protein levels for MFN2 and OPA1, compared with control group.

RES Improved the Negative Effect on Mitochondrial Respiration by DON *in vitro*

Our results have shown that RES ameliorates the damage challenged with DON through mitophagy, then we want to find out whether RES and DON affect the mitochondrial respiration *in vitro*. We found that supplementation with 0.5 μmol/L or 1 μmol/L DON gradually decreased ($P < 0.05$) individual parameters for basal respiration, proton leak, maximal respiration, spare respiratory capacity, non-mitochondrial respiration, and ATP production in cells. While supplementation with 15 μM RES elevated the rate of spare respiratory capacity in 0.5 μmol/L DON-treated cells ($P < 0.05$) but not normal cells (**Figure 4**).

The results of total NAD and ATP in IPEC-J2 cells are shown in **Figure 5**. supplementation with 0.5 μmol/L DON and 15 μM RES increased ($P < 0.05$) for total NAD, while supplementation with DON or RES alone decreased ($P < 0.05$) for ATP. However, addition of both 0.5 μmol/L DON and 20 μM RES increased ($P < 0.05$) the content of ATP.

RES Did Not Relieve Injury on Mitochondrial Respiration Caused by DON When Knockout Atg5

The results of mitochondrial respiration in IPEC-J2 cells are shown in **Figure 6**. Supplementation with 0.5 μmol/L and 1 μmol/L DON gradually decreased ($P < 0.05$) individual parameters for basal respiration, proton leak, maximal respiration, spare respiratory capacity, non-mitochondrial respiration, and ATP production in cells. While supplementation

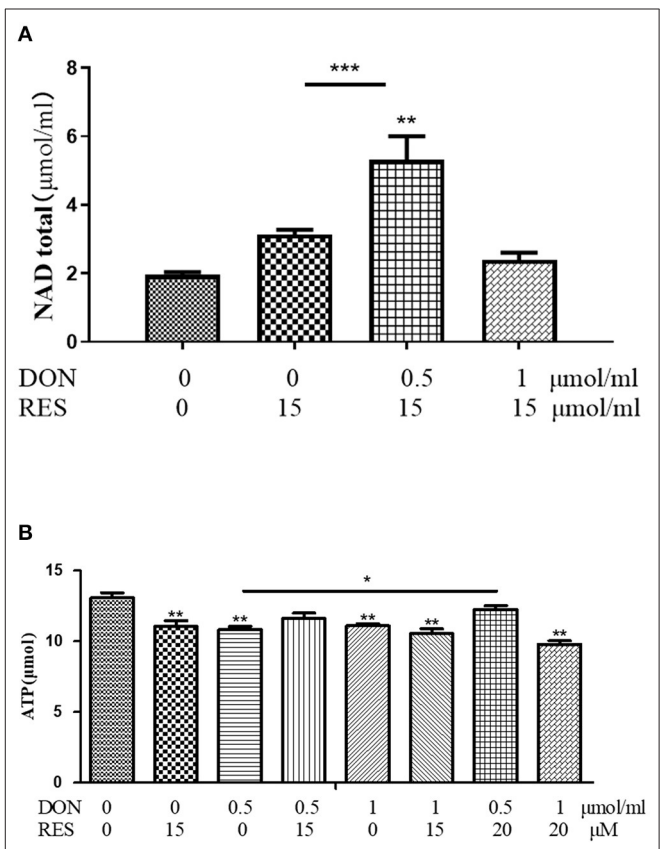

FIGURE 5 | (A,B) Effect of DON and RES on Total NAD and ATP. Cells were treated with 0 (NC) or 0.5 μmol/L or 1 μmol/L DON and 0 or 15 μM or 20 μM RES, respectively. Data were expressed as means ± SEM of at least three independent experiments. *Values with different letters are significantly different ($P < 0.05$), *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

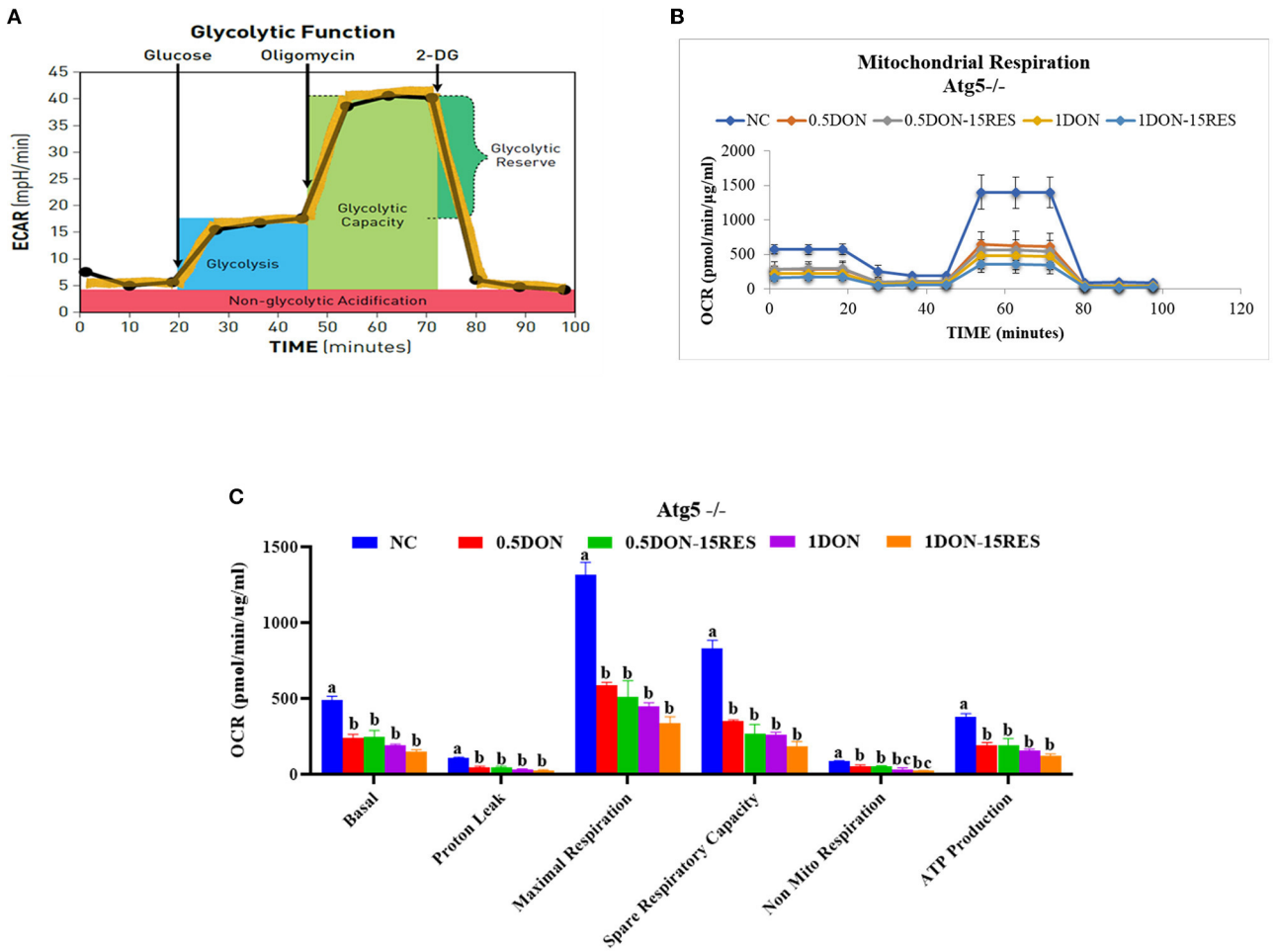

FIGURE 6 | RES did not relieve injury on mitochondrial respiration caused by DON when knockout Atg5. **(A)** Schematic and **(B)** oxygen consumption rate (OCR) assessed by extracellular flux analysis. OCR was measured under basal conditions followed by the sequential addition of oligomycin (0.5 μM), FCCP (1 μM), rotenone (1 μM), or antimycin A (1 μM). Each data point represents an OCR measurement. **(C)** Individual parameters for basal respiration, proton leak, maximal respiration, spare respiratory capacity, nonmitochondrial respiration, and ATP production were determined. Cells were treated with 0 (NC) or 0.5 μmol/L or 1 μmol/L DON and 0 or 15 μM RES, respectively. Data were expressed as means ± SEM of at least three independent experiments. **(A–C)** Values with different letters are significantly different ($P < 0.05$).

with 15 μM RES there is no significant difference between the mitochondrial respiration.

DISCUSSION

DON is a common source of grain pollution, and has a negative impact on intestinal function and reduce growth performance for animals (14). Supplementation with 1 mg kg/DON damaged the intestinal morphology and impaired intestinal mucosa and permeability, accompanied by an inflammation response (15). 0.5 μg/ml DON cultured for 6 h in IPEC-J2 cells induced oxidative stress, inflammation and apoptosis. It has been reported that RES, as an effective antioxidant, can significantly increase cellular antioxidant enzyme activity, reduce intracellular ROS content, and decrease oxidative stress in intestinal epithelial cells, indicating that RES can be used as an effective feed additive to prevent intestinal damage in livestock production (16). Consistent with their research, the results of our study suggests that RES could improve the intestinal damage by DON. It shows RES plays an important role in protecting animal health.

RES regulates biological functions, such as anti-oxidative stress, anti-inflammatory, and antibacterial through molecular regulatory mechanisms (SIRT1, Nrf2, and NF-κB, etc). Moreover, RES is effective in preventing diseases like cardiovascular disease, diabetes, neurodegeneration, and cancer (17). In addition, RES can be used as an alternative to antibacterial feed additives to regulate piglets' intestinal flora, enhance antioxidant capacity in piglets' serum, reduce oxidative stress on piglets at weaning, and significantly improve growth performance of weaned

piglets (18, 19). Recent study has shown that RES could be able to mitigate diquat-induced intestinal oxidative stress in piglets through mitochondrial autophagy (10). Interestingly, RES also enhances the transcription of BNIP3, a mitochondrial autophagy-related gene, through HIF1α and AMPK, thereby maintaining mitochondrial homeostasis and alleviating high-fat-induced endothelial function impairment (20).

VDAC is a class of pore-protein ion channels located in the outer mitochondrial membrane and plays a key role in regulating metabolism and energy fluxes across the outer mitochondrial membrane: it is involved in the transport of ATP, ADP, pyruvate, malate and other metabolites (21). VDAC expression was decreased in the DON group in our experiments, and there was an imbalance in the functions regulating mitochondrial outer membrane metabolism and energy. This situation was alleviated by the addition of RES. Cytochrome c plays a role in the electron transport chain and apoptosis, and also acts as an antioxidant enzyme in mitochondria to remove superoxide and hydrogen peroxide from mitochondria (22). Our results are consistent with previous studies, in that the expression of Cytochrome c protein was significantly reduced in the DON group, suggesting that the addition of DON caused oxidative damage to the cells. The damage was alleviated by the addition of RES. PHB1 has an important role in mitochondrial function and morphology and promotes cell proliferation in mice (23, 24). Consistent with the results in this experiment, PHB1 protein expression was decreased and cell proliferation was impaired in the DON group, and the addition of RES was associated with a recovery in PHB1 expression. All these proteins are closely associated with autophagy, and because the previous RT-PCR results showed that RES alleviates intestinal damage in relation to autophagy genes, we further validated the role of RES by testing the levels of these proteins. To sum up, our results have suggested that RES might alleviate DON-induced intestinal damage by improving mitochondrial autophagy.

Cellular respiration results in the conversion of nutrients into, for example, ATP, and then the release of a series of metabolic reaction products. In eukaryotic cells, mitochondria are important organelles for cellular respiration and are involved in the process of aerobic respiration. The nutrients protein, fat and carbohydrates in aerobic respiration are degraded by pyruvate to enter the tricarboxylic acid cycle to produce energy. In this paper, we examined the changes in the oxygen consumption rate of cells under RES treatment by Seahorse and found that 15 μM RES elevated the rate of spare respiratory capacity in 0.5 μmol/L DON-treated cells ($P < 0.05$) but not normal cells, however there is no significant change in basal respiration, proton leak, maximal respiration, non-mitochondrial respiration. When the autophagy-related gene ATG-5 was knocked out, there were no significant changes in the indicators of mitochondrial respiratory metabolism. In this experiment, the total intracellular ATP and NAD were also tested, and it was found that the addition of 0.5 μmol/L DON and 20 μM RES significantly increase for ATP, while addition of DON or RES alone significantly decrease. This indicates that RES could relieve DON-induced mitochondrial damage through mitophagy.

CONCLUSIONS

RES alleviated the negative effects on mRNA and protein expression levels of mitophagy-related genes challenged with DON in piglets, elevated the rate of spare respiratory capacity, increased for ATP, and improved DON-induced mitochondrial damage *in vitro*. In conclusion, we have suggested that resveratrol would ameliorate the intestinal damage challenged with deoxynivalenol may through mitophagy in weaning piglets.

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 the Animal Care and Use Committee of Guangdong Academy of Agricultural Sciences and followed the Guidelines for the Care and Use of Animals for Research and Teaching. Written informed consent was obtained from the owners for the participation of their animals in this study.

AUTHOR CONTRIBUTIONS

YH and HX performed experiments, analyzed data, and wrote the manuscript. LW wrote and edited the manuscript. HX supervised the project, developed the study concept, and wrote and edited the manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fvets.2021.807301/full#supplementary-material

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frontiers in Veterinary Science

ORIGINAL RESEARCH
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Effects of Different Supplemental Levels of *Eucommia ulmoides* Leaf Extract in the Diet on Carcass Traits and Lipid Metabolism in Growing–Finishing Pigs

Yuhuan Yang[1], Fengna Li[2,3], Qiuping Guo[2], Wenlong Wang[2], Lingyu Zhang[2,3], Yunju Yin[1], Saiming Gong[1], Mengmeng Han[2,3] and Yulong Yin[2]*

[1] College of Animal Science and Technology, Hunan Agricultural University, Changsha, China, [2] Hunan Provincial Key Laboratory of Animal Nutritional Physiology and Metabolic Process, National Engineering Laboratory for Pollution Control and Waste Utilization in Livestock and Poultry Production, Key Laboratory of Agro-Ecological Processes in Subtropical Region, Hunan Provincial Engineering Research Center for Healthy Livestock and Poultry Production, Scientific Observing and Experimental Station of Animal Nutrition and Feed Science in South-Central, Ministry of Agriculture, Institute of Subtropical Agriculture, Chinese Academy of Sciences, Changsha, China, [3] College of Advanced Agricultural Sciences, University of Chinese Academy of Sciences, Beijing, China

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***Correspondence:**
Fengna Li
lifengna@isa.ac.cn

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This study examined the effects of dietary *Eucommia ulmoides* leaf extract (ELE) supplements on carcass traits and lipid metabolism in growing–finishing pigs. A total of 144 crossbred (Duroc × Landrace × Yorkshire) piglets with an average initial weight of 10.11 ± 0.03 kg were randomly allotted to four treatment groups, each with six replicates and six piglets per replicate. Each group of pigs was fed a basal diet or a diet supplemented with increasing levels of ELE (0.1, 0.2, or 0.3%). The results showed that adding ELE had no negative effect on the growth performance of pigs. Dietary supplements of 0.1% ELE significantly increased carcass weight ($p < 0.01$), dressing percentage ($p < 0.01$), carcass length ($p < 0.05$), and eye muscle area ($p < 0.05$). Compared with the control group, a 0.2% ELE supplement significantly increased ($p < 0.01$) the levels of adiponectin, insulin-like growth factor 1, and hormone-sensitive lipase and lipoprotein lipase activity in the serum. Histological examination showed that ELE inhibited fat deposition in the backfat tissue. Lipid metabolism-related biochemical indices and mRNA expression levels were improved after supplementing diets with ELE. Moreover, all three levels of ELE dramatically upregulated ($p < 0.05$) the protein levels of p-AMPK-α and p-ACC. In summary, adding ELE to pig diets could improve the carcass traits of growing–finishing pigs and exert a lipid-lowering effect by activating the AMPK-ACC pathway and regulating mRNA expression levels related to lipid metabolism. Supplementing the diet with 0.1–0.2% ELE is the optimal range to reduce fat deposition in pig backfat tissue.

Keywords: *Eucommia ulmoides* leaf extract, DLY growing-finishing pigs, growth performance, carcass trait, lipid metabolism, AMPK-ACC signal pathway

INTRODUCTION

Obesity is becoming one of the most important health problems in several countries, affecting scores of people, as it increases the risk of various diseases, such as fatty liver, diabetes, and coronary heart disease (1). In recent years, an antiobesity effect of *Eucommia ulmoides* has been supported by an increasing number of studies. Two prevenient studies have reported that *Eucommia ulmoides* improved hyperglycemia in diabetic rats (2) and type 2 diabetes patients (3). Moreover, *Eucommia ulmoides* promoted the recovery of lipid metabolism disorders caused by a high-fat diet in rats (4).

Eucommia ulmoides (Chinese: Duzhong), also known as Gutta-percha tree, Sixian, and Sizhong, is a perennial deciduous tree of the Eucommiaceae family (5). *Eucommia ulmoides* is widely distributed in China, with a high annual yield (6). Its medicinal history can be traced back thousands of years and is now widely used in clinics (5). *Eucommia ulmoides* is rich in lignans, iridoid terpenoids, flavonoids, polysaccharides, and other active components, with antihypertensive, hypoglycemic, anti-inflammatory, liver protection, antitumor, and other pharmacological effects (5, 7, 8). Studies into the potential of *Eucommia ulmoides* as a feed supplement in Chinese herbal medicine have been gradually developed. Previous studies focused on the effects of *Eucommia ulmoides* leaf and its extracts on growth performance and antioxidant activity in pigs (9–11). However, at present, there are few studies on the effect of *Eucommia ulmoides* leaf extracts (ELE) on lipid metabolism in growing–finishing pigs, and the optimal supplement level is also unknown. Since there are many similarities between pigs and humans in terms of structure and function, the effect of ELE as a dietary supplement in pigs can be used as a model for the study of human nutrition and metabolism (12).

Following from previous research, we added dietary supplements of 0, 0.1, 0.2, or 0.3% ELE to growing–finishing pig diets and recorded the effects on growth performance, carcass traits, and lipid metabolism. This provides a basis for the wider application of ELE in animal husbandry and reducing the incidence of human obesity.

MATERIALS AND METHODS

Preparation of ELE

ELE were purchased from Zhangjiajie Hengxing Biotechnology Co., Ltd. (Zhangjiajie, China). Data provided by the company show that the main active ingredients include 5% chlorogenes, 8% EL flavonoids, and 20% EL polysaccharides.

Animals and Diets

The animal experiments were approved by the Committee on Animal Care of the Institute of Subtropical Agriculture, Chinese Academy of Sciences. A total of 144 crossbred barrows (Duroc × Landrace × Yorkshire, DLY, 10.11 ± 0.03 kg) were randomly divided into four treatments, six replicates in each treatment, and six pigs in each replicate. The experimental diets were as follows: (1) control diet; (2) control diet + 0.1% ELE; (3) control diet + 0.2% ELE; (4) control diet + 0.3% ELE. All the growing–finishing pigs were raised in pens and had *ad libitum* access to diets and clean drinking water. All pigs were weighed when the pigs in the control group weigh 10, 30, 70, and 115 kg, and feed intake was recorded every week to calculate the average daily gain (ADG), average daily feed intake (ADFI), and the ratio of feed to gain (F/G). The experiment used a corn–soybean meal diet referred to NRC (1998, 2012). The ingredients and nutritional composition of basal diet are shown in **Table 1**.

TABLE 1 | Ingredients and nutritional composition of basic diets.

Ingredients (%)	Dietary treatment		
	10–30 kg	30–70 kg	70–115 kg
Corn	63.70	58.60	67.00
Soybean meal	19.80	29.00	23.76
Dried whey	4.30	–	–
Wheat bran	–	7.80	6.00
Fish meal	9.00	–	–
Soybean oil	0.80	1.55	0.88
Lys	0.38	0.18	0.01
Met	0.10	0.00	0.00
Thr	0.09	0.01	0.00
Trp	0.01	0.00	0.00
$CaHPO_4$	0.00	0.69	0.50
Limestone	0.52	0.87	0.55
Salt	0.30	0.30	0.30
Premix[a]	1.00	1.00	1.00
Total	100.00	100.00	100.00
Nutrient content (%)			
DE[b] (MJ/kg)	14.60	14.20	14.20
CP	20.27	18.27	16.30
Total Lys	1.52	1.15	0.88
Total (Met + Cys)	0.79	0.61	0.55
Total Thr	0.94	0.77	0.68
Total Trp	0.26	0.25	0.21
Total Ca	0.69	0.60	0.52
Total P	0.57	0.51	0.45

[a] *Supplied per kg of diet (10–30 kg): vitamin A, 18,000 IU; vitamin D_3, 5,000 IU; vitamin E, 40 IU; vitamin K_3, 4 mg; vitamin B_1, 6 mg; vitamin B_2, 12 mg; vitamin B_6, 6 mg; vitamin B_{12}, 0.05 mg; biotin, 0.2 mg; folic acid, 2 mg; niacin, 50 mg; D-calcium pantothenate, 25 mg; Cu (as copper sulfate), 20 mg; Fe (as ferrous sulfate), 90 mg; Mn (as manganese oxide), 15 mg; Zn (as zinc oxide), 80 mg; I (as potassium iodide), 0.3 mg; and Se (as sodium selenite), 0.3 mg. Supplied per kg of diet (30–115 kg): vitamin A, 15,000 IU; vitamin D_3, 3,000 IU; vitamin E, 40 IU; vitamin K_3, 4 mg; vitamin B_1, 3 mg; vitamin B_2, 10 mg; vitamin B_6, 4 mg; vitamin B_{12}, 0.03 mg; biotin, 0.2 mg; folic acid, 2 mg; niacin, 35 mg; D-calcium pantothenate, 20 mg; Cu (as copper sulfate), 15 mg; Fe (as ferrous sulfate), 80 mg; Mn (as manganese oxide), 15 mg; Zn (as zinc oxide), 70 mg; I (as potassium iodide), 0.5 mg; and Se (as sodium selenite), 0.3 mg.*

[b] *Calculated value for DE.*

Sample Collection

At the end of the trial, all the pigs were fasted overnight (12 h), and one or two pigs of each replicate with average final body weight was selected (8 pigs/treatment) to slaughter by electrical stunning in a commercial abattoir. Before slaughter, blood samples were collected into a plain tube and placed at room temperature for 30 min, then centrifuged at 3,000

× g for 10 min at 4°C. Serum was collected and stored at −80°C for further analysis (13). The backfat samples were immediately excised and stored at −20°C for determination of the chemical composition or placed in liquid N_2 and then stored at −80°C for the analysis of quantitative real-time PCR. Fresh samples of backfat (1 cm^3) were fixed in paraformaldehyde fixative for paraffin sections and hematoxylin and eosin staining.

Carcass Trait Analysis

At slaughter, the carcass and the left side of carcass were weighted so that slaughter rate could be calculated. Other carcass traits including carcass length (carcass straight length and carcass slant length), average backfat thickness (the 3rd−4th lumbar spine, the 10th−11th lumbar spine, and the last rib), and loin–eye area were measured from the left side of the carcass. The left side of the carcass was split up into skeletal muscle and fat as previously described (14). The fat mass rate percentage and lean mass percentage were weighed and calculated.

Serum Biochemical Index Measurements

Total protein (TP), albumin (ALB), urea nitrogen (BUN), blood glucose (GLU), total cholesterol (TC), triglyceride (TG), high-density lipoprotein cholesterol (HDL-C), low-density lipoprotein cholesterol (LDL-C), and very low-density lipoprotein cholesterol (VLDL-C) in serum were measured with cobas C311 Analyzer (Roche Diagnostics, Basel, Switzerland) and commercial kits (Leadman Biotech Limited, Beijing, China) as specified by the manufacturer.

Measurement of Serum Cytokine Levels

The concentrations of leptin (LEP), adiponectin (ADPN), insulin (INS), and insulin-like growth factor 1 (IGF-1) were performed by using ELISA kits (Changsha Aoji Biotechnology Co., Ltd., Changsha, China).

Measurement of Serum Enzyme Activity

The activity of acetyl-coa carboxylase (ACC), hormone sensitive lipase (HSL), lipoprotein lipase (LPL), adipose triacylglyceride lipase (ATGL), and acyl CoA cholesterol acyltransferase (ACAT) in serum was detected by ELISA kits (Changsha Aoji Biotechnology Co., Ltd., Changsha, China).

Backfat Tissue Histological Analysis

The mean cross-sectional area and quantity of adipocyte in backfat tissue were measured by classic hematoxylin and eosin staining. Serial tissue sections of 4 μm were sliced using a paraffin slicer (RM 2016, Shanghai Leica Instrument Co., Ltd., Shanghai, China). The slices were dyed with hematoxylin dye solution for 3–5 min, washed with tap water and dehydrated with 85 and 95% gradient alcohol for 5 min, respectively, then dyed with eosin dye solution for 5 min, dehydrated with absolute ethanol, and finally sealed with neutral gum. The stained slides are scanned with a panoramic slice scanner of Pannoramic DESK/MIDI/250/1000 (3DHISTECH, Budapest, Hungary), the scanned slices are opened with CaseViewer 2.4 software (3DHISTECH, Hungary), the field of view is intercepted, and Image-Pro Plus 6.0 (Media Cybernetics, Rockville, MD, USA) is used for calculation and analysis.

Total RNA Isolation and Quantitative Real-Time PCR Analysis

Total RNA isolation and real-time quantitative PCR were conducted as previously described (15). In brief, total RNA was extracted from backfat tissue samples using TRIzol Reagent (Hunan Aikerui Bioengineering Co., Ltd., Changsha, China). The purity of the total RNA was verified using a NanoDrop ND2000 (NanoDrop Technologies Inc., Wilmington, DE, USA) at 260 and 280 nm, and the OD260/OD280 ratios of the RNA samples were all between 1.8 and 2.0. The total RNA was treated with DNase I (Hunan Aikerui Bioengineering Co., Ltd., Changsha, China) to remove DNA and reverse transcribed to complementary deoxyribonucleic acid (cDNA) using Evo M-MLV RT Kits with gDNA clean for qPCR (Hunan Aikerui Bioengineering Co., Ltd., Changsha, China) following the manufacturer's protocol. Quantitative real-time PCR was performed using an ABI 7900HT Real-Time PCR system (Applied Biosystems, Branchburg, NJ, USA) with SYBR Green Premix Pro Taq HS qPCR Kits (Hunan Aikerui Bioengineering Co., Ltd., Changsha, China). The PCR system consisted of 5 μl SYBR Green Pro Taq HS Premix, 2 μl cDNA, 2.2 μl RNase-free water, and 0.4 μl primer pairs (forward and reverse) in a total volume of 10 μl. The PCR protocols included one cycle at 95°C for 30 s, 40 cycles at 95°C for 5 s, and 60°C for 30 s. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as the endogenous control gene to normalize the expression of target genes according to the comparative Ct method as follows: $2^{-\Delta\Delta Ct}$ ($\Delta\Delta Ct = \Delta Ct_{\text{gene of interest}} - \Delta Ct_{\text{GAPDH}}$) (16). Primer sequences are shown in the **Table 2**.

Western Blot Analysis

An appropriate amount of backfat tissue sample was weighed and added to RIPA lysate for ice lysis, and then BCA protein assay kits (Beyotime Biotechnology, Shanghai, China) were used to measure the protein concentration. Next, SDS-PAGE electrophoresis was carried out. Firstly, the glass plate was cleaned, and then the glue with an appropriate concentration was prepared according to the protein concentration of the sample. The loading amount was calculated, and β-mercaptoethanol was added to the equal-volume buffer and 1/10-volume buffer, mixed well, put into the Mastercycler nexus PCR instrument (Eppendorf, Hamburg, Germany), and mixed well. After adding samples, electrophoresis was carried out, and then the membrane was transferred. After sealing the membrane, the primary antibody and secondary antibody were incubated for color development.

Statistical Analysis

All experimental data were analyzed using one-way analysis of variance (ANOVA) of SPSS (version 26.0, SPSS Inc., Chicago, IL, USA), and then the Duncan multiple-comparison test was performed. Results were expressed as mean and SEM, $p < 0.05$

TABLE 2 | Primers used for quantitative real-time PCR.

Genes[a]	Primers	Sequences (5′ to 3′)	Product size, bp
ACC	Forward	AGCAAGGTCGAGACCGAAAG	169
	Reverse	TAAGACCACCGGCGGATAGA	
FAS	Forward	CTACCTTGTGGATCACTGCATAG	114
	Reverse	GGCGTCTCCTCCAAGTTCTG	
SREBP-1c	Forward	GCGACGGTGCCTCTGGTAGT	218
	Reverse	CGCAAGACGGCGGATTTA	
HSL	Forward	CACAAGGGCTGCTTCTACGG	167
	Reverse	AAGCGGCCACTGGTGAAGAG	
LPL	Forward	CTCGTGCTCAGATGCCCTAC	148
	Reverse	GGCAGGGTGAAAGGGATGTT	
ATGL	Forward	TCACCAACACCAGCATCCA	95
	Reverse	GCACATCTCTCGAAGCACCA	
CPT1B	Forward	GACAAGTCCTTCACCCTCATCGC	170
	Reverse	GGGTTTGGTTTGCCCAGACAG	
PPAR γ	Forward	CCAGCATTTCCACTCCACACTA	124
	Reverse	GACACAGGCTCCACTTTGATG	
AMPK α	Forward	GCATAGTTGGGTGAGCCACA	105
	Reverse	CCTGCTTGATGCACACATGA	
FATP1	Forward	ACCACTCCTACCGCATGCAG	78
	Reverse	CCACGATGTTCCCTGCCGAGT	
FAT/CD36	Forward	CTGGTGCTGTCATTGGAGCAG	160
	Reverse	CTGTCTGTAAACTTCCGTGCCTGTT	
FABP4	Forward	CAGGAAAGTCAAGAGCACCA	227
	Reverse	TCGGGACAATACATCCAACA	
GAPDH	Forward	CAAAGTGGACATTGTCGCCATCA	123
	Reverse	AGCTTCCCATTCTCAGCCTTGACT	

[a]*ACC, acetyl CoA carboxylase; FAS, fatty acid synthase; HSL, hormone-sensitive lipase; LPL, lipoprotein lipase; ATGL, adipose triacylglyceride lipase; CPT1B, carnitine palmitoyl transferase 1B; FATP1, fatty acid transport protein 1; FAT/CD36, fatty acid translocase; FABP4, fatty acid-binding protein 4; SREBP1c, sterol regulatory element-binding protein-1c; PPAR γ, translocase peroxisome proliferator-activated receptor γ; AMPK α, adenosine monophosphate-activated protein kinase α.*

was considered significant, and $0.05 \leq p < 0.10$ was considered as trend.

RESULTS

Growth Performance

Table 3 shows that from 10 to 30 kg, there was no significant difference ($p > 0.05$) in ADG, ADFI, or F/G with increasing levels of ELE supplements. At the 10–70-kg stage, ADG was higher in the group supplemented with 0.1% ELE ($p < 0.05$) than in the other groups. F/G was lower in the 0.1% ELE group ($p < 0.05$) than in the other groups, but there was no dramatic discrepancy ($p > 0.05$) compared with the control group. Adding 0.2 or 0.3% ELE to the diet could markedly improve ADFI ($p < 0.05$). Over the whole period of the experiment, ADFI was higher ($p < 0.05$) in the 0.1% ELE group than in the 0.3% group, but there were no observable change in ADG or F/G among the different treatments.

TABLE 3 | Growth performance of growing and growing–finishing pigs fed the diets with various levels of ELE.

Item[1]	ELE[2] levels, %				SEM	*p-value*
	0	0.1	0.2	0.3		
10–30 kg						
Initial weight, kg	10.08	10.11	10.12	10.11	0.03	0.76
Final weight, kg	29.23	28.76	29.18	28.77	0.27	0.46
ADG, kg·day^{-1}	0.54	0.54	0.55	0.54	0.01	0.83
ADFI, kg·day^{-1}	0.90	0.93	0.93	0.91	0.01	0.07
F/G	1.68	1.72	1.71	1.71	0.02	0.37
10–70 kg						
Final weight, kg	69.65	71.60	68.24	68.83	1.13	0.20
ADG, kg·day^{-1}	0.63^{ab}	0.65^{a}	0.62^{b}	0.61^{b}	0.01	0.04
ADFI, kg·day^{-1}	1.41^{b}	1.45^{a}	1.46^{a}	1.42^{b}	0.01	0.02
F/G	2.26^{ab}	2.22^{b}	2.32^{a}	2.32^{a}	0.02	0.02
10–115 kg						
Final weight, kg	114.33	116.18	115.33	114.95	1.51	0.39
ADG, kg·day^{-1}	0.69	0.71	0.70	0.68	0.01	0.44
ADFI, kg·day^{-1}	1.96^{ab}	1.99^{a}	1.96^{ab}	1.95^{b}	0.01	0.06
F/G	2.85	2.82	2.82	2.88	0.03	0.39

[ab]*Different superscript letters on the same line are significant differences ($p < 0.05$) ($n = 6$).*
[1]*ADG, average daily weight gain; ADFI, average daily feed intake; F/G, the ratio of feed intake to body weight gain.*
[2]*ELE, Eucommia ulmoides leaf extract.*

Carcass Trait

Table 4 shows that carcass weight ($p < 0.05$), slaughter rate ($p < 0.01$), and carcass straight length ($p < 0.05$) in the 0.1% ELE group were markedly higher than those in the other three groups. Meanwhile, compared with the control group, dietary supplemented with 0.1% ELE observably aggrandized the loin–eye area ($p < 0.05$) of growing–finishing pigs. In addition, adding 0.3% ELE notably increased the lean meat rate ($p < 0.01$) of growing–finishing pigs compared with the other three groups.

Serum Biochemical Indexes

Table 5 shows that adding ELE in the diet memorably descended the content of TP ($p < 0.05$), TG ($p < 0.05$), and VLDL-C levels ($p < 0.01$) compared with the control group; meanwhile, it increased serum ALB ($p < 0.01$) and HDL-C levels ($p < 0.01$).

Serum Cytokine Levels

Table 6 shows that dietary supplementation with 0.2% ELE could signally elevate the levels of ADPN ($p < 0.01$) and IGF-1 ($p < 0.01$) in serum. Serum leptin and insulin levels were not changed dramatically ($p > 0.05$) among the groups.

Activity of Enzymes Related to Lipid Metabolism

The activities of HSL, LPL, and ACAT first increased and then decreased as the level of ELE increased in the diet

TABLE 4 | Carcass trait of growing–finishing pigs fed the diets with various levels of ELE.

Item	ELE[1] levels, %				SEM	*p-value*
	0	0.1	0.2	0.3		
Carcass weight, kg	82.63^{b}	87.00^{a}	84.27^{b}	83.73^{b}	0.93	0.02
Slaughter rate, %	73.37^{b}	75.39^{a}	73.46^{b}	72.85^{b}	0.39	<0.01
Carcass straight length, cm	95.91^{b}	98.64^{a}	95.64^{b}	95.74^{b}	0.79	0.03
Carcass slant length, cm	82.29	83.45	82.14	82.64	0.62	0.50
Average backfat thickness, mm	25.63	24.94	24.46	23.74	1.11	0.55
Loin–eye area, cm^2	27.17^{b}	31.60^{a}	29.70^{ab}	30.96^{a}	0.94	0.01
Lean mass percentage, %	55.00^{b}	55.54^{b}	55.46^{b}	58.40^{a}	0.75	<0.01
Fat mass percentage, %	16.19	15.37	15.65	16.00	0.56	0.85

^{ab}Different superscript letters on the same line are significant differences ($p < 0.05$) ($n = 8$).
[1]ELE, Eucommia ulmoides leaf extract.

TABLE 5 | Effects of dietary ELE on serum biochemical indexes of growing–finishing pigs.

Item[1]	ELE[2] levels, %				SEM	*p-value*
	0	0.1	0.2	0.3		
TP, g·L^{-1}	74.39^{a}	72.90^{ab}	72.54^{ab}	70.61^{b}	0.84	0.03
ALB, g·L^{-1}	52.67^{a}	55.39^{a}	53.41^{a}	46.64^{b}	1.05	<0.01
BUN, mmol·L^{-1}	5.20^{ab}	5.44^{ab}	6.03^{a}	4.63^{b}	0.28	0.01
GLU, mmol·L^{-1}	6.36	6.45	6.30	6.27	0.21	0.93
TG, mmol·L^{-1}	0.58^{a}	0.46^{b}	0.49^{ab}	0.43^{b}	0.04	0.03
TC, mmol·L^{-1}	2.73	3.05	2.78	2.99	0.14	0.30
LDL-C, mmol·L^{-1}	1.86	1.92	1.79	2.05	0.09	0.25
VLDL-C, mmol·L^{-1}	15.11^{a}	14.11^{ab}	11.81^{c}	12.60^{bc}	0.57	<0.01
HDL-C, mmol·L^{-1}	0.56^{b}	0.84^{a}	0.71^{a}	0.55^{b}	0.05	<0.01

$^{a-c}$Different superscript letters on the same line are significant differences ($p < 0.05$) ($n = 8$).
[1]TP, total protein; ALB, albumin; BUN, urea nitrogen; Glu, blood glucose; TC, total cholesterol; TG, triglyceride; HDL-C, high-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol; VLDL-C, very low-density lipoprotein cholesterol.
[2]ELE, Eucommia ulmoides leaf extract.

(**Table 7**). ACC activity was lower ($p < 0.01$) at 0.1% ELE than at other levels. Compared with the control group, diet supplemented with 0.1% and 0.2% ELE notably enhanced ($p < 0.01$) HSL and LPL activities. Besides, the ATGL activity of the 0.1% ELE group exceeded ($p < 0.05$) that of the other two treatment groups, but there was no marked difference compared with the control group. In addition, ACAT activity was no marked discrepancy among the four groups.

TABLE 6 | Effects of dietary ELE on serum cytokine levels of growing–finishing pigs.

Item[1]	ELE[2] levels, %				SEM	*p-value*
	0	0.1	0.2	0.3		
LEP, ng·mL^{-1}	11.60	10.68	10.06	11.90	0.62	0.16
ADPN, μg·mL^{-1}	25.45^{b}	26.75^{b}	35.35^{a}	24.54^{b}	1.62	<0.01
INS, mIU·L^{-1}	26.80	26.08	24.40	25.82	1.91	0.85
IGF-1, ng·mL^{-1}	371.34^{b}	449.79^{b}	604.92^{a}	543.78^{a}	31.40	<0.01

^{ab}Different superscript letters on the same line are significant differences ($p < 0.05$) ($n = 8$).
[1]LEP, leptin; ADPN, adiponectin; INS, insulin; IGF-1, insulin-like growth factor 1.
[2]ELE, Eucommia ulmoides leaf extract.

TABLE 7 | Effects of different levels of ELE on key enzyme activity-related lipid metabolism in backfat tissue of growing–finishing pigs.

Item[1]	ELE[2] levels, %				SEM	*p-value*
	0	0.1	0.2	0.3		
ACC, U·L^{-1}	27.57^{b}	22.21^{c}	26.40^{b}	33.16^{a}	1.27	<0.01
HSL, U·L^{-1}	843.40^{b}	1189.28^{a}	1287.42^{a}	975.86^{b}	57.32	<0.01
LPL, U·L^{-1}	454.19^{c}	603.63^{ab}	682.70^{a}	556.42^{b}	28.38	<0.01
ATGL, mIU·mL^{-1}	315.63^{ab}	345.28^{a}	290.35a^{b}	263.12^{b}	23.06	0.01
ACAT, U·L^{-1}	85.94	86.71	94.64	87.97	5.03	0.60

$^{a-c}$Different superscript letters on the same line are significant differences ($p < 0.05$) ($n = 8$).
[1]ACC, acetyl CoA carboxylase; HSL, hormone-sensitive lipase; LPL, lipoprotein lipase; ATGL, adipose triacylglyceride lipase; ACAT, acyl CoA cholesterol acyltransferase.
[2]ELE, Eucommia ulmoides leaf extract.

Mean Cross-Sectional Area and Quantity of Adipocyte in Backfat Tissue

Figure 1 shows that all the supplementary levels of ELE significantly decreased ($p < 0.05$) the mean cross-sectional area of adipocytes and increased ($p < 0.05$) the total number of adipocytes in backfat tissue.

Relative MRNA Expression Levels of the Key Genes Related to Lipid Metabolism in Backfat Tissue

Figure 2 shows that dietary supplementation with 0.1% and 0.2% ELE could downregulate ($p < 0.05$) the mRNA expression levels of adipogenic genes such as ACC, FAS and SREBPS1c (**Figure 2A**) and upregulate ($p < 0.05$) the mRNA expression levels of lipid-lowering genes, such as HSL, ATGL and SREBP1c, but there was no dramatic variation ($p > 0.05$) in LPL in this study (**Figures 2A–C**). In the 0.2% ELE group, the mRNA expression levels of CPT1 and AMPK-α increased significantly ($p < 0.05$), but that of PPARγ did not change dramatically ($p > 0.05$) (**Figures 2B,C**). In addition, compared with the control group, supplementing with ELE markedly increased ($p < 0.05$)

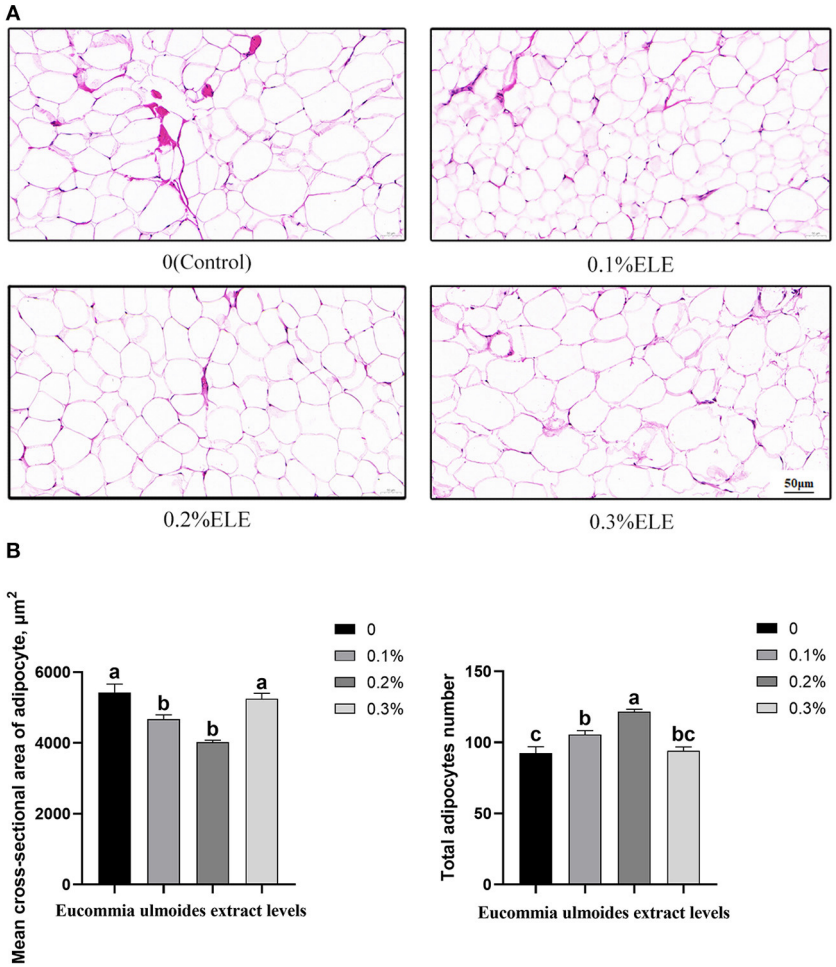

FIGURE 1 | Histological analysis of the mean cross-sectional area and quantity of adipocyte of growing–finishing pigs fed the diets of different levels of *Eucommia ulmoides* leaf extract (ELE). **(A)** Representative cross-sectional HE staining photos of adipocytes in backfat tissue (magnification ×100, bar = 50 μm). **(B)** Quantitative analysis of adipocyte number in backfat tissue. Data are expressed as means ± SEM. $^{a-c}$Values with different letters are significantly different among dietary ELE treatments ($p < 0.05$) ($n = 4$).

the mRNA expression levels of FAT/CD36 and FABP4, and 0.2% ELE decreased the mRNA expression level of FATP1 (**Figure 2D**).

Western Blotting Analysis

Relative protein expression levels for AMPK-α, p-AMPK-α, ACC, and p-ACC were determined by using Western blotting. The results showed that all three different levels of ELE upregulated ($p < 0.05$) the relative protein expression levels of p-AMPK-α and p-ACC (**Figures 3A,B**).

DISCUSSION

Previous studies have shown that ELE is rich in amino acids, minerals, and other nutrients. The essential amino acid content in ELE is high, of which leucine is the highest, followed by valine (5). In addition, iridoids, phenols, and flavonoids are abundant in ELE, which reduces blood lipids (17, 18) and improves diabetes (19) and antioxidation (7). In recent years, *Eucommia ulmoides* is considered to be a very useful feed additive in healthy livestock and poultry breeding.

This study compared the effects of different supplementary levels of ELE in the diet on growth performance, carcass traits, and lipid metabolism in pigs. Growth performance directly affects the meat growth performance of growing–finishing pigs, thus affecting the economic return. These results showed that supplementing pig diets with different levels of ELE had no significant effect on ADG, ADFI, or F/G in piglets, which was consistent with a previous study (9). This might have been due to the strong aromatic compounds in *E. ulmoides* leaf, which might have affected the palatability of the feed. However, our results differ from some previous studies (10, 11), which might have been due to different processing technologies and amounts of ELE supplements used, while ELE supplements had no negative effects on growth performance.

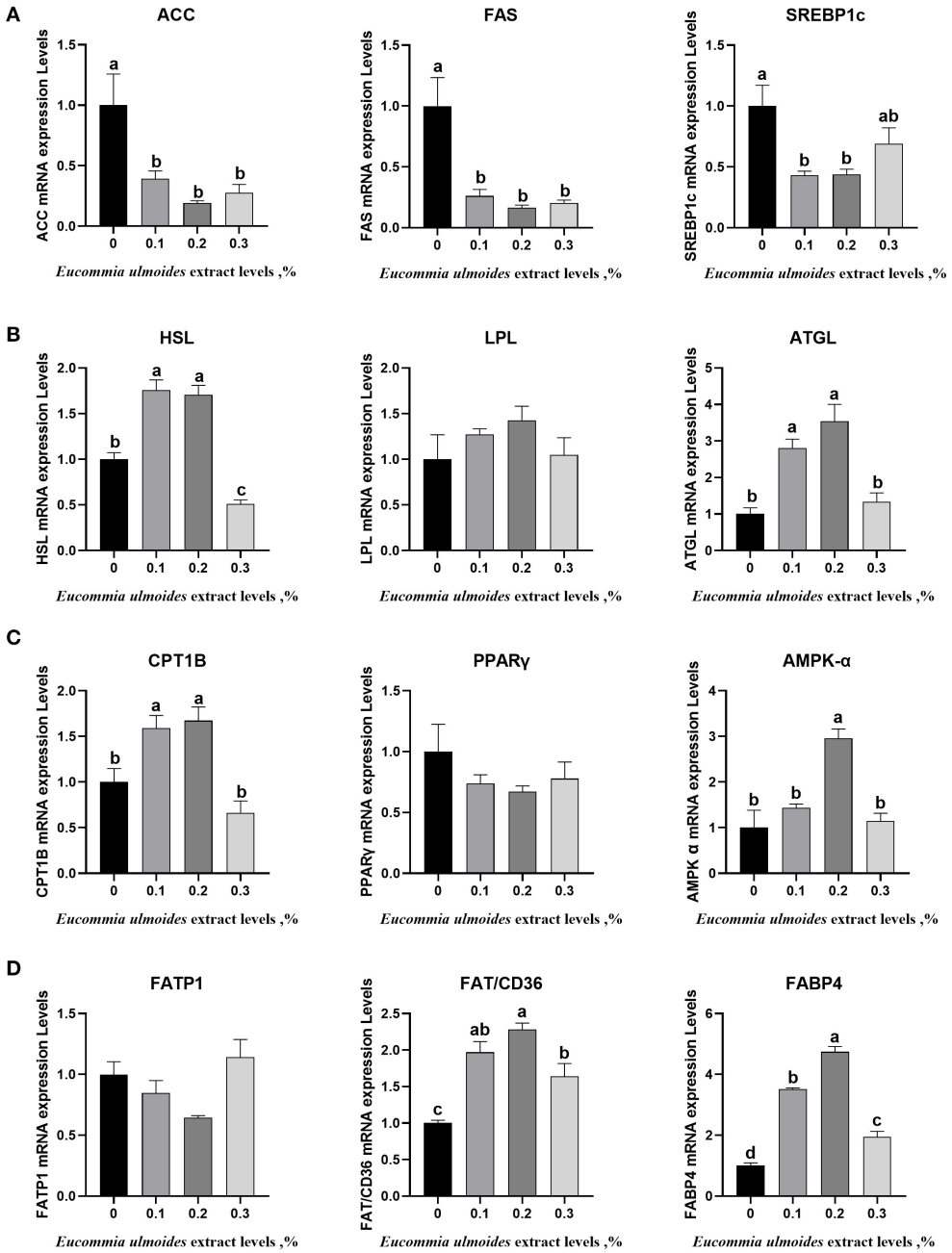

FIGURE 2 | (A) The relative mRNA expression levels of the key genes related to lipogenesis including acetyl-CoA carboxylase α (ACC), fatty acid synthase (FAS), and sterol regulatory element-binding protein-1c (SREBP1c), $n = 8$. **(B)** The relative mRNA expression levels of the key genes related to lipolysis including HSL, LPL, and ATGL, $n = 8$. **(C)** The relative mRNA expression levels of the key genes related to fatty acid oxidation including CPT1B, translocase peroxisome proliferator-activated receptor γ (PPARγ), and adenine monophosphate-activated protein kinase α (AMPK-α), $n = 8$. **(D)** The relative mRNA expression levels of the key genes related to fatty acid transport including FATP1, FAT/CD36, and FABP4, $n = 8$. Data are expressed as means ± SEM ($n = 8$). $^{a-d}$Values with different letters are significantly different among dietary ELE treatments ($p < 0.05$).

Human consumption of meat products containing a large amount of fat may pose a threat to health; long-term consumption may induce cardiovascular diseases and obesity (20). Compared with the control group, the average backfat thickness of the three treatment groups decreased by 2.7, 4.6, and 7.4%, respectively. Meanwhile, fat mass percentage in the three treatment groups decreased by 5.1, 3.2, and 1.2%, respectively. Unfortunately, none of them reached a significant level. However, the histomorphological analysis of backfat tissue showed that ELE significantly reduced the average cross-sectional

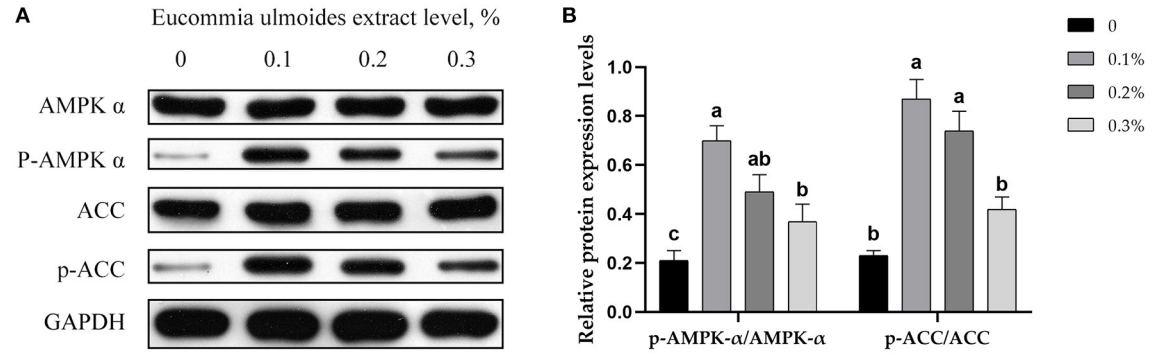

FIGURE 3 | (A) Representative immunoblots of protein levels and phosphorylation degrees of AMPK-α, p-AMPK-α, ACC, and p-ACC, in backfat tissue of growing–finishing pigs. **(B)** Relative protein expression levels of p-AMPK-α/AMPK-α and p-ACC/ACC. Data are expressed as means ± SEM. $^{a-c}$Values with different letters are significantly different among dietary ELE treatments ($p \leq 0.05$) ($n = 4$).

area of adipocyte; the more mature a fat cell is, the larger it is (21), indicating that ELE effectively inhibited the growth and maturation of adipose cells. In conclusion, ELE had a potential inhibitory effect on fat accumulation in back fat tissue; this might be related to chlorogenic acid, the most important active ingredient in ELE. Dietary supplementation with 0.5% and 1% chlorogenic acid was previously reported to reduce the accumulation of visceral fat and lipid content in rats (22). Moreover, dietary supplementation with 0.2% ELE significantly increased carcass weight, slaughter rate, carcass straight length, and loin–eye area, which was consistent with our expectations, indicating that low-dose ELE could improve the carcass traits of growing–finishing pigs.

The changes in serum biochemical indexes can affect the metabolism and nutrient deposition of animals and are affected by the growth stage, endocrine status, and dietary nutrient level (23). We examined the indexes related to lipid and nitrogen metabolism in serum of growing–finishing pigs. Our results showed that adding ELE to the diet increased the serum ALB content and decreased the TP content in growing–finishing pigs, indicating that ELE was beneficial to the overall health of pigs. HDL, a "vascular scavenger," has an anti-atherosclerosis function (24) and can prevent coronary heart disease (25). VLDL is known as an atherogenic factor. It is reported that *Eucommia ulmoides* reduce the levels of triglycerides and total cholesterol in the plasma of finishing pigs (9). In this study, ELE markedly improved HDL content and decreased the levels of VLDL and TC, which indicated that ELE effectively improved lipid metabolism and cardiovascular health in growing–finishing pigs and that the chlorogenic acid and geniposidic acid contained in ELE could play a vital role in antiobesity. Studies have shown that both chlorogenic acid and geniposidic acid from ELE reduce serum TG and TC in obese mice (17, 18).

Adiponectin is secreted by adipocytes and has insulin-sensitizing, anti-atherosclerotic, and anti-inflammatory effects (26). Previous studies have shown that adiponectin promotes the oxidation of fatty acids in muscle and adipose tissue (27). Adiponectin can increase HDL levels and decrease TG levels (28). IGF-1 is a hormone that is closely related to metabolic syndrome and is mainly secreted by the liver cells. This is related to carbohydrate and lipid metabolism (29). Recombinant IGF-1 enhances the lipolysis of adipose tissue, increases the rate of lipid oxidation (30), and promotes the use of free fatty acids in muscle (29). In this study, supplementation with ELE boosted the concentrations of adiponectin and IGF-1 in circulation, which indicates that it plays a lipid-lowering role by regulating hormone levels in growing–finishing pigs.

To explore whether ELE had a similar effect on enzymes related to lipid metabolism, we measured the activities of enzymes related to lipid metabolism in the serum of fattening pigs. AAC is a well-known rate-limiting enzyme (31–33). HSL and ATGL are two important lipases in the animal body. HSL can hydrolyze TG, diglyceride, monoglyceride, cholesterol ester, retinol ester, and other lipids and produce glycerol and free fatty acids (34, 35). ATGL is highly expressed in adipose tissue and is highly specific for TG (36). Moreover, LPL is a rate-limiting enzyme for the degradation of blood triglycerides to glycerol and free fatty acids (37). In addition, ACAT is the only enzyme in the body that can catalyze cholesterol to produce cholesterol esters. Excessive cholesterol esters may lead to atherosclerosis (38). In the present study, although ELE did not dramatically change ACAT activity, dietary supplementation with 0.1% ELE increased HSL, LPL, and ATGL activities while decreasing ACC activity, enlightening the effect of ELE on reducing serum TG which could be achieved by regulating the activities of these enzymes. In addition, we also analyzed the histomorphology of backfat, and the results showed that the addition of ELE markedly reduced the mean cross-sectional area of adipocyte and increased the number of backfat adipocytes. It is proved that ELE can inhibit fat deposition.

We further examined the mRNA expression levels of enzymes and cytokines related to lipogenesis (ACC and FAS), lipolysis (HSL, LPL, and ATGL), fatty acid oxidation (CPT1B, AMPK α), fatty acid transportation (FATP1, FAT/CD36, and FABP4), and lipid deposition (SREBP-1c, PPARγ) to determine the molecular mechanism by which ELE regulates lipid metabolism in growing–finishing pigs. In this study, all three levels of ELE downregulated the mRNA expression of ACC and FAS,

and 0.2% ELE significantly downregulated the expressions of SREBP1c and FATP1. The level of PPARγ mRNA showed a downward trend but did not reach a memorable level. These results showed that 0.1 and 0.2% ELE could effectively reduce the mRNA expression levels of adipogenesis genes. In addition, supplementing with 0.1 and 0.2% ELE upregulated the mRNA level expressions of HSL, ATGL, CPT1B, AMPK, FAT/CD36, and FABP4. Compared with the control group, the 0.3% ELE supplement also significantly upregulated the expressions of FAT/CD36 and FABP4 mRNA. Additionally, the 0.3% ELE supplement downregulated the mRNA expression level of HSL, suggesting that the lipid-lowering effect of ELE may decrease when the dosage exceeds 0.2%. The mRNA expression levels of HSL and ATGL were consistent with the observations that ELE increased HSL and ATGL enzyme activities in growing–finishing pigs. These results also reveal that ELE can exert a lipid-lowering effect by downregulating the mRNA expression levels of lipid-producing genes and upregulating the mRNA expression levels of lipid-lowering genes.

AMPK and ACC are not only the key links in their metabolic regulation but also closely related to each other, which can form upstream and downstream signal pathways in cells. The AMPK-ACC signaling pathway formed by AMPK and its downstream target ACC has important physiological significance in the process of fat synthesis and oxidation (39). When activated by adiponectin, AMPK phosphorylation inactivates ACC phosphorylation, which catalyzes the formation of malonyl-CoA. Malonyl-CoA is the substrate for fatty acid biosynthesis, which inhibits fatty acid oxidation (40). Therefore, we speculate that ELE has a lipid-lowering effect through the AMPK-ACC pathway. We measured the protein expression level of AMPK-α and ACC and the expression level of phosphorylated proteins. As expected, all the three levels of ELE remarkably improved the protein levels of p-AMPK-α and p-ACC and showed a downward trend with the increase in dosage.

CONCLUSIONS

The addition of ELE < 0.3% in growing–finishing pigs could partially improve the carcass traits of growing–finishing pigs and had no adverse effect on growth performance. The 0.1% ELE supplement improved carcass traits, and the 0.1 and 0.2% ELE supplement can reduce the level of TG in serum and increase the level of hormones and enzyme activity that promote fat catabolism. The mRNA and protein expression levels of the key genes related to lipid metabolism showed that the lipid-lowering mechanism of ELE may be through the activation of the AMPK-ACC pathway to inhibit fat deposition in backfat tissue, and the lipid-lowering effect of the 0.1 and 0.2% ELE supplement was the best. However, when the supplemental level was 0.3%, there was no significant effect on carcass traits and lipid metabolism of growing–finishing pigs, and some indexes even had negative effects. In conclusion, the supplemental range of 0.1 to 0.2% ELE is the optimal addition. ELE contains a variety of bioactive components; which component plays a leading role needs our further study using the cell culture model.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/supplementary material.

ETHICS STATEMENT

The animal study was reviewed and approved by Committee on Animal Care of the Institute of Subtropical Agriculture, Chinese Academy of Sciences.

AUTHOR CONTRIBUTIONS

YuhY, FL, QG, and YulY designed the experiments. YuhY and YunY conducted the experiments. WW, QG, LZ, YunY, MH, and SG helped with animal experiments. YuhY analyzed the data and wrote the original draft. QG and FL revised the manuscript. All authors have read and approved the final manuscript.

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Maternal Supplementation With Different Probiotic Mixture From Late Pregnancy to Day 21 Postpartum: Consequences for Litter Size, Plasma and Colostrum Parameters, and Fecal Microbiota and Metabolites in Sows

Li Han[1], *Md. Abul Kalam Azad*[1], *Pan Huang*[1], *Wei Wang*[2], *Wenming Zhang*[3], *Francois Blachier*[4] *and Xiangfeng Kong*[1]*

[1] Hunan Provincial Key Laboratory of Animal Nutritional Physiology and Metabolic Process, National Engineering Laboratory for Pollution Control and Waste Utilization in Livestock and Poultry Production, Institute of Subtropical Agriculture, Chinese Academy of Sciences, Changsha, China, [2] The Institute of Cell Transplantion and Gene Therapy, Centra-South University, the Engineering Center for Xenotransplantation, Changsha, China, [3] Evonik (China) Co., Ltd., Beijing, China, [4] UMR PNCA, INRAE, AgroParisTech, Université Paris-Saclay, Paris, France

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Paola Sacchi,
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Reviewed by:
Renli Qi,
Chongqing Academy of Animal Science, China
Yu-Hsiang Yu,
National Ilan University, Taiwan

***Correspondence:**
Xiangfeng Kong
nnkxf@isa.ac.cn

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The present study determined the effects of different probiotic mixture supplementation to sows from late pregnancy to day 21 postpartum on reproductive performance, colostrum composition, plasma biochemical parameters, and fecal microbiota and metabolites. A total of 80 pregnant sows were randomly assigned to one of four groups (20 sows per group). The sows in the control group (CON group) were fed a basal diet, and those in the BS-A+B, BS-A+BL, and BS-B+BL groups were fed basal diets supplemented with 250 g/t of different probiotic mixture containing either 125 g/t of *Bacillus subtilis* A (BS-A), *Bacillus subtilis* B (BS-B), and/or *Bacillus licheniformis* (BL), respectively. The trial period was from day 85 of pregnancy to day 21 postpartum. The results showed that different dietary probiotic mixture supplementation increased ($P < 0.05$) the average weaning weight and average daily gain of piglets, while dietary BS-A+BL supplementation increased the number of weaned piglets ($P < 0.05$), litter weight ($P = 0.06$), litter weight gain ($P = 0.06$), and litter daily gain ($P = 0.06$) at weaning compared with the CON group. Different dietary probiotic mixture supplementation improved ($P < 0.05$) the colostrum quality by increasing the fat and dry matter concentrations, as well as the protein and urea nitrogen concentrations in the BS-A+BL group. Dietary probiotic mixture BS-B+BL increased the plasma total protein on days 1 and 21 postpartum while decreased the plasma albumin on day 1 postpartum ($P < 0.05$). In addition, the plasma high-density lipoprotein-cholesterol was increased in the BS-A+B and BS-B+BL groups on day 21 postpartum, while plasma ammonia was decreased in the BS-A+B and BS-A+BL groups on day 1 and in the three probiotic mixtures groups on day 21 postpartum ($P < 0.05$). Dietary supplementation with different

probiotic mixture also modified the fecal microbiota composition and metabolic activity in sows during pregnancy and postpartum stages. Collectively, these findings suggest that maternal supplementation with *Bacillus subtilis* in combination with *Bacillus licheniformis* are promising strategies for improving the reproductive performance and the overall health indicators in sows, as well as the growth of their offspring.

Keywords: fecal microbiota, litter size, metabolites, pregnant sows, probiotics

INTRODUCTION

Reproductive performance can be influenced by the health status of sows during pregnancy, and such a parameter is closely associated with the economic efficiency of pig farms (1). However, sows are susceptible to various stress factors (including factors associated with service staff, environment, physiological stages, etc.) during pregnancy and lactation. Such situation of stress may cause imbalance of intestinal microbiota composition and metabolic activity, lower nutrient utilization, and lead to sows body weight loss (2). The gut microbiota composition of sows during pregnancy and lactation can impact the enteric nutrient absorption and immunity (3), which consequently influences the body weight (BW) of piglets at birth and weaning, the number of piglets born alive, and the number of living piglets at weaning (4). Moreover, the BW loss of sows during lactation can influence the lactation performance, as well as the subsequent weaning-to-service interval and reproductive cycle (5). Therefore, in order to maximize the reproductive potential and the body health of sows, such objectives might be achieved by different dietary strategies, including supplementation with antibiotics, probiotics, prebiotics, and enzymes in sow diets (6). Recent concerns regarding antibiotic resistance in animals and humans has led to the use of antibiotic alternatives such as probiotic strains in livestock production. Such alternative has attracted increased attention to improve the reproductive performance and overall health of animals.

The most commonly used probiotics in livestock production include the *Bifidobacterium*, *Lactobacillus*, *Bacillus* spp., *Enterococcus* spp., and *Saccharomyces cerevisiae* (7). Among these probiotics, *Bacillus* spp. is differentiated by its ability to survive in the intestinal tract, form spores, secrete bacteriostatic substances, withstand adverse conditions of feed processing, and maintain stability. Moreover, *Bacillus* spp. produces different kinds of digestive enzymes and stimulates peristalsis of the host intestine, thereby enhancing nutrient digestion (8, 9). Therefore, it is deemed to be a beneficial feed additive for animal intestinal health (10). *Bacillus* spp. are also widely used as probiotics in humans, as they may bring a health benefit to the host gastrointestinal physiology (11). Concurrently, Cai et al. (12) have also shown that dietary *Bacillus* spp. supplementation has positive effects on pigs, such as improving growth performance and feed conversion ratio, reducing the incidence of diarrhea and mortality, as well as increasing the BW and number of piglets born alive and kept alive up to weaning time. Moreover, previous studies also revealed that a probiotic mixture of *Bacillus subtilis* and *Bacillus licheniformis* in growing-finishing pigs increased the digestibility and fecal *Lactobacillus* counts while decreased fecal NH_3 and total mercaptan emissions (13). The decreased NH_3 concentration is also considered beneficial for colonocyte mitochondrial energy metabolism, as this bacterial metabolite inhibits oxygen consumption in colonocytes when present in excess (14).

Although various *Bacillus* spp. are used as probiotics for animals and humans, the mixture of different *Bacillus* strains (such as *B. subtilis* and *B. licheniformis*) has been little studied in pigs, and the mechanisms involved in the effects observed are not yet fully understood. In addition, most studies and applications of *Bacillus* spp. are mostly concentrated on the stages of piglets at nursery, weaned piglets, and growing pigs. However, the studies are relatively limited on the effects of *Bacillus* spp. used during pregnancy and lactation, regarding the impact on the sow reproductive performance and the profiles of the sow's intestinal microbiota. In addition, the effects of maternal supplements on their offspring piglets have been little documented. Our previous study found that dietary supplementation with a probiotic mixture of *B. subtilis* and *B. licheniformis* to piglets at weaning could improve several indicators of intestinal health through improving intestinal morphology and altering intestinal microbiota and metabolite concentrations (15). In addition, dietary supplementation with *B. subtilis* increased the amounts of intestinal microbes with presumed beneficial effects, and the fecal concentrations of several bioamines and short-chain fatty acids (SCFA) of perinatal sows (15, 16). In this context, we hypothesized that dietary probiotic mixture (*B. subtilis* A, BS-A; *B. subtilis* B, BS-B; and/or *B. licheniformis*) supplementation from late pregnancy to day 21 postpartum would be beneficial for sow health and thus influence their reproductive performance. The BS-A is a product containing a single *B. subtilis* strain and has a strong capability of *Clostridium perfingens* inhibition. The BS-B is a product containing a single pure *Bacillus* strain with strong *Escherichia coli F18* inhibition ability. Both strains show significant pathogens inhibition through multiple secondary metabolites production. Moreover, *B. licheniformis* is a product containing a single pure *B. licheniformis* strain, which has the potential to improve intestinal morphology in broilers. Therefore, the combination of these strains might have synergic beneficial effects in animals. Thus, the present study was conducted to determine the effect of dietary supplementation with probiotic mixture containing *B. subtilis* and/or *B. licheniformis* from late pregnancy to day 21 postpartum on reproductive performance, biochemical parameters in blood and colostrum, and intestinal microbiota composition and their metabolites.

MATERIALS AND METHODS

Animals, Housing, and Treatments

A total of 80 Large White sows close to day 85 of pregnancy with 2–4 parities were used and randomly assigned to one of four groups (20 sows per group). The sows in the control group were fed the basal diet (CON group), and those in the experimental groups received the basal diet supplemented with 250 g/t complex probiotics (4.0×10^9 CFU/kg). The diets of the BS-A+B, BS-A+BL, and BS-B+BL groups contained 125 g/t *B. subtilis* A (BS-A) + 125 g/t *B. subtilis* B (BS-B), 125 g/t BS-A + 125 g/t *B. licheniformis* (BL), and 125 g/t BS-B + 125 g/t BL, respectively. The complex probiotics were prepared by *Evonik* (*China*) *Co., Ltd*. The trial lasted from day 85 of pregnancy to day 21 postpartum.

The pregnant sows were housed in individual pens (2.50 × 0.85 m) during late pregnancy (days 85–110), and were moved to the farrowing facilities (2.50 × 2.70 m) on day 110 of pregnancy, where they were housed individually with a hard plastic slatted bedding, together with their litters until weaning. The room temperature was maintained at 21–24°C with 60% relative humidity. In addition, heating lights were used to maintain the temperature of the piglets. The sows were fed a pregnancy diet between days 85 to 107 of pregnancy and a lactation diet from day 108 of pregnancy to day 21 postpartum. The sows were fed twice daily (8:00 a.m. and 5:00 p.m.) with ∼2.0–3.0 kg of diets and changed according to their body condition. Sows and piglets had available *ad libitum* access to water throughout the trial *via* the individual nipple. The composition and nutrient levels of the basal diets for pregnant and lactating sows are shown in **Table 1**.

TABLE 1 | Ingredients and nutrient levels of basal diets of sows during late pregnancy and lactation (as-fed basis).

Items	Pregnancy diet	Lactation diet
Ingredients (%)		
Corn	60.30	58.65
Wheat bran	23.50	5.00
Wheat flour		2.00
Soybean oil		4.00
Soybean meal	12.00	20.50
Enzymic protein powder		3.00
Fish meal		2.50
L-Lysine·HCl	0.12	0.15
L-Threonine	0.03	0.05
L-Valine		0.10
Anti-mildew agent	0.05	0.05
Pregnancy compound premix[a]	4.00	
Lactation compound Premix[b]		4.00
Total	100.00	100.00
Nutrient levels (%)[c]		
Digestible energy (MJ/kg)	15.23	15.56
Dry matter	98.00	97.74
Crude fiber	3.60	3.54
Crude protein	14.17	19.78
Lysine	0.98	1.53
Methionine	0.12	0.16
Threonine	0.68	0.99

[a] *Provided the following for one kilogram diet: VA 10,000 IU, VD 2,500 IU, VE 100 IU, VK 2.0 mg, VB_2 10 mg, VB_6 1.0 mg, VB_{12} 50 μg, choline chloride 1,500 mg, Fe 80 mg, Cu 20 mg, Zn 100 mg, Mn 45 mg, I 0.7 mg, and Se 0.25 mg.*

[b] *Provided the following for one kilogram diet: VA 15,000 IU, VD 3,200 IU, VE 50 IU, VK 4.0 mg, VB_1 4.0 mg, VB_2 10 mg, VB_6 3.0 mg, VB_{12} 20 μg, choline chloride 800 mg, Fe 120 mg, Cu 20 mg, Zn 112 mg, Mn 24 mg, I 0.5 mg, and Se 0.4 mg.*

[c] *Digestible energy is a calculated value, and others are analyzed values.*

Sample Collection and Preparation

On day 1 postpartum, the litter size, number of born alive, and litter weight were recorded, as well as the number and weight of weaned piglets per litter on day 21 postpartum, to calculate the daily gain of litters at weaning and average daily gain of piglets. The backfat thickness was measured at the level of the last rib at 5–8 cm from the midline of each sow using ultrasonography (Renco Lean-Meater®, Minneapolis, MN, USA) on days 85 and 112 of pregnancy, and again on day 21 postpartum. The colostrum samples (∼10 mL) of eight sows per group were collected within 12 h after farrowing and stored at −80°C for the analysis of colostrum composition. The fresh fecal samples were randomly collected in 50 mL sterile centrifuge tubes from eight sows per group on days 100 and 112 of pregnancy and on days 7, 14, and 21 postpartum, and then stored at −20°C for analysis of the microbiota composition and metabolite concentrations. On days 1 and 21 postpartum, the blood samples were randomly collected from the precaval vein into 10 mL heparin coated-tubes, and plasma was separated by centrifuging at 3,500 × g for 10 min at 4°C and immediately stored at −20°C for the analysis of biochemical parameters.

Analysis of Plasma Biochemical Parameters

The plasma biochemical parameters, including total protein (TP), albumin (ALB), urea nitrogen (UN), ammonia (AMM), alkaline phosphatase (ALP), triglyceride (TG), total cholesterol (TC), high-density lipoprotein-cholesterol (HDL-C), and low-density lipoprotein-cholesterol (LDL-C) were measured using a Roche Automatic Biochemical Analyzer (Cobas c311, F. Hoffmann-La Roche Ltd, Basel, Switzerland) and commercially available kits (F. Hoffmann-La Roche Ltd, Basel, Switzerland).

Analysis of Colostrum Composition

The colostrum composition, including somatic cells, milk fat, milk protein, lactose, urea nitrogen, defatted dry matter, and total dry matter, were determined using MilkoScan FT+200 Type 76150 (FOSS electric, Hilleroed, Denmark).

DNA Extraction and Analysis of Fecal Microbiota Quantity

Total microbial DNA was extracted and purified according to the manufacturer's instructions of QIAamp DNA Stool Mini Kit (QIAgen, Hilden, Germany). The concentration of each extracted DNA was measured using a NanoDrop ND-1000 instrument (NanoDrop Technologies Inc., Wilmington, DE, USA) and stored at −80°C. An absorption ratio (260/280 nm) of all samples

TABLE 2 | Group-specific primer sequences for bacteria.

Bacteria	Sequence (5′-3′)	Product size (bp)
Bifidobacterium	F: TCGCGTCYGGTGTGAAAG	128
	R: GGTGTTCTTCCCGATATCTACA	
Clostridium cluster IV	F: GCACAAGCAGTGGAGT	240
	R: CTTCCTCCGTTTTGTCAA	
Escherichia coli	F: CATGCCGCGTGTATGAAGAA	95
	R: CGGGTAACGTCAATGAGCAAA	
Firmicutes	F: GGAGYATGTGGTTTAATTCGAAGCA	126
	R: AGCTGACGACAACCATGCAC	
Lactobacillus	F: AGCAGTAGGGAATCTTCCA	345
	R: ATTCCACCGCTACACATG	
Total bacteria	F: GTGSTGCAYGGYYGTCGTCA	123
	R: ACGTCRTCCMCNCCTTCCTC	

within 1.8–2.0 was deemed to be of sufficient purity to be used for subsequent analyses. The 16S rRNA gene sequences of *Bifidobacterium* spp., *Clostridium* cluster IV, *Escherichia coli*, *Firmicutes*, *Lactobacillus*, and total bacteria were cloned into the pMD19-T vector (17). Gene sequences by references (18) were amplified from total DNA using the primers listed in **Table 2**. A total of six clones with 16S rRNA gene sequences belonging to different taxa were used as templates to test primer specificity. Standard curves were constructed with DNA from representative species of a concentration range of 10^2-10^{10} DNA copies per mL using 384-well plates in the Lightcycler® 480 instrument II (Applied Biosystems, Carlsbad, CA, USA). The microbial DNA extracted from the fecal samples and specific DNA from recombinant microbiota were quantified by RT-PCR. Reaction conditions were at 50°C for 2 min, an initial denaturation step at 95°C for 5 min, and then 20 s denaturation at 94°C for 40 cycles, primer annealing at a species-specific temperature for 30 s, and primer extension at 60°C for 1 min (19). The specific primers for RT-PCR were synthesized by Sangon Biotech (Shanghai) Co., Ltd. Data were analyzed using the Roche Lightcycler 480 software 1.5.0. Microbiota quantities were expressed as a logarithm of the number of microbe copies contained per gram of samples [lg (copies/g)].

Analysis of Fecal Bacterial Metabolites

The concentrations of fecal SCFA, including acetate, propionate, butyrate, isobutyrate, valerate, and isovalerate, were measured as described previously by Zhou et al. (20). The fresh fecal samples (0.900–1.000 g) were homogenized and centrifuged in sealed tubes at 10,000 × g for 10 min at 4°C. A mixture of the supernatant fluid and 25% metaphosphoric acid solution (1 mL: 0.25 mL) were then filtered through a 0.45-μm polysulfone microporous membrane filter and analyzed using Agilent 6890 gas chromatography (Agilent Technologies, Inc, Palo Alto, CA, USA) (21). The concentrations of bioamines, including tryptamine, putrescine, cadaverine, 1,7-heptyl diamine, tyramine, spermidine, and spermine, were measured as described previously by Kong et al. (22).

Statistical Analyses

Statistical data analysis was performed with one-way ANOVA using SPSS 18.0 software (SPSS, Inc, Chicago, IL, USA). Levene's test for homogeneity of variance was used, followed by Duncan's multiple-range test (in the case of variance homogeneity). Values are expressed as means ± standard error (SE). P-values < 0.05 were taken to indicate statistical significance, with a trend toward significance at $0.05 \leq P < 0.10$.

RESULTS

Reproductive Performance

The effects of different dietary probiotic mixture supplementation on the reproductive performance of sows are presented in **Table 3**. The average weaning weight and average daily gain of weaned piglets were increased ($P < 0.05$) by supplementing the sows' diet with different probiotic mixture. However, dietary supplementation with different probiotic mixture did not affect neither the litter size nor the number of piglets born alive. Also, the litter weight at birth remains unchanged compared with the CON group. The number of weaned piglets was higher ($P < 0.05$) in the BS-A+BL group compared with the CON and BS-A+B groups. In addition, the BS-A+B group displayed a trend for an increased ($P = 0.06$) average piglets' birth weight. Moreover, the BS-A+BL group also displayed a trend for an increased litter weight ($P = 0.07$), litter weight gain ($P = 0.06$), and litter daily gain ($P = 0.06$) at weaning.

Backfat Thickness and Colostrum Composition

The effects of different dietary probiotic mixture supplementation on the backfat thickness of sows are presented in **Table 4**. The backfat thickness of the BS-A+BL group was increased ($P < 0.05$) from day 85 to day 112 of pregnancy compared with the CON and BS-B+BL groups. However, there were no significant changes in the backfat thickness of sows from day 112 of pregnancy to day 21 postpartum among the different dietary treatment groups.

The effects of different dietary probiotic mixture supplementation on nutrient compositions of colostrum are summarized in **Table 5**. Compared with the CON group, the concentrations of milk fat and total dry matter in colostrum were increased ($P < 0.05$) when sows were supplemented with different probiotic mixture. The concentrations of protein and UN of colostrum were higher ($P < 0.05$) in the BS-A+BL group compared with the CON group.

Plasma Biochemical Parameters

The effects of different dietary probiotic mixture supplementation on plasma biochemical parameters of sows are presented in **Table 6**. On day 1 postpartum, the plasma ALB concentration was decreased ($P < 0.05$) in the BS-B+BL group compared with the other three groups, while the plasma TP concentration was increased ($P < 0.05$) in the BS-B+BL group compared with the CON group. In addition, the plasma ALP activity was decreased ($P < 0.05$) in the BS-A+B group

TABLE 3 | Effects of dietary probiotic mixture supplementation on reproductive performance of sows.

Items	CON group	BS-A+B group	BS-A+BL group	BS-B+BL group	*P*-values
Litter size (*n*)	11.84 ± 0.68	10.32 ± 0.54	12.42 ± 0.86	11.42 ± 0.66	0.19
Born alive (*n*)	11.53 ± 0.73	10.26 ± 0.52	12.05 ± 0.79	11.26 ± 0.63	0.30
Weaned piglets (*n*)	10.00 ± 0.26^{b}	9.85 ± 0.25^{b}	10.90 ± 0.28^{a}	10.40 ± 0.26^{ab}	0.02
Litter weight at birth (kg)	17.07 ± 0.95	17.25 ± 0.74	18.56 ± 1.03	17.99 ± 0.72	0.60
Average birth weight (kg)	1.50 ± 0.04	1.70 ± 0.05	1.57 ± 0.05	1.64 ± 0.06	0.06
Litter weight at weaning (kg)	61.72 ± 3.02	68.24 ± 2.69	72.02 ± 2.47	69.10 ± 2.52	0.06
Litter weight gain at weaning (kg)	46.65 ± 2.47	52.66 ± 2.36	55.20 ± 2.07	52.20 ± 2.11	0.06
Litter daily gain at weaning (kg/d)	2.22 ± 0.12	2.51 ± 0.11	2.63 ± 0.10	2.49 ± 0.10	0.06
Average weaning weight (kg)	6.15 ± 0.16^{b}	7.09 ± 0.17^{a}	6.75 ± 0.18^{a}	6.72 ± 0.16^{a}	<0.01
Average daily gain (kg)	0.22 ± 0.01^{b}	0.26 ± 0.01^{a}	0.25 ± 0.01^{a}	0.24 ± 0.01^{a}	<0.01

Data are presented as means with SE (n = 20). ^{a,b}Mean values in the same row with different superscripts were significantly different (P < 0.05). The BS-A+B, BS-A+BL, and BS-B+BL groups contained 125 g/t Bacillus subtilis A (BS-A), 125 g/t Bacillus subtilis B (BS-B), and/or 125 g/t Bacillus licheniformis (BL), respectively.

TABLE 4 | Effects of dietary probiotic mixture supplementation on backfat thickness of sows.

Items	CON group	BS-A+B group	BS-A+BL group	BS-B+BL group	*P*-values
Backfat thickness (mm)					
Day 85 of pregnancy	18.75 ± 1.02	19.85 ± 1.15	18.50 ± 0.88	19.30 ± 0.95	0.79
Day 112 of pregnancy	19.55 ± 0.87	21.25 ± 1.07	21.45 ± 0.94	21.00 ± 1.01	0.51
Day 21 postpartum	16.55 ± 0.80	17.35 ± 0.96	19.20 ± 0.82	18.00 ± 0.90	0.19
Backfat thickness changes (mm)					
Day 85 to day 112 of pregnancy	1.71 ± 0.22^{b}	2.64 ± 0.46^{ab}	3.39 ± 0.39^{a}	2.00 ± 0.40^{b}	0.01
Day 112 of pregnancy to day 21 postpartum	−3.33 ± 0.61	−4.39 ± 0.50	−3.31 ± 0.50	−3.76 ± 0.58	0.47

Data are presented as means with SE (n = 20). ^{a,b}Mean values in the same row with different superscripts were significantly different (P < 0.05). The BS-A+B, BS-A+BL, and BS-B+BL groups contained 125 g/t Bacillus subtilis A (BS-A), 125 g/t Bacillus subtilis B (BS-B), and/or 125 g/t Bacillus licheniformis (BL), respectively.

TABLE 5 | Effects of dietary probiotic mixture supplementation on nutrient composition of colostrum in sows.

Items	CON group	BS-A+B group	BS-A+BL group	BS-B+BL group	*P*-values
Somatic cells (×10^3 piece/mL)	1,399 ± 373.8	1,591 ± 369.1	1,324 ± 472.5	2,337 ± 1,134.3	0.70
Fat (%)	2.66 ± 0.23^{b}	3.89 ± 0.38^{a}	3.92 ± 0.21^{a}	3.66 ± 0.46^{a}	0.04
Protein (%)	14.76 ± 0.42^{b}	15.59 ± 0.64^{ab}	17.19 ± 0.37^{a}	16.23 ± 0.68^{ab}	0.03
Lactose (%)	3.96 ± 0.13	3.86 ± 0.18	3.72 ± 0.07	3.62 ± 0.12	0.30
Urea nitrogen (mg/dL)	50.13 ± 2.08^{b}	56.13 ± 2.41^{ab}	62.25 ± 2.91^{a}	56.75 ± 2.3^{ab}	0.02
Defatted dry matter (%)	22.73 ± 0.34	23.48 ± 0.5	24.41 ± 0.46	23.87 ± 0.57	0.11
Total dry matter (%)	28.62 ± 0.51^{b}	30.76 ± 0.69^{a}	31.65 ± 0.41^{a}	30.91 ± 0.92^{a}	0.02

Data are presented as means with SE (n = 8). ^{a,b}Mean values in the same row with different superscripts were significantly different (P < 0.05). The BS-A+B, BS-A+BL, and BS-B+BL groups contained 125 g/t Bacillus subtilis A (BS-A), 125 g/t Bacillus subtilis B (BS-B), and/or 125 g/t Bacillus licheniformis (BL), respectively.

compared with the CON and BS-B+BL groups, and the plasma AMM concentration was decreased ($P < 0.05$) in the BS-A+B and BS-A+BL groups compared with the other two groups on day 1 postpartum. Moreover, the plasma UN concentration was higher ($P = 0.06$) in the BS-A+BL group on day 1 postpartum. On day 21 postpartum, the plasma AMM concentration was decreased ($P < 0.05$) in the three probiotic mixture groups compared with the CON group, while the plasma HDL-C concentration was increased ($P < 0.05$) in the BS-A+B and BS-B+BL groups compared with the other two groups. Moreover, the plasma TC concentration ($P = 0.06$) in the BS-A+B group and TP concentration ($P = 0.06$) in the BS-B+BL group tended to increase on day 21 postpartum.

Amount and Composition of Fecal Microbiota

The effects of different dietary probiotic mixture supplementation on fecal microbiota composition in sows are summarized in **Table 7**. No significant differences ($P > 0.05$) were observed in the amounts of *Bifidobacterium* spp., *E. coli*, and total bacteria in the fecal samples from the different treatment groups. The ratio of fecal *Lactobacillus* to *E. coli* on day

TABLE 6 | Effects of dietary probiotic mixture supplementation on plasma biochemical parameters of sows.

Items	Day postpartum	CON group	BS-A+B group	BS-A+BL group	BS-B+BL group	*P*-values
ALB (g/L)	1	41.76 ± 0.63^{a}	42.39 ± 0.83^{a}	43.68 ± 0.43^{a}	39.05 ± 1.26^{b}	0.01
	21	41.05 ± 0.72	41.16 ± 0.82	41.14 ± 0.88	41.23 ± 1.19	0.99
ALP (U/L)	1	49.88 ± 2.39^{a}	38.50 ± 1.92^{b}	42.38 ± 2.19^{ab}	47.75 ± 4.55^{a}	0.04
	21	37.88 ± 2.66	44.88 ± 6.26	38.63 ± 2.67	44.63 ± 3.58	0.47
AMM (μmol/L)	1	123.34 ± 4.40^{a}	74.89 ± 6.69^{b}	62.10 ± 6.66^{b}	107.64 ± 3.35^{a}	<0.01
	21	93.73 ± 1.45^{a}	52.00 ± 2.06^{b}	58.53 ± 3.84^{b}	59.26 ± 7.20^{b}	<0.01
HDL-C (mmol/L)	1	0.64 ± 0.03	0.63 ± 0.03	0.62 ± 0.03	0.71 ± 0.03	0.16
	21	0.72 ± 0.04^{b}	0.91 ± 0.06^{a}	0.72 ± 0.04^{b}	0.95 ± 0.04^{a}	<0.01
LDL-C (mmol/L)	1	0.80 ± 0.06	0.86 ± 0.07	0.81 ± 0.04	0.80 ± 0.03	0.83
	21	1.10 ± 0.13	1.19 ± 0.05	0.96 ± 0.08	1.08 ± 0.05	0.31
TC (mmol/L)	1	1.41 ± 0.07	1.40 ± 0.09	1.35 ± 0.04	1.47 ± 0.05	0.67
	21	1.82 ± 0.11	2.02 ± 0.09	1.63 ± 0.11	1.91 ± 0.07	0.06
TG (mmol/L)	1	0.28 ± 0.03	0.23 ± 0.02	0.25 ± 0.02	0.24 ± 0.04	0.74
	21	0.19 ± 0.03	0.21 ± 0.03	0.17 ± 0.01	0.15 ± 0.02	0.27
TP (g/L)	1	67.85 ± 1.00^{b}	70.15 ± 0.99^{ab}	70.45 ± 0.63^{ab}	73.40 ± 1.65^{a}	0.02
	21	75.66 ± 1.42	78.83 ± 1.61	76.79 ± 2.02	82.33 ± 1.82	0.06
UN (mmol/L)	1	4.19 ± 0.30	4.54 ± 0.16	5.35 ± 0.30	4.69 ± 0.35	0.06
	21	4.83 ± 0.19	5.88 ± 0.54	5.65 ± 0.65	5.79 ± 0.32	0.37

Data are presented as means with SE (n = 8). ^{a,b}Mean values in the same row with different superscripts were significantly different (P < 0.05). The BS-A+B, BS-A+BL, and BS-B+BL groups contained 125 g/t Bacillus subtilis A (BS-A), 125 g/t Bacillus subtilis B (BS-B), and/or 125 g/t Bacillus licheniformis (BL), respectively.
ALB, albumin; ALP, alkaline phosphatase; AMM, ammonia; HDL-C, high-density lipoprotein-cholesterol; LDL-C, low-density lipoprotein-cholesterol; TC, total cholesterol; TG, triglyceride; TP, total protein; UN, urea nitrogen.

7 postpartum was increased ($P < 0.05$) in the BS-A+BL group, and the amount of *Lactobacillus* tended to increase ($P = 0.07$), when compared with the CON and BS-B+BL groups. The fecal amount of *Firmicutes* was decreased ($P < 0.05$) in the BS-B+BL group compared with the CON and BS-A+BL groups on day 7 postpartum and tended to increase ($P = 0.07$) in the probiotic mixture supplemented groups on day 21 postpartum. The fecal amount of *Clostridium cluster* IV in the BS-B+BL group tended to increase ($P = 0.05$) on day 112 of pregnancy compared with the CON group.

Fecal Concentrations of Bacterial Metabolites

The effects of different dietary probiotic mixture supplementation on fecal SCFA concentrations in sows are presented in **Table 8**. On day 100 of pregnancy, the fecal valerate concentration was higher ($P < 0.05$) in the BS-A+BL and BS-B+BL groups compared with the CON group. The fecal isobutyrate and branched-chain fatty acid (BCFA) concentrations were higher ($P < 0.05$) in the three probiotic mixture supplemented groups compared with the CON group. Moreover, the fecal isobutyrate concentration in the BS-B+BL group and the BCFA concentration in the BS-A+BL and BS-B+BL groups were higher ($P < 0.05$) compared with the BS-A+B group. However, the fecal acetate ($P = 0.08$) concentration tended to increase in the probiotic mixture supplemented groups compared with the CON group. On day 112 of pregnancy, a higher ($P < 0.05$) propionate concentration was observed in the BS-A+B group compared with the CON group. The fecal straight-chain fatty acids, isovalerate, and total SCFA concentrations in the BS-A+BL and BS-B+BL groups and the valerate and BCFA concentrations in the BS-A+BL group were lower ($P < 0.05$) when compared with the BS-A+B group. Moreover, the acetate concentration tended to increase in the BS-A+B group ($P = 0.09$) compared with the other three groups. On day 7 postpartum, the fecal acetate, isovalerate, and BCFA concentrations were higher ($P < 0.05$) in the BS-A+B and BS-A+BL groups compared with the CON group. Moreover, the straight-chain fatty acid concentration in the BS-A+B and the total SCFA concentration in the probiotic mixture supplemented groups were higher ($P < 0.05$) when compared with the CON group. However, no significant differences ($P > 0.05$) were observed in the fecal bacterial metabolites on days 14 and 21 postpartum among the different treatment groups.

The effects of different dietary probiotic mixture supplementation on fecal bioamine concentrations in sows are presented in **Table 9**. There were no significant differences in the bioamine concentrations among the different treatment groups on day 100 of pregnancy except that the tryptamine concentration tended to increase ($P = 0.06$) in the BS-A+B and BS-A+BL groups. On day 112 of pregnancy, the spermine concentration was higher ($P < 0.05$) in the BS-B+BL group compared with the other three groups. On day 7 postpartum, the fecal 1,7-heptanediamine ($P < 0.05$) and spermidine ($P = 0.07$) concentrations were higher in the BS-A+BL group compared with the other three groups. The fecal tryptamine ($P = 0.08$) concentration in the BS-B+BL group and the spermine ($P <$

TABLE 7 | Effects of dietary probiotic mixture supplementation on fecal microbiota quantity in sows.

Items (Lg copies/g)	CON group	BS-A+B group	BS-A+BL group	BS-B+BL group	*P*-values
Bifidobacterium					
Day 100 of pregnancy	5.63 ± 0.38	5.93 ± 0.39	6.10 ± 0.30	6.03 ± 0.34	0.80
Day 112 of pregnancy	4.93 ± 0.27	5.37 ± 0.40	4.60 ± 0.48	5.00 ± 0.33	0.56
Day 7 postpartum	4.60 ± 0.41	4.57 ± 0.42	4.76 ± 0.41	4.03 ± 0.31	0.59
Day 14 postpartum	4.30 ± 0.27	4.43 ± 0.30	4.71 ± 0.25	3.97 ± 0.30	0.32
Day 21 postpartum	4.37 ± 0.44	4.47 ± 0.27	4.89 ± 0.37	4.46 ± 0.27	0.72
Lactobacillus					
Day 100 of pregnancy	7.12 ± 0.28	7.58 ± 0.31	7.09 ± 0.45	7.04 ± 0.48	0.75
Day 112 of pregnancy	6.28 ± 0.32	6.70 ± 0.27	5.99 ± 0.47	6.40 ± 0.31	0.56
Day 7 postpartum	5.54 ± 0.41	6.13 ± 0.41	6.75 ± 0.31	5.56 ± 0.24	0.07
Day 14 postpartum	6.90 ± 0.32	6.66 ± 0.23	6.80 ± 0.23	6.78 ± 0.33	0.95
Day 21 postpartum	6.61 ± 0.41	6.39 ± 0.33	5.59 ± 0.53	7.08 ± 0.25	0.08
Escherichia coli					
Day 100 of pregnancy	6.49 ± 0.36	6.27 ± 0.32	6.17 ± 0.35	6.01 ± 0.28	0.77
Day 112 of pregnancy	7.37 ± 0.18	7.17 ± 0.18	7.65 ± 0.15	7.52 ± 0.18	0.25
Day 7 postpartum	7.95 ± 0.24	7.65 ± 0.18	7.43 ± 0.14	7.70 ± 0.12	0.45
Day 14 postpartum	7.46 ± 0.24	7.33 ± 0.18	7.03 ± 0.19	7.27 ± 0.22	0.52
Day 21 postpartum	6.89 ± 0.24	6.52 ± 0.22	6.36 ± 0.28	6.90 ± 0.32	0.41
Lactobacillus/E. coli					
Day 100 of pregnancy	1.13 ± 0.09	1.23 ± 0.09	1.15 ± 0.06	1.20 ± 0.12	0.85
Day 112 of pregnancy	0.86 ± 0.05	0.94 ± 0.06	0.78 ± 0.06	0.86 ± 0.06	0.31
Day 7 postpartum	0.70 ± 0.06^{b}	0.80 ± 0.05^{ab}	0.91 ± 0.05^{a}	0.73 ± 0.04^{b}	0.02
Day 14 postpartum	0.94 ± 0.07	0.92 ± 0.06	0.97 ± 0.05	0.94 ± 0.06	0.92
Day 21 postpartum	0.97 ± 0.07	1.00 ± 0.07	0.91 ± 0.11	1.04 ± 0.07	0.69
***Clostridium cluster* IV**					
Day 100 of pregnancy	7.50 ± 0.14	7.83 ± 0.08	7.70 ± 0.10	7.75 ± 0.08	0.16
Day 112 of pregnancy	6.69 ± 0.14	6.90 ± 0.13	6.94 ± 0.17	7.24 ± 0.09	0.05
Day 7 postpartum	7.10 ± 0.19	6.93 ± 0.08	6.86 ± 0.12	6.90 ± 0.10	0.58
Day 14 postpartum	6.95 ± 0.10	7.01 ± 0.11	6.91 ± 0.19	6.72 ± 0.16	0.54
Day 21 postpartum	6.79 ± 0.16	6.85 ± 0.15	6.84 ± 0.12	7.04 ± 0.10	0.59
Firmicutes					
Day 100 of pregnancy	9.20 ± 0.23	9.70 ± 0.09	9.70 ± 0.10	9.25 ± 0.42	0.32
Day 112 of pregnancy	8.46 ± 0.23	8.75 ± 0.15	8.74 ± 0.06	8.60 ± 0.21	0.62
Day 7 postpartum	8.85 ± 0.16^{a}	8.60 ± 0.14^{ab}	8.91 ± 0.17^{a}	8.26 ± 0.14^{b}	0.02
Day 14 postpartum	8.98 ± 0.09	9.11 ± 0.07	8.68 ± 0.24	8.72 ± 0.09	0.12
Day 21 postpartum	7.40 ± 0.43	8.30 ± 0.20	8.36 ± 0.51	8.78 ± 0.17	0.07
Total bacteria					
Day 100 of pregnancy	9.09 ± 0.22	9.39 ± 0.11	8.88 ± 0.42	8.97 ± 0.41	0.69
Day 112 of pregnancy	9.01 ± 0.14	9.12 ± 0.07	9.05 ± 0.14	9.28 ± 0.07	0.35
Day 7 postpartum	9.30 ± 0.07	9.02 ± 0.08	9.10 ± 0.15	9.02 ± 0.05	0.16
Day 14 postpartum	9.29 ± 0.06	9.33 ± 0.06	9.30 ± 0.11	8.74 ± 0.32	0.10
Day 21 postpartum	9.31 ± 0.10	9.13 ± 0.09	9.34 ± 0.08	9.30 ± 0.10	0.36

Data are presented as means with SE (n = 8). ^{a,b}*Mean values in the same row with different superscripts were significantly different (P < 0.05). The BS-A+B, BS-A+BL, and BS-B+BL groups contained 125 g/t Bacillus subtilis A (BS-A), 125 g/t Bacillus subtilis B (BS-B), and/or 125 g/t Bacillus licheniformis (BL), respectively.*

0.05) concentration in the BS-A+BL and BS-B+BL groups were higher compared with the CON and BS-A+B groups on day 14 postpartum. Moreover, the 1,7-heptanediamine concentration was higher ($P < 0.05$) in the BS-A+BL group compared with the CON and BS-B+BL groups on day 21 postpartum. However, the spermine concentration tended to increase ($P = 0.07$) in the BS-B+BL group on day 21 postpartum.

DISCUSSION

Dietary probiotics supplementation can maintain or even improve indicators of gut health, leading to an overall better performance and health status in animal production. Therefore, this area of research has become more and more active in the field of animal nutrition (23). The present

TABLE 8 | Effects of dietary probiotic mixture supplementation on fecal short-chain fatty acids (SCFA) concentrations in sows.

Items (mg/g)	CON group	BS-A+B group	BS-A+BL group	BS-B+BL group	*P*-values
Acetate					
Day 100 of pregnancy	3.87 ± 0.35	4.61 ± 0.21	4.81 ± 0.50	5.15 ± 0.22	0.08
Day 112 of pregnancy	5.59 ± 0.43	6.20 ± 0.12	5.07 ± 0.32	4.87 ± 0.56	0.09
Day 7 postpartum	5.36 ± 0.36^{b}	6.33 ± 0.18^{a}	6.56 ± 0.29^{a}	6.10 ± 0.24^{ab}	0.03
Day 14 postpartum	7.16 ± 0.78	7.74 ± 0.42	7.30 ± 0.50	7.06 ± 0.50	0.84
Day 21 postpartum	5.29 ± 0.29	5.57 ± 0.26	5.30 ± 0.17	5.27 ± 0.21	0.78
Propionate					
Day 100 of pregnancy	1.96 ± 0.14	2.34 ± 0.13	2.18 ± 0.11	2.23 ± 0.12	0.20
Day 112 of pregnancy	2.30 ± 0.24^{b}	3.00 ± 0.15^{a}	2.07 ± 0.10^{b}	2.31 ± 0.14^{b}	<0.01
Day 7 postpartum	2.32 ± 0.21	3.12 ± 0.32	2.50 ± 0.16	2.41 ± 0.26	0.11
Day 14 postpartum	3.24 ± 0.60	3.81 ± 0.26	3.59 ± 0.42	3.10 ± 0.23	0.58
Day 21 postpartum	2.28 ± 0.13	2.56 ± 0.17	2.36 ± 0.13	2.42 ± 0.12	0.54
Butyrate					
Day 100 of pregnancy	0.41 ± 0.13	0.47 ± 0.20	0.07 ± 0.01	0.48 ± 0.27	0.36
Day 112 of pregnancy	0.19 ± 0.10	0.10 ± 0.01	0.11 ± 0.01	0.32 ± 0.13	0.23
Day 7 postpartum	0.34 ± 0.12	0.44 ± 0.22	0.11 ± 0.01	0.17 ± 0.06	0.29
Day 14 postpartum	1.60 ± 0.38	1.70 ± 0.10	1.75 ± 0.32	1.76 ± 0.36	0.98
Day 21 postpartum	1.25 ± 0.11	1.45 ± 0.06	1.16 ± 0.09	1.41 ± 0.09	0.10
Valerate					
Day 100 of pregnancy	0.22 ± 0.02^{b}	0.25 ± 0.02^{ab}	0.30 ± 0.04^{a}	0.33 ± 0.02^{a}	0.04
Day 112 of pregnancy	0.32 ± 0.04^{ab}	0.38 ± 0.04^{a}	0.25 ± 0.01^{b}	0.29 ± 0.02^{ab}	0.03
Day 7 postpartum	0.26 ± 0.02	0.41 ± 0.03	0.44 ± 0.11	0.33 ± 0.02	0.19
Day 14 postpartum	0.45 ± 0.05	0.49 ± 0.02	0.44 ± 0.07	0.42 ± 0.04	0.83
Day 21 postpartum	0.33 ± 0.02	0.37 ± 0.03	0.30 ± 0.01	0.37 ± 0.02	0.12
Straight-chain fatty acids					
Day 100 of pregnancy	5.60 ± 0.51	6.24 ± 0.63	6.06 ± 1.09	5.70 ± 0.28	0.91
Day 112 of pregnancy	8.40 ± 0.64^{ab}	9.68 ± 0.23^{a}	7.51 ± 0.40^{b}	7.22 ± 0.75^{b}	0.02
Day 7 postpartum	8.29 ± 0.39^{b}	10.30 ± 0.46^{a}	9.61 ± 0.47^{ab}	9.01 ± 0.45^{ab}	0.02
Day 14 postpartum	12.44 ± 1.51	13.72 ± 0.71	13.07 ± 1.27	12.34 ± 1.07	0.83
Day 21 postpartum	9.16 ± 0.48	9.94 ± 0.46	9.12 ± 0.33	9.46 ± 0.36	0.48
Isobutyrate					
Day 100 of pregnancy	0.73 ± 0.19^{c}	1.32 ± 0.17^{b}	1.69 ± 0.15^{ab}	2.09 ± 0.08^{a}	<0.01
Day 112 of pregnancy	0.31 ± 0.02	0.34 ± 0.01	0.25 ± 0.01	0.41 ± 0.11	0.24
Day 7 postpartum	0.27 ± 0.01	0.37 ± 0.03	0.35 ± 0.05	0.29 ± 0.02	0.06
Day 14 postpartum	0.43 ± 0.05	0.48 ± 0.03	0.40 ± 0.05	0.39 ± 0.03	0.41
Day 21 postpartum	0.32 ± 0.02	0.36 ± 0.03	0.31 ± 0.01	0.36 ± 0.02	0.23
Isovalerate					
Day 100 of pregnancy	0.40 ± 0.04	0.46 ± 0.03	0.57 ± 0.08	0.57 ± 0.04	0.06
Day 112 of pregnancy	0.57 ± 0.05^{ab}	0.66 ± 0.02^{a}	0.46 ± 0.02^{b}	0.49 ± 0.04^{b}	<0.01
Day 7 postpartum	0.51 ± 0.04^{b}	0.75 ± 0.04^{a}	0.73 ± 0.11^{a}	0.58 ± 0.04^{ab}	0.04
Day 14 postpartum	0.85 ± 0.10	0.93 ± 0.06	0.80 ± 0.12	0.77 ± 0.06	0.59
Day 21 postpartum	0.61 ± 0.05	0.70 ± 0.07	0.58 ± 0.02	0.71 ± 0.04	0.21
Branched-chain fatty acids (BCFA)					
Day 100 of pregnancy	1.13 ± 0.19^{c}	1.78 ± 0.17^{b}	2.26 ± 0.16^{a}	2.66 ± 0.10^{a}	<0.01
Day 112 of pregnancy	0.87 ± 0.07^{ab}	1.00 ± 0.03^{a}	0.71 ± 0.03^{b}	0.90 ± 0.08^{a}	0.02
Day 7 postpartum	0.78 ± 0.05^{b}	1.13 ± 0.07^{a}	1.09 ± 0.16^{a}	0.88 ± 0.05^{ab}	0.03
Day 14 postpartum	1.28 ± 0.14	1.41 ± 0.09	1.20 ± 0.17	1.16 ± 0.09	0.54
Day 21 postpartum	0.93 ± 0.07	1.06 ± 0.10	0.89 ± 0.03	1.07 ± 0.06	0.19
Total SCFA					
Day 100 of pregnancy	6.74 ± 0.44	8.02 ± 0.55	8.31 ± 1.07	8.36 ± 0.27	0.28
Day 112 of pregnancy	9.27 ± 0.68^{ab}	10.68 ± 0.26^{a}	8.22 ± 0.41^{b}	8.12 ± 0.69^{b}	<0.01

(Continued)

TABLE 8 | Continued

Items (mg/g)	CON group	BS-A+B group	BS-A+BL group	BS-B+BL group	*P*-values
Day 7 postpartum	9.07 ± 0.40^{c}	11.43 ± 0.51^{a}	10.70 ± 0.58^{ab}	9.89 ± 0.46^{bc}	0.04
Day 14 postpartum	13.71 ± 1.62	15.13 ± 0.77	14.27 ± 1.42	13.50 ± 1.15	0.81
Day 21 postpartum	10.08 ± 0.54	11.01 ± 0.49	10.01 ± 0.34	10.52 ± 0.39	0.38

Data are presented as means with SE (n = 8). [a–c]Mean values in the same row with different superscripts were significantly different (P < 0.05). The BS-A+B, BS-A+BL, and BS-B+BL groups contained 125 g/t Bacillus subtilis A (BS-A), 125 g/t Bacillus subtilis B (BS-B), and/or 125 g/t Bacillus licheniformis (BL), respectively.

TABLE 9 | Effects of dietary probiotic mixture supplementation on fecal bioamine concentrations in sows.

Items (μg/g)	CON group	BS-A+B group	BS-A+BL group	BS-B+BL group	*P*-values
Tryptamine					
Day 100 of pregnancy	2.92 ± 0.24	3.27 ± 0.38	4.39 ± 1.27	1.88 ± 0.29	0.06
Day 112 of pregnancy	3.84 ± 0.59	5.51 ± 0.95	4.35 ± 0.51	3.26 ± 0.47	0.12
Day 7 postpartum	2.16 ± 0.40	2.33 ± 0.48	2.05 ± 0.21	2.40 ± 0.30	0.90
Day 14 postpartum	1.47 ± 0.53	1.52 ± 0.29	1.86 ± 0.39	3.17 ± 0.71	0.08
Day 21 postpartum	1.55 ± 0.45	1.69 ± 0.44	1.78 ± 0.32	1.42 ± 0.08	0.93
1,7-Heptanediamine					
Day 100 of pregnancy	0.44 ± 0.03	0.38 ± 0.04	0.38 ± 0.07	0.33 ± 0.06	0.42
Day 112 of pregnancy	0.48 ± 0.07	0.57 ± 0.09	0.75 ± 0.18	0.64 ± 0.13	0.48
Day 7 postpartum	0.37 ± 0.05^{b}	0.38 ± 0.04^{b}	0.57 ± 0.05^{a}	0.34 ± 0.06^{b}	<0.01
Day 14 postpartum	0.29 ± 0.05	0.29 ± 0.02	0.28 ± 0.01	0.34 ± 0.04	0.34
Day 21 postpartum	0.30 ± 0.04^{b}	0.37 ± 0.02^{ab}	0.47 ± 0.07^{a}	0.26 ± 0.03^{b}	<0.01
Spermidine					
Day 100 of pregnancy	10.44 ± 1.71	7.13 ± 1.69	9.60 ± 2.47	13.62 ± 2.73	0.27
Day 112 of pregnancy	12.00 ± 1.28	11.51 ± 1.30	14.61 ± 2.14	$15..93 \pm 1.41$	0.17
Day 7 postpartum	6.08 ± 0.68	6.66 ± 0.43	8.98 ± 0.78	6.82 ± 1.11	0.07
Day 14 postpartum	10.06 ± 0.98^{b}	8.60 ± 0.69^{b}	16.82 ± 1.78^{a}	10.56 ± 1.00^{b}	<0.01
Day 21 postpartum	15.27 ± 1.84	15.97 ± 1.64	16.17 ± 2.37	17.60 ± 1.81	0.86
Spermine					
Day 100 of pregnancy	0.72 ± 0.11	0.30 ± 0.06	0.61 ± 0.16	0.69 ± 0.18	0.13
Day 112 of pregnancy	0.67 ± 0.10^{b}	0.93 ± 0.11^{b}	0.95 ± 0.19^{b}	1.78 ± 0.24^{a}	<0.01
Day 7 postpartum	0.62 ± 0.11	0.69 ± 0.08	0.88 ± 0.10	0.55 ± 0.08	0.11
Day 14 postpartum	0.70 ± 0.06^{c}	0.54 ± 0.08^{c}	1.61 ± 0.26^{a}	1.17 ± 0.13^{b}	<0.01
Day 21 postpartum	1.18 ± 0.16	1.50 ± 0.23	1.20 ± 0.23	1.92 ± 0.21	0.07

Data are presented as means with SE (n = 8). [a–c]Mean values in the same row with different superscripts were significantly different (P < 0.05). The BS-A+B, BS-A+BL, and BS-B+BL groups contained 125 g/t Bacillus subtilis A (BS-A), 125 g/t Bacillus subtilis B (BS-B), and/or 125 g/t Bacillus licheniformis (BL), respectively.

study showed that maternal supplementation with different probiotics mixture from late pregnancy to day 21 postpartum increased the average body weight and average daily gain of weaned piglets, and BS-A+BL supplementation increased the number of weaned piglets. Similarly, Alexopoulos et al. (24) also demonstrated that 400 g/t *B. licheniformis* and *B. subtilis* supplementation from 14 days prior to the expected farrowing to weaning periods increased the number of weaned piglets per litter and the BW of piglet at weaning. In addition, maternal intestinal microflora can affect the colonization and development of gut microbiota of offspring, which is associated with the weight gain of offspring (25). Therefore, these findings indicated that different probiotic mixture supplementation to sows during late pregnancy to day 21 postpartum are able to improve the reproductive performance of sows, and thus influence the growth performance of piglets.

The nutrient composition of sows' milk is closely related to the survival rate and the growth and development of piglets (4). Several studies have reported that *Bacillus* spp. such as *B. subtilis* and *B. licheniformis* inclusion in sow diets during late gestation and lactation are able to influence the colostrum or milk composition (24, 26). The present study shows that dietary supplementation with different probiotic mixture increased the concentrations of fat and dry matter in the colostrum, as well as the concentrations of protein and UN in the BS-A+BL group. Consumption of milk with better quality has also been reported to increase the piglets' weaning weight when sows were fed *B. subtilis* during lactation (24, 26). Therefore, these findings suggest that the improvement in the reproductive performance

of sows might be related to the dietary probiotic supplementation (27), which improves the sows' milk quality and quantity and promotes fat deposition and growth of suckling piglets up to a certain extent.

Plasma biochemical parameters can partly reflect the nutritional status, tissue and organ functions, and metabolic status of animals. In addition, the plasma AMM concentration may reflect the liver function of animals (28). The present study showed that dietary supplementation with different probiotic mixture decreased plasma AMM concentration on day 21 postpartum, suggesting that the nitrogen metabolism of sows was elevated. The HDL-C is responsible for transporting TC to liver cells for oxidation, the plasma concentration of which is markedly related to lipoprotein metabolism (29). The present study showed that dietary supplementation with probiotic mixture BS-A+B and BS-B+BL increased the plasma HDL-C concentration on day 21 postpartum, indicating that these probiotic mixtures improved the lipoprotein metabolism of sows. Moreover, lactating sows need higher energy reserves and nutrients to maintain body tissues and support milk production (30). Research evidence showed that dietary probiotic supplementation can improve the intestinal environment and nutrient metabolism (31), as well as backfat thickness at birth and weaning (32, 33). The present study showed that dietary BS-A+BL supplementation increased the backfat thickness with changes recorded from days 85 to 112 of pregnancy. This suggests that in sows, the recovery of physical condition postpartum is promoted up to a certain extent by the supplementation used.

The intestinal microbiota composition plays a key role in maintaining health and regulating pathogenesis in the host (34). Studies have found that the quantity of intestinal *Firmicutes* has the potential to increase the energy intake from the diet and the body weight in humans (35). Moreover, *Clostridium clusters* IV, *Lactobacillus*, and *Bifidobacterium* can participate in nutrient metabolism and energy recycling and play important roles in the trophic, metabolic, and protective functions of the host (36). Dietary probiotic supplementation could regulate the balance and activity of gut microbes and thereby affect the metabolism and utilization of nutrients (37), the physiology and immune processes, the protection against pathogens, and the resistance to disease (38). In the present study, dietary supplementation with probiotic BS-A+BL increased the *Lactobacillus* to *E. coli* ratio, which might have a beneficial effect on the reproductive performance of sows and the intestinal health of offspring. Moreover, dietary supplementation with probiotic mixture of BS-A+BL on day 7 postpartum and BS-B+BL on day 21 postpartum trended to increase the abundance of *Lactobacillus* in sows. This is in agreement with the previous study by Kaewtapee et al. (39), which reported that *Bacillus* spp. (*B. subtilis* and *B. licheniformis*) supplementation in diets with low- and high-protein content increased the abundances of *Bifidobacterium* spp. and *Lactobacillus* spp. However, these findings are not in line with those of Bohmer et al. (40), who found that the fecal bacterial counts of sows were not affected by probiotics supplementation. This discrepancy might be explained by the differences in genetic background, breeds, and ages of the sows, as well as the dose and periods of prebiotic supplementation in the different studies.

The gut microbial metabolites influence nutrient metabolism, immunity, and health of the host through various regulatory mechanisms (41, 42). Some of anaerobic bacteria in the colon ferment the complex carbohydrates, indigestible fibers, or amino acids released from proteins, producing the SCFA, such as acetate, butyrate, and propionate (43). Among these metabolites, acetate can be metabolized by peripheral tissues (44) and provide energy for the host. Propionate is primarily used by the liver and can regulate cholesterol synthesis (45). Our results showed that dietary BS-A+B supplementation increased the fecal concentrations of propionate and valerate on day 112 of pregnancy and acetate and straight-chain fatty acids on day 7 postpartum. Moreover, the concentrations of acetate on day 7 postpartum and valerate on day 100 pregnancy were higher in the BS-A+BL group, as well as the concentration of valerate on day 100 pregnancy in the BS-B+BL group. These findings suggest that dietary probiotic mixture supplementation may modulate the SCFA production in the colon of sows. A previous study reported that obesity has been found to be associated with the increase in fecal total SCFA concentration (46). However, it is unknown if a causative link exists between these two parameters. Therefore, it has been postulated that the probiotic strains may provide the additional energy for the host to promote weight gain in sows (47). In another study, Ohigashi et al. (48) reported that the increase in SCFA production is accompanied by a decrease in the luminal pH, which resulted in the suppression of intestinal pathogens and increased nutrient absorption. Thus, these findings indicate that the intestinal microflora balance could be improved by dietary probiotic mixture supplementation.

The BCFA, including isobutyrate and isovalerate, are the products of L-leucine, L-isoleucine, and L-valine obtained from protein breakdown. The BCFA concentrations are the markers of protein catabolism in the intestinal cavity (49). The present study showed that dietary probiotics BS-A+B and BS-A+BL supplementation increased the fecal BCFA concentrations on day 7 postpartum, suggesting that there are more indigestible proteins in the small intestine which entered the colon, and that the catabolism of L-leucine, L-isoleucine, or L-valine was increased in the colon (22). However, the underlying mechanisms need to be further clarified.

Bioamines are mainly produced through the decarboxylation of different amino acid precursors (including methionine, tryptophan, arginine, and ornithine) by bacterial metabolism (50, 51). These metabolites have some known physiological functions in different tissues of the body, including regulation of gene expression, nucleic acid and protein synthesis, cell signaling, cell proliferation and differentiation, and placental growth and embryonic development in animals (52). Tryptophan is linked to tryptamine via tryptophan decarboxylase, and putrescine is synthesized indirectly from arginine or directly from ornithine, which can occur simultaneously in many bacteria (53). Polyamines synthesized by the intestinal microbiota are

known to be involved in intestinal epithelium renewal (54). The present study showed that the fecal concentrations of tryptamine, 1,7-heptanediamine, spermidine, and spermine were increased in the BS-A+BL group, as well as tryptamine and spermine in the BS-B+BL group. These changes may be due to an increased metabolic capacity of the intestinal microbiota for amino acid decarboxylation. Previous studies demonstrated that higher levels of bioamines may contribute to decreased colonic chronic inflammation by inhibiting inflammatory cytokine synthesis in macrophages (55, 56). Further studies are necessary to determine whether the parameters of intestinal mucosal inflammation were modified by *B. subtilis* or *B. licheniformis* supplementation.

CONCLUSION

Collectively, dietary supplementation with different probiotic mixture of *Bacillus* spp. in sows from late pregnancy to day 21 postpartum can increase the BW and average daily gain of offspring piglets, while only *B. subtilis* A in combination with *B. licheniformis* can increase the number of piglets. The colostrum composition was also found to be improved following dietary probiotic supplementation, an improvement that may be linked to the positive effect of piglet's growth and development. Furthermore, dietary supplementation with *B. subtilis* A in combination with *B. licheniformis* altered the intestinal microbiota and different bacterial metabolite concentrations. Further future studies will help to understand better the causal links between these different biological and biochemical parameters. Finally, it is worth noting that dietary supplementation with *B. subtilis* A in combination with *B. licheniformis* from day 85 of pregnancy to day 21 of postpartum was the optimum probiotic mixture beneficial for both sows and piglets.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/supplementary material.

ETHICS STATEMENT

The animal study was reviewed and approved by Animal Care and Use Committee of Institute of Subtropical Agriculture, Chinese Academy of Sciences.

AUTHOR CONTRIBUTIONS

LH and PH performed the experiments. LH and MA performed the statistical analyses and wrote the manuscript. WZ, WW, and XK contributed to experimental concepts and design, provided scientific direction, and finalized the manuscript with the help of FB. All authors read and approved the final manuscript.

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REVIEW
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Metabolic Reprogramming, Gut Dysbiosis, and Nutrition Intervention in Canine Heart Disease

*Qinghong Li**

Nestlé Purina Research, Saint Louis, MO, United States

This review provides a state-of-the-art overview on recent advances in systems biology in canine cardiac disease, with a focus on our current understanding of bioenergetics and amino acid metabolism in myxomatous mitral valve disease (MMVD). Cross-species comparison is drawn to highlight the similarities between human and canine heart diseases. The adult mammalian heart exhibits a remarkable metabolic flexibility and shifts its energy substrate preference according to different physiological and pathological conditions. The failing heart suffers up to 40% ATP deficit and is compared to an engine running out of fuel. Bioenergetics and metabolic readaptations are among the major research topics in cardiac research today. Myocardial energy metabolism consists of three interconnected components: substrate utilization, oxidative phosphorylation, and ATP transport and utilization. Any disruption or uncoupling of these processes can result in deranged energy metabolism leading to heart failure (HF). The review describes the changes occurring in each of the three components of energy metabolism in MMVD and HF. It also provides an overview on the changes in circulating and myocardial glutathione, taurine, carnitines, branched-chain amino acid catabolism and tryptophan metabolic pathways. In addition, the review summarizes the potential role of the gut microbiome in MMVD and HF. As our knowledge and understanding in these molecular and metabolic processes increase, it becomes possible to use nutrition to address these changes and to slow the progression of the common heart diseases in dogs.

Keywords: mitral valve disease (MVD), energy metabolism, amino acids, nutrition, disease, heart failure, microbiome, cardiac metabolism

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Edited by:
Yehui Duan,
Institute of Subtropical Agriculture, Chinese Academy of Sciences (CAS), China

Reviewed by:
Kris Helke,
Medical University of South Carolina, United States
Dan Wang,
Wuhan Polytechnic University, China

***Correspondence:**
Qinghong Li
qinghong.li@rd.nestle.com

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INTRODUCTION

The adult mammalian heart has a very high demand for energy in order to sustain its constant contractile activities and meet its basal metabolic needs (1). More than 70% of ATPs in the normal adult heart are produced by fatty acid oxidation (FAO) in the complex mitochondrial machinery while the remaining balance comes from the oxidation of other substrates including glucose (1). The heart is metabolically flexible and shifts its preference in energy substrates in accordance with different developmental stages, physiological, or pathological conditions (2). The concept of the failing heart as an energy starved engine that runs out of fuel was initially proposed by Herrmann and Decherd almost one century ago and continues to attract considerable research interests today (1–7). The failing heart can exhibit an energy deficit of up to 40% less ATP than a healthy heart (5, 8), increasing its reliance on glucose and other energy substrates as fuel in the context of reduced

capacity of FAO (1, 2, 9). Inside the cardiac myocytes, glucose is either converted to sorbitol by the polyol pathway or phosphorylated by hexokinase to glucose 6-phosphate, which subsequently goes through several metabolic pathways including glycolysis (10). Recently, a growing body of evidence indicates ketone bodies as a significant fuel source in the failing and diseased heart (7, 11–14). Pathological alternations of these energy metabolic pathways are associated with impaired signal transductions and altered energy and redox homeostasis leading to contractile dysfunction. Although the pathophysiology of heart failure (HF) is complex and multifactorial (15), strategies that aim to improve cardiac energy metabolism, as an example, by switching to a more efficient myocardial energy substrate, have begun to show promise (7, 13, 16–19).

The concentrations of myocardial and circulating amino acids change in the failing heart in humans and animal models (20–22). Total free amino acids were increased in the humans failing right ventricles (23). Branched-chain amino acid (BCAA) catabolic deficiency is associated with the failing heart in humans and animal models (24–27). Several uremic toxins, many of which are amino acid metabolic products, are associated with heart disease (22, 28). However, the contribution of amino acid metabolic reprogramming to cardiac health and disease has been understudied and underappreciated. In addition, several gut microbiota-produced metabolites have been associated with the cardiovascular disease although no causal relationship has been established (29–31). Myxomatous mitral valve disease (MMVD), the most common naturally occurring heart disease in dogs, is characterized as a slow progressive MV degeneration, which causes mitral regurgitation and, in some cases, can lead to congestive heart failure (CHF) (13, 32, 33). Canine MMVD is very similar to the primary MV prolapse in humans at the morphological, pathophysiological, and molecular levels, and is considered as a model for MV prolapse (34–37). The TGF-β and serotonin (5-HT) signaling pathways have been implicated in the physiopathogenesis of MMVD in both humans and dogs. The observations included increased valvular expressions in genes and proteins in both pathways, increased 5-HT concentrations in circulation, myocardial and valvular tissues in dogs with MMVD. The comparative pathophysiology and the underlying signaling mechanisms by TGF-β and 5-HT have been extensively reported and reviewed (36–42). This review will summarize current advances in cardiac energy and amino acid metabolic reprogramming, associations between gut dysbiosis and heart disease, and opportunities for nutritional intervention.

CARDIAC ENERGY METABOLISM

The failing heart undergoes extensive metabolic remodeling (43, 44). Cardiac energy metabolism is composed of three interconnected components: substrate utilization and transfer, ATP production by oxidative phosphorylation (OXPHOS), and ATP transfer and utilization by myofibrils (**Figure 1**). Disruptions or uncoupling of these components may cause derangements in cardiac energy metabolism. This review will describe changes in each of the three components in the failing heart and MMVD in dogs.

Energy Substrate Utilization

Fatty Acid Utilization

In a normal mammalian heart, 70–90% of energy requirement comes from FAO, while the remaining balance comes from glycolysis and oxidation of lactate, and to a small degree, from ketolysis and amino acid oxidation (44, 45). However, the relative contribution of each substrate to the cardiac energy production can vary greatly depending on substrate availability, metabolic demand, and cardiac health condition (44). In the early phase of HF, minor reductions in fatty acid uptake and oxidation are observed, while significant decreases in FAO are detected in advanced HF (6, 45–47). Circulating free fatty acids (FAs) cross the sarcolemmal membrane either through passive diffusion or a carrier protein-assisted pathway (**Figure 2**). These protein carriers include FA binding protein (FABP), FA transporter protein (FATP), and FA translocase (CD36/FAT). Cytosolic FAs are esterified to become fatty acyl CoA, from which its acyl group is transferred to carnitine to form acylcarnitine by carnitine palmitoyltransferase 1 (CPT1). The acylcarnitine enters the mitochondrial inner matrix *via* the carnitine shuttle and is converted to fatty acyl CoA by CPT2. The fatty acyl CoA goes through several cycles of β-oxidation producing the reduced forms of both nicotinamide adenine dinucleotide (NADH) and flavin adenine dinucleotide ($FADH_2$), and acetyl CoA, which enters the TCA cycle for ATP production. The complex regulation of FAO pathway occurs at essentially every step, including the availabilities of circulating free fatty acids, fatty acid uptake and transport across cardiac sarcolemma, fatty acid esterification to become fatty acyl-CoA esters, mitochondrial update *via* the carnitine shuttle, and sequential β-oxidations of long-chain acyl-CoA into acetyl-CoA, and biochemical reactions in the TCA cycle and electron transport chain (ETC) (1).

In dogs with MMVD, fatty acid uptake and transport to cytoplasm and fatty acid conversion to fatty acyl-CoA esters are altered (48). In an RNA-seq transcriptomics study, fatty acid binding protein was downregulated in the MV of preclinical MMVD dogs compared with non-MMVD dogs (48). In addition, long-chain acyl-CoA synthetase, the enzyme that converts long-chain fatty acids to acyl CoA esters, was downregulated in both the left ventricle (LV) and MV (48). These changes suggest impairments in the fatty acid transport and utilization pathway that may lead to deranged bioenergetics.

Glucose Utilization

In cardiac hypertrophy, there is a significant metabolic shift from FAO to glucose (10, 43). Glucose oxidation is more oxygen efficient than FAO, but produces less ATP per molecule. The complete oxidation of 1 palmitate (C16:0) molecule generates 105 ATP molecules, and consumes 46 oxygen atoms, whereas the complete oxidation of 1 glucose molecule generates 31 ATP molecules and consumes only 12 oxygen atoms. The fluxes of glucose and fatty acids are regulated by a feedback mechanism known as the Randle cycle or the glucose-fatty acid cycle (49), which involves the competition between glucose

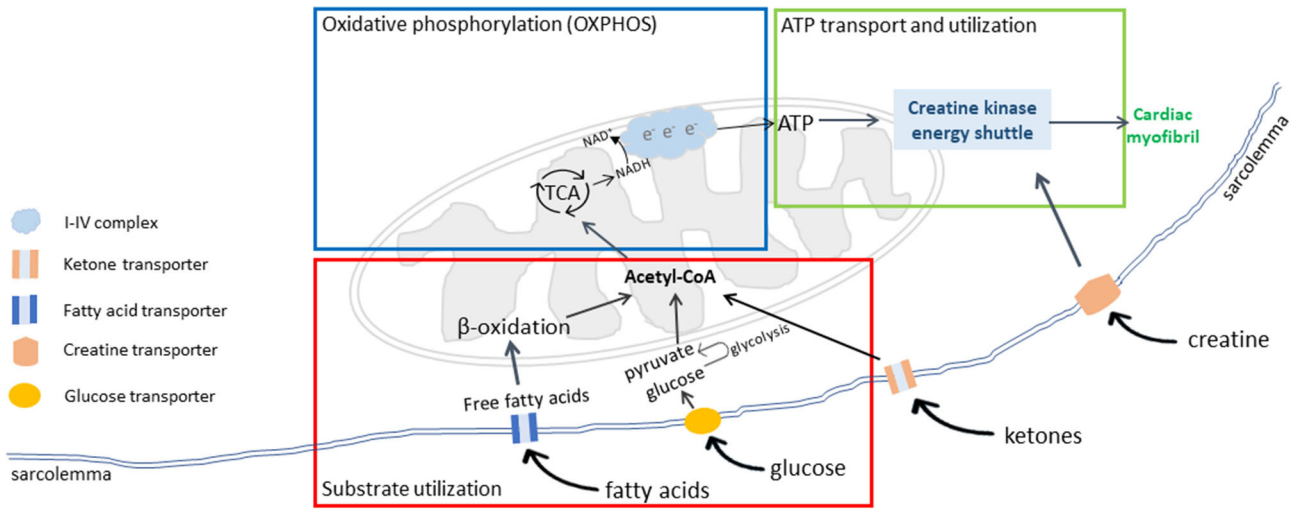

FIGURE 1 | Cardiac energy metabolism. There are three interconnected components of cardiac energy metabolism: substrate transport and utilization (red box), mitochondrial oxidative phosphorylation (OXPHOS) for energy production (blue box), and ATP transport and utilization by cardiac myofibrils (green box). Adapted from Neubauer (6).

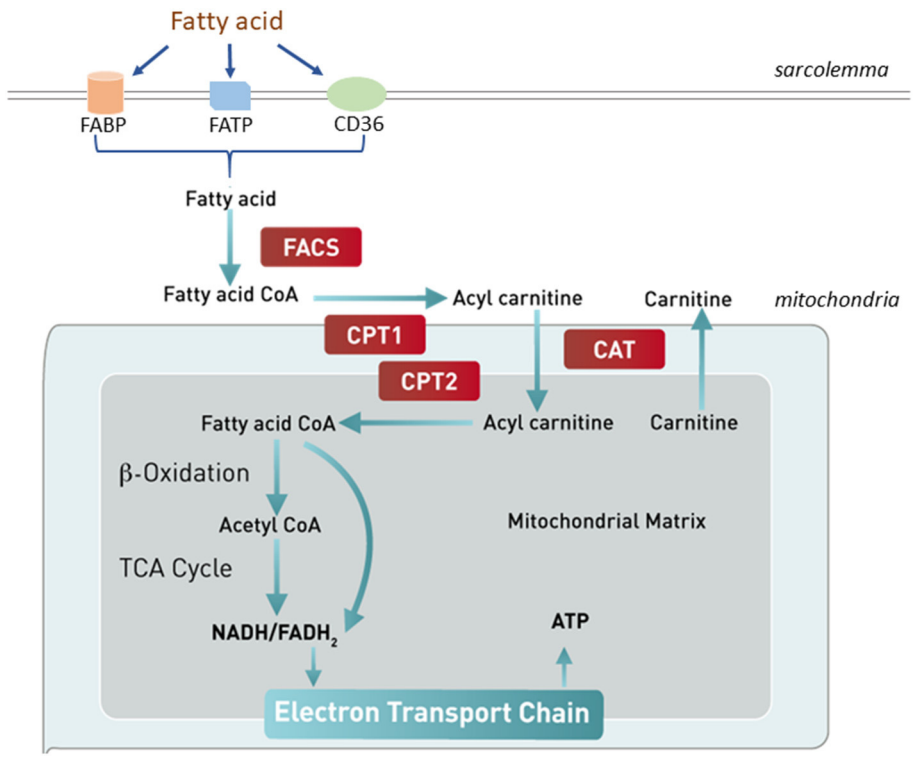

FIGURE 2 | Fatty acid uptake and oxidation. Free fatty acids in circulation enter the cardiomyocytes through either passive diffusion or a carrier protein-facilitated pathway, which involves fatty acid binding protein (FABP), fatty acid translocase (CD36/FAT), or fatty acid transporter protein (FATP). Cytosolic fatty acids are esterified to become fatty acyl CoA by fatty acyl CoA synthase (FACS). The acyl group of fatty acyl-CoA is transferred to carnitine to form acylcarnitine by carnitine palmitoyltransferase 1 (CPT1). The acylcarnitine enters the mitochondrial inner matrix *via* the carnitine shuttle and is converted to fatty acyl CoA by CPT2. The fatty acyl CoA goes through several cycles of β-oxidation producing NADH, $FADH_2$, and acetyl CoA, which enters the TCA cycle for ATP production. CAT, carnitine translocase.

and fatty acids for oxidation. In cardiomyocytes, the majority of glucose is metabolized through glycolysis, which produces pyruvate and ATP. Pyruvate can be reduced to lactate by lactate dehydrogenase in cytosol or oxidized to acetyl-CoA by pyruvate dehydrogenase to fuel the TCA cycle in mitochondria (10) (**Figure 3**). During hypertrophied growth and remodeling, FAO

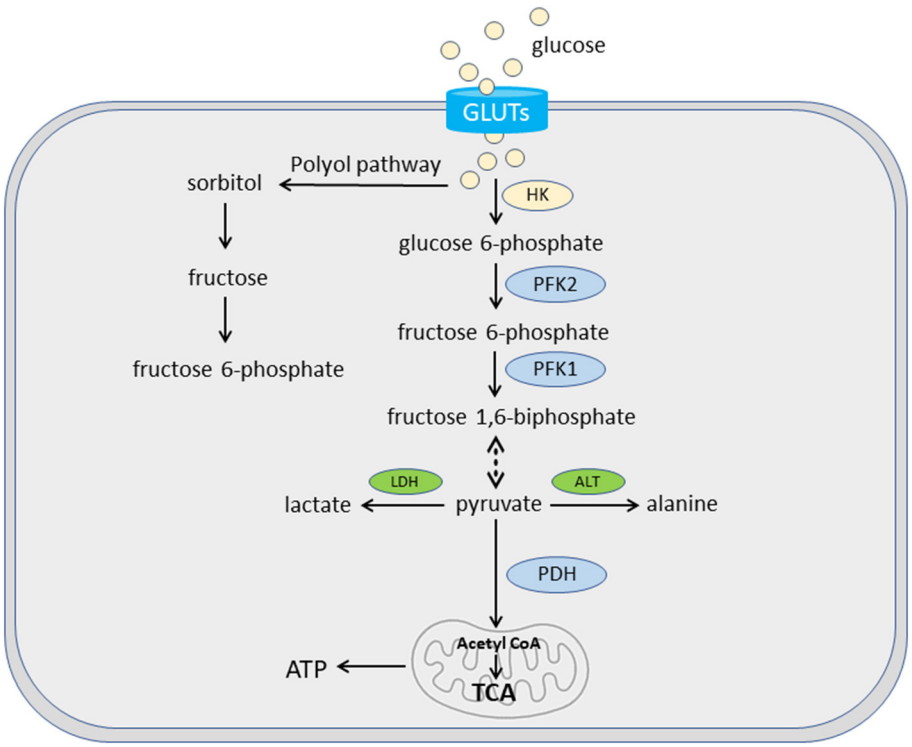

FIGURE 3 | Glucose uptake and oxidation. Glycolysis plays an important role in cardiac bioenergetics. In cardiomyocytes, glucose is transported *via* glucose transporters (GLUTs). Some glucose is converted to fructose and then fructose 6-phosphate for glycolysis *via* polyol pathway. The majority of glucose goes through a series of enzymatic reactions of glycolysis to be converted to pyruvate. These enzymes include hexokinase (HK) for glucose 6-phosphate, phosphofructokinase 2 (PFK2) for fructose 6-phosphate, and PFK1 for fructose 1,6-biphosphate. Pyruvate can be reduced to lactate by lactate dehydrogenase (LDH) in cytosol or oxidized to acetyl-CoA by pyruvate dehydrogenase (PDH) in the mitochondria. A small amount of pyruvate can be converted into alanine by alanine transaminase (ALT).

is decreased with a concomitant increase in glucose utilization (6, 10). However, decreased glucose oxidation was also reported in the development of HF (50). Glucose enters mammalian cells *via* facilitated diffusion, a process regulated by transmembrane glucose transporters (GLUTs) (51). Both GLUT1 and GLUT4 have a well-established role in myocardium. GLUT1 is abundant in the fetal heart whereas GLUT4 is the predominant isoform in the adult heart (10). In dogs with preclinical MMVD, transcriptional changes in GLUT3 and GLUT6 were reported: increased expression of GLUT3 was observed in both the LV and MV, while GLUT6 expression was upregulated in the MV (48). No change was found in either GLUT1 or GLUT4. One possibility is that dogs use different GLUT isoforms than humans or rodents. Interspecies expression difference in GLUT was reported. For example, human β-cells predominantly express GLUT1 while its expression of GLUT2 is 100-fold lower than in rat β-cells (52). Nevertheless, the study did not rule out the possible involvement of other GLUT isoforms in myocardial glucose utilization in dogs. GLUT3, a high-affinity GLUT isoform and a major glucose transporter for the brain, is also present in human adult and fetal myocardium (53, 54). GLUT6 knockout mutant mice show little metabolic effect (55), suggesting a redundant role of GLUT6 in the murine heart. In an untargeted serum metabolomics study, circulating glucose concentration was lower while lactate level was higher in preclinical MMVD dogs vs. non-MMVD dogs (48). The data supported the hypothesis of increased glucose utilization in dogs with MMVD.

Ketone Utilization

Acetoacetate and β-hydroxybutyrate (BHB) are the two main forms of ketone bodies. Under normal, non-fasting conditions, ketones contribute little to myocardium energy metabolism. Recently, emerging evidence demonstrates the importance of ketones as an alternate fuel source for the failing heart (7, 11, 12, 14). Ketone bodies are mainly produced in the liver cells from circulating fatty acids (**Figure 4**) (56). After a series of enzymatic reactions, two molecules of acetyl-CoA are converted to one molecule of acetoacetate, which is further reduced to BHB by β-hydroxybutyrate dehydrogenase 1 (BDH1) in the mitochondria. These ketone bodies reach other tissues *via* circulation and are taken up by other organs by monocarboxylate transporters. In cardiomyocytes, BHB is oxidized to be reconverted into acetoacetate by BDH1, a key enzyme for ketone utilization. Acetoacetate is activated by succinyl-CoA:3 ketoacid-CoA transferase (SCOT), the rate-limiting enzyme of ketolysis, to become acetoacetyl-CoA, which undergoes a final round of thiolysis to produce 2 molecules of acetyl-CoA. Acetyl-CoA enters the TCA cycle to fuel energy production.

In humans, blood ketone bodies are elevated in patients with CHF, and are inversely correlated with LV ejection fraction

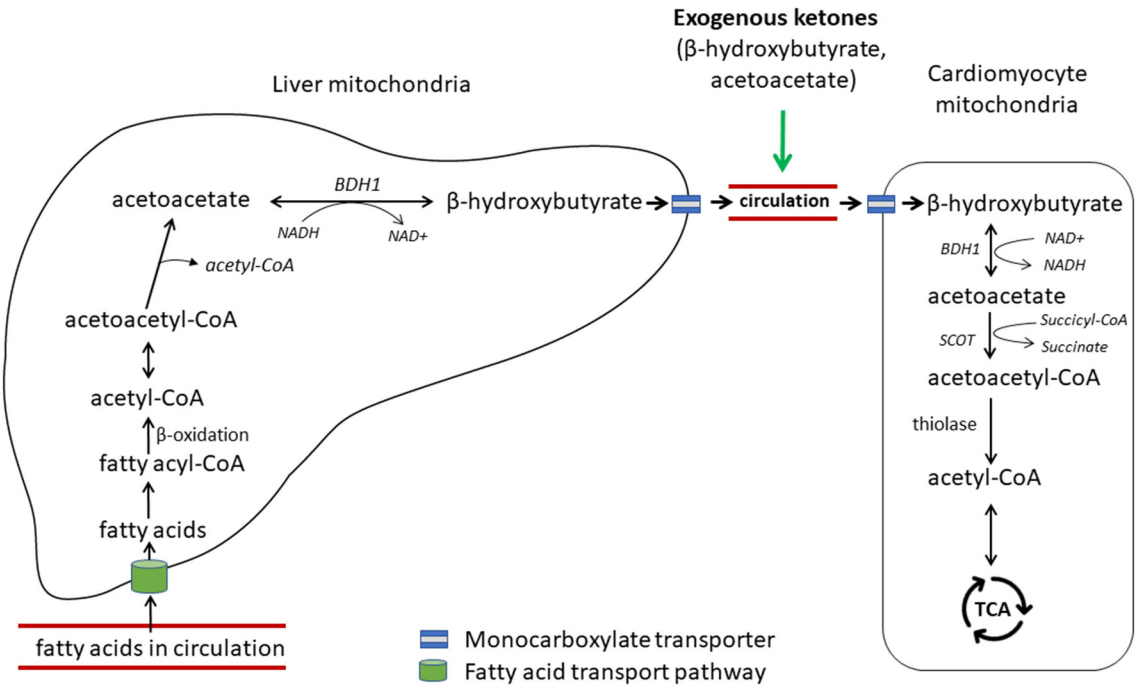

FIGURE 4 | Hepatic ketogenesis and cardiac ketolysis. Free fatty acids in circulation are transported into the liver *via* a carrier protein-assisted transport pathway. These fatty acids undergo fatty acid oxidation in the hepatic mitochondria to produce acetyl-CoA, which after a series of enzymatic reactions produces ketone bodies, acetoacetate and β-hydroxybutyrate. Ketones are transported to the heart and other extrahepatic organs *via* blood portal by the monocarboxylate transporters 1 and 1 (MCT1/2). β-hydroxybutyrate is converted to acetoacetate by β-hydroxybutyrate dehydrogenase 1 (BDH1), one of the key enzymes for ketone utilization. Acetoacetate is activated to acetoacetyl-CoA by succinyl-CoA:3 ketoacid-CoA transferase (SCOT), the rate-limiting enzyme for ketolysis. Acetoacetyl-CoA undergoes a final thiolysis reaction to be separated into 2 molecules of acetyl-CoA. Adapted from Selvaraj et al. (7).

(57, 58). The gene expressions of myocardial BHD1 and SCOT were upregulated in human HF patients compared with non-HF controls (12). In a well-defined mouse model of HF, the expression of BHD1 protein was increased in the hypertrophied and failing heart, and ketone oxidation was increased in the context of reduced FAO (11). More recently, Horton and colleagues demonstrated that the shift to ketone utilization in the failing heart is adaptive, and that BHD1-deficient mice unable to utilize ketones in the heart resulted in worsened HF in response to insults (14). Additionally, mice with increased delivery of ketone bodies by a ketogenic diet or direct ketone infusion to the heart ameliorated pathological cardiac remodeling and dysfunction. These authors further demonstrated ketone bodies as a metabolic stress defense, rendering protective effects on pathologic cardiac remodeling and dysfunction in dogs. Although still in its early development (59), therapeutic ketosis to treat HF starts to gain considerable attention (7, 60).

Similar to human HF patients, the levels of circulating BHB and acetoacetate are also increased in dogs with preclinical MMVD as well as those with CHF compared with healthy dogs (22). Increases in circulating ketone bodies may be the result of a compensatory increase in ketone production in the liver or a decrease in myocardium ketone utilization or both. To date, numerous myocardial and heart valve gene expression studies have been reported in dogs with MMVD (48, 61–63). The gene expression of SCOT was downregulated in the MV in dogs with preclinical MMVD (48). No myocardial expressional change in BDH1 or SCOT gene or protein has been reported. One possibility is that the myocardium and heart valve adapt to different energy substrates in the early phase of MMVD pathogenesis: while the myocardium can metabolize both glucose and ketones for fuel, the heart valve relies primarily on glucose. It would be interesting to test the hypothesis on more MV and myocardial samples.

OXPHOS in Mitochondria

Mitochondria, the "powerhouse" of the cell, supplies 95% of energy to cardiomyocytes (16, 45). The catabolic products of fatty acids, glucose, ketone bodies, and amino acids are used to fuel the TCA cycle to generate energy substrates, which enter the electron transport chain (ETC) for OXPHOS. Electron transport induces proton pumping from the inner mitochondrial matrix to the mitochondrial intermembrane space, a process that generates the membrane potential for ATP production (64, 65). The levels of circulating citrate and aconitate, both of which are TCA cycle intermediates, are increased in canine MMVD (22). Accumulation of these intermediates in circulation may signify impaired or inefficient TCA cycle. In addition, the concentration of inorganic phosphate (Pi) is elevated in circulation in proportion to the severity of MMVD in dogs (22). Pi is an important regulator of cytosolic ATP production (66, 67). An *in vitro* study demonstrated that Pi plays a

complex regulatory role in multiple sites of OXPHOS, including electron flow, generation of reduced forms of nicotinamide adenine dinucleotide (NAD+), distribution of energy flow in the cytochrome chain, as well as serving as the primary substrate for the ATPase to produce ATP in the cardiac mitochondria (68). It is likely that the increased Pi level in MMVD dogs is a part of the cytosolic feedback signaling to preserve energy metabolism homeostasis in the energy-deprived failing heart.

Creatine Kinase Shuttle and Energy Transfer to Myofibrils

The high-energy phosphate bond in ATP is transferred to creatine by the mitochondrial creatine kinase to generate phosphocreatine (PCr) and ADP. PCr, a molecule smaller than ATP, can rapidly diffuse from mitochondria to myofibrils, where myocardial creatine kinase catalyzes the reconversion from PCr to ATP and release free creatine. Free creatine is recycled in the mitochondria. In mammals, the majority of creatine is obtained from the diet or biosynthesized in the liver and kidneys and is taken up by the heart from circulation against a large concentration gradient using the specific creatine transporter (**Figure 1**) (69, 70).

In HF, the total myocardial creatine pool size is consistently decreased regardless of species or etiology, possibly due to reduced sarcolemmal creatine uptake (71, 72). Total creatine kinase as well as mitochondrial creatine kinase activity are also reduced in human HF patients and animal models of HF (43). However, the causal relationship between the impaired creatine kinase shuttle pathway and reduced myocardial ATP levels has not yet been established. In dogs with MMVD, the concentrations of circulating creatine are increased as the disease advances (22). Notably, the level of circulating creatine in dogs with stage B1 MMVD is higher than that of healthy dogs. Because the degradation of creatine to creatinine is a slow unregulated process, creatine levels are determined by creatine transporter activity (43). One hypothesis is that increased serum creatine levels are likely the result of reduced sarcolemmal creatine transporter activities, and that myocardial creatine level is decreased at the very early stage of MMVD. This observation is consistent with the hypothesis that cardiac energy deficiency has already begun in the early preclinical stage of canine MMVD. Moderate augmentation of creatine kinase activity to increase creatine and PCr levels in myocardium through pharmaceutical or nutritional intervention has been considered as an attractive strategy (73, 74). However, caution should be taken because massive increases in the creatine transporter function can have detrimental effects (75).

AMINO ACID METABOLISMS

Glutathione

Oxidative stress is an imbalanced state between generation and elimination of reactive oxygen species (ROS). Increased production and decreased removal of ROS play a causal role in the pathophysiology of HF (76). In the heart, mitochondria function as a redox hub (77). Superoxide (O_2^-) is generated in the ETC but is quickly converted to oxygen (O_2) and hydrogen peroxide (H_2O_2) by superoxide dismutase. Free H_2O_2 is further reduced to water by glutathione peroxidase, consuming two molecules of reduced glutathione (GSH) and generating 1 molecule of oxidized glutathione (GSSG): $2GSH + H_2O_2 \rightarrow GSSG + 2H_2O$. Glutathione peroxidase activity is inversely correlated to the risk of coronary artery disease (78).

Glutathione, a tripeptide of glutamine, cysteine, and glycine, determines intracellular redox state (79). Systemic glutathione relates to HF progression and cardiac remodeling. Myocardial and circulating glutathione levels are depleted in cardiac patients compared with healthy controls (80, 81). In dogs with CHF, the plasma ratio of reduced to oxidized glutathione (GSH:GSSG) is significantly lower than that of healthy controls (82). Circulating GSSG is also higher in dogs with preclinical MMVD compared with healthy dogs (48). For many mammals including humans and dogs, methionine is an essential amino acid that must be supplied through diets, while glycine is a conditionally essential amino acid that cannot not be sufficiently synthesized endogenously and has to be supplemented *via* diets. Methionine serves as the precursor for cysteine, taurine, and carnitine biosynthesis. The concentration of circulating methionine is lower in dogs with preclinical MMVD and CHF compared with healthy dogs (22, 48). Seral concentrations of glycine and glutamine are also reduced in MMVD vs. healthy dogs (22). The key determinants of GSH synthesis are the availability of cysteine and the activity of the rate-limiting enzyme, glutamate cysteine ligase. Decreased methionine, glycine, and glutamine in circulation may signify reduced myocardial GSH biosynthesis in dogs with MMVD.

Carnitine, Deoxycarnitine, and Acylcarnitines

L-carnitine plays an important role in fatty acid metabolism and oxidation and is concentrated in the skeletal and cardiac muscles. Myocardium can synthesize deoxycarnitine, an immediate precursor of L-carnitine, but lacks the hydroxylase that catalyzes the final conversion from deoxycarnitine to carnitine (83, 84). In mammals, L-carnitine is synthesized from lysine and methionine in the liver, brain, and in human kidneys. There is a bidirectional exchange between carnitine and deoxycarnitine across cardiac sarcolemma: the heart uses its deoxycarnitine to exchange for L-carnitine from the blood stream (83). In human patients with dilated cardiomyopathy (DCM) and CHF, total and free myocardial carnitine levels, and carnitine palmitoyl-transferase (CPT) activities are significantly lower, while plasma total and free carnitine concentrations are higher when compared with healthy controls (85–87). In dogs, myocardial carnitine deficiency was first associated with a family of dogs with DCM (88). Reduced myocardial carnitine and increased plasma carnitine concentration were reported in pacing-induced CHF in adult mongrel dogs (89). Circulating deoxycarnitine is lower in dogs with preclinical MMVD than healthy dogs (48), while total and free carnitine levels are increased in proportion to the severity of MMVD (22, 28). It is possible that the myocardium's ability to synthesize deoxycarnitine is impaired in dogs with MMVD and that its ability to exchange carnitine from the

blood stream is compromised, resulting in reduced myocardial carnitine uptake and increased levels of circulating carnitine. Nevertheless, the causal relationship between carnitine deficiency and cardiac disease in dogs has not been established. The benefit of carnitine supplementation in canine heart disease remains observational (90, 91).

Acylcarnitines are intermediates of FAO. Accumulation in acylcarnitines in the blood signifies disorders in mitochondrial or peroxisomal FAO (92, 93). Elevated levels of plasma long-chain (C14–C21), median-chain (C6–C13), and short-chain (C2–C5) acylcarnitines were documented in human HF patients (94–96). Accumulation of long-chain acylcarnitines in circulation is thought to contribute to the pathogenesis of HF by stimulating ROS production and releasing inflammatory mediators (95). Chen et al. showed that human patients with acute HF had higher plasma levels of acylcarnitines of all types, compared with normal controls (96). Improved FAO was associated with improved cardiac function along with substantial decreases in plasma long-chain and short-chain acylcarnitines (96). In dogs with MMVD, twenty-two long-chain, medium-chain, and short-chain acylcarnitines are accumulated in circulation in MMVD dogs vs. healthy dogs (22). Short-chain acylcarnitines are the degradation products of BCAAs, derived from muscular breakdown or gut microbiota metabolism. Accumulation of adipoylcarnitine (C6-DC), a dicarboxylcarnitine and several hydroxyl-acylcarnitines suggests activation of ω-FAO in peroxisome, which is a rescue pathway in response to impaired mitochondrial β-oxidation (97). Carnitine and acylcarnitines are positively correlated with one another, and with left atrial dimension in dogs (22). Remarkably, in a 6-month diet intervention study where improvements in left atrial enlargement and mitral regurgitation were observed in dogs with preclinical MMVD (18), three circulating acylcarnitines, oleoylcarnitine (C18), adipoylcarnitine (C6-DC), and margaroylcarnitine (C17), were decreased in dogs fed the intervention diet, while little change was observed in dogs fed the control diet (98). In the same study, the seral level of deoxycarnitine was increased in response to the diet intervention (98). The utility of free carnitine or carnitine esters as diagnostic or prognostic biomarkers for canine MMVD warrants further investigation.

Tryptophan Metabolism

Tryptophan (Trp) is another essential amino acid that must be acquired through diet in both humans and dogs (99, 100). In addition to protein synthesis, dietary Trp is metabolized by three pathways. The main kynurenine pathway *via* indoleamine 2,3-dioxygenase (IDO) and tryptophan 2,3-dioxygenase (TDO) leads to the production of important metabolites, such as kynurenine (Kyn), kynurenic acid (KA), quinolinic acid (QA), and eventually nicotinamide adenine dinucleotide (NAD+), and picolinic acid; the minor serotonin (5-HT) pathway *via* Trp hydroxylase (TPH), and the third the microbiota-dependent pathways to produce several key metabolites including ligands for the aryl hydrocarbon receptor (AhR)-mediated signaling, indole and its derivatives (**Figure 5**) (101–103). Human patients with cardiovascular disease and CHF often have accelerated Trp catabolism leading to lower circulating Trp levels and higher Kyn/Trp ratios compared with healthy individuals (104–108). In dogs with MMVD, although no change in Trp or Kyn is observed, the concentrations of QA are increased in MMVD dogs compared with healthy dogs, suggesting an upregulation in the Trp-Kyn pathway (22).

Upregulation of the Trp-Kyn pathway may also lead to increased production of NAD+, an essential cofactor for bioenergetics and an important coenzyme for FAO, glycolysis, TCA cycle, and ETC. In mammalian cells, the majority of NAD+ is produced by the salvage pathway that recycles nicotinamide and nicotinamide riboside to NAD+, while the remaining balance comes from *de novo* biosynthesis *via* the Trp-Kyn pathway, and the Preiss-Handler pathway using nicotinic acid (109). Seral concentration of nicotinamide, precursor for the salvage pathway, is decreased, while that of QA, a key intermediate of the *de novo* pathway, is increased in dogs with MMVD compared with healthy dogs (22). These results suggest that the main salvage pathway for NAD+ production may be compromised, while the *de novo* biosynthesis *via* the Trp-Kyn pathway is activated to rescue (22). However, QA and 3-hydroxykynurenine, both of which are Kyn metabolites with cytotoxicity, may directly interfere with mitochondrial function by AhR activation, and intensify the ROS production leading to mitochondrial impairment (110–112).

Trp is a substrate for TPH, a rate-limiting enzyme that hydroxylates Trp to form 5-HT, which enters the cells through serotonin transporter (SERT) (113). Serotonin has been associated with pathological remodeling in mature human heart valves (114–116). In particular, serotonergic 5-HT_2 receptors are implicated in heart disease (117, 118). The 5-HT signaling pathway has been linked to the pathogenesis of MMVD in dogs (39). Circulating 5-HT is increased in early stage MMVD but decreased as the disease progresses to end stage (38, 41). However, in the untargeted serum metabolomics studies comparing healthy dogs and dogs with different stages of MMVD, no difference in 5-HT was observed (22, 48). More than 95% of 5-HT in the body is produced in the gut. *Turicibacter sanguinis*, a spore-forming bacteria in the gut, signals intestinal enterochromaffin cells to produce 5-HT (102, 119). In a recent fecal microbiome study using the 16S rRNA gene sequencing, at the genus level the abundances of *Turicibacter* are reduced in dogs with MMVD compared with healthy dogs (120). However, the sequencing method did not provide enough resolution to identify the species of *Turicibacter*. The nature of the association requires further investigation.

BCAA in Heart Failure

Energy substrate readaptation is one of the hallmarks of the failing heart. While much attention has been focused on the regulatory mechanism and functional impacts of fatty acids and carbohydrates, the contribution of amino acid metabolism in the development of HF is largely understudied (25). In an early study, Peterson et al. demonstrated that myocardial free amino acids were increased in human HF (23). Several amino acids including BCAAs, were increased in circulation in a rat model of hypertension (121). Metabolomics and transcriptomics studies also revealed changes in BCAAs and key amino acid metabolic

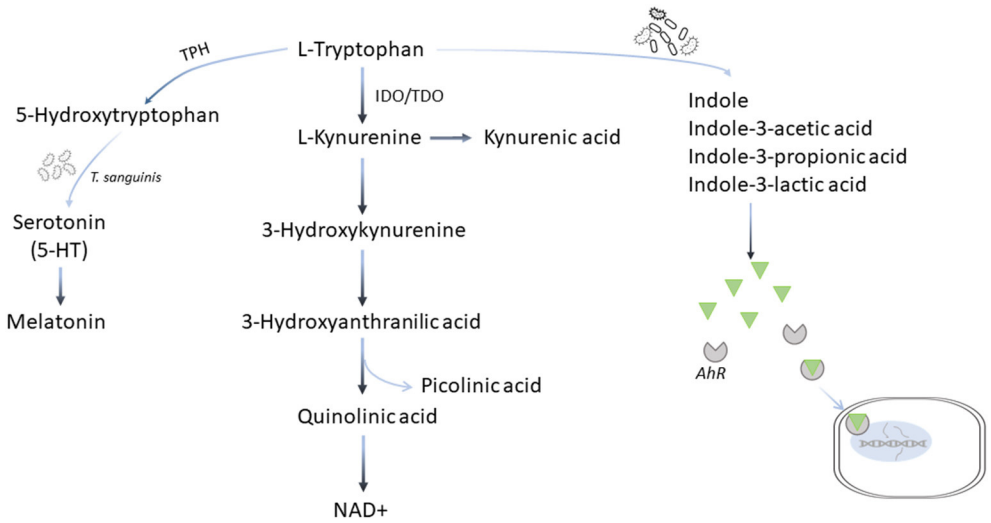

FIGURE 5 | Tryptophan metabolic pathways. Tryptophan is metabolized by three pathways. More than 90% of dietary tryptophan is metabolized *via* the main kynurenine pathway mediated by the indoleamine 2,3-dioxygenase (IDO) and tryptophan 2,3-deoxygenase (TDO) leading to the production of important metabolites, such as kynurenine, kynurenic acid, quinolinic acid, and eventually nicotinamide adenine dinucleotide (NAD+), and picolinic acid; the minor serotonin (5-HT) pathway *via* tryptophan hydroxylase (TPH), and the third the microbiota-dependent pathways to produce several key metabolites including ligands for the aryl hydrocarbon receptor (AhR)-mediated signaling, indole and its derivative.

pathways in murine models of HF (21, 122). Sun and colleagues reported that catabolic deficiency of BCAA, including leucine, isoleucine, and valine, is a metabolic hallmark for murine failing heart and human DCM (24). It was postulated that BCAA catabolic deficiency leads to accumulation of branched-chain alpha-keto acids, induces ROS, and activates mTOR (25). In dogs, a recent untargeted serum metabolomics study reported accumulations of numerous intermediates of BCAA metabolism in MMVD (22). The quantitative Metabolite Set Enrichment Analysis indicated enrichment of oxidation of BCAAs in MMVD dogs with CHF vs. healthy dogs (22). Circulating valine concentration was slightly lower in preclinical dogs with MMVD compared to healthy controls (48). The nature of the association between BCAA metabolism and canine heart disease, if any, warrants further investigation.

Taurine

Taurine is one of the sulfur-containing amino acids that is not incorporated into proteins but found to be in high concentrations in the heart and skeletal muscles (123). In mammals, the susceptibility to taurine deficiency varies by species: while taurine can be synthesized endogenously and is considered non-essential or conditionally-essential in humans, rodents, and dogs, it is essential for cats (91, 124–127). Taurine has been implicated in the maintenance of normal contractile function, modulation of myocardial calcium homeostasis, and potentially acts as an antioxidant and anti-inflammatory agent (123, 128). Schaffer et al. demonstrated that the taurine-deficient heart is associated with reduced ATP generation and is energy starved, possibly due to impaired mitochondrial respiratory chain activity, and NADH utilization (129). Although taurine deficiency causes reversible cardiomyopathy in cats (130), it does not play a significant role in the development of cardiomyopathy in dogs (91). Freeman et al. found no correlation between dietary and circulating taurine concentrations (131). A retrospective study on DCM in dogs suggested that taurine supplementation was not associated with survival or echocardiographic changes although the study did not rule out the possibility of a breed-specific role of taurine (132). In untargeted serum metabolomics studies, no difference in taurine concentration was found between healthy dogs and dogs with various stages of MMVD (22, 48).

GUT DYSBIOSIS AND MMVD

The human gastrointestinal tract is colonized with 10–100 trillions of typically non-pathogenic commensal microorganisms, collectively known as microbiota (133). These microorganisms encode >4 million non-redundant genes, which is more than 100 times of human genomes (134, 135). The additional pool of microbial genes aid in food digestion and absorption, xenobiotic metabolism, development of immune system (136), and contribute to the pathogenesis of metabolic disorders, including cardiovascular disease (29, 30). Since the establishment of an initial link between gut microbiota and cardiovascular disease (CVD), numerous gut microbiota-dependent metabolites and pathways, including the trimethylamine N-oxide (TMAO) pathway, short-chain fatty acid pathways, and bile acid pathways, have been implicated in the pathogenesis of CVD and HF (29, 30, 137–139). The gut hypothesis of HF postulates that impaired intestinal mucosal integrity in HF patients allows gut bacteria and their endotoxins to leak into circulation, and resultant chronic and low-grade systemic inflammation characteristic of HF (31, 138, 139).

TMAO, a diet-derived metabolite that is coproduced by microbiota and host, has been associated with cardiovascular diseases including HF (29, 30, 139). Dietary precursors, including L-carnitine, choline/phosphatidylcholine, and to a less degree, betaine, are converted to trimethylamine (TMA) by gut microbiota. TMA enters the portal circulation to be further oxidized to form TMAO by host hepatic enzymes known as flavin-containing monooxygenases (FMOs) (30, 139). Trimethyllysine, a methylated derived of amino acid lysine, is another source for the endogenous TMAO synthesis although less efficient than TMA (140). In dogs with preclinical MMVD and CHF, circulating TMAO as well as its nutritional precursors, L-carnitine, phosphatidylcholines and betaines, are increased compared with healthy controls (22, 28). A recent study analyzing fecal microbiome in healthy dogs and dogs with MMVD shows that gut microbial diversities are significantly different between healthy dogs and dogs with CHF (120). The dysbiosis index, which is measured using quantitative PCR on a panel of eight fecal bacterial groups, shows increases at the pre-clinical stages and becomes significantly higher in dogs with CHF when compared with healthy dogs. The study suggests that gut microbiota change has begun at the early preclinical MMVD. Significant differences in the abundance of *E. coli*, were found between dogs with MMVD vs. healthy dogs (120, 141). The *E. coli* genome shares 99% sequence identity with carnitine oxygenase (*cntA*), the key gene for TMA biosynthesis (142). It is possible that *E. coli* contributes to the increase in TMAO in MMVD dogs. However, the causal link between cardiovascular diseases and TMAO or its dietary precursors has yet to be determined. The abundance of *C. hiranonis*, a gut bacterium capable of converting primary bile acid to secondary bile acid, is inversely associated with dysbiosis index. Strikingly, the bile acid conversion was complete in dogs with high levels *C. hiranonis*, but incomplete in those without (120). The preliminary data indicate an interplay among host, gut microbiota, and signaling pathways mediated by the gut microbe-dependent metabolites in MMVD in dogs.

NUTRITION INTERVENTION TO ADDRESS METABOLIC CHANGES

Nutrition plays an important role in heart health (143). Sodium restriction has been recommended to human patients with HF due to its ability to lower blood pressure and prevent hypertension (144). However, multiple randomized controlled studies in humans demonstrated that sodium restrictions activate renin-angiotensin-aldosterone system (RAAS) and increase insulin resistance. The existing evidence does not support a universal reduction in sodium intake in CVD patients (145, 146). Roles of caloric restriction, omega-3 PUFAs, taurine, carnitine, B vitamins, magnesium, potassium, coenzyme Q10, and antioxidants in human and canine CVD have also been extensively discussed (90, 131, 143, 147–150), but most of the benefits remain observational or from case reports. Rigorous randomized controlled studies are warranted. The use of low-sodium diets in dogs with HF is a common practice for veterinarians, but the advantages and disadvantages of sodium restriction on canine patients with CHF warrants further investigations (151). One concern is that the RAAS signaling has vasoconstrictor properties and is thought to contribute to renal injury. In one study, low salt diet induces RAAS, increases oxidative stress and attenuates nitric oxide bioavailability in the canine heart (152). In a double-blinded, crossover study, 18 dogs with HF were randomized into either a low-sodium diet or a moderate-sodium diet for 4 weeks. Among the dogs that completed the study, maximal left ventricular size showed a marginal decrease on the low-sodium diet ($P = 0.05$) (151). The same research group followed up with a 4-week randomized placebo-controlled study to test the efficacy of a moderately reduced sodium diet enriched with omega-3 PUFAs, carnitine, taurine, arginine, and several antioxidants in 29 dogs with asymptomatic preclinical MMVD (153). The report didn't state whether the study was blinded or not. Dogs fed the test diet had significant reductions in maximal left atrial diameter (both weight-based and non-weight-based) and left ventricular internal dimension in diastole (non-weight-based) compared with the placebo controls (153). In recent years, the systems biology approach has been increasingly used to probe the molecular and metabolic pathways underlying cardiovascular diseases and to generate testable hypotheses to address those changes (48, 154). A cardiac protection blend of nutrients (CPB), including medium-chain triglycerides, fish oil, amino acids taurine, methionine, lysine, magnesium, and vitamin E, was designed based on the results of a multi-omics study on canine MMVD (18, 48). In a 6-month, single-blinded, randomized, placebo-controlled dietary intervention study, Li et al. tested the clinical efficacy of CPB on preclinical dogs with MMVD (18). Dogs supplemented with CPB had significant reductions in left atrial diameter, left atrial to aortic root ratio, and the severity of mitral regurgitation when compared with dogs fed the placebo diet. Notably, several dogs in the placebo group advanced from B1 stage to B2 stage at 6 months, while no dog in the CPB group progressed from B1 to B2 ($P < 0.05$). Untargeted metabolomics study using the serum samples from these dogs supported the hypothesis that CPB improves energy metabolism and reduces inflammation and oxidative stress (98). Large studies with more dogs from different breeds should be conducted to confirm the results. Several micronutrients are essential for mitochondrial health, energy metabolism and production. In the TCA cycle, vitamin B1 (thiamine) is part of pyruvate dehydrogenase complex for the conversion from pyruvate to acetyl-CoA, vitamin B5 (pantothenic acid) is a precursor for coenzyme A biosynthesis, and vitamin B12 (cobalamin) is a cofactor for succinyl-CoA formation. Some nutrients are also crucial for the activities of the ETC complex: vitamin B3 (niacin) is a precursor of NAD+ biosynthesis, vitamin B2 (riboflavin) is a building block for ETC complex I and II, and coenzyme Q10 and taurine are associated with the activities of ETC complex. Amino acid metabolic readaptation in the failing heart provides additional opportunities for nutrition intervention. The level of methionine, an essential amino acid, is lower in dogs with MMVD than control dogs. The catabolism of glucogenic and ketogenic amino acids can generate glucose, ketones and other

energy substrates important for energy homeostasis. Finally, supplementation of certain prebiotic fibers can be used to reduce uremic toxins including TMAO and restore gut symbiosis, and provides an alternative therapeutic option for canine heart patients.

Many nutrition intervention studies were designed to test combinations of nutrients, which can perform better than individual supplements (155, 156). *In vitro or in vivo* models may be used to understand the roles each nutrient plays or how they interact. These models can also be used to screen for nutrients or combination of nutrients for synergistic effects before clinical testing.

CONCLUDING REMARKS

Recent advances in systems biology and high-throughput multi-omics technologies make it possible to explore molecular and metabolic changes at the systems level in canine MMVD and HF. Some of the cellular and metabolic pathways are excellent targets for nutritional or pharmaceutical interventions. As our knowledge in systems biology and nutrition science continues to grow and with the new technologies and diagnostics available, there will exist significant opportunities to deliver breakthrough nutritional interventions to support dogs with MMVD and other cardiac diseases.

AUTHOR CONTRIBUTIONS

QL is responsible for the conception and writing of the manuscript.

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Differences in Metabolic Profiles of Healthy Dogs Fed a High-Fat vs. a High-Starch Diet

Yang Lyu[1], Daisy Liu[1], Patrick Nguyen[2], Iain Peters[3], Romy M. Heilmann[4], Veerle Fievez[5], Lieselot Y. Hemeryck[6†] and Myriam Hesta[1*†]*

[1] ECAN Equine and Companion Animal Nutrition, Faculty of Veterinary Medicine, Ghent University, Merelbeke, Belgium, [2] Nutrition, Physiopathology and Pharmacology Unit, National College of Veterinary Medicine, Food Science and Engineering, Nantes, France, [3] SYNLAB VPG, Exeter Science Park, Exeter, United Kingdom, [4] Gastrointestinal Laboratory, Department of Small Animal Clinical Sciences, College of Veterinary Medicine and Biomedical Sciences, Texas A&M University, College Station, TX, United States, [5] Department of Animal Sciences and Aquatic Ecology, Faculty of Bioscience Engineering, Ghent, Belgium, [6] Laboratory of Chemical Analysis, Faculty of Veterinary Medicine, Ghent University, Merelbeke, Belgium

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Edited by:
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South China Agricultural University, China

***Correspondence:**
Myriam Hesta
Myriam.Hesta@UGent.be
Lieselot Y. Hemeryck
LieselotY.Hemeryck@UGent.be

† These authors have contributed equally to this work and share senior authorship

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Obesity is a common problem in dogs and overconsumption of energy-rich foods is a key factor. This study compared the inflammatory response and fecal metabolome of dogs fed a high-fat vs. a high-starch diet. Ten healthy lean adult beagles were equally allocated into two groups in a cross-over design. Each group received two diets in which fat (horse fat) and starch (pregelatinized corn starch) were exchanged in an isocaloric way to compare high fat vs. high starch. There was a tendency to increase the glucose and glycine concentrations and the glucose/insulin ratio in the blood in dogs fed with the high-fat diet, whereas there was a decrease in the level of Non-esterified fatty acids and a tendency to decrease the alanine level in dogs fed with the high-starch diet. Untargeted analysis of the fecal metabolome revealed 10 annotated metabolites of interest, including L-methionine, which showed a higher abundance in dogs fed the high-starch diet. Five other metabolites were upregulated in dogs fed the high-fat diet, but could not be annotated. The obtained results indicate that a high-starch diet, compared to a high-fat diet, may promote lipid metabolism, anti-oxidative effects, protein biosynthesis and catabolism, mucosal barrier function, and immunomodulation in healthy lean dogs.

Keywords: starch, fat, fecal metabolome, obesity, dogs

INTRODUCTION

Obesity is one of the largest health challenges nowadays in dogs. Studies report a prevalence of canine overweight and obesity ranging from 34 to 60% (1–3). Obesity in dogs has been linked to not only a decreased vitality, emotional wellbeing, and longevity but also an increased risk of certain health issues such as insulin resistance, hypertension, cardiovascular disease, and osteoarthritis (4, 5). While multiple molecular mechanisms might link obesity to its complications, inflammation is a common feature that has been implicated in the pathophysiology of many obesity-associated disorders (6). Similarly to these findings in humans, it has been revealed that obese and overweight dogs showed a higher inflammatory state (7), as indicated by increased concentrations of serum interleukin-6 (IL-6) (7), C-reactive protein (CRP), and tumor necrosis factor alpha (TNF-α) (8).

The increase in adiposity is often attributed to high dietary fat intake (9). Many studies have shown that high-fat diets (>30% of energy from fat) can easily induce obesity in humans (10, 11), mice (12, 13), and dogs (14, 15). When the average amount of fat in the diet increases, the incidence of obesity also rises (11, 12). Furthermore, in mice and humans, consumption of high-fat diets leads to alterations in the composition and function of the gut microbiota, promoting metabolic endotoxemia and triggering an inflammatory response (13, 16). In dogs, feeding a high-fat diet is associated with insulin resistance (17), reduced brain insulin transport (14), decreased microbiota α-diversity (15), and reduced abundance of *Prevotella*, *Solobacterium*, and *Coprobacillus* (18). However, the majority of studies on the intake and possible adverse health effects of a high-fat diet in dogs focused on increased inflammation and alterations in the gut microbiome, but no study so far has investigated the effect of a high-fat diet on the gut metabolome.

Starch is the most abundant dietary nutrient globally, and provides energy to a rapidly growing human population (19), and is also a main nutritional source for pet dogs (20). However, this highly digestible energy source could also lead to nutrition-related health problems. Studies have suggested that typical starch-rich diets can also contribute to obesity (19, 21), hyperglycaemia (21), pathogenesis and difficulty of managing type 2 diabetes mellitus (22), as well as cardiovascular disease (23). In dogs, nutritional research on dietary starch mainly concerns its digestibility and fermentation, but not the effect of high-starch diets on metabolic changes and inflammatory responses.

Metabolomics is the study of all small molecules detectable in a biological sample. It provides information on subclinical metabolic alterations associated with (patho)physiological changes and disease outcomes (24). Metabolome analysis has revealed previously unknown alterations in amino acid, lipid, and carbohydrate metabolism across species, with underlying links to several conditions like obesity, inflammation, and oxidative stress (25). Metabolomics is rather emerging in canine nutrition, with current research being limited to study the metabolomic profile in the healthy vs. obese or overweight dogs (26), and in dogs fed with different protein levels (27), with far more pending to be explored. The present study aimed to compare the inflammatory status and fecal metabolome of lean dogs fed a high-fat vs. a high-starch diet, providing new insights and basis for a theoretical framework for high-fat vs. high-starch induced metabolic and inflammatory effects in relation to obesity.

MATERIALS AND METHODS

Animals and Experimental Design

Ten healthy adult research beagles of ideal body weight (BW) and condition (4 intact females, 3 intact males and 3 neutered males; 4.2 ± 2.6 y; 10.5 ± 1.2 kg; body condition score (BCS) 4–5/9) were equally allocated into two groups in a cross-over study design with two periods of 6 weeks each. All dogs were housed individually and under a 12-h light and 12-dark cycle with a room temperature of 17°C. Prior to the study, a commercial standard diet (Hill's Science Plan Advanced Fitness; Hill's Pet Nutrition, Inc., Topeka, KS, USA) mixed with experimental diets was fed to the dogs for 1 week adaption −75% standard with 25% experimental diets for 3 days, 50% of each for 2 days, and 75% experimental with 25% standard diets for 2 days. During the first period of the study (P1), five dogs in group A were fed a high-starch (HS; pregelatinized corn starch; ∼63.5% carbohydrate and 9.4% fat) diet and five dogs in group B were fed a high-fat (HF; horse fat; ∼12.9% carbohydrate and 46.9% fat) diet. After P1, a mixture of two experimental diets was fed to the dogs for one-week transition–group A: 75% HS with 25% HF diet for 3 days, 50% of each for 2 days, and 25% HS with 75% HF for 2 days; same proportion but reverse diets for group B. Experimental diets were then completely switched during the second period of the study (P2).

TABLE 1 | Formulation of the diets (g/kg).

Item	High Starch diet (HS)	High Fat diet (HF)
Horse hearts	701.5	827.6
Corn starch (pregelatinized)	287.6	0.0
Corn oil	3.9	4.6
Horse fat	1.4	161.4
Premix	5.6	6.4
KJ/100g Dry matter	1,811	2,579

Diets were formulated to be isonitrogenous on energy basis, so that for a given energy allocation the protein intake was similar regardless of the diet. The formulation of both diets is presented in **Table 1**. The HF and HS diet contained 18.2 g crude fat, 13.9 g crude protein, and 5.0 g nitrogen-free extract per MJ, and 5.2 g crude fat, 13.1 g crude protein, and 35.0 g nitrogen-free extract per MJ, respectively. Dogs were fed once a day at 10:00 a.m., and had free access to water. Body weight and BCS were assessed weekly. Food intake was recorded daily, and the amount was adjusted weekly to maintain a stable body weight, if needed.

All samples were collected at the end of each study period. Fasting blood samples (∼30 mL) were drawn from the jugular vein. An aliquot of ∼4 mL was collected in PAXgene Blood RNA tubes (PreAnalytiX GmbH, Erembodegem, Belgium) to analyse mRNA expression of TLR-4, CD14, IL-10, IL-18, IL-1B, IL-1RA, IL-8, and TNF-α. Serum and plasma for assessing mRNA expression, acylcarnitine and amino acid profiles were obtained by centrifuging blood at 2000 x g for 15 min at 4°C, which was stored at −20°C until analysis. Fresh fecal samples (∼10 g) were collected within 10 min after spontaneous defaecation. The samples were scored for fecal consistency (1 = watery liquid feces that can be poured; 2 = soft, unformed stool that assumes the shape of the recipient; 3 = soft, formed, moist stool; 4 = hard, formed, dry stool; 5 = hard, dry stool), and fecal pH was measured with a portable pH meter (Hanna Instruments Ltd., Temse, Belgium). An aliquot of ± 2 g was placed into a sterile plastic tube, frozen immediately on dry ice, lyophilized as soon as possible, and stored at −80°C in preparation of metabolomic

analysis. The remainder of the fecal sample was stored at −20°C for chemical analyses.

Analytical Methods

Body composition was determined by the deuterium dilution method using Fourier-transform infrared spectroscopy as described by (28).

Proximate analysis was performed on the diets using standard methods, ISO 1442:1997 for dry matter, ISO 936:1998 for crude ash, Kjeldahl nitrogen (6.25 × N, ISO 5983–1, 2005) for dietary crude protein, and ISO 5498:1981 for crude fiber. Nitrogen-free extract was calculated by subtracting crude ash, crude protein, crude fat, and crude fiber from the dry matter content. A Total Dietary Fiber Assay Kit (Sigma–Aldrich Co., Overijse, Belgium) was used to determine total dietary fiber and insoluble dietary fiber using procedures based on a combination of enzymatic and gravimetric methods (29). Soluble dietary fiber was calculated by subtracting insoluble dietary fiber from total dietary fiber.

Serum concentrations of glucose, triglyceride, cholesterol, and total protein were determined using the Architect C16000 analyser (Abbott Max-Planck-Ring, Wiesbaden, Germany). Fibrinogen concentration was determined using the Sysmex CS-5100 analyser (Siemens Healthcare Diagnostics Products GmbH, Marburg, Germany), insulin concentration was determined by a commercially available kit (INS-Irma, DIAsource ImmunoAssays S.A., Louvain-la-Neuve, Belgium), and the insulin-to-glucose ratio was calculated as described in German et al., (30) to assess insulin sensitivity. Serum leptin concentration was measured using a validated, commercially available canine ELISA kit (Millipore Corp., Billerica, MA, USA) following the manufacturer's instructions. Serum Non-esterified fatty acids (NEFA) concentrations were analyzed by spectrophotometry (EZ Read 400 Microplate Reader, Biochrom Ltd., Cambridge, United Kingdom). Free carnitine, acylcarnitine and amino acid profiles were determined on lithium-heparin plasma by quantitative electrospray tandem mass spectrometry as previously described (31, 32). Blood lipopolysaccharides (LPS) concentrations were determined using a kinetic turbidimetric Limulus amoebocyte lysate (LAL) assay.

S100A12 concentration in serum and feces was determined by a species-specific ELISA (33). Fecal short-chain fatty acid [SCFA; i.e., acetate, propionate, butyrate, iso-butyrate, iso-valerate], and NH_3 concentrations were determined first by extracting samples with 10% formic acid, containing 1 mg/ml 2-ethyl butyric acid as internal standard (3 g sample + 15 ml extraction fluid; shake for 5 min, centrifugate, and filtrate). The determination of respectively the volatile fatty acids and ammonia was carried out using gas chromatography as previously described (34, 35).

mRNA Expression

Total RNA was isolated from the PAXgene tubes using the PAXgene blood RNA kit (Qiagen, Manchester, UK) according to the manufacturer's instructions. RNA concentration was measured using the Qubit RNA Assay Kit (Invitrogen, Paisley, Scotland). Primers and probes for the assay were designed using Primer 3 (www.genome.wi.mit.edu/cgibin/primer/primer3_www.cgi.) and M-Fold using the canine specific GenBank sequence for IL-1β (EU249360) and IL-1α (AF216526) as described previously (36). The assays for the 3 housekeeper genes (succinate dehydrogenase complex, subunit A [SDHA], TATA box binding protein [TBP], and tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein zeta polypeptide [YWAZ]) and the remaining genes (**Supplementary Table 1**) were the same as those used previously (37).

Synthesis of cDNA was carried out using the ImProm II Reverse Transcription System (Promega Corporation) with 500 ng of total RNA in a final volume of 20 μL. Quantitative PCR (qPCR) was performed using GoTaq Colorless Master Mix (Promega). Gene specific amplification was performed using 0.2 μM of each primer, 0.1 μM of the probe, ROX (1:5000, Invitrogen) and 5 μl of diluted cDNA in a final volume of 25 μl. Sample incubations were performed in an MxPro 3005P (Agilent) at 95°C for 2 min and then 45 cycles of 95°C for 10 sec and 60°C for 30 sec during which the fluorescence data were collected. Threshold values (Ct) for the samples were calculated using the MxPro qPCR software 4.1 (Agilent Technologies Co., Santa Clara, CA, USA). Relative copy number expression values were calculated for each sample and normalized against the housekeeper gene results using the qBase applet for Microsoft Excel (http://medgen.ugent.be/qbase/) as described by Vandesompele et al. (38).

Untargeted Metabolomics Analysis

Freeze-dried fecal samples were subjected to generic extraction as optimized and described previously (39). Analysis of extracted fecal samples was performed using a Dionex UltiMate 3000 XRS UHPLC system (Thermo Fisher Scientific, San José, CA, USA) coupled to a Q-Exactive™ bench top Quadrupole-Orbitrap HRMS (Thermo Fisher Scientific, San José, CA, USA) (39, 40). Calibration of the Q-Exactive HRMS system was performed according to the instructions of the manufacturer. Internal (each 10 samples) and external QC (quality control) samples (pool of samples made from aliquots of the study samples) were analyzed prior to and after analysis of the samples to stabilize the system and monitor (and if needed, correct for) instrumental drift. Samples were analyzed in 1 batch, in a randomized order.

Raw data was Pre-processed using Sieve™ 2.1 (Thermo Fisher Scientific, San José, USA), as described by De Paepe et al., (40). Simca™ 13 (Umetrics AB, Umeå, Sweden) was used for multivariate statistical data processing. PCA-X and OPLS-DA modeling were performed following logarithmic data transformation and Pareto scaling, with further validation assessed by assessment of R2 and Q2 goodness (>0.5), permutation testing ($n = 100$) and cross-validated analysis of variance (CV-ANOVA, $p < 0.05$). Discriminative/predictive ions were selected based on their eccentric position in the S-plot [p (corrected) > |0.5|] and a Variable Importance in Projection-score (VIP score) >2. Prediction of chemical formula was based on accurate mass and the full scan spectrum using Xcalibur™; i.e., obtained through calculation and evaluation of isotopic signature (carbon and sulfur) and allowing a max mass deviation of 5 ppm. Putative identification was achieved using the Human Metabolome Database (HMDB), PubChem and Kyoto

Encyclopedia of Genes and Genomes (KEGG) databases (freely available online). A heatmap with dendrogram was generated using TBtools software (https://github.com/CJ-Chen/TBtools) to illustrate metabolite abundances and sample clustering.

Statistical Analyses

The metabolomic data were processed as described above. The effect of diets, periods and their interaction were analyzed by two-way ANOVA using MetaboAnalyst 3.0 software (McGill University, Canada).

To evaluate the effect of both the diet and the two-period dietary exchange, the remainder of the data was analyzed by Wilcoxon-Mann–Whitney test with diet, period and diet*period as factors and dog as random effect. These analyses were processed by R version 3.1.0 (The R Foundation for Statistical Computing) using the Coin package (version 1.0–23). Summary statistics were expressed as mean values ± SD. A p-value of < 0.05 was considered statistically significant and a p-value < 0.10 was considered as a significant trend. All p-values were corrected by the false discovery rate.

RESULTS

Food Intake and Body Composition

All dogs remained healthy throughout the study. All diets were well tolerated and did not affect the dogs' food intake. Daily energy intakes did not differ between diets. There was no significant diet and period effect on BW and BCS at the end of each study period. Furthermore, neither diet nor period significantly affected the dogs' absolute and relative body fat mass.

Fecal Parameters

There was no significant effect of diet and period on the fecal concentration of NH3, acetate, propionate, butyrate, iso-butyrate, and iso-valerate. The diets also did not affect the fecal S100A12 concentrations, and no significant effect was observed on the fecal score and pH. These results are summarized in **Supplementary Table 2**.

Blood Parameters

Significant findings in blood parameters are presented in **Table 2**, while blood parameters which did not significantly differ between diets or periods are available in **Supplementary Table 3**. A significant diet effect ($p = 0.041$) was observed for the Pre-prandial NEFA concentration, with dogs being fed the high-starch diet showing a higher level of NEFA than dogs being fed the high-fat diet. Diet × period trends were observed for the plasma concentration of glycine ($p = 0.077$) and tyrosine ($p = 0.058$). Additionally, significant trends according to diet were observed for the plasma concentration of glucose (GLU; $p = 0.054$), glycine (Gly; $p = 0.094$), alanine (Ala; $p = 0.089$), and the ratio of glucose to insulin (GLU/INS; $p = 0.063$). More specifically, dogs on the high-fat diet displayed a trend for a decreased Ala concentration and an increased GLU concentration and GLU/INS ratio compared to dogs on the high-starch diet. Significant effects of period were also observed for the GLU/INS ratio ($p = 0.036$) and NEFA concentration ($p = 0.042$).

No significant dietary effect or trend was observed for LPS concentration, acylcarnitine profiles and mRNA expression levels in the blood (**Supplementary Table 3**).

Fecal Metabolome

A total of 4391 and 1934 ions were obtained in the positive and negative ionization mode, respectively. PCA-X score plots revealed good clustering of fecal samples according to diet in both positive and negative ionization mode (**Supplementary Figure 1**), as well as good clustering of QC samples. The characteristics of the OPLS-DA model (**Supplementary Figure 2**) were good to excellent: $R^2Y = 0.884$ and $Q^2 = 0.661$ for the "positive" model and $R^2Y = 0.861$ and $Q^2 = 0.670$ for the "negative" model, also obtaining successful cross-validation (CV-ANOVA with $p < 0.01$), as well as a valid permutation test. A total of 15 fat/starch associated metabolites could be retained (**Supplementary Figure 3**), with 5 unidentified metabolites and 9 putatively annotated metabolites. The identity of one metabolite marker; i.e., L-methionine, was confirmed by means of an analytical standard. An overview of the (characteristics of the) retrieved discriminative metabolites is presented in **Table 3**.

The results obtained for investigating the interaction among diet, period and their interaction are summarized in **Supplementary Table 4**. All 15 metabolites were significantly influenced by the two diets, while none of them was significantly influenced by the study periods. One unidentified metabolite (Unidentified_1) was subject to the interaction between diet and period.

Normalized abundances of metabolites discriminating for the high-fat vs. high-starch diet are presented in a heatmap in **Figure 1**. Clear clustering of the samples according to diets was observed (except for one sample HF(P1)3). Moreover, dogs fed the high-fat diet displayed a significantly higher abundance of 5 Non-annotated molecules, whereas dogs fed the high-starch diet displayed a significantly higher abundance of L-methionine, 6 molecules tentatively identified as (iso)leucyl-threoninyl-valine; (iso)leucyl-(iso)valine or (iso)valyl-(iso)leucine; L-lysopine or (iso)leucyl-serine/seryl-(iso)leucine or valyl-threonine or threoninyl-valine; glycyl-valine or valyl-glycine or gly-norvaline or L-theanine or N-acetylornithine; valyl-valine; and (iso)leucyl-(iso)leucine, as well as 3 molecules putatively identified as spermic acid 2 or (iso)leucyl-threonine or threoninyl-(iso)leucine.

DISCUSSION

Most nutritional studies on the inflammatory response and metabolism in dogs have focused on the effects of protein (27, 42, 43). However, there is less information concerning dietary fat and nonfibrous carbohydrates, which are the main energy-delivering nutrients in traditional dog food (44). Health issues, particularly obesity-related problems, associated with diets rich in fat and starch, have received increasing attention in recent

TABLE 2 | Blood parameters (significant findings; $n = 5$).

Item	HS	HF	Period 1	Period 2	*p* value		
					Diet	Period	Diet*Period
GLU (μM)	78.40 ± 6.28	85.70 ± 5.21	83.10 ± 5.99	81.00 ± 7.60	0.054	0.324	0.134
GLU/INS	16.25 ± 5.54	46.46 ± 59.15	45.24 ± 59.59	17.48 ± 7.53	0.063	0.036	0.145
Gly (μM)	190.1 ± 24.12	186.9 ± 37.32	201.0 ± 33.47	176.0 ± 22.61	0.094	0.220	0.077
Ala (μM)	358.6 ± 118.5	239.5 ± 44.27	335.4 ± 129.1	262.8 ± 65.54	0.089	0.159	0.347
NEFA (μM)	1.13 ± 0.25	0.94 ± 0.23	1.13 ± 0.31	0.95 ± 0.15	0.041	0.042	0.108
Phe (μM)	85.11 ± 18.94	84.04 ± 16.98	80.64 ± 11.61	88.51 ± 21.88	0.139	0.077	0.120
Tyr (μM)	54.87 ± 8.93	51.45 ± 10.59	53.82 ± 8.63	52.49 ± 11.09	0.113	0.085	0.058

GLU, glucose; INS, insulin; Gly, glycine; Ala, alanine; NEFA, Non-esterified fatty acid; Phe, phenylalanine; Tyr, tyrosine.

TABLE 3 | Overview of characteristics of L-methionine, putatively annotated and unidentified metabolite markers.

Compound n°	Putative identification*	ID level	Formula	*m/z*	ppm	RT	Ionization mode	VIP score	Reference
1	Unidentified_1	–	–	223.1234	/	0.91	H^+	2.485	–
2	Unidentified_2	–	–	415.2172	/	0.97	H^+	2.373	–
3	L-Methionine	1	$C_5H_{11}NO_2S$	150.0580	2.04	1.57	H^+	2.996	HMDB
4	Glycyl-Valine/Glycyl-Norvaline/Valyl-Glycine/L-Theanine/N-acetylornithine	4	$C_7H_{14}N_2O_3$	173.0931	0.44	1.65	H^-	3.816	HMDB
5	Unidentified_3	–	–	356.1478	/	1.75	H^-	2.481	–
6	Spermic acid 2/(Iso)leucyl-threonine/ Threoninyl-(Iso)leucine	4	$C_{10}H_{20}N_2O_4$	231.1357	2.72	2.22	H^-	2.321	HMDB
7	L-Lysopine/(Iso)leucyl-serine/seryl-(Iso)leucine/Valyl-Threonine/Threoninyl-Valine	4	$C_9H_{18}N_2O_4$	219.1333	2.89	2.32	H^+	3.379	HMDB
8	Valyl-valine	4	$C_{10}H_{20}N_2O_3$	217.1542	2.34	4.24	H^+	3.160	HMDB
9	Spermic acid 2/(Iso)leucyl-threonine/ Threoninyl-(Iso)leucine	4	$C_{10}H_{20}N_2O_4$	231.1358	3.33	4.88	H^-	3.526	HMDB
10	Spermic acid 2/(Iso)leucyl-threonine/ Threoninyl-(Iso)leucine	4	$C_{10}H_{20}N_2O_4$	231.1357	3.03	5.16	H^-	4.124	HMDB
11	(Iso)leucyl-valine/Valyl-(Iso)leucine	4	$C_{11}H_{22}N_2O_3$	229.1566	3.47	5.54	H^-	3.426	HMDB
12	(Iso)leucyl-Threoninyl-Valine	4	$C_{15}H_{29}N_3O_5$	332.2173	2.01	6.88	H^+	3.430	PubChem
13	(Iso)leucyl-(Iso)leucine	4	$C_{12}H_{24}N_2O_3$	243.1722	3.39	6.98	H^-	3.553	HMDB
14	Unidentified_4	–	–	283.1200	/	7.12	H^-	3.096	–
15	Unidentified_5	–	–	263.0809	/	9.17	H^+	2.466	–

ID level, metabolite identification level according to Sumner et al. (41); ppm, absolute difference theoretical vs. detected m/z; RT, retention time (min.); VIP, variable importance in projection-score.

IUPAC names are provided in **Supplementary Table 5.*

canine studies (14, 18, 45). For the first time, the present study investigated the inflammatory response and fecal metabolome in healthy lean dogs fed a high-fat or a high-starch diet.

No Effect on Inflammatory Related mRNA Expression

Obesity is characterized as a state of low-grade systemic inflammation, in which many inflammatory cytokines appear to play a role. They might also be linked to co-morbidities of obesity (46). Recent evidence suggests that a high-fat diet (60% fat) can induce obesity and exacerbate obesity-related inflammation and metabolic disorders in mice (47, 48). Moreover, gut inflammation is more severe in obesity-prone rats compared to obesity-resistant rats when both were fed a high-fat diet (45.3% fat) (49). Inflammation was also selectively more marked in the short-term high-fat-fed mice (60% fat) (50), which might depend on the level of weight gain. However, no significant changes were noted in the inflammatory related mRNA expression in this study. Overall, this result is not surprising as the dogs were fed to keep an ideal body condition and body weight. In fact, in our study, the two diets were isoenergetically exchanged, with the high-fat diet not exceeding the safe upper limit for dogs (70% for adult maintenance) (51). Next to this, except for the species differences and susceptibility to obesity, as genetically modified mice are more prone to developing morbid obesity (52), the discrepancy may be ascribed to the study's limited experimental duration, tested parameters or sample types. The study was not designed to investigate potential long-term effects. Tissue samples could not be collected, or more invasive parameters tested (e.g., inflammatory markers in fat or intestinal mucosal) in the dogs due to ethical reasons. Also, differences in the fat source and different levels of dietary fat intake between dogs and mice

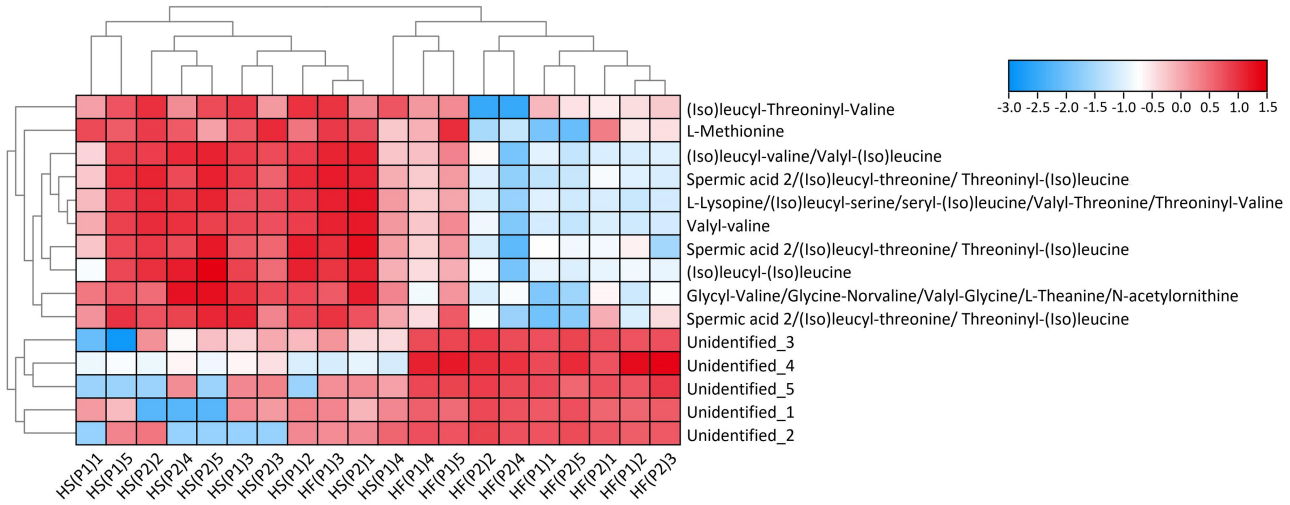

FIGURE 1 | Heatmap with cluster dendrogram of retrieved discriminative metabolites. Colors represent log-transformed peak intensity of metabolites. HF = high-fat diet, HS = high-starch diet, P1 = study period 1 and P2 = study period 2 for 5 dogs per group.

studies might also influence the experimental results. Therefore, future research investigations need to evaluate an extended set of inflammatory parameters under a variety of experimental conditions to unravel the inflammatory response of dogs fed a high-fat vs. a high-starch diet.

Observed Metabolic Effects in Blood

Vast differences were found in the metabolic profiles in blood our study. When dogs were fed with the high-fat diet, there is a tendency to increase glucose concentration and the ratio of glucose to insulin in the blood. This result suggests that the high-fat diet can affect glucose metabolism in healthy dogs. Previously, a high-starch diet (43% energy from starch and 26% from fat) has also been shown to increase glucose and insulin concentrations in healthy dogs compared to isoenergetic low- (12% energy from starch and 40% from fat) and moderate-starch diets (30% energy from starch and 34% from fat) (53). However, both fat and protein contents were adjusted to formulate the low- and moderate-starch diets, making it difficult to evaluate the effect of only fat in this study (53). Another study did not find any significantly changes in plasma glucose and insulin concentrations in dogs fed with a high-fat diet (63% energy from fat and 12% from starch) at two energetic intake levels (100% and 150% of maintenance energy requirements [MER]). Insulin sensitivity, however, was lower in the dogs of 150% MER group, which also had higher BW and BCS (15). The present study was not designed to analyse the effect on insulin sensitivity as only Pre-prandial samples were drawn. Thus, future research is needed to explore the possibility of such an effect. In the present study, the high-starch diet was associated with different metabolic effects compared to the high-fat diet in healthy lean dogs, as indicated by the increased NEFA concentration and a trend for increased Ala concentrations in the blood. Previous studies have reported that the increased Ala and NEFA levels are associated with increased glucose and lipid metabolism. Alanine is the key protein-derived gluconeogenic precursor, and plasma NEFA arise mainly from hydrolysis of triacylglycerol within the adipocyte (54, 55). This result suggests that compared to a high-fat diet, a high-starch intake could influence host glucose and lipid metabolism in healthy dogs. Future studies are needed to further investigate the metabolic effects of high-starch in exchange for high-fat diets.

Observed Metabolic Effects in Feces

Metabolomic research in dogs is still at a preliminary stage, with a limited number of published studies. Most of those studies focus on studying the metabolome in the context of disease or following dietary supplementation (56–59). However, the current study investigated shifts in the fecal metabolome of dogs on a high-starch vs. high-fat diet for the very first time. Polar metabolomics revealed a distinctively different fecal metabolomic profile in dogs fed a high starch vs. high fat diet. Specifically, the high-starch diet increased the abundance of L-methionine, and several molecules that were tentatively annotated as (iso)leucyl-threoninyl-valine, (Iso)leucyl-(Iso)valine/(Iso)valyl-(Iso)leucine, L-lysopine/(iso)leucyl-serine/seryl-(iso)leucine/ valyl-threonine/threoninyl-valine, valyl-valine, (iso)leucyl-(iso)leucine, glycyl-valine/valyl-glycine/gly-norvaline/L-theanine/N-acetylornithine, and spermic acid 2/(iso)leucyl-threonine/threoninyl-(iso)leucine.

Of the (putatively) annotated fecal metabolites, several are known to exert anti-oxidative and immunomodulatory effects. Methionine for example plays a critical role in the metabolism and health of many species, including dogs (60), as it is an essential amino acid involved in protein as well as aminoacyl-tRNA biosynthesis. Interestingly, there is accumulating evidence for aminoacyl-tRNA synthetases being involved in a wide range of physiological and pathological processes, including different types of immune responses (61). Recent research has furthermore

demonstrated that methionine intervenes in lipid metabolism and anti-oxidation (61, 62).

Based on accurate mass, we assume compound n°4 may either be a dipeptide, L-theanine or N-acetylornithine, although confident identification could not be achieved. L-theanine is an L-glutamate and L-glutamine analog. Interestingly, glutamine analogs are known to improve intestinal mucosal repair function (63), whilst recent studies furthermore reported that L-theanine supplement affects intestinal mucosal immunity by regulating SCFA metabolism in rats (64) and broilers (65). These studies implied that increased abundance of L-theanine in this study may have beneficial effects on intestinal reparation and immune. N-Acetylornithine is an intermediate in the enzymatic biosynthesis of the amino acid L-arginine from L-glutamate (66), which involves protein synthesis, anti-oxidative and immunomodulatory effects as well as e.g., improved mucosal barrier function (67).

Besides compound *n*° 4, eight other marker molecules (*n*° 6–13) were also putatively identified as di- or tripeptides. Di- or tripeptides are incomplete breakdown products of protein digestion or intermediates in protein catabolism (68).

For compounds n° 6, 9 and 10, alternative tentative annotations include spermic acid 2, and for compound n° 7, putative identification as L-lysopine is a possibility besides being a dipeptide. Spermic acid 2 is an metabolite of putrescine and spermine (https://contaminantdb.ca/contaminants/CHEM041097, ContaminantDB, McGill University, Canada), which are produced by the collective microbiome (69). Putrescine and spermine are required for several physiological functions including protein synthesis, cell growth and differentiation (70), and spermine is furthermore known to supress inflammation (71). Increased spermic acid levels in this study thus implied the improvement of these roles by the high-starch diet. Regarding L-lysopine, a marker molecule with the same accurate mass was previously detected and putatively annotated in healthy suckling piglets (72). More specifically, the tentatively annotated L-lysopine was downregulated ($p > 0.05$) in piglet plasma following supplementation with an additional 0.12% methionine in the basal diet of sows during late gestation and lactation. Methionine supplementation showed a positive effect on piglet growth performance, which was hypothesized to be due to an increased antioxidant capacity of the piglets. This does not align with the proposed anti-oxidative effects of the high-starch vs. high-fat diet in this study since the putatively annotated L-lysopine molecule was higher following the intake of the high-starch vs. high-fat diet. According to HMDB, L-lysopine originates from food (or feed) (73), but there is very limited knowledge on spermic acid 2 and L-lysopine either dogs or any other species. Therefore, these findings warrant further investigation.

Five metabolite markers were upregulated following feeding of the high-fat diet compared to the high-starch diet. For compound n° 14 ("Unidentified_4"), a potential match with karalicin was retrieved in the PubChem database. There is however no existing prior knowledge about this compound either in any species, although it has been observed that it can produced by certain bacteria (74). Another potential HMDB match for compound n° 14 is 2-Phenylethyl beta-D-glucopyranoside (C14H20O6). This compound has previously been detected in caraway and citrus (73), but it is unclear whether this compound could therefore also present in the dog feed. Due to these uncertainties, compound n° 14 was not annotated as either karalicin or 2-Phenylethyl beta-D-glucopyranoside. Overall, metabolite identification is a major bottleneck in metabolomics research (75), and even more so for studies in dogs since there is no existing dog metabolome database.

General Discussion

In summary, no difference was found in the inflammatory response in dogs fed with a high-starch vs. a high-fat diet, whereas different metabolic profiles were observed for the two diets. The high-starch diet in this study might be associated with several effects that indicated by the altered metabolic profiles, including protein biosynthesis, lipid metabolism, as well as exert anti-oxidation and/or immunomodulation. Future studies should encompass investigation of both short and long-term effects of high-starch in exchange for high-fat diets furthermore taking into account the source and level of fat intake. Moreover, in order to better understand the link between fecal metabolome and host metabolism, the analysis of microbiome is warranted in future studies, as well as multiple-omics analyses such as proteomics and lipidomics, which would enable studying the formation and/or degradation of proteins and the more a polar fraction of the metabolome. Lastly, it should be noted that Beagles in breeds are more prone to developing obesity (76) and therefore, follow-up studies should furthermore explore the effect of a high-fat vs. high-starch diet in relation to obesity and related problems, not only in Beagles, but also in other dog breeds, and in different age stages.

CONCLUSION

Inflammatory and metabolic responses of dogs fed a high-fat and high-starch diet were evaluated in the present study. The inflammatory response did not differ between the two diets. The high-starch diet was associated with increased blood NEFA level, a tendance for increased blood Ala level and showed a profound impact on the fecal metabolomic profile with alterations of the abundance of 15 fecal metabolites including methionine, the high-fat dietary intake was associated with a trend to for increase the glucose concentration, and the glucose/insulin ratio in the blood and significantly increase in the abundance of 5 other metabolites. These alterations might be linked to promotion of lipid metabolism, anti-oxidative effects, protein biosynthesis and catabolism, mucosal barrier function and immunomodulation in healthy dogs.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article can be found in online repositories (Figshare, doi: 10.6084/m9.figshare.17185376.v1).

ETHICS STATEMENT

The animal study was reviewed and approved by Ethical Committee of the Faculty of Veterinary Medicine, Ghent University, Belgium.

AUTHOR CONTRIBUTIONS

YL analyzed and interpreted the data and drafted the first version of the manuscript. DL performed the feeding trial and collected the samples. PN analyzed the proximate analysis. RH analyzed the S100A12 concentrations. VF analyzed fecal SCFA, BCFA, and NH3 concentrations. LH performed the metabolomic analysis and was a major contributor in writing the manuscript. MH was the project administration who conceived and designed the experiment. All authors read and approved the final manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fvets.2022.801863/full#supplementary-material

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Conflict of Interest: IP was employed by the company SYNLAB VPG.

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Effects of *Phragmites australis* Shoot Remainder Silage on Growth Performance, Blood Biochemical Parameters, and Rumen Microbiota of Beef Cattle

Qiye Wang[1,2], *Xianglin Zeng*[1], *Yutong Zeng*[1], *Xiaoruowei Liu*[1], *Yancan Wang*[1], *Xin Wang*[1], *Jianzhong Li*[1], *Yiqiang Wang*[3], *Zhi Feng*[3], *Pengfei Huang*[1], *Jia Yin*[1], *Jing Huang*[1], *Mingzhi Zhu*[4]* and *Huansheng Yang*[1,2]*

[1] Hunan Provincial Key Laboratory of Animal Intestinal Function and Regulation, Hunan International Joint Laboratory of Animal Intestinal Ecology and Health, Laboratory of Animal Nutrition and Human Health, College of Life Sciences, Hunan Normal University, Changsha, China, [2] Hunan Provincial Key Laboratory of Animal Nutritional Physiology and Metabolic Process, Key Laboratory of Agro-Ecological Processes in Subtropical Region, Hunan Provincial Engineering Research Center of Healthy Livestock, Scientific Observing and Experimental Station of Animal Nutrition and Feed Science in South-Central, Ministry of Agriculture, Institute of Subtropical Agriculture, Chinese Academy of Sciences, Changsha, China, [3] Key Lab of Non-wood Forest Nurturing and Protection of National Ministry of Education, Hunan Provincial Key Laboratory for Forestry Biotechnology, Central South University of Forestry and Technology, Changsha, China, [4] Key Laboratory of Tea Science of Ministry of Education, National Research Center of Engineering Technology for Utilization of Functional Ingredients From Botanicals, College of Horticulture, Hunan Agricultural University, Changsha, China

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***Correspondence:**
Mingzhi Zhu
mzzhucn@hotmail.com
Huansheng Yang
yhs@hunnu.edu.cn

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The objective of the present study was to assess the effects of replacing corn silage with *Phragmites australis* shoot remainder (PSR) silage on intake, growth performance, serum biochemical parameters, and rumen microbial diversity of growing-finishing beef. Fifteen Angus beef cattle with an average body weight of 253 ± 2.94 kg were randomly divided into three groups (five replicas vs. each group vs. Angus beef cattle). The three treatments were group A fed 60% PSR silage + 40% concentrate, group B fed 30% PSR silage + 30% corn silage + 40% concentrate, and group C fed 60% corn silage + 40% concentrate. The adaptation period was 15 days, and the trial period lasted for 45 days. Results showed that the ADG was significantly higher, and FCR was significantly lower both in groups A and B compared with group C. The results of serum biochemical parameters showed that the concentration of GLU was significantly lower in group B than both groups A and C. Microbial diversity results showed that the OTUs, Shannon, Chao1, and ACE indices were significantly lower in group A compared with groups B and C. At the phyla level, the relative abundances of *Tenericutes* and *Melainabacteria* had significant differences among the three groups, and the relative abundances of *Papillibacter*, *Anaeroplasma*, and *Anaerovorax* had significant differences among the three groups at the genus level. Additionally, *Rikenellaceae* was the unique biomarker among the three groups. Furthermore, the results of function prediction showed that the gene families associated with metabolism of cofactors and vitamins, cellular processes and signaling, metabolism, biosynthesis of other secondary metabolites, infectious diseases, signaling molecules and interaction, nervous system, and digestive system

were significantly decreased, while lipid metabolism was dramatically increased from groups A to C at KEGG level 2. At KEGG level 3, 11 metabolic pathways were significantly influenced among the three groups. In summary, these findings indicated that PSR silage substituted the corn silage totally or partially improved the growth performance, and altered the rumen microbial composition and diversity and the corresponding change in prediction function of rumen bacteria in Angus beef cattle.

Keywords: *Phragmites australis* feed, growth performance, rumen microbiota, rumen bacterial function, beef cattle

INTRODUCTION

Phragmites australis is a kind of native perennial grass, which is a very good non-competitive feed resource. *P. australis* has excellent nutritional value and a broad ecological distribution and adaptation in the world, and other characteristics (1). Therefore, rational utilization of *P. australis* resources is one of the effective methods to enlarge the feed source and relieve the shortage of roughage when ensuring that *P. australis* has rich nutrient content and high yield. According to statistics, China is rich in *P. australis* resources, and the distribution area is about 800,000 ha, among which Hunan has about 80,000 ha and with an annual output of up to 400,000 tons, mainly distributed in Dongting Lake and along the Yangtze River (2). *P. australis* feed has good palatability, which contains high crude protein and comprehensive mineral nutrition, and also contains a variety of amino acids and vitamins. In particular, the organic matter of starch, protein, and cellulose in *P. australis* feed degrades into monosaccharide, disaccharide, amino acid, and trace elements after fermentation, which makes the feed become soft, fragrant, and more palatable (1). Therefore, *P. australis* has the potential to be an important roughage for livestock. According to the determination (data from the American Feed Regulation Society NRC2-01-113), the dry matter of the stem and leaves of the young *P. australis* contained metabolizable energy 9.20 MJ/kg, crude protein 12.2%, crude fiber 26.8%, calcium 0.4%, and phosphorus 0.3%, which was higher than that of the common forage (3, 4). Existing studies have found that adding a certain amount of dried reed to the diet can accelerate the growth of livestock and improve the feed utilization rate (4).

Kadi et al. (5) reported that *P. australis* feed contained high N content, neutral detergent fiber (NDF), potassium, and magnesium. Tanaka et al. (1) investigated the timing of harvest and nutritive value of *P. australis* for ruminants in Lake Dianchi of China, which found that *P. australis* harvested in the early growing stage had relatively high concentrations of total digestible nutrients and demonstrated that *P. australis* can use a high-quality roughage for ruminants. Generally, there are three feed types of *P. australis* feed used in livestock: fresh, sun-dried, and ensiled. *P. australis* shoot remainder (PSR) is a by-product of processing *P. australis* shoots. By analyzing the nutrient composition of PSR, we detected that the crude protein and crude fiber contents reached 14.93 and 19.27%, respectively, which have high nutritional value, but barely have been utilized (unpublished data). While fresh PSR cannot be preserved for a long time, ensiled PSR is considered to be an effective long-term preservation method for beef cattle breeding. Different roughage may influence production performance and rumen microbial structure and function in ruminants (6–8). However, little research has indicated whether PSR can replace traditional feed ingredients in ruminants, especially affecting the rumen microbiota of beef cattle. Furthermore, effects of PSR silage on growth performance, blood biochemical indices, and rumen microbiota of beef cattle have no reports. Thus, the aim of this study was to explore the effect of PSR silage substitution for corn silage, totally or partially, on growth, serum biochemical indices, rumen microbial diversity, and predicted function in beef cattle.

MATERIALS AND METHODS

Animals, Treatments, and Experimental Procedures

Fifteen Angus beef cattle with an average initial body weight (IBW) of 253 ± 2.94 kg were chosen and randomly allotted to three experimental treatments consisting of three dietary levels of PSR silage (DM basis): 60% (group A), 30% (group B), and 0 (group C) as a substitute of corn silage, respectively. Experimental diets were composed of 60% of silage and 40% of concentrate (DM basis) and were formulated to meet nutritional requirements (9) of beef cattle, and feed ingredients, and the nutritional composition are shown in **Table 1**. Each bull was fed in individual pens with automatic drinking and free feeding intake, five pens per group. Before the trial, all bulls were weighed, dewormed, and vaccinated (foot and mouth disease vaccine and anthrax vaccine). Cattle were adapted to the diets for 15 days, and the experimental period lasted for 45 days.

Bulls were fed three times daily at 07:00, 12:00, and 17:00 h with total mixed diets. Residual feed was evaluated at 06:00 h each day to quantify and adjust daily feed allowance to a maximum of 5% residues. Feed samples were collected from each pen every 15 days and then composited, and were frozen at −20°C for nutritional ingredients analysis.

Growth Performance and Blood Biochemical Parameters

Each bull was weighed individually in the morning on an empty stomach at the beginning and end of the experiment. The ADG (average daily gain) was calculated by the weight gain per bull

TABLE 1 | Diet ingredients and nutrition levels.

Item	Groups		
	A	B	C
Ingredient, %			
PSR silage	60	30	0
Corn silage	0	30	60
Corn	16.00	16.00	16.00
Wheat bran	14.30	14.30	14.30
Soybean meal	7.20	7.20	7.20
NaCl	0.50	0.50	0.50
Premix[a]	2.00	2.00	2.00
Total	100	100	100
Nutrient levels[b]			
NE_{mf}, MJ /kg	7.99	8.04	8.07
Crude protein, %	14.39	13.30	12.22
Crude fat, %	2.89	2.84	2.79
Neutral-detergent fiber, %	33.85	34.76	35.66
Acid-detergent fiber, %	23.56	25.40	27.24
Ca, %	0.37	0.36	0.35
P, %	0.30	0.29	0.27

[a] *Premix provides the following per kg: vitamin A 160,000 IU, vitamin D3 22,000 IU, vitamin E 1,200 mg, Cu 380 mg, Fe 1,100 mg, Zn 1,900 mg, Mn 1,600 mg, I 20 mg, Se 5.8 mg, Co 2.5 mg.*

[b] *Except for NE_{mf}, which was the predicted value referring to the related formulas of Feeding Standard of Beef Cattle (NY/T 815-2004), the rest was measured value.*

divided by the trial days. The ADFI (average daily feed intake) was calculated by the amount of diet offered minus the residues per pen and then divided by the total trial days. The FCR (feed conversion ratio) was calculated as ADFI per ADG (10). At the end of the experiment, blood samples were collected in a 5-ml vacuum tube without anticoagulant (Changsha Yiqun Medical Equipment) from the caudal vein of each bull in the morning on an empty stomach. After standing for 2–3 h, blood samples were centrifuged by the model TG16-WS H1650 centrifuges (Hunan Xiangyi Laboratory Instrument Development Co. Ltd.) at 3,000 r/min for 10 min, and then the supernatant was separated and stored at −20°C for further analysis. A TBA-120FR automatic biochemistry analyzer (Toshiba Corporation) was used to measure the concentrations of serum biochemical parameters (11–13).

DNA Extraction and Amplification of 16S rRNA Genes

The total microbial genomic DNA was extracted using the CTAB/SDS method. The V3–V4 regions of 16S rRNA genes were amplified with forward primer V515F (5′-GTGYCAGCMGCCGCGGTAA-3′) and reverse primer V806R (5′-GGACTACHVGGGTWTCTAAT-3′). The PCR reactions were performed in 30-μl systems. For specific PCR amplified procedures, refer to Wang et al. (14, 15). The sequencing libraries were constructed by Ion Plus Fragment Library Kit 48 rxns (Thermo Scientific). The Ion S5^{TM} XL platform to sequence was further used, and 407- to 412-bp single-end reads were generated (14, 15).

Sequencing and Bioinformatics Analysis

The raw reads were cleaned by the Cutadapt quality control process (16). The UCHIME algorithm (17) was used to detect and remove the chimera sequences and finally to obtain the clean reads. Sequence analysis was performed by Uparse software (Uparse v7.0.1001) (18) to cluster the operational taxonomic units (OTUs) with ≥97% similarity. The Silva Database (19) was used to annotate taxonomic information and normalize the OTU abundant information. The alpha diversity and beta diversity were analyzed subsequently by QIIME (Version 1.7.0) and displayed by R Software (Version 2.15.3). Phylogenetic Investigation of Communities by Reconstruction of Unobserved States (PICRUSt) was utilized to predict the metabolic function of the microbiota. The raw sequencing data of this study were submitted to the Sequence Read Archive (SRA) with accession numbers SRR15662882–SRR15662896.

Statistical Analysis

The experimental data were analyzed on SPSS 22.0 software packages (SPSS, Chicago, IL, USA). Using the one-way ANOVA and *t*-tests to test the significance of growth performances and serum biochemical parameters, and the non-parameter test was performed to analyze the rumen microbial diversities, relative abundance, and function prediction. Final results were presented with meaning values. Differences were considered to have a tendency at $0.05 < p < 0.10$ and statistically significant at $p \leq 0.05$.

RESULTS

Growth Performance

The results of growth performance are shown in **Table 2**. There was no significant difference in IBW (initial body weight) and ADFI among treatments, although the FBW (final body weight) did not differ among treatments and was higher in groups A and B than in group C. The ADG was significantly greater (linear, $p = 0.032$) in groups A and B than in group C. Similarly, the FCR was significantly lower (linear, $p = 0.006$) in groups A and B than in group C. Notably, the ADG and FCR were not different between A and B treatments.

Serum Biochemical Parameters

The concentrations of serum TP, ALT, AST, TG, CHOL, HDL, LDLC, and NH3 did not differ among treatments. The concentration of serum GLU was quadratically affected (quadratic, $p = 0.004$) among treatments, and the concentration of BUN showed a linearly downward trend (linear, $p = 0.096$) among treatments (**Table 3**).

Rumen Bacterial Communities

The results of sequencing analysis are presented in **Figure 1**. The difference in bacterial composition among the three groups was analyzed by the PCoA, and the PCoA plots showed that the group A data had a tendency to be separated from both the B and C groups (**Figure 1A**). Similarly, there were no significant differences in diversity and uniformity by the level of species richness existing between B and C based on the observed species

TABLE 2 | Effects of PSR silage on growth performance of Angus beef cattle ($N = 5$).

Item[2]	Groups[1]			SEM[3]	*p*-value	
	A	B	C		Linear	Quadratic
Initial weight, kg	250.00	252.00	257.00	8.78	0.767	0.942
Final weight, kg	313.00	315.00	304.00	9.10	0.719	0.764
ADG, kg/day	1.40[a]	1.40[a]	1.04[b]	0.07	0.032	0.186
ADFI, kg/day	6.58	7.11	6.77	0.29	0.800	0.517
FCR	4.71[b]	5.09[b]	6.63[a]	0.33	0.006	0.269

Values within a row with different superscripts (a, b) differ significantly at $p < 0.05$.
[1]*A, fed 60% PSR silage; B, fed 30% PSR silage + 30% corn silage; C, fed 60% corn silage.*
[2]*ADG, average daily gain; ADMI, average daily feed intake; FCR, feed conversion rate.*
[3]*SEM, standard error of the mean; PSR, phragmites australis shoots remainder.*

TABLE 3 | Effects of PSR silage on serum biochemical parameters of Angus beef cattle ($N = 5$).

Item[2]	Groups[1]			SEM[3]	*p*-value	
	A	B	C		Linear	Quadratic
TP, g/L	70.28	69.10	73.02	1.05	0.300	0.267
GLU, mmol/L	3.76[a]	3.14[b]	4.06[a]	0.14	0.246	0.004
BUN, mmol/L	6.04	5.82	5.18	0.20	0.096	0.619
ALT, U/L	31.28	30.94	29.38	1.36	0.604	0.847
AST, U/L	82.40	82.40	67.20	5.19	0.256	0.505
TG, mmol/L	0.24	0.26	0.30	0.02	0.239	0.776
CHOL, mmol/L	2.26	2.46	2.15	0.11	0.672	0.297
HDL, mmol/L	2.11	2.25	1.93	0.10	0.486	0.313
HDLC, mmol/L	0.65	0.72	0.54	0.04	0.344	0.200
NH3, mmol/L	177.70	162.04	162.24	4.67	0.193	0.430

Values within a row with different superscripts (a, b) differ significantly at $p < 0.05$.
[1]*A, fed 60% PSR silage; B, fed 30% PSR silage + 30% corn silage; C, fed 60% corn silage.*
[2]*TP, total protein; GLU, glucose; BUN, blood urea nitrogen; ALT, alanine aminotransferase; AST, aspartate aminotransferase; TG, triglyceride; CHOL, cholesterol; HDL, high-density lipoprotein; LDLC, low-density lipoprotein–cholesterol; NH3, ammonia.*
[3]*SEM, standard error of the mean.*

(OTUs), Shannon, Chao1, and Ace index analyses (**Figure 1B**). Compared with both groups B and C, the A group had less OTUs ($p < 0.001$), Chao1 ($p < 0.001$), ACE ($p < 0.001$), and Shannon ($p < 0.01$) unexpectedly (**Figure 1B**).

A total of 22 bacterial phyla were identified by taxonomic analysis in the rumen samples. The relative abundance of more than 1% were *Bacteroidetes*, *Firmicutes*, *Proteobacteria*, and *Fibrobacteres* (**Figures 1C,D**). Notably, the relative abundance of *Bacteroidetes* and *Firmicutes* was the richest in the three trial groups (**Figures 1C,D**). Additionally, the relative abundance of *Tenericutes* ($p = 0.015$) and Melainabacteria ($p = 0.025$) was significantly lower in group A than that in group B and group C, respectively, at the phyla level (**Figure 1D**).

A total of 122 bacterial genera were detected at the genus level. Twenty representative genera were elucidated in all the rumen samples (**Figure 1E**). Among these genera, the relatively high abundance (>1%) *unidentified_Bacteroidales* belonged to *Bacteroidetes* in the phylum, *Fibrobacter* is one of *Fibrobacteres*, *unidentified_Ruminococcaceae*, and *unidentified_Lachnospiraceae* belong to *Clostridia* in members of *Firmicutes*. *Unidentified_Prevotellaceae* belongs to *Bacteroidetes*, *Succiniclasticum* is also a member of *Firmicutes*. The relative abundances of *Papillibacter* ($p = 0.008$), *Anaeroplasma* ($p = 0.017$), and *Anaerovorax* ($p = 0.043$) had significant differences among the three groups, and the relative abundance of *unidentified_Rikenellaceae* ($p = 0.088$) also had a notable change in group B in the genus level (**Figure 1F**). Additionally, LEfSe analysis results showed the dominant bacteria species for each group by LDA score, the *Prevotellaceae* and *Rikenellaceae* showed statistical differences and were considered as biomarkers between groups A and B, the *Prevotellaceae*, *Ruminococcaceae*, and *Rikenellaceae* were the biomarkers between groups A and C, and the *Rikenellaceae* was the unique biomarker among the three groups (**Figure 1G**). Furthermore, the phylogenetic tree of the top 100 genera was obtained through multisequence alignment, as shown in **Figure 2**, in which the phylogenetic relationship of rumen bacteria species at the genus level could be presented more intuitively among the three groups.

Predicted Metabolic Pathways and Functions of Rumen Bacterial Communities

Metabolic functions of rumen bacteria were predicted by PICRUSt in the present study (**Figure 2**). The result showed that "metabolism" was in the highest relative abundance with more than 49% of all sequence reads among three groups at KEGG level 1 (**Figure 3A**). At KEGG level 2, the most relatively abundant gene families (relative abundance > 0.10%) from all rumen samples are present in **Figure 3B**. Genes belonging to amino acid metabolism, carbohydrate metabolism, replication and repair, membrane transport, translation, and energy metabolism were the most relative abundant among the three groups (**Figure 3B**). Among these gene families, the genes associated with metabolism of cofactors and vitamins ($p = 0.028$), cellular processes and signaling ($p = 0.049$), metabolism ($p = 0.001$), biosynthesis of other secondary metabolites ($p = 0.024$), infectious diseases ($p = 0.006$), signaling molecules and interaction ($p = 0.013$), nervous system ($p = 0.041$), and digestive system ($p = 0.014$)

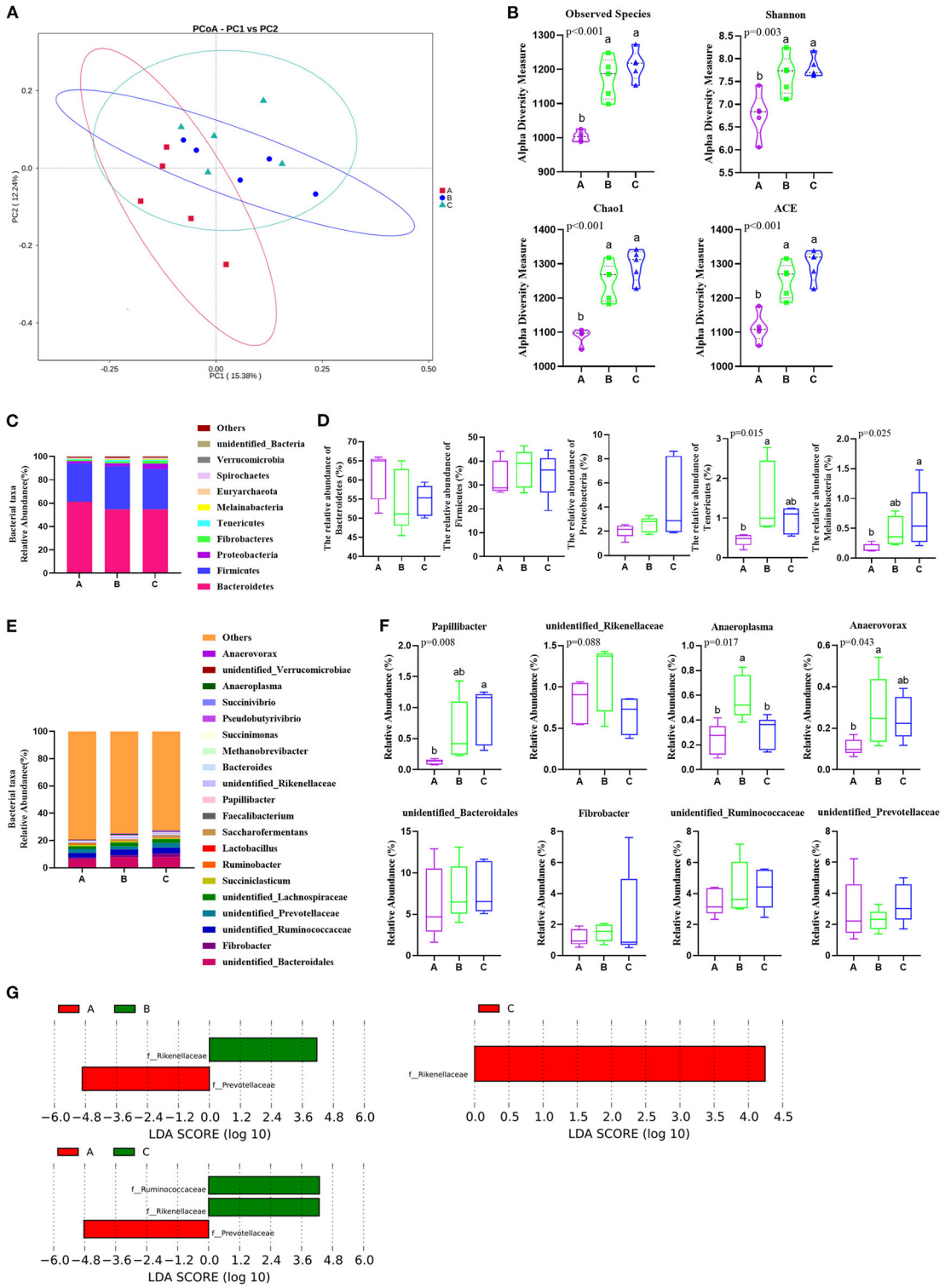

FIGURE 1 | *Phragmites australis* shoot remainder (PSR) silage totally or partially substituting the corn silage alters rumen microbiota composition in Angus beef cattle ($N = 5$). Group A, fed 60% PSR silage; Group B, fed 30% PSR silage + 30% corn silage; and Group C, fed 60% corn silage. **(A)** PCoA analysis of rumen microbiota based on operational taxonomic unit (OTU) abundance. **(B)** Assessment of alpha diversity. **(C)** Rumen microbiota taxonomic profiling at the phylum level. **(D)** Relative abundances of Bacteroidetes, Firmicutes, Proteobacteria, Tenericutes, and Melainabacteria. Bars with different letters (a, b) indicate significant differences ($p < 0.05$) among different groups (the same below). **(E)** Rumen microbiota taxonomic profiling at the genus level. **(F)** Relative abundances of representative and significant difference genera. **(G)** LDA score of rumen microbiota composition according to LEfSe analysis by three treatments.

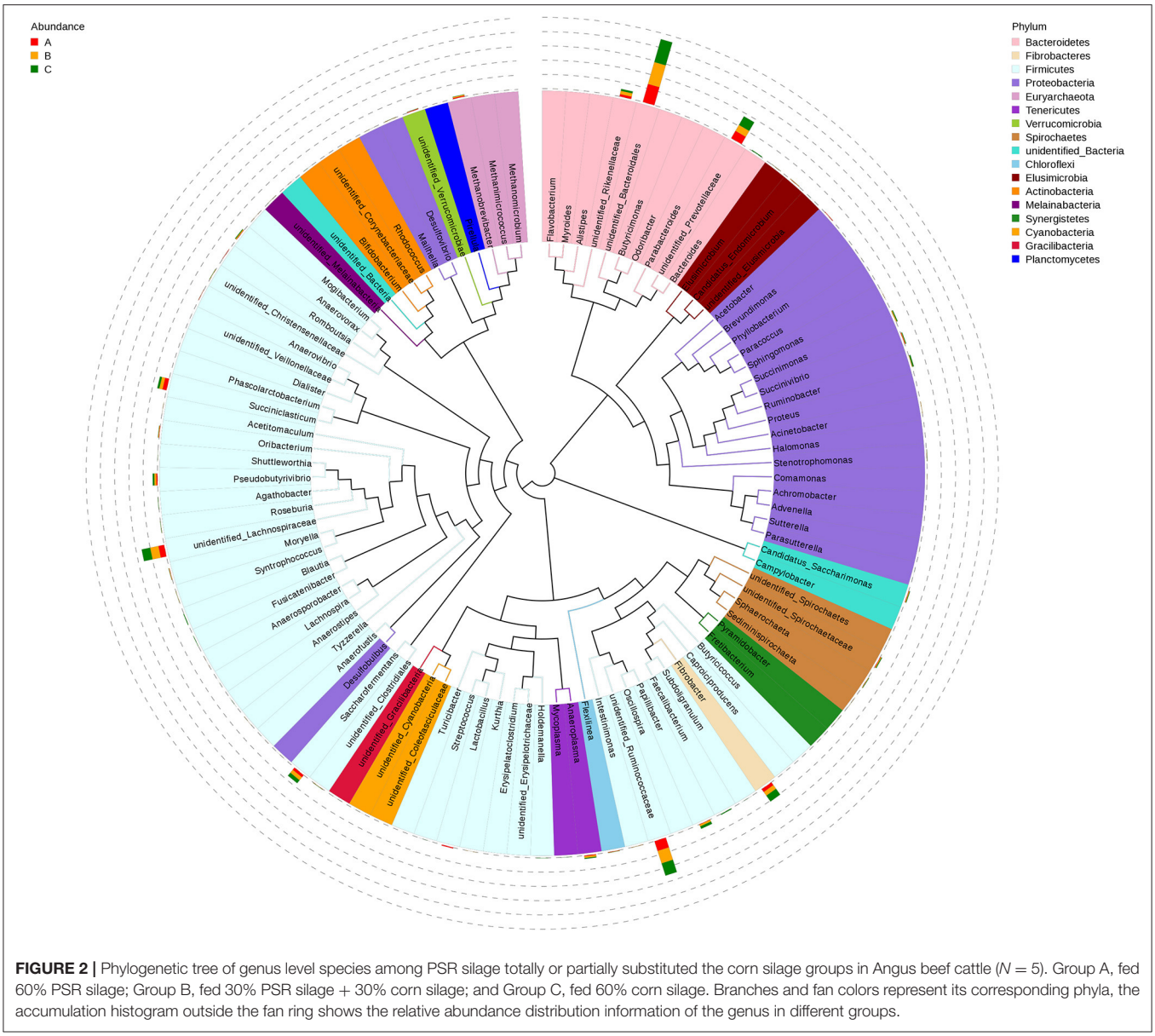

FIGURE 2 | Phylogenetic tree of genus level species among PSR silage totally or partially substituted the corn silage groups in Angus beef cattle ($N = 5$). Group A, fed 60% PSR silage; Group B, fed 30% PSR silage + 30% corn silage; and Group C, fed 60% corn silage. Branches and fan colors represent its corresponding phyla, the accumulation histogram outside the fan ring shows the relative abundance distribution information of the genus in different groups.

were significantly decreased from groups A to C, amino acid metabolism ($p = 0.066$) and nucleotide metabolism ($p = 0.057$) showed the descent tendency, while lipid metabolism ($p = 0.039$) was dramatically increased, and signal transduction had an increasing tendency ($p = 0.092$) from groups A to C (**Figure 3C**). At KEGG level 3, the majority of relatively abundant pathways were transporters, general function prediction only, DNA repair and recombination proteins, ribosome, purine metabolism, and ABC transporters (**Figure 3D**). Notably, the relative abundance of 11 pathways showed significant variation among the three groups (**Figure 3E**). The pathways involved in the pyrimidine metabolism ($p = 0.023$), DNA replication proteins ($p = 0.038$), glycine, serine, and threonine metabolism ($p = 0.019$), arginine and proline metabolism ($p = 0.015$), other ion coupled transporters ($p = 0.005$), alanine, aspartate, and glutamate metabolism ($p = 0.019$), cysteine and methionine metabolism ($p = 0.003$), transcription machinery ($p = 0.029$), energy metabolism ($p = 0.010$), and general function prediction only ($p = 0.042$) were significantly increased, and DNA repair and recombination proteins ($p = 0.094$) and peptidases ($p = 0.074$) had increased trend in group A compared with the other two groups. Inversely, secretion system ($p = 0.030$) was significantly decreased, and pyruvate metabolism ($p = 0.079$) had a decreased trend in group A than in the other two groups B and C.

DISCUSSION

Depending on the results of animal feeding trial, PSR silage group (group A), mixed group (group B), and corn silage (group C)

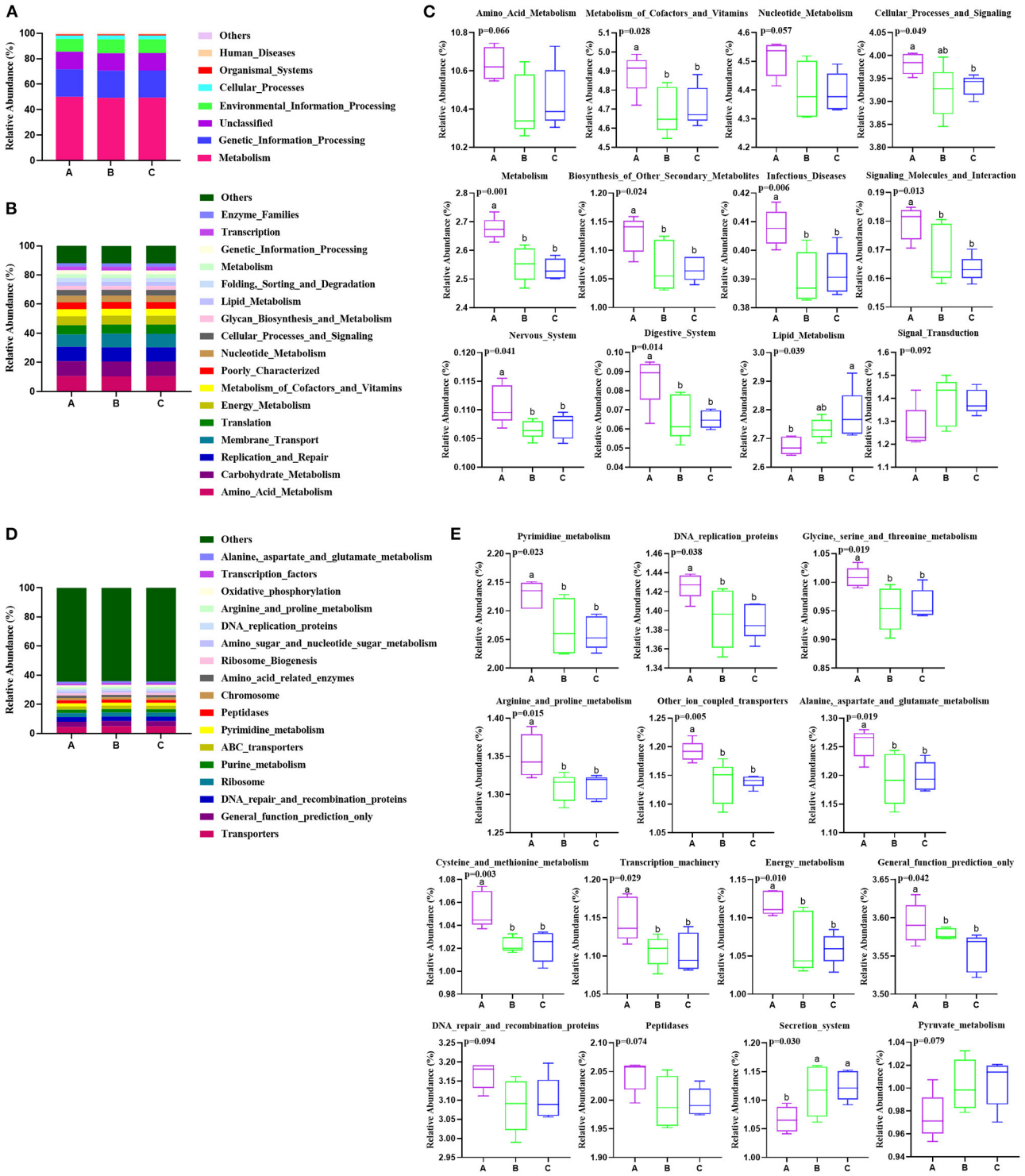

FIGURE 3 | Effects of PSR silage totally or partially substituted the corn silage on the predicted functional composition of rumen bacterial community in Angus beef cattle ($N = 5$). Group A, fed 60% PSR silage; Group B, fed 30% PSR silage 30% corn silage; and Group C, fed 60% corn silage. **(A)** The majority of the gene sequences annotated to KEGG level 1. **(B)** The majority of the gene sequences annotated to KEGG level 2. **(C)** The gene families of significant differences at KEGG level 2. **(D)** The majority of the gene sequences annotated to KEGG level 3. **(E)** The relative abundant pathways with significant differences at KEGG level 3. Bars with different letters (a,b) indicate significant differences ($p < 0.05$) among different groups.

affected the growth performance of Angus beef cattle. The ADG was significantly affected by PSR silage, which in PSR silage group was the highest, followed by the mixed group, equally 34.62% higher than corn silage group. Inversely, the FCR in the PSR silage group and mixed group was obviously lower than in the corn silage, 31.52 and 23.23% lower, respectively, while the ADFI had no significant difference among the three groups. These results indicated that PSR silage substitution for corn silage totally or partially could improve the growth performance of beef cattle, mainly by improving feed utilization efficiency to increase the ADG in the growing–fattening of Angus beef cattle. Thus, it can be observed that PSR silage might completely replace corn silage for beef cattle breeding. Therefore, the effective use of *P. australis* and other unconventional feed materials may not only expand feed sources and reduce feeding costs but also further improve the weight gain rate and meat production performance of beef cattle, and finally increase the economic benefits of breeding.

Nutrients are digested and absorbed by the body and carried through the bloodstream to tissues, organs, and cells. Therefore, blood biochemical indicators can be a good response to the intake of nutrition levels of the body. GLU concentration can reflect the energy metabolism of animals (20). In the current study, the concentration of GLU in the mixed group was significantly lower than in the PSR silage and corn silage treatments, while the higher ADG was observed in the mixed group. These results were inconsistent with previous studies, and this might be related to species and feed composition (11–15). The concentration of BUN is perceived as an effective indicator to measure the metabolism of protein and amino acid, low BUN level indicates high nitrogen metabolism capacity (21). Diets supplemented with PSR silage increased the concentrations of BUN in the present study, which might be related to the high protein content of *P. australis* shoots remainder.

Rumen is the most powerful digestive organ, in which complex microbial communities are closely related to degrade and convert plant materials in ruminants (22). More than 70% of the energy was provided by rumen bacteria fermentation to ensure the host growth and reproduction performance (23), and the compositions of rumen microorganism are influenced by diets (24). In the present study, the core microbiome accounts for more than 78% of total OTUs among the three groups, and this result was similar with other researches (11–13). The core microbiome plays crucial roles in maintaining the "functional redundancy" of rumen ecosystem, and this redundancy further guarantees its major functional properties (25). The present study showed that the observed species (OTUs), Shannon, Chao1, and ACE indices were significantly decreased in the PSR silage diet, indicating that the rumen microbiome was altered by PSR silage.

The present study systematically revealed the composition and structure of rumen microbiome in Angus beef cattle fed PSR silage. Bacteroidetes and Firmicutes were the most two relatively abundant phyla in the current study, which was referred to the degradation of protein and carbohydrates (26), and these findings were consistent with previous studies (27). de Menezes et al. (28) have also found that the dairy cows fed pasture or TMR diets did not have obvious differences on the rumen microbiome at the phylum level, with the sequences of *Bacteroidetes* and *Firmicutes* representing approximately 80% of the total rumen microbiome. Consistently, the relative abundance of *Bacteroidetes* and *Firmicutes* were over 80% in all three diets in the current study. Proteobacteria was detected as the third most relatively abundant phylum in this study, which was similar to previous studies (29). In general, the composition and structure of rumen microbial community might be related to the feed efficiency and animal breed. Studies have found that several members belonged to the phylum *Tenericutes* related to being animal pathogens and parasites, and the greater abundance of *Tenericutes* was associated with the reduced intramuscular fat deposition of longissimus in Angus steers (30, 31). The relative abundance of *Tenericutes* was the lowest in the PSR silage diet in the present study, indicating that PSR silage might improve gastrointestinal health and further promotes intramuscular fat deposition. Actually, the growth performance was the greatest by feeding PSR silage diet. *Melainabacteria* is a newly identified gut bacteria, whose relative abundance in the rectal contents of diabetic model rats was significantly increased (32). The relative abundance of *Melainabacteria* was significantly decreased (especially in group A) after adding PSR silage to the diets in the present study, which indicated that PSR silage-substituted corn silage might improve host sugar, fat, and protein metabolism.

At the genus level, the effects of PSR silage on the rumen bacterial population were further identified in this study. The relative abundance of *Papillibacter* in the PSR silage group was significantly lower than that in the corn silage and mixed groups. *Papillibacter* is known as a butyrate producer (33). The decreased abundance of *Papillibacter* in PSR silage indicated that the butyrate production was relatively decreased by rumen microbiota. The relative abundance of *Anaeroplasma* in the mixed group was significantly higher than the groups of corn silage and PSR silage. *Anaeroplasma* is a genus characterized by its anaerobic fermentation, which produces fatty acids as propionate (34). All of these taxa were previously reported as members of the regular and efficient microbiota from rumen, and their increased abundance may indicate an improved ability of digestion or, at least, a need for more specialized fermentation in rumen due to, for example, more food intake. Besides, *Anaeroplasma* was highly correlated with high weight gain, and may be important for cattle nutrition either individually or in a consortium (35).

Some studies indicate that *Anaerovorax* can generate more SCFAs to provide additional energy sources and maintain feed efficiency (36). Unexpectedly, the relative abundance of *Anaerovorax* in the PSR silage group is significantly lower than that in the corn silage and mixed groups in the present study. The relative abundance of *unidentified_Rikenellaceae* in the corn silage group was obviously lower than that in the PSR silage and mixed groups. To date, all cultured members of the family *Rikenellaceae* are described as anaerobic, mesophilic, and rod-shaped bacteria that usually ferment carbohydrates or proteins. Su et al. (37) isolated a carbohydrate-fermenting and hydrogen-producing *Rikenellaceae* from a reed swamp in China (37). It

remains to identify and examine the functions of the *unidentified Rikenellaceae* to understand the roles in the present study. Additionally, the PSR silage diet increased the relative proportion of *Rikenellaceae* at the family level, and *Rikenellaceae* might be one of the biomarkers between PSR silage feed and corn silage feed using LEfSe.

The composition of rumen microorganisms affects host metabolic function and physiological health. The relative abundance of the dominant microbial phyla is stable in ruminants. The dominant three microbial phyla were *Bacteroidetes*, *Firmicutes*, and *Proteobacteria* in the rumen among the three groups, indicating that the rumen microbiota in cattle was also relatively stable at the phyla level. These results were in agreement with previous studies (38, 39), and the most dominant phyla *Firmicutes* and *Bacteroidetes* were closely related to carbohydrate and protein metabolism (40, 41). At the genus level, the dominant four genera were *unidentified Bacteroidales*, *Fibrobacter*, *unidentified Ruminococcaceae*, and *unidentified Prevotellaceae*, and their relative abundance all did not have significant difference among different diets in the present study, which was similar to previous studies (41, 42). Menni et al. (43) found that the abundance of *Ruminococcaceae* might play a significant role in energy and lipid metabolism, and which was negatively associated with vascular sclerosis. These results indicated that the PSR silage diet might not affect host health in cattle.

The PICRUSt prediction results showed that amino acid metabolism, carbohydrate metabolism, replication and repair, membrane transport, translation, and energy metabolism were the dominant gene families at KEGG level 2, all of which are essential for survival, growth, and reproduction of gastrointestinal microbes (44). These results were similar to our previous studies in sheep (11–15). Among these gene families, unexpectedly, the genes associated with metabolism of cofactors and vitamins, cellular processes and signaling, metabolism, biosynthesis of other secondary metabolites, infectious diseases, signaling molecules and interaction, nervous system, and digestive system were significantly higher in the PSR silage diet than in the mixed and corn silage diets, while lipid metabolism was dramatically lower in the PSR silage diet than in the corn silage diet. Furthermore, the majority of gene families were transporters, general function prediction only, DNA repair, and recombination proteins, ribosome, purine metabolism, and ABC transporters at KEGG level 3. Notably, the relative abundance of 11 pathways showed significant variation among the three groups. The pathways involved in the pyrimidine metabolism, DNA replication proteins, glycine, serine, and threonine metabolism, arginine and proline metabolism, other ion coupled transporters, alanine, aspartate, and glutamate metabolism, cysteine and methionine metabolism, transcription machinery, energy metabolism, and general function prediction only were significantly higher, and secretion system was significantly lower in the PSR silage diet than in the mixed and corn silage diets. These results indicated an enhanced fermentation activity performed by rumen microorganisms in the PSR silage diet. The current study also implied that feeding only a roughage of PSR silage diet altered the ruminal microbial functions.

In conclusion, the present study mainly investigated that the growth performance, blood biochemical parameters, and the composition and function of rumen microbiota of PSR silage feed totally or partially substituted the corn silage in Angus beef. The results suggest that the PSR silage diet and mixed diet increase ADG and decrease FCR, reduce serum glucose levels, and alter the rumen microbiota and inferred metabolic functions. These findings indicated that PSR silage could partially substitute corn silage for beef cattle breeding, replacing 30% of corn silage in the diet has good feeding effect in cattle.

CONCLUSIONS

Feeding different PSR silage level diets improved growth performance, changed the contents of serum glucose and urea nitrogen and, furthermore, might affect the energy and protein metabolic efficiency of Angus beef. Moreover, the rumen bacterial diversity indices decreased significantly by feeding PSR silage, the relative abundances of *Tenericutes* and *Melainabacteria* were significantly reduced by feeding PSR silage, and *Papillibacter*, *Anaeroplasma,* and *Anaerovorax* had significantly decreased by feeding PSR silage at the genus level, and furthermore, the metabolic pathways were significantly influenced by related bacteria for PSR silage. The results indicated that feeding PSR silage could improve the growth performance and alter the rumen bacteria diversity and the corresponding function, and PSR silage could partially substitute (30%) corn silage for beef cattle breeding.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: https://www.ncbi.nlm.nih.gov/, SRR15662882-SRR15662896.

ETHICS STATEMENT

The experimental procedures of this study were approved by the Animal Care Committee of Hunan Normal University in reference to the Administration of Affairs Concerning Experimental Animals.

AUTHOR CONTRIBUTIONS

HY and QW conceptualized the study. XZ and XL handled the methodology. PH and ZF were in charge of the software. MZ was responsible for the validation. YZ and YaW performed the formal analysis. QW conducted the investigation, prepared and wrote the original draft, and handled the project administration. HY handled the resources and acquired the funding. XW and JH were responsible for the data curation. MC and YiW assisted in

the writing, review, and editing of the draft. MZ and JY handled the visualization. JL was in charge of the supervision. All authors have read and agreed to the published version of the manuscript.

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Dietary *Moringa oleifera* Alters Periparturient Plasma and Milk Biochemical Indicators and Promotes Productive Performance in Goats

Ali Afzal[1], *Tarique Hussain*[1]*, *Amjad Hameed*[2], *Muhammad Shahzad*[1], *Muhammad Usman Mazhar*[1] and *Guan Yang*[3]

[1] *Animal Sciences Division, Nuclear Institute for Agriculture and Biology College, Pakistan Institute of Engineering and Applied Sciences (NIAB-C, PIEAS), Faisalabad, Pakistan,* [2] *Nuclear Institute for Agriculture and Biology College, Pakistan Institute of Engineering and Applied Sciences (NIAB-C, PIEAS), Faisalabad, Pakistan,* [3] *Department of Infectious Diseases and Public Health, City University of Hong Kong, Kowloon, China*

The purpose of the current study was to explore the supplementing effects of *Moringa oleifera* leaf powder (MOLP) on plasma and milk biochemical indices and productive/reproductive performance of goats. A total of 30 healthy pregnant goats were randomly distributed ($n = 10$) into three experimental groups: control (M_0) group (basal diet without MOLP), $M_{2\%}$ group (basal diet + 2% MOLP), and $M_{3.5\%}$ group (basal diet + 3.5% MOLP). The experiment started 2 months before parturition and continued till the first month of lactation. The plasma flavonoids were significantly increased in the $M_{3.5\%}$ group during the entire experiment, whereas the total phenolic contents were enhanced only during the lactation period depending on the supplementation percentage. The amount of vitamin C increased significantly in $M_{2\%}$ and $M_{3.5\%}$ groups as compared to the M_0 group. Supplementation of MOLP improved the plasma total antioxidant capacity by declining malondialdehyde concentration and total oxidant status values. The activities of superoxide dismutase and peroxidase enzymes were modified in $M_{2\%}$ and $M_{3.5\%}$ supplemented groups throughout the experiment, while the catalase activity was significantly influenced only during the lactation stage. The protein and lycopene contents in plasma were significantly improved in the $M_{3.5\%}$ group, whereas the total sugars and carotenoid level was increased in both $M_{2\%}$ and $M_{3.5\%}$ groups. Dietary supplementation with 3.5% MOLP more effectively enhanced protease and amylase activities as compared to 2% supplementation. MOLP also improved the biochemical indices and antioxidant status of colostrum and milk. The milk yield, weight gain of the kids, and reproductive performance were high in $M_{2\%}$ and $M_{3.5\%}$ groups in comparison to the M_0 group. These findings disclose that supplementing the diet with 3.5% MOLP improves antioxidant status, milk yield, and reproductive performance in goats.

Keywords: *Moringa oleifera*, plasma biochemistry, antioxidant status, milk composition, productive performance, goat

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Elsa Lamy,
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Ioannis S. Pappas,
University of Thessaly, Greece
F. Capela e Silva,
University of Evora, Portugal

***Correspondence:**
Tarique Hussain
drtariquerahoo@gmail.com

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INTRODUCTION

Reproductive performance is a key factor in goat production and is directly related to maternal nutrition. The pariparturient period (late gestation and early lactation) is characterized by depressed feed intake, endocrine, and metabolic changes that interrupt energy balance and anti-oxidant status of the body (1, 2). Nutritional requirements are high during this period due to accelerated digestion rate, tissue mobilization for mammary development, and fetus growth (3). However, maternal malnutrition is common in small ruminants in most parts of the world due to scarcity and high cost of feed stuffs. The farmers, in these regions, mostly depend on conventional grazing and crop residues to meet the requirements of their animals. The natural grazing pastures and crop residues have fluctuating nutritional status, and their feeding alone is not sufficient to satisfy the energy needs of pregnant and lactating animals (4). The energy deficit feed makes the pregnant animals more prone to oxidative stress with enormous production of reactive oxygen species (ROS) that results in a disturbance of the balance between oxidant and antioxidant defense systems of the body (5). All biomolecules including lipids, carbohydrates, and proteins are adversely affected by oxidative stress, which ultimately leads to a decline in reproductive and productive performance (6). Furthermore, the newborn kids may suffer from a variety of diseases that will negatively influence their survival and growth rate (7).

Colostrum is the first secretion produced soon after parturition and is a source of immunity for newborns. Immunoglobulins, minerals, and many other biologically active substances are transferred passively through the colostrum to the kids of sheep, goat, cattle, and horse as they do not get into the embryo's bloodstream (8). The composition and quality of colostrum and milk predominantly depend on the nutrition of the mother (9, 10). The feed should fulfill the nutritional requirements of pregnant animals to get good quality of colostrum and milk from them after parturition. The inadequate supply of nutrients will adversely affect the synthesis and composition of colostrum and milk.

The diet manipulation with phytobiotics (plant-derived feed additives) has been proposed to be an effective approach in managing nutrition-induced oxidative stress during pregnancy and lactation in both small and large ruminants (11–14). Some herbal plants have medicinal values and are nutritionally important to enhance the health status and reproductive performance of goats. Their supplementation with a basal diet can minimize nutrition-related problems in goat production (15).

Moringa oleifera (MO) is an evergreen tree fodder, also known as a "miracle tree," and is one of the most useful, multi-purpose, fast-growing, and drought-resistant trees. It is well-known for its medicinal importance and nutritional characteristics. Moringa leaves contain a sufficient quantity of vitamins, minerals, and proteins according to the nutritional demands of pregnant and lactating animals (16, 17). Moreover, MO leaves are also a rich source of different bioactive compounds, especially abundant in antioxidants including flavonoids (kaempferol, myricetin, and quercetin), phenolic acids (gallic, ellagic, and chlorogenic acid), Vit C, Vit E, β-carotene, Se, and Zn (18). These substances have been detected separately in various plants, but MO is distinct in having them all in substantial amounts (19).

The MO leaves are readily adapted and easily digested by animals. The favorable impacts of MO have been observed on the anti-oxidant status and reproductive performance in mice and sows (20, 21). However, there is little information about dietary supplementation effects of *Moringa oleifera* leaf powder (MOLP) during the nutritionally critical stages (late pregnancy and early lactation) in goats. Therefore, the study was designed to evaluate the effects of MOLP as a nutritional supplement on productive and reproductive performance, plasma, and milk biochemical indices in Beetal goats. The results of this study will enlighten the knowledge about the development of different bioactive compounds from MO leaf in the field of goat reproduction.

MATERIALS AND METHODS

Ethical Statement

The procedures used for study and ethical clearance were approved and granted by Animal Use and Care Research Committee at Nuclear Institute for Agriculture and Biology (NIAB), Faisalabad, Pakistan.

Experimental Design and Animal Husbandry

The experiment was conducted at the goat research farm of Nuclear Institute for Agriculture and Biology (NIAB), situated ∼7 km from the nucleus of the city Faisalabad, Pakistan (longitude 73.0791° E and latitude 31.4287° N) at an altitude of 184 m from sea level. The average rainfall and temperature were 15.50 mm and 41°C during the months (April to July 2020) of the experiment.

A total of 30 pregnant Beetal goats of 2–3 years age and weighing about 40 kg ± 2.3 were selected exactly 2 months before their predicted delivery. The ultrasonographic examination of goats was performed by the Department of Theriogenology, University of Agriculture, Faisalabad for confirmation of pregnancy. All the goats were randomly divided ($n = 10$) into the control (M_0) group (250 g basal diet without MOLP/animal/day), $M_{2\%}$ group (250 g basal diet with 5 g MOLP/animal/day), and $M_{3.5\%}$ group (250 g basal diet with 8.75 g MOLP/animal/day). The basal diet was composed of wheat, corn, rice bran, sugarcane molasses, minerals, and soybean meal formulated according to the nutritional requirements of pregnant goats (**Table 1**) (23). The experiment was initiated 2 months before the estimated due date of kidding and continued till the first month of lactation. All the animals were acclimatized 1 week for the basal diet. Before the start of the experiment, deworming was performed with Albendazole (Zoben, Prix, Lahore, Pakistan) at a dose rate of 2.5 mg/5 kg/BW. The goats were kept in well-ventilated semi-open sheds and offered two times free pasture grazing (*Chloris gayana, Leptochloa fusca, Carduus nutans, Chenopodium album,* and *Cirsium arvense*) during morning and evening schedule with free access to clean drinking water.

TABLE 1 | Formulation of basal diet (% DM).

Ingredients	Amount (%)
Wheat grains	30
Corn	20
Wheat bran	15
Rice bran	15
Wheat straw	10
Soyabean meal	5
Molasses	3
Dicalcium phosphate	1.8
Vitamin and mineral premix[a]	0.2
Total	100

[a] *Vitamin and mineral premix per kg containing: Vit E 300 IU, Vit A 50,000 IU, Vit D3 80,000 IU, Ca 18.5%, Mg 8.2%, P 3.5%, Na 3%, Zn 3,200 mg, Mn 3,333 mg, Cu 800 mg, Se 24 mg, Iodate 68 mg, Co 16 mg (22).*

TABLE 2 | Chemical composition of basal diet and MOLP (DM basis).

Constituents	Basal diet	MOLP
	Mean (%) ± SE	Mean (%) ± SE
Dry matter	94.8 ± 0.43	92.5 ± 0.52
Crude protein	13.5 ± 0.16	18.2 ± 0.06
Crude fat	4.4 ± 0.23	5.5 ± 0.05
Ash	16.7 ± 0.37	11.3 ± 0.03
Nitrogen free extract	47.3 ± 0.33	38.4 ± 0.29
Neutral detergent fiber	53.2 ± 0.26	32.4 ± 0.12
Acid detergent fiber	18.06 ± 0.13	19.1 ± 0.08

MOLP, Moringa oleifera dried leaf powder (22).

Plant Material

The fresh green leaves of MO (PKM1) were collected from a breeder's farm (Lahore, Pakistan) during the month of November 2019, and their authenticity was assured by an experienced botanist at NIAB. A representative sample of leaves was sent to the institute's herbarium for reference in the future. The leaves were cleaned properly by washing and dried under shade at room temperature for 4 days. The dried leaves were processed to make powder and then stored in airtight containers for use in the experiment.

Compositional Analysis of Diet

The MOLP and basal diet were subjected to proximate chemical compositional analysis by using standardized methods of analytical chemists (24) as also used in our previous study (22). The MOLP was also analyzed for its different biochemical elements spectrophotometrically (UV-VIS U2800, Hitachi, Japan). The compositional analysis of MOLP and basal diet are presented in **Tables 2**, **3**.

Collection of Blood and Milk Samples

Blood samples (5 ml) were collected in sterile EDTA tubes (Vacutainer, Xinle) from the jugular vein with 20-day intervals after the start of the experimental diet from day 90 of gestation. Plasma was separated from blood samples by centrifugation at 3,000 rpm/4°C and stored at −20°C till further analysis. Colostrum samples were obtained within 2 h after parturition. Milk samples were collected with 1-week interval for 4 consecutive weeks and stored at −20°C for further biochemical analyses. Blood and milk samples were collected at 7–8 a.m. during the whole experiment. The defatting of colostrum and milk samples was done by centrifugation at 2,500 × *g* for 15 min for enzymatic and non-enzymatic antioxidant estimation.

TABLE 3 | Nutritional constituent analysis of MOLP.

Biochemical constituents	Mean ± SE
Total phenolic contents (μM/g)	36,000 ± 3.21
Total Flavonoids (μg/g)	258.58 ± 2.28
Vitamin C (μg/g)	546.16 ± 3.06
Lycopene (mg/g)	9.95 ± 0.17
Total carotenoids (mg/g)	13.87 ± 0.33
Total sugars (mg/g)	27.51 ± 1.52
Methionine (% of DM)	0.42 ± 0.012
Cysteine (% of DM)	0.52 ± 0.014
Sodium (mg/g)	2.13 ± 0.075
Calcium (mg/g)	180 ± 1.154
Potassium (mg/g)	8.99 ± 0.571
Selenium (mg/g)	0.31 ± 0.057
Iron (mg/g)	0.16 ± 0.034

MOLP, Moringa oleifera leaf powder (22).

Analysis of Blood Plasma

Non-enzymatic Antioxidants

Total Flavonoids

Total flavonoids (TF) in plasma samples were estimated by $AlCl_3$ colorimetric assay and rutin was used as standard (25). The samples were mixed with 100 μl of 10% $AlCl_3$, 100 μl of 1 M potassium acetate, and 275 μl of deionized water. The contents were incubated for 40 min at room temperature and then absorbance was measured at 415 nm by using a double beam spectrophotometer (UV-VIS U2800, Hitachi, Japan). The TFs were computed with the help of a standard curve and expressed as μg RE (retinol equivalents) per milliliter of sample.

Total Phenolic Content

Total phenolic contents (TPC) were assessed using a modified Folin-Ciocalteu procedure (26). The 100-μl blood plasma sample was vortexed with 100 μl of Folin-Ciocalteu reagent for 15 s and then incubated for 1 h at room temperature after adding 700 mM Na_2CO_3 (800 μl). The absorbance of reaction was read at 765 nm and TPCs were calculated from a linear regression equation.

Vitamin C

Vitamin C was determined by using a standardized protocol of Moeslinger et al. (27). Briefly, vitamin C causes reduction of a colored compound 2,6 Dichlorophenolindophenol (DCPIP) into $DCPIPH_2$ (colorless compound). This reaction was monitored

by fall-off absorbance at 520 nm. The concentration of vitamin C in plasma samples was measured by using a standard calibration curve.

Malondialdehyde

Malondialdehyde (MDA) concentration in blood plasma samples was assessed colorimetrically by using MDA as standard (28). Plasma sample (25 μl) was homogenized in 0.1% trichloroacetic acid and centrifuged exactly for 5 min at 14,000 × *g*. Then, trichloroacetic acid (20%) containing thiobarbituric acid (0.05%) was added in 1 ml aliquot of the supernatants and heated for 30 min by placing in boiling water bath. The reaction mixture was cooled after removing from the water bath and centrifuged for 10 min at 14,000 × *g*. The absorbance of clear supernatants was read at 535 nm and the value of non-specific absorbance (600 nm) was subtracted from it. The MDA contents were measured by a coefficient of extinction 155 per mM per cm.

Total Antioxidant Capacity

Total antioxidant capacity (TAC) of plasma samples was measured by an assay based on the reduction of a blue radical cation ($ABTS^{\bullet+}$) to its original ABTS colorless form by antioxidants (29). The assay solution to measure TAC is composed of reagent R_1 (CH3COONa buffer and glacial acetic acid), R_2 (H_2O_2, Na3PO4 buffer, glacial acetic acid, and ABTS), and sample. The contents of the reaction solution were incubated for about 6 min at room temperature and then absorbance was read at 660 nm. The TAC value was computed from a standard ascorbic acid calibration curve and represented as ascorbic acid (μM) equivalent per milliliter of sample.

Total Oxidant Status

The method for the determination of total oxidant status (TOS) values of plasma samples is based on the oxidation of Fe^{2+} into Fe^{3+} by oxidants present in the sample (29). A specific color appeared when Fe^{3+} ions react with xylenol and the magnitude of color is directly related to the quantity of oxidant molecules that were measured spectrophotometrically. The reaction solution for the determination of TOS value is composed of R_1, xylenol solution (0.38 g in 500 μl of 25 mM H_2SO_4), R_2 [ferrous ammonium sulfate (II) 0.0196 g, o-dianisidine 0.0317 g, glycerol 500 μl, and NaCl 0.4 g], and sample. The absorbance value was read after 5 min of adding R_2. Hydrogen peroxide (H_2O_2) was used to calculate the final value of TOS that was expressed in μM H_2O_2 equivalent per milliliter.

Enzymatic Antioxidants

Superoxide Dismutase Activity

The plasma samples were assayed for superoxide dismutase (SOD) activity by an inhibition assay that works on the base of SOD ability to inhibit the photochemical reduction of nitroblue tetrazolium (NBT) into formazan (30). The reaction solution for the study of inhibition assay is composed of 50 mM potassium phosphate buffer (pH 7.8), 13 mM L-methionine, 57 μM NBT, Triton X-100 (0.025%), riboflavin (0.004%), and 50 μl of blood plasma sample in a total volume of 3 ml. The photoreaction was performed in a box lined with aluminum (Al) foil and having a 15-W lamp as a light source. The absorbance of the reduction reaction of NBT to formazan was taken at 560 nm and a unit of SOD activity was defined as the amount of enzyme required to cause 50% inhibition of NBT.

Peroxidase Activity

Plasma peroxidase (POD) activity was estimated by using the method of Agostini et al. (31) with some necessary modifications and using guaiacol as substrate. The assay solution to measure POD activity contained guaiacol (200 mM), H_2O_2 (400 mM), 545 μl of distilled water, 200 mM potassium phosphate buffer (pH 7.0), and 15 μl of blood plasma sample. The reaction was initiated immediately after the addition of the plasma sample, and the absorbance of the reaction solution was measured after every 20 s for 1 min at 470 nm. One unit of POD activity was narrated as the quantity of enzyme that catalyzed the oxidation of guaiacol.

Catalase Activity

Catalase (CAT) activity of blood plasma samples was measured by mixing the samples with 50 mM potassium phosphate buffer (pH 7.0) and dithiothreitol (1 mM) as described by Beers and Sizer (32). The reaction mixture to study CAT activity contained 59 mM H_2O_2, 50 mM phosphate buffer (pH 7), and a 100-μl plasma sample. The decreasing pattern of absorbance was measured after every 20 s for 1 min at 240 nm and a unit of CAT activity was described as a change in absorbance in 0.01 min.

Biochemical Parameters

Total Soluble Protein

The quantitative protein estimation of plasma samples was performed by dye-binding method as described by Bradford (33). The plasma sample (5 μl) was mixed and homogenized with 0.1 N NaCl. The reaction solution was incubated for 5 min at room temperature after adding 1 ml Bradford dye to form a protein–dye complex. Thereafter, absorbance was measured at 420 nm.

Total Sugar

The total sugar level of plasma samples was enacted by Folin's (34) protocol with few desired modifications. Briefly, the samples were mixed with sulfuric acid (H_2SO_4) and neutralized by using sodium carbonate (Na_2CO_3). The contents were then filtered and absorbance was read spectrophotometrically at 415 nm for estimation of sugar contents.

Lycopene and Carotenoids

Lycopene and carotenoids in blood plasma samples were assayed according to the standardized procedure of Nagata and Yamashita (35). For estimation of lycopene and carotenoids, 1 ml of blood plasma sample was thoroughly homogenized with 10 ml of hexane and acetone solution (6:4). The assay solution was incubated for 5 min at 37°C and then filtered. The absorbance was measured at 453, 505, and 663 nm, and finally the quantities of lycopene and carotenoids were calculated with the help of the following formulae:

$$\text{Lycopene} = -0.0458A_{663} + 0.372A_{505}$$

$$\text{Carotenoids} = 0.216A_{663} - 0.304A_{505} + 0.452A_{453}$$

Hydrolytic Enzymes

Protease

The protease activity was measured by the casein digestion method (36). Protease enzyme releases an amino acid "tyrosine" after digestion of casein. The reaction of tyrosine Folin's reagent results in the formation of a blue color product that is quantified at 660 nm. A standard calibration curve of tyrosine was used to compute the protease activity in plasma samples. One unit of enzyme activity was defined as the amount of enzyme that causes the release of soluble acid fragments equivalent to 0.001 A 280 nm in 1 min at pH 7.8.

Esterase

The esterase (alpha and beta) enzyme activity was estimated by utilizing naphthyl acetate (α and β) as substrate as described by Van Asperen (37). The reaction mixture consisting of plasma (enzyme extract), phosphate buffer (0.04 M, pH 7), 30 mM naphthyl acetate (α and β), and 1% acetone was incubated in the dark at 37°C for 15 min. Then, 1 ml of staining solution composed of fast blue BB (1%) and sodium dodecyl sulfate (5%) was added in both blank control (phosphate buffer and substrate solution) and reaction mixture and again incubated for 15 min in the dark at room temperature. The absorbance of the assay was recorded at 590 nm, and the enzyme activity was calculated in $\mu M\ min^{-1}\ ml^{-1}$ of a sample using a standard curve.

Amylase

The activity of the amylase enzyme in plasma samples was assessed by using 0.2 M tris-malate (pH 7.2) buffer as an extraction cum assay medium (38). For estimation of enzyme activity, 1 ml of substrate solution (0.15% starch) was homogenized with 1 ml of plasma and incubated at 37°C for 10 min. The OD of the reaction mixture was measured at 620 nm after adding quenching reagent and enzyme activity was represented in milligrams of starch degraded per minute per milliliter of blood plasma sample.

Analysis of Milk

Milk samples were analyzed for chemical composition by a milk analyzer (Julie Z7, Scope Electric, Regensburg, Germany). The defatted milk samples were used for estimation of non-enzymatic (TPC, TAC, and vitamin C) and enzymatic (SOD, POD, and CAT) antioxidants by a spectrophotometer (UV-VIS U2800, Hitachi, Japan) as described above for plasma samples.

Reproductive and Productive Performance

The reproductive and productive performance was evaluated in terms of birth weight, weight gain per week, survival rate of newly born kids, shedding time of placenta, the onset of first postpartum estrus, and milk yield in goats.

Statistical Analysis

All the statistical analyses were carried out by using SPSS version 20. The experimental procedures were performed in triplicate, and data obtained were analyzed by one-way analysis of variance (ANOVA) with repeated measures under the shade of LSD to access the differences among different treatment means on specific days. The results were expressed in the tables as mean ± SE, and the values with $p < 0.05$ were considered statistically significant.

RESULTS

Non-enzymatic Antioxidant Parameters

The response of plasma non-enzymatic antioxidant parameters to MOLP supplementation during pregnancy and early lactation period is shown in **Table 4**. Plasma TFs were increased significantly from day 110 of pregnancy to day 20 of lactation in the $M_{3.5\%}$ group ($p < 0.05$). The impact of supplementation was non-significant on $M_{2\%}$ group plasma TFs as compared to the control (M_0) group ($p > 0.05$). The values of plasma TPC of the $M_{3.5\%}$ and $M_{2\%}$ groups were non-significant in the pregnancy stage, while they became significant during the early lactation period of the experiment ($p < 0.05$). The increase in plasma Vit C contents was significant ($p < 0.05$) from the 130th day of pregnancy till day 20 of lactation in both $M_{3.5\%}$ and $M_{2\%}$ supplemented groups. The MOLP supplementation significantly improved the plasma TAC by declining MDA concentration and TOS values near parturition and early lactation stage of the experiment in contrast to the control (M_0) group ($p < 0.05$).

Enzymatic Antioxidants

The MOLP supplementation impacts on plasma enzymatic antioxidants are presented in **Table 5**. The SOD activity of $M_{3.5\%}$ and $M_{2\%}$ groups increased significantly throughout the experiment in response to supplementation ($p < 0.05$) and reached its peak on day 20 of lactation in the $M_{3.5\%}$ group. The effect of supplementation levels (3.5 and 2%) was non-significant on POD activity ($p > 0.05$). However, there was a significant difference between the POD activities of supplemented ($M_{3.5\%}$ and $M_{2\%}$) and control (M_0) groups during the entire experiment ($p < 0.05$). A non-significant increase in the plasma CAT activity was noticed till day 150 of gestation ($p > 0.05$), but soon after kidding, the CAT activity was enhanced significantly during the lactation period according to the supplementation levels in $M_{3.5\%}$ and $M_{2\%}$ groups as compared to the control (M_0) group ($p < 0.05$).

Biochemical Indices

The change in plasma biochemical indicators in response to MOLP supplementation is given in **Table 6**. The supplementation initially showed no pronounced effect on plasma TSP contents till the 130th day of pregnancy. Thereafter, the plasma TSP contents increased significantly on day 150 of pregnancy and day 20 of lactation in $M_{3.5\%}$ group in comparison to $M_{2\%}$ and M_0 groups ($p < 0.05$). The plasma sugar level of both $M_{3.5\%}$ and $M_{2\%}$ supplemented groups increased significantly from the beginning of the experiment till day 20 of lactation ($p < 0.05$). A drastic increase in plasma sugar level was observed soon after kidding in supplemented groups in contrast to the control group. The concentration of carotenoids in plasma was significantly enhanced throughout the experiment in the $M_{3.5\%}$ group as compared to the control (M_0) group ($p < 0.05$), whereas the lycopene contents were improved from day 130 of

TABLE 4 | Plasma non-enzymatic antioxidant indices of Beetal goats.

Non-enzymatic antioxidants	Levels of *Moringa oleifera* leaf powder supplementation			SEM	*p*-value
	M_0	$M_{2\%}$	$M_{3.5\%}$		
Gestation day 110					
Total flavonoids (μg/ml)	239.93 ± 0.91[b]	241.06 ± 0.74[b]	253.91 ± 0.72[a]	0.43	0.030
Total phenolic contents (μM/ml)	5,821.75 ± 0.85[b]	5,832.61 ± 1.60[a]	5840 ± 2.45[a]	1.19	0.028
Vitamin C (μg/ml)	803.75 ± 1.55	805.50 ± 1.04	810.25 ± 1.44	0.84	0.221
Malondialdehyde (μM/ml)	5.90 ± 0.33	5.76 ± 0.37	5.59 ± 0.26	0.17	0.627
Total anti-oxidant capacity (μM/ml)	1.41 ± 0.02[c]	1.55 ± 0.01[b]	1.67 ± 0.01[a]	0.01	0.002
Total oxidant status (μM/ml)	1,566.25 ± 1.11	1,559.75 ± 0.63	1,555.50 ± 2.10	1.04	0.065
Gestation day 130					
Total flavonoids (μg/ml)	251.31 ± 0.59[b]	256.14 ± 0.76[b]	272.79 ± 1.07[a]	0.29	0.013
Total phenolic contents (μM/ml)	5,819 ± 0.82[b]	5,844.75 ± 1.31[a]	5,848.25 ± 0.95[a]	0.65	0.007
Vitamin C (μg/ml)	795 ± 1.08[c]	811.25 ± 1.32[b]	819.50 ± 0.96[a]	0.42	0.008
Malondialdehyde (μM/ml)	7.26 ± 0.09[b]	5.28 ± 0.23[a]	5.06 ± 0.27[a]	0.18	0.024
Total anti-oxidant capacity (μM/ml)	1.49 ± 0.01[c]	1.73 ± 0.02[b]	1.86 ± 0.02[a]	0.01	0.002
Total oxidant status (μM/ml)	1,585.25 ± 0.63[c]	1,533 ± 0.71[b]	1,502.5 ± 0.96[a]	0.39	<0.001
Gestation day 150					
Total flavonoids (μg/ml)	258.93 ± 1.13[b]	264.66 ± 0.68[b]	278.84 ± 0.58[a]	0.67	0.001
Total phenolic contents (μM/ml)	5,796.50 ± 0.86[b]	5,853 ± 0.57[a]	5,859.25 ± 2.05[a]	1.14	<0.001
Vitamin C (μg/ml)	780 ± 0.41[c]	816.75 ± 0.85[b]	827.75 ± 0.48[a]	0.39	<0.001
Malondialdehyde (μM/ml)	8.31 ± 0.12[b]	4.55 ± 0.22[a]	4.19 ± 0.18[a]	0.06	0.004
Total anti-oxidant capacity (μM/ml)	1.37 ± 0.02[c]	1.81 ± 0.01[b]	1.96 ± 0.04[a]	0.02	0.007
Total oxidant status (μM/ml)	1,605 ± 1.08[c]	1,515.75 ± 0.85[b]	1,481 ± 0.82[a]	0.86	<0.001
Lactation day 20					
Total flavonoids (μg/ml)	265.28 ± 1.23[b]	273.19 ± 2.46[b]	291.44 ± 0.63[a]	1.06	0.013
Total phenolic contents (μM/ml)	5,811.50 ± 0.64[c]	5,866 ± 0.71[b]	5,884 ± 1.08[a]	0.68	<0.001
Vitamin C (μg/ml)	783 ± 0.39[c]	825.51 ± 0.48[b]	838.69 ± 1.25[a]	0.44	<0.001
Malondialdehyde (μM/ml)	7.94 ± 0.25[c]	4.01 ± 0.04[b]	2.74 ± 0.29[a]	0.11	0.013
Total anti-oxidant capacity (μM/ml)	1.53 ± 0.01[c]	1.94 ± 0.01[b]	2.16 ± 0.04[a]	0.02	0.001
Total oxidant status (μM/ml)	1,598.50 ± 0.65[c]	1,492.50 ± 1.04[b]	1,459.75 ± 0.48[a]	0.42	<0.001

Means with superscript letters (a, b, c) within the same row differ significantly at $p \leq 0.05$.

pregnancy to day 20 of lactation in the $M_{3.5\%}$ as well as $M_{2\%}$ group ($p < 0.05$).

Hydrolytic Enzymes

The activities of plasma hydrolytic enzymes in supplemented ($M_{3.5\%}$ and $M_{2\%}$) and control (M_0) groups are illustrated in **Table 7**. The protease enzyme activity was modified more effectively from day 130 to 150 of gestation in the $M_{3.5\%}$ group in comparison to the $M_{2\%}$ group. However, the supplementation of MOLP significantly influenced plasma protease activity in the $M_{2\%}$ group during the lactation period ($p < 0.05$). However, the supplementation did not show any significant impact on esterase activity during the experiment ($p > 0.05$). The amylase activity was slightly improved in the $M_{3.5\%}$ group from day 150 of gestation in comparison to the control (M_0) group ($p < 0.05$).

Milk Biochemical Composition

The alterations in milk biochemical composition as a result of MOLP supplementation are expressed in **Table 8**. The colostrum and milk protein contents were increased significantly in $M_{3.5\%}$ group. However, the supplementation in the $M_{2\%}$ group exhibited a significant impact on days 21 and 28 milk protein contents ($p < 0.05$). The milk fat percentage was not affected by supplementation in either the $M_{3.5\%}$ or $M_{2\%}$ group up to day 14 of lactation ($p > 0.05$), but on days 21 and 28, the milk fat was significantly increased in the $M_{3.5\%}$ group as compared to the control (M_0) group ($p < 0.05$). There was no influence of supplementation on the lactose contents of colostrum and mature milk samples of $M_{3.5\%}$ and $M_{2\%}$ groups ($p > 0.05$). Total carotenoids were significantly high in the milk samples of the $M_{3.5\%}$ group as compared to $M_{2\%}$ and M_0 groups from day 0 to day 28 of lactation ($p < 0.05$).

Milk Enzymatic and Non-enzymatic Antioxidants

The enzymatic and non-enzymatic antioxidant parameters of the colostrum and milk samples are presented in **Table 9**. The TPCs in the colostrum and milk samples of the $M_{3.5\%}$ group were significantly increased in response to supplementation. There was no effect of supplementation on the TPCs of colostrum from

TABLE 5 | Plasma enzymatic antioxidant indices of Beetal goats.

Enzymatic antioxidants (Units/ml)	Levels of *Moringa oleifera* leaf powder supplementation			SEM	*p*-value
	M_0	$M_{2\%}$	$M_{3.5\%}$		
Gestation day 110					
SOD	21.29 ± 0.52^c	25.82 ± 0.28^b	32.08 ± 0.39^a	0.23	0.011
POD	195.82 ± 0.61^b	207.16 ± 0.79^a	212.24 ± 1.30^a	0.45	0.002
CAT	40 ± 0.82	41 ± 1.29	44.75 ± 0.48	0.52	0.062
Gestation day 130					
SOD	19.88 ± 0.63^c	28.20 ± 0.57^b	37.77 ± 0.73^a	0.29	0.015
POD	209.65 ± 0.88^b	231.79 ± 1.16^a	238.17 ± 1.06^a	0.34	0.001
CAT	42 ± 0.41	44.75 ± 0.85	45.25 ± 0.25	0.24	0.100
Gestation day 150					
SOD	13.76 ± 0.38^c	28.88 ± 0.64^b	39.54 ± 0.89^a	0.47	0.001
POD	215.21 ± 0.74^b	244.11 ± 0.89^a	247.07 ± 0.98^a	0.51	0.005
CAT	45.50 ± 0.96	47 ± 0.71	49.50 ± 0.94	0.49	0.154
Lactation day 20					
SOD	17.97 ± 0.43^c	36.17 ± 1.05^b	42.76 ± 0.36^a	0.30	0.001
POD	226.91 ± 0.69^b	261.79 ± 1.55^a	268.11 ± 0.72^a	0.67	0.001
CAT	50.48 ± 0.87^c	55.50 ± 0.29^b	63.39 ± 0.86^a	0.38	0.023

Means with superscript letters (a, b, c) within the same row differ significantly at $p \leq 0.05$. SOD, superoxide dismutase; POD, peroxidase; CAT, catalase.

TABLE 6 | Plasma biochemical parameters of Beetal goats.

Biochemicals	Levels of *Moringa oleifera* leaf powder supplementation			SEM	*p*-value
	M_0	$M_{2\%}$	$M_{3.5\%}$		
Gestation day 110					
Total soluble proteins (mg/ml)	60.98 ± 0.73	63.87 ± 0.34	65.09 ± 1.12	0.59	0.118
Total sugars (mg/ml)	4.58 ± 0.05^c	5.40 ± 0.04^b	6.18 ± 0.03^a	0.03	0.003
Carotenoids (μg/ml)	165.77 ± 0.79^b	169.09 ± 1.07^b	176.56 ± 0.62^a	0.25	0.013
Lycopene (μg/ml)	98.75 ± 2.66	103 ± 1.15	108 ± 3.02	0.53	0.207
Gestation day 130					
Total soluble proteins (mg/ml)	59.56 ± 3.17	66.23 ± 0.57	69.16 ± 1.78	1.63	0.066
Total sugars (mg/ml)	4.43 ± 0.05^c	5.80 ± 0.04^b	6.63 ± 0.06^a	0.02	0.004
Carotenoids (μg/ml)	170.79 ± 0.96^b	177.45 ± 0.83^b	185.41 ± 0.61^a	0.49	0.001
Lycopene (μg/ml)	95 ± 1.08^c	111 ± 0.57^b	119.75 ± 0.85^a	0.51	0.010
Gestation day 150					
Total soluble proteins (mg/ml)	55.96 ± 3.04^b	61.19 ± 1.49^b	71.02 ± 0.69^a	1.56	0.042
Total sugars (mg/ml)	3.88 ± 0.13^c	5.90 ± 0.04^b	6.93 ± 0.05^a	0.03	0.005
Carotenoids (μg/ml)	168 ± 2.19^b	175.93 ± 0.68^b	187.13 ± 1.30^a	0.85	0.032
Lycopene (μg/ml)	89.75 ± 0.47^c	116.78 ± 1.03^b	125.69 ± 0.75^a	0.47	0.001
Lactation day 20					
Total soluble proteins (mg/ml)	63.50 ± 1.56^b	67.18 ± 0.35^b	75.40 ± 0.73^a	0.78	0.016
Total sugars (mg/ml)	4.03 ± 0.04^c	6.30 ± 0.05^b	7.35 ± 0.07^a	0.04	0.002
Carotenoids (μg/ml)	172.42 ± 1.02^b	178.74 ± 1.32^b	193.55 ± 0.89^a	0.73	<0.001
Lycopene (μg/ml)	93 ± 0.91^c	118.25 ± 0.85^b	129.5 ± 0.95^a	0.69	<0.001

Means with superscript letters (a, b, c) within the same row differ significantly at $p \leq 0.05$.

the $M_{2\%}$ group, while these TPCs were improved significantly in the milk samples of the $M_{2\%}$ group from day 14 to 28 of lactation ($p < 0.05$). The TAC of the milk from $M_{3.5\%}$ and $M_{2\%}$ groups was significantly increased from the beginning to day 28 of lactation in comparison to the control (M_0) group ($p < 0.05$). The significant impact of MOLP on colostrum and milk Vit C level was noticed only in the $M_{3.5\%}$ group ($p < 0.05$). Both the supplementation levels (3.5 and 2%) showed a significant impact

TABLE 7 | Plasma hydrolytic enzymes activities of Beetal goats.

Enzymes	Levels of *Moringa oleifera* leaf powder supplementation			SEM	*p*-value
	M_0	$M_{2\%}$	$M_{3.5\%}$		
Gestation day 110					
Protease (U/ml)	233.25 ± 0.95	235.50 ± 0.64	239.25 ± 1.18	0.79	0.065
Esterase (μM/min/ml)	643.75 ± 0.85	641 ± 1.08	645.75 ± 0.63	0.37	0.084
Amylase (mg/min/ml)	1.31 ± 0.02	1.34 ± 0.01	1.39 ± 0.02	0.01	0.369
Gestation day 130					
Protease (U/ml)	235 ± 1.08^b	238.50 ± 0.29^b	243 ± 0.41^a	0.38	0.009
Esterase (μM/min/ml)	638.25 ± 0.95	639.50 ± 0.96	641.75 ± 0.48	0.41	0.252
Amylase (mg/min/ml)	1.35 ± 0.02	1.39 ± 0.03	1.42 ± 0.01	0.02	0.110
Gestation day 150					
Protease (U/ml)	238.50 ± 0.51^b	240.75 ± 0.75^b	247 ± 0.82^a	0.44	0.036
Esterase (μM/min/ml)	634.50 ± 0.87	636.50 ± 0.64	637 ± 0.91	0.25	0.486
Amylase (mg/min/ml)	1.36 ± 0.03^b	1.43 ± 0.01^b	1.51 ± 0.01^a	0.01	0.036
Lactation day 20					
Protease (U/ml)	242.75 ± 0.85^c	249.75 ± 0.63^b	254.50 ± 0.29^a	0.47	0.008
Esterase (μM/min/ml)	639.25 ± 0.63	642 ± 0.82	644 ± 0.56	0.33	0.059
Amylase (mg/min/ml)	1.41 ± 0.01^b	1.44 ± 0.01^b	1.57 ± 0.02^a	0.04	0.016

Means with superscript letters (a, b, c) within the same row differ significantly at $p \leq 0.05$.

TABLE 8 | Effect of *Moringa oleifera leaf powder* supplementation on milk composition.

Biochemical	Levels of *Moringa oleifera* leaf powder supplementation			SEM	*p*-value
	M_0	$M_{2\%}$	$M_{3.5\%}$		
Day 0 (Colostrum)					
Protein (%)	8.93 ± 0.37^b	10.18 ± 0.28^b	13.53 ± 0.53^a	0.24	0.043
Fat (%)	8.95 ± 0.40	9.18 ± 0.48	9.53 ± 0.34	0.37	0.092
Lactose (%)	2.93 ± 0.29	3.09 ± 0.31	3.27 ± 0.13	0.21	0.663
Carotenoids (μg/ml)	15.22 ± 0.83^b	16.71 ± 0.40^b	18.53 ± 0.39^a	0.49	0.031
Day 7					
Protein (%)	6.84 ± 0.09^b	7.11 ± 0.31^b	8.51 ± 0.25^a	0.16	0.015
Fat (%)	7.38 ± 0.86	7.94 ± 0.62	8.72 ± 0.30	0.61	0.233
Lactose (%)	3.43 ± 0.07	3.62 ± 0.21	3.71 ± 0.16	0.05	0.514
Carotenoids (μg/ml)	6.91 ± 0.19^b	7.36 ± 0.07^b	9.82 ± 0.36^a	0.15	0.041
Day 14					
Protein (%)	5.08 ± 0.36^b	5.40 ± 0.25^b	7.94 ± 0.46^a	0.28	0.045
Fat (%)	6.56 ± 0.35	6.87 ± 0.39	7.19 ± 0.52	0.28	0.645
Lactose (%)	3.97 ± 0.14	4.01 ± 0.23	4.14 ± 0.20	0.09	0.273
Carotenoids (μg/ml)	4.68 ± 0.41^b	4.94 ± 0.16^b	6.37 ± 0.08^a	0.2	0.022
Day 21					
Protein (%)	4.03 ± 0.13^c	5.15 ± 0.08^b	6.56 ± 0.20^a	0.08	0.012
Fat (%)	4.94 ± 0.34^b	5.32 ± 0.11^b	6.29 ± 0.16^a	0.17	0.024
Lactose (%)	4.37 ± 0.51	4.51 ± 0.58	4.89 ± 0.78	0.1	0.748
Carotenoids (μg/ml)	2.73 ± 0.21^b	3.02 ± 0.13^b	4.36 ± 0.11^a	0.09	0.007
Day 28					
Protein (%)	3.43 ± 0.19^c	4.27 ± 0.11^b	5.82 ± 0.40^a	0.22	0.027
Fat (%)	4.87 ± 0.13^b	3.76 ± 0.15^b	3.41 ± 0.02^a	0.09	0.003
Lactose (%)	4.95 ± 0.80	5.08 ± 0.19	5.35 ± 0.49	0.15	0.917
Carotenoids (μg/ml)	1.38 ± 0.36^b	1.61 ± 0.25^b	3.04 ± 0.08^a	0.17	0.025

Means with superscript letters (a, b, c) within the same row differ significantly at $p \leq 0.05$.

TABLE 9 | Effect of *Moringa oleifera leaf powder* supplementation on milk antioxidant parameters.

Non-enzymatic antioxidants	Levels of *Moringa oleifera* leaf powder supplementation			SEM	*p*-value
	M_0	$M_{2\%}$	$M_{3.5\%}$		
Day 0 (Colostrum)					
Total phenolic contents (μM/ml)	2,826.75 ± 3.49^b	2,838.50 ± 0.64^b	2,875.50 ± 1.55^a	1.06	0.002
Total anti-oxidant capacity (μM/ml)	0.92 ± 0.02^c	1.42 ± 0.08^b	1.78 ± 0.13^a	0.07	0.005
Vitamin C (μg/ml)	457.75 ± 1.65^b	464.75 ± 1.79^b	483 ± 0.82^a	0.97	0.005
SOD (Units/ml)	69.86 ± 0.46^c	88.65 ± 0.69^b	96.53 ± 0.32^a	0.18	0.001
POD (Units/ml)	291.25 ± 0.63^b	309.22 ± 0.85^a	314.02 ± 1.04^a	0.37	0.003
CAT (Units/ml)	71.75 ± 0.85	76.75 ± 1.11	84 ± 1.78	1.01	0.060
Day 7					
Total phenolic contents (μM/ml)	2,608.85 ± 2.38^b	2,622 ± 0.71^b	2,669.25 ± 1.88^a	0.21	0.005
Total anti-oxidant capacity (μM/ml)	0.81 ± 0.05^c	1.39 ± 0.12^b	1.73 ± 0.11^a	0.09	0.016
Vitamin C (μg/ml)	421.75 ± 1.97^b	427.25 ± 1.32^b	436.75 ± 0.75^a	0.78	0.035
SOD (Units/ml)	75.75 ± 0.63^c	81.52 ± 0.26^b	94.4 ± 0.39^a	0.16	0.001
POD (Units/ml)	263.45 ± 0.87^b	277.56 ± 1.32^a	285 ± 1.65^a	0.28	0.015
CAT (Units/ml)	66.75 ± 1.37	69.75 ± 1.18	74 ± 0.41	0.35	0.103
Day 14					
Total phenolic contents (μM/ml)	2,471.50 ± 1.32^c	2,493.25 ± 1.03^b	2,547 ± 2.55^a	1.03	<0.001
Total anti-oxidant capacity (μM/ml)	0.74 ± 0.10^c	1.29 ± 0.13^b	1.64 ± 0.12^a	0.12	0.017
Vitamin C (μg/ml)	412.70 ± 1.31^b	416.40 ± 1.28^b	429.60 ± 0.93^a	0.71	0.016
SOD (Units/ml)	61.52 ± 0.31^c	74.40 ± 0.41^b	83.45 ± 0.55^a	0.33	<0.001
POD (Units/ml)	251.67 ± 0.79^b	270.92 ± 1.37^a	276.05 ± 0.96^a	0.19	0.008
CAT (Units/ml)	50.61 ± 1.51	56.75 ± 1.43	62 ± 1.92	0.69	0.072
Day 21					
Total phenolic contents (μM/ml)	2,289 ± 1.47^c	2,326 ± 0.91^b	2,391.50 ± 0.65^a	0.32	0.001
Total anti-oxidant capacity (μM/ml)	0.65 ± 0.04^c	1.24 ± 0.11^b	1.53 ± 0.09^a	0.08	0.013
Vitamin C (μg/ml)	385.75 ± 2.46^b	391.50 ± 1.50^b	408.25 ± 0.75^a	0.57	0.021
SOD (Units/ml)	58.65 ± 0.69^c	63.45 ± 0.91^b	75.04 ± 0.48^a	0.51	0.003
POD (Units/ml)	227.02 ± 1.08^c	243.01 ± 0.56^b	259.66 ± 0.73^a	0.25	0.008
CAT (Units/ml)	37.95 ± 1.31^b	41.98 ± 0.32^b	53.30 ± 0.61^a	0.21	0.007
Day 28					
Total phenolic contents (μM/ml)	2,133 ± 0.82^c	2,173.25 ± 1.70^b	2,205.75 ± 1.25^a	0.59	<0.001
Total anti-oxidant capacity (μM/ml)	0.61 ± 0.13^c	1.11 ± 0.04^b	1.37 ± 0.07^a	0.07	0.007
Vitamin C (μg/ml)	374.45 ± 1.04^b	378.70 ± 0.85^b	395.25 ± 0.69^a	0.52	0.001
SOD (Units/ml)	51.70 ± 0.77^c	59.65 ± 1.05^b	66 ± 0.41^a	0.57	0.003
POD (Units/ml)	202.66 ± 0.68	216.02 ± 1.12	231.01 ± 1.31	0.55	0.006
CAT (Units/ml)	29.75 ± 0.43^b	31.36 ± 0.59^b	46 ± 0.42^a	0.26	0.002

Means with superscript letters (a, b, c) within the same row differ significantly at p ≤ 0.05. SOD, superoxide dismutase; POD, peroxidase; CAT, catalase.

on the SOD activity of colostrum and milk samples as compared to the non-supplemented (M_0) group ($p < 0.05$). Initially, the POD activity was increased significantly irrespective of the level of supplementation. However, from day 21 to 28 of lactation, a significant difference was observed in the improvement of POD activity depending on levels of supplementation. The increase in the activity of CAT enzyme was non-significant up to day 14 of lactation in both $M_{3.5\%}$ and $M_{2\%}$ groups ($p > 0.05$). However, on days 21 and 28, the supplementation resulted in a significant enhancement in the CAT activity of milk samples from the $M_{3.5\%}$ group in comparison to the control (M_0) group ($p < 0.05$).

Productive and Reproductive Performance

The supplementation of MOLP increased the milk production in $M_{3.5\%}$ and $M_{2\%}$ groups as compared to the control (M_0) group from day 7 to 28 of lactation ($p < 0.05$) as is presented in **Table 10**. Similarly, the weight gain of kids in supplemented groups was significantly high (**Table 11**). The results of reproductive parameters (**Figures 1**, **2**) showed that the shedding time of the placenta and the time of onset of first postpartum estrus was less in the goats of $M_{3.5\%}$ and $M_{2\%}$ groups as compared to the goats of the (M_0) control group. The survival rate and initial birth weight of the kids of $M_{3.5\%}$- and $M_{2\%}$-supplemented goats were higher than M_0 group goats (**Figures 3**, **4**).

TABLE 10 | Effect of *Moringa oleifera leaf powder* supplementation on milk production (liters) in Beetal goats.

Days	Levels of *Moringa oleifera* leaf powder supplementation			SEM	*p*-value
	M_0	$M_{2\%}$	$M_{3.5\%}$		
07	1.01 ± 0.03^c	1.27 ± 0.05^b	1.41 ± 0.04^a	0.04	0.007
14	1.32 ± 0.14^c	1.48 ± 0.11^b	1.65 ± 0.09^a	0.11	0.033
21	1.54 ± 0.07^c	1.73 ± 0.06^b	2.01 ± 0.07^a	0.06	0.031
28	1.87 ± 0.16^c	2.11 ± 0.13^b	2.72 ± 0.15^a	0.14	0.04

Means with superscript letters (a, b, c) within the same row differ significantly at $p \leq 0.05$.

TABLE 11 | Effect of feeding *Moringa oleifera* leaf powder supplemented goat milk on body weight (kg) of their kids.

Days	Levels of *Moringa oleifera leaf powder* supplementation			SEM	*p*-value
	M_0	$M_{2\%}$	$M_{3.5\%}$		
0	2.14 ± 0.49^c	2.81 ± 0.52^b	3.53 ± 0.36^a	0.45	0.037
7	2.91 ± 0.17^c	3.77 ± 0.32^b	4.71 ± 0.28^a	0.25	0.016
14	3.64 ± 0.22^c	4.51 ± 0.29^b	5.88 ± 0.12^a	0.2	0.001
21	4.49 ± 0.42^c	5.72 ± 0.18^b	6.96 ± 0.14^a	0.24	0.021
28	5.03 ± 0.16^c	6.61 ± 0.24^b	8.36 ± 0.35^a	0.16	0.044

Means with superscript letters (a, b, c) within the same row differ significantly at $p \leq 0.05$.

DISCUSSION

The animals are more prone to oxidative stress during the transition period because an increase in energy requirements to support developing fetuses and subsequent lactation coincide with depressed feed intake. The nutritional quality of feedstuffs is very important to regulate the pregnancy and lactation performance of goats. The MO leaves are well-known for their nutritional and therapeutic properties that have been attributed to their various phytochemical constituents (39, 40).

The pregnancy and lactation stress results in the excessive production of hydroxyl (OH) and nitric oxide (NO) radicals. The animal body is well-equipped with different protective mechanisms to neutralize the harmful effects of OH and NO radicals. The antioxidant protective system is naturally suppressed near parturition due to certain physiological changes in the body (41). The MOLP supplementation to pregnant goats resulted in an increase in their plasma TFs and TPCs that control the immense production of OH and NO radicals *via* Haber-Weiss and Fenton reactions to minimize the detrimental effects of oxidative stress during pregnancy and lactation (42). The regulation of antioxidant defense system of the body under different stress conditions through MO leaf extract supplementation was also reported in rats (43).

Vitamin C is generally regarded as the first line of defense to provide protection from the damaging effects of oxidative stress in pregnancy especially near parturition (44). A constant decline in plasma vit C level near parturition aggravates the situation that makes the animals more vulnerable to oxidative damage (45). A significant improvement in plasma vit C level during this study in supplemented goats showed that MOLP as a rich source of vit C has the ability to protect the pregnant goats from the deleterious effects of oxidative stress by suppressing the action of free radicals. The increase in plasma vit C concentration in response to MOLP supplementation is in accordance with the findings of a study on Aardi goats (14).

Lipids act as substrate for reactive nitrogen and oxygen species (RNS and ROS) to start the process of lipid peroxidation (LPO) (46, 47). The balance between the production and elimination of ROS and RNS from the body is sustained by antioxidant defense system in healthy animals. Any untoward disturbance in this balance may enhance the plasma TOS and MDA concentration. The high values of TOS and MDA represent the state of oxidative stress (48). It is evident from the results of this study that the supplementation of MOLP increased the plasma TAC in treated goats by suppressing the process of LPO. This consequently reduces the values of plasma TOS and MDA. The enzymatic (SOD, POD, and CAT) antioxidants also have a key role in limiting LPO, as both enzymatic and non-enzymatic components of antioxidant defense system work in collaboration to maintain the conditions suitable for mother and developing fetus by minimizing the parturition and early lactation stress (49).

The physiological changes in maternal body during the transition period especially near parturition result in excessive production of ROS (50). These ROS are converted into H_2O_2 by SOD enzyme, while POD and CAT enzymes further degrade the H_2O_2 into water and molecular oxygen (51, 52). The successful completion of parturition stage and start of healthy lactation depend on the activities of SOD, POD, and CAT enzymes (53). High antioxidant (SOD, POD, and CAT) enzyme activities indicate that the supplementation of MOLP in late pregnancy improved the plasma enzymatic antioxidants that was also reported in some other studies conducted with different supplementation levels of MO in rabbits, poultry, and dairy cows (54–56).

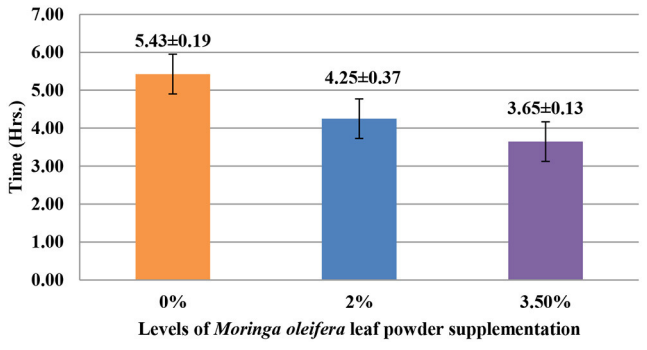

FIGURE 1 | Effect of *Moringa oleifera* leaf powder supplementation on shedding time (hours) of placenta in Beetal goats.

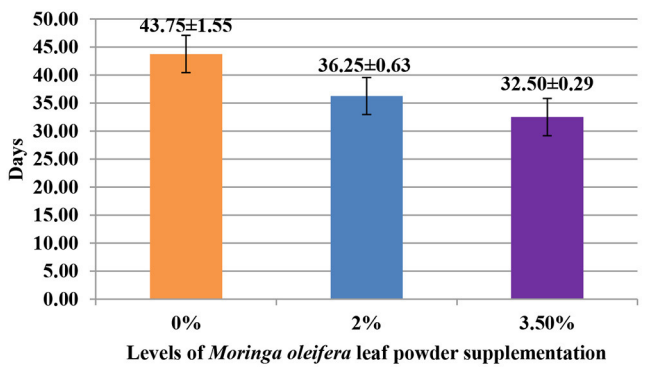

FIGURE 2 | Effect of *Moringa oleifera* leaf powder supplementation on onset time (days) of first postpartum estrus after kidding in Beetal goats.

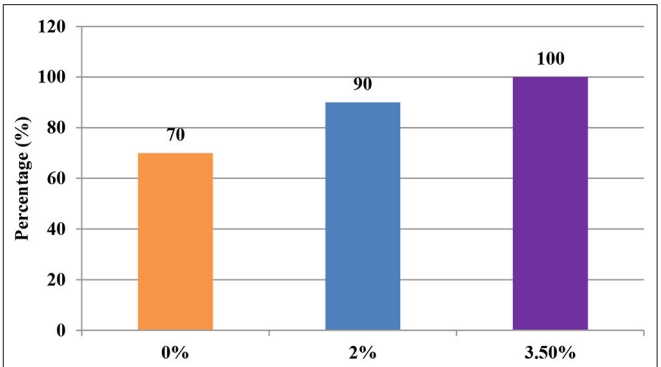

FIGURE 3 | Survival rate (%) of kids in response to milk feeding of goats supplemented with *Moringa oleifera* leaf powder.

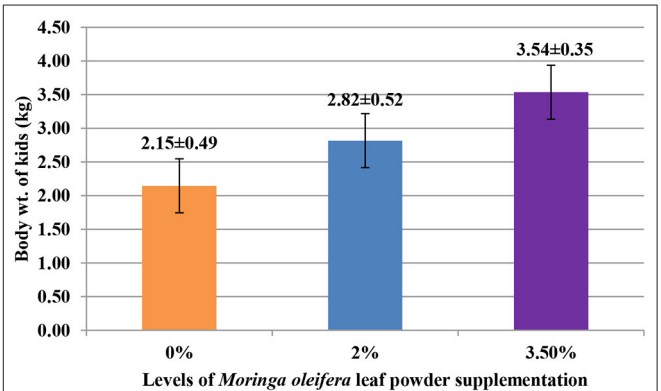

FIGURE 4 | Effect of *Moringa oleifera* leaf powder supplementation on birth weight (kg) of kids in Beetal goats.

Blood biochemical parameters are the established indicators to provide information about the health status of pregnant animals for their successful transfer from gestation to lactation stage (57). Late pregnancy is characterized by severe metabolic changes and a rapid decrease in plasma protein level was also noticed in different other animal species during this period (58–60). The plasma protein level drops promptly in the last trimester due to high amino acid requirements for developing fetus and preparation of the mammary system for subsequent lactation stage (61). However, after parturition, the plasma protein level starts to increase due to immense production of immunoglobulins (62). It is evident from the findings of this study that the plasma protein level was high in supplemented groups as compared to the control group of the experiment during the peripartum period. The supplementation of MOLP increased the plasma protein contents to satisfy the high protein requirements of pregnancy and lactation. These results support the findings of other studies in Jersey cattle and sows (21, 63). The presence of high amount of protein in MOLP enhances the synthesis of selenocysteine-based selenoproteins. These proteins have been reported to play a role in the modification of antioxidant defense system and improvement of reproductive functions (64).

The decrease in plasma sugar level near parturition is typical for goats and ewes. A reduction in feed intake occurs during late pregnancy due to the squeezing of the rumen by rapid fetal growth (65). The mobilization of the body fats starts if the energy requirements of the animals are not fulfilled with the advancement in pregnancy through provision of appropriate feed supplement (66). The negative energy balance in this stage may lead to the development of ketosis and some other metabolic diseases. The feeding of MOLP increased the plasma sugar level of supplemented groups to provide sufficient amount of energy for parturition and early lactation. The revival of gluconeogenesis process after parturition in response to certain endocrine changes rapidly increase the plasma sugar level (67, 68). Furthermore, the supplementation also improved the plasma lycopene and carotenoids status to regulate the synthesis of inflammatory cytokines that reduces the chances of complications at the time of parturition (69, 70).

A proteolytic enzyme system in the body helps in the removal of worthless and damaged biomolecules to maintain hemostasis during pregnancy and lactation (71). The significant increase in the activity of protease enzyme in MOLP-supplemented groups depicted its defensive effects at the cellular level. The proteolytic enzyme system is of great importance because it also has the ability to act as a secondary antioxidant defense system when the primary antioxidant system is unable to protect the body from oxidative stress (72). The supplementation of MOLP also resulted

in an improvement in the amylase activity. This enzyme enhances the conversion of carbohydrates into glucose to produce energy according to the requirements of body during pregnancy and lactation (66, 73).

The major outcome of the study was that supplementing the goats' ration with MOLP markedly influenced their colostrum and milk composition. The preparations for colostrum synthesis start in the last month of pregnancy (74). Colostrum provides energy and maternal immunity along with different other growth factors to maintain health status and development of newly born kids. The findings of this study showed that supplementation of MOLP increased the protein contents of colostrum and milk in supplemented groups. The concentration of protein is generally high in colostrum than normal milk due to the presence of Igs in huge amount (75). The consumption of Igs protects the newly born kids from different diseases and thereby enhances their survival rate. The supplementation also improved the fat percentage of mature milk, which is in accordance with the findings of Kholif et al. (76). MOLP stimulates the production of acetate that acts as a major precursor for the biosynthesis of fat (77). Similarly, a significant increase in carotenoids of colostrum and milk was also noticed with 3.5% supplementation. The concentration of carotenoids in milk mostly depends on the type of feed and MOLP being a rich source of carotenoids has positive effects on the carotenoid contents of colostrum and milk in both supplemented groups. The carotenoids have been reported to play an important role in the improvement of milk quality by preventing the process of auto-oxidation (78).

The presence of antioxidants in appropriate amount prolongs the shelf life of milk and reduces its chances of microbial spoilage. The antioxidants in milk also protect the suckling kids from various health hazards by strengthening their immune system (79). The supplementation of diet with MOLP increased the colostrum and milk TAC as was also reported previously in cows by Kekana et al. (63). This could be due to the synergistic effects of flavonoids, phenolics, Se, and vit C present in MOLP. Generally, milk is not considered a good source of vit C. However, the results of this study showed the presence of an appreciable amount of vit C in the milk of goats supplemented with MOLP. Thus, feeding the goats with MOLP-supplemented diet positively influenced the vit C contents in their milk. The presence of functional antioxidants in MOLP also improved the enzymatic (SOD, POD, and CAT) antioxidant status of the milk to fulfill the demands of both milk producers and consumers for healthier dairy products. The favorable impacts of MO supplementation on milk composition were also reported in some other studies performed on dairy animals (80, 81).

The beneficial effects of MOLP supplementation on reproductive performance parameters observed in this study were due to its high nutritional profile. The birth weight of kids in supplemented groups was high as compared to the control group. The supplementation of MOLP in advance stage of pregnancy increases the provision of protein to developing fetus for its further growth and also improves the protein contents of colostrum and milk (82). The high level of protein contents in the milk of supplemented goats promoted the weight gain in their kids. The presence of therapeutic compounds in MOLP increased the survival rate of the kids of supplemented goats by protecting them from various diseases (83). Similar findings were reported by Qwele et al. (84), who disclosed that feeding of MO-supplemented diet is beneficial for the animals to protect them from oxidative stress-induced diseases. High milk production in early lactation stage often results in negative energy balance (NEB), if the nutrient supply is inadequate to lactating animals. The NEB is the major cause of delay in shedding of placental membranes and resumption of postpartum estrus after parturition (85). MOLP supplementation improved the energy status of the body to ensure the revival of ovarian activity and reduces postpartum anestrus interval. The shortening of postpartum anestrus duration by dietary modifications has also been reported previously in cows (86). The high proportion of protein and Se contents in MO strengthened the uterine muscle contractions for timely shedding of placenta and thus protects the reproductive tract from different infections.

CONCLUSION

The results of the current study revealed that supplementing 3.5% MOLP improved maternal health and milk quality in terms of antioxidant status and biochemical composition. Furthermore, this supplementation level also increased the milk yield, kids' growth rate, and reproductive performance of goats. These findings propose that MOLP has the potential to improve the productive/reproductive performance of goats. However, further studies are required with different feeding levels of MOLP to explore the molecular aspects of improving productive and reproductive performance in large herds of animals.

AUTHOR'S NOTE

This experiment/research paper is a part of Ali Afzal PhD study.

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 procedures used for study and ethical clearance was approved and granted by Animal Use and Care Research Committee at Nuclear Institute for Agriculture and Biology (NIAB), Faisalabad, Pakistan.

AUTHOR CONTRIBUTIONS

AA: conduct experiment, data aggregation, statistical analysis, and wrote manuscript. AH: lab analysis. TH: design experiment, monitoring experiment, and revised manuscript. MS and MM: methodology. GY: manuscript editing and revision. All authors contributed to the article and approved the submitted version.

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