INTEGRATED ROLE OF NUTRITION AND DIGESTIVE PHYSIOLOGY FOR ANIMAL HEALTH

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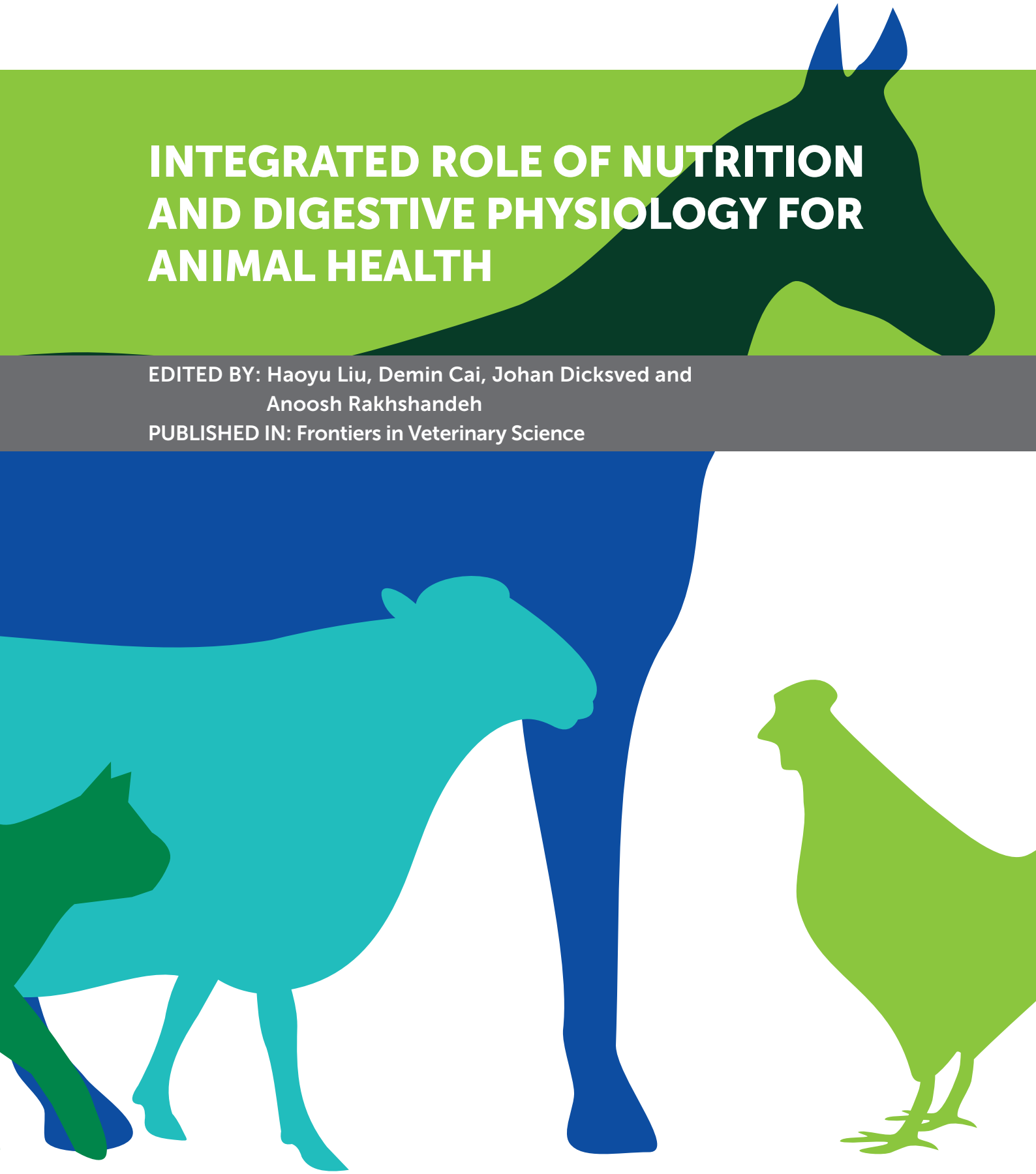

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INTEGRATED ROLE OF NUTRITION AND DIGESTIVE PHYSIOLOGY FOR ANIMAL HEALTH

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EDITORIAL
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Editorial: Integrated Role of Nutrition and Digestive Physiology for Animal Health

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Keywords: diet integration, digestive physiology, host-microbe interaction, integrated nutrition, macronutrients

Editorial on the Research Topic

Integrated Role of Nutrition and Digestive Physiology for Animal Health

Consisting of nutrition, digestive physiology, and the microbiota, each point of the gut triangle influences the animal metabolic health and performance. The mammalian gastrointestinal (GI) tract is composed of the intestine and multiple accessory organs. To ensure effective digestion, it is organized into a functional structure with specialized and region-specific anatomical, histological, and functional diversities. The morphological and functional features of the digestive system generally reflect the nutritional status of animals. In addition, the abundance of indigenous microbiota plays a pivotal role in the interplays of the triangle of intestine. Given the complexity of the GI tract, we argue that a more holistic perspective should be applied in this research field. Fortunately, with the advent of the next generation sequencing (NGS) technology and other molecular tools, it becomes feasible to gain a better understanding of the bioactive components present in food and feed, and the mechanisms of action toward desired metabolism and overall animal health. This Research Topic "Integrated role of nutrition and digestive physiology for animal health" in Frontiers in Veterinary Science collected 13 scientific contributions from high qualified research groups focusing on large animal species. The articles within this Research Topic detail the gut microbiome signatures in relation to animal metabolism and growth, and cover a wide-range of alternative feed ingredients from their effects on the digestive physiology optimization to the molecular regulation mechanisms, as well as motivations for and use of dietary supplements in livestock industry.

Gopi et al. describe how an early boosting of nutrients can shape the gut physiology and maximize the metabolic potential of broilers. An *in ovo* administration of nucleoside results in a better developed GI function, thus a higher energy metabolizability in chickens when growing up (Gopi et al.). The liver physiology of piglets at birth and at weaning is studied in details by Li et al. where the importance of lipid homeostasis is addressed with an emphasis on epigenetic regulations (Li et al.). In their article, the hepatic cholesterol metabolism of a commercial pig breed Large White is analyzed and compared with a local breed Erhualian, showing differences in several aspects. Biodiversity of livestock populations is necessary for adaptation to climate changes, as well as for consumer demand. However, the metabolic traits of local livestock breeds and their accustomed dietary regimens are often overlooked. Along this line, Meng et al. specifically characterize the beef quality of the Chinese native cattle Yunling, Wenshan, in comparison with Simmental. And provide insights into novel metabolic pathways and the underlying regulatory mechanisms (Meng et al.).

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As aforementioned, nutrition is a major key to the efficiency of digestive process and growth performance in animal husbandry. Several different dietary regimens and novel feed resources worldwide are explored. Chen et al. and Shi, Xun et al. critically examine the utilization of dietary antimicrobial alternatives tea tree oil and the combination of curcumin and piperine, their anti-inflammatory properties and the potential to improve animal produce quality (Chen et al.; Shi, Xun et al.). The latter study specifically describes the antioxidant activity of the feed additive and the associated changes of GI function in Wuzhishan piglets (Shi, Xun et al.). This is also underscored in broiler chickens fed onions (*Allium cepa* L.) extract from Egypt and shows less stressful behavior, better GI health and digestibility (Omar et al.). Indeed, oxidative stress can cause overt inflammation and severe damages in animals (Sun et al.). The Egyptian leek (*Allium ampeloprasum var. kurrat*) leaf extract is evaluated as a replacement of antibiotic growth promoter in chickens by Al-khalaifah et al.. Remarkably, both studies in poultry show improved growth performance parameters and economic efficiency including better return and profit. In many developing countries, in-feed antimicrobial usage as a growth promoter is a standard practice in livestock. Repeatedly exposing food animals to small doses of antibiotics contributes significantly to antimicrobial resistance in humans. In this regard, Lourenco et al. investigate the effects of feeding carbadox on the gut microbiome of weanling piglets, showing an antibiotic-dependent shift without growth promoting effects (Lourenco et al.). Nevertheless, researches on antibiotics and the alternatives in livestock science in relation to digestive physiology and metabolic health should be encouraged, especially in countries or areas where antimicrobials can still be purchased over the counter. In addition to feed supplements, non-typical nutrition such as the physi-chemical properties of feed is described by Liermann et al. where interactive effects between dietary rapeseed proportion and technical treatments are shown to alter performance, GI morphometric traits and immune responses in broilers (Liermann et al.).

As the major target of nutrition, and the essential mediator of metabolism, the gut microbiota becomes one area of intense interest. Indeed, livestock health depends on a beneficial host-microbe interaction. Interestingly, Shi et al. reveal a dietary level switch regulation of digestive physiology in black goat kids (Shi, Zhang et al.). Supplementations of coconut oil above the optimal level do not promote growth but suppress rumen microorganisms and their activities, therefore disrupt the intestinal microenvironment. The temporospatial gut microbiota signature is described in angus steers across life span (Welch et al.) and in sheep from the world's highest elevation (Fan et al.), respectively. Apparently, the gut microbiome plasticity supports animals to survive and thrive. In these studies of livestock digestive physiology and microbiota, NGS technologies are widely used. With the advancement of this technology and many other molecular tools readily available for farm animal studies, we are all set to gain a better understanding of the bioactive components present in food and feed, and an integrated perspective of their actions toward desired physiological responses.

AUTHOR CONTRIBUTIONS

H-YL wrote the manuscript. JD, AR, and DC edited and contributed to the organization of the editorial articles. All authors contributed to the article and approved the submitted version.

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ORIGINAL RESEARCH
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Epigenetic Regulation of Key Enzymes CYP7a1 and HMGCR Affect Hepatic Cholesterol Metabolism in Different Breeds of Piglets

Xian Li[1], *Hanyang Xiao*[1], *Xiaoqian Jian*[1], *Xiangyin Zhang*[1], *Hui Zhang*[1], *Yang Mu*[1], *Hua Wang*[2], *Shulin Chen*[1] *and Rihua Cong*[1]*

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Liver is the place where cholesterol is synthesized, transported, secreted, and transformed, thus liver takes an irreplaceable role in cholesterol homeostasis. Hepatic cholesterol metabolism differs between breeds, yet the molecular mechanism is unclear. In this study Large White (LW) and Erhualian (EHL) piglets (at birth and 25-day-old) were used, 6 each time point per breed. Erhualian piglets had significantly lower body and liver weight compared with Large White at birth and weaning, but the liver/ body weight ratio was higher at weaning, associated with increased serum and liver cholesterol and triglyceride content. The mRNA expression of Cholesterol-7alpha-hydroxylase *(CYP7a1)* and Recombinant Acetyl Coenzyme Acetyltransferase 2 (*ACAT2)* were down-regulated in Erhualian piglets at birth, while hepatic Sterol-regulatory element binding protein 2 (*SREBP2)* mRNA expression was up-regulated in Erhualian piglets at weaning, as well as SREBP2 protein content, compared with Large White piglets. At birth, the depressed CYP7a1 transcription in Erhualian piglets was associated with decreased Histone H3 (H3) and increased Histone H3 lysine 27 trimethylation (H3K27me3). While the results revealed significant promoter hypermethylation of 3-Hydroxy-3-methylglutaryl-CoA reductase *(HMGCR)* promoter in Erhualian piglets at weaning, together with increased Histone H3 lysine 9 monomethylation (H3K9me1) and Histone H3 lysine 4 trimethylation (H3K4me3). These results suggest that epigenetic modification may be an important mechanism in hepatic cholesterol metabolism among different species, which is vital for maintaining cholesterol homeostasis and decreasing risk of cardiovascular disease.

Keywords: piglets, cholesterol metabolism, liver, epigenetic regulation, CYP7a1, HMGCR

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BACKGROUND

Cholesterol participates in the formation of cell membrane (1) and can synthesis bile acid and vitamin D as a precursor, which is of great significance to the life activities of the body (2, 3). Liver cholesterol content is determined by the net balance of cholesterol synthesis, transport and catabolism, i.e., cholesterol can be converted into bile acids (4). HMGCR is the key enzymes

in cholesterol biosynthesis (5). HMGCR transcription is regulated by Sterol-regulatory element binding proteins (SREBPs), of which SREBP2 is the major subtype that regulates liver cholesterol (6, 7). Meanwhile, the content of cholesterol in the cell can affect the activity of HMGCR and SREBP2, thereby forming a feedback loop that forms the homeostasis of cholesterol (8). The transport of cholesterol is that into and out of the liver. Low-density lipoprotein (LDL) molecules are the main operator of cholesterol in the blood which are responsible for transporting liver cholesterol to other peripheral tissues, while High-density lipoprotein (HDL) transports cholesterol from extrahepatic tissues back to the liver. Low-density lipoprotein receptor (LDL-R) mediates the uptake of low-density lipoprotein cholesterol (LDL-C), which is very important in regulating serum concentrations of total cholesterol (Tch) and LDL-C. The key enzyme of cholesterol synthesis of bile acid in liver through the predominant pathway is CYP7a1 (9), however Cholesterol-27alpha-hydroxylase (CYP27a1) is the rate-limiting enzyme in the alternative pathway of bile acid synthesis.

The content of cholesterol in liver and serum is different vary from species to species and even within the same species. Studies have shown that the serum cholesterol content of different breeds of pigs was different (10, 11). Serum cholesterol concentration was lower in Full blood Chianina compared with crossbreds (Hereford × Angus crossbred), which was affected by sex class and dietary (12). There are significant differences in the sensitivity of different breeds of rabbits to hypercholesterolemia caused by a high cholesterol diet. Polymorphisms at genetic loci may be associated with significant heterogeneity in sensitivity to dietary cholesterol. These polymorphisms include absorption of dietary cholesterol, conversion of liver cholesterol to bile acids, feedback inhibition of endogenous cholesterol synthesis, or regulation of the LDL-R pathway (13–15). The expression level of *LDL-R* mRNA was different in muscle of pigs from different genetic groups, which was correlated with Intramuscular fat of longus dorsi (IMF) content in the Longissimus dorsi muscle of pigs (16). Indeed, while there are many reports about the difference of liver cholesterol content among varieties, molecular mechanisms of the difference were seldom studied.

HMGCR is essential for cholesterol synthesis. CYP7a1 is the rate-limiting enzymes in the classical pathway of bile acids synthesis in liver (17). Epigenetic regulation including DNA methylation and histone modification on fetal gene expression during cholesterol metabolism have been well-demonstrated (18). In angiocardiopathy and cancer researches, *CYP7a1* and *HMGCR* gene are described to be vulnerable to epigenetic regulation including DNA methylation and histone modification (18). However, whether interspecific differences affects gene expression via epigenetics has not been well-explored. Meaney provide a clue that DNA acylation has a key role in the overall coordination of cholesterol homeostasis (19). Different species modifies hepatic cholesterol metabolic gene transcription by DNA methylation and histones modification like H3K4me3, H3K27me3, and H3K9me1 (19–21). Activation of the *CYP7a1* gene is associated with an increase in H3 acetylation and H3K4me3 and a decrease in H3K9me1 and H3K27me3. Meanwhile, the activation of HMGCR transcription is accompanied by an increase in H3 acetylation and a decrease in histones H3, H3K9me1, and H3K27me3 (21–24). Based on these studies, we hypothesized that epigenetic events have a crucial function in modifying liver cholesterol metabolism in Large White and Erhualian piglets.

MATERIALS AND METHODS

Animal and Sampling

The experimental animals include Large White and Erhualian pig, and the animal experiments were performed in the Conservation and Breeding Farm at Jiangsu Polytechnic College of Agriculture and Forestry, Jurong, Jiangsu Province, China. LW and EHL piglets (at birth and 25-day-old) were used in the study, 6 each time point per breed. All piglets were killed and sampled after weighing. Serum specimens were prepared and stored at −20°C. Liver specimens were collected within 20 min of death, quickly frozen in liquid nitrogen and stored at −80°C until further analysis. Animal experiments were conducted under the guidance of the Animal Ethics Committee of Northwest A&F University, China.

Cholesterol in Serum and Liver

Total cholesterol in serum and liver was measured using a commercial cholesterol test kit (AO10027, Jinma Biotechnology Co., Ltd., Wenzhou, China). Respective assay kits (006340 and 006328, respectively, Beijing BHKT Clinical Reagent Co., Ltd., Beijing, China) were used to detect the concentrations of LDL-C and High-density lipoprotein cholesterol (HDL-C) in the serum. Liver cholesterol was extracted according to previously reported methods (Gibney and Nolan 2010). Briefly, 200 mg frozen liver sample was pipette in 1 ml of lysis buffer (18 mmol/L Tris, pH 7.5, 300 mmol/L mannitol, 50 mmol/L Ethylene Diamine Tetraacetic Acid(EDTA), 0.1 mmol/L PMSF) by a Polytron homogenizer (PT1200E, Brinkman Instruments, Littau, Switzerland). Two hundred microliter of homogenate were vigorously mixed with 800 μl chloroform/methanol (2:1, vol/vol) and centrifuged at 3,000 g for 5 min.

Cholesterol was extracted from the organic phase, air-dried and reconstituted in 30 μl of a mixture of tert-butanol and methanol (13:2, vol/vol). The cholesterol content was determined by cholesterol assay kit (Jinma Biotechnology Co., Ltd., Wenzhou, China).

Total RNA Isolation and mRNA Quantification

Total RNA was isolated from liver samples using Trizol Reagent (Tiangen Biotech Co., Ltd., Beijing, China). Each sample was synthesized from total RNA using iScript cDNA Synthesis Toolkit (Promega, Madison, WI, USA) according to the manufacturer's instructions. Diluted 2 μl of cDNA (1:50) for real-time polymerase chain reaction (PCR). Primer sequences are shown in **Table 1** and were synthesized by Invitrogen (Shanghai, China). Real-time PCR was performed in Mx3000P (Stratagene, USA). Simulated reverse transcription (RT) and no template controls were set to monitor the possible contamination of genomic and exogenous DNA both at RT and PCR. The

TABLE 1 | Primer sequences of the target genes.

Target	Primer sequence (5′-3′)	Product (bp)	GenBank No.
SREBP1	F: GCGACGGAGCCTCTGGTAGT	217	NM214157.1
	R: GCAAGACGGCGGATTTATTCA		
SREBP2	F: GCCTACCGCAAGGTGTTTC	305	DQ020476.1
	R: GTCATTTGCTGGCAGTCGTT		
HMGCR	F: CAGGCTGAAGTAAGGGAGA	174	DQ432054.1
	R: CACGAAGTAGGTGGCGAGA		
LDL-R	F: ACTGCTCATCCTCCTCTT	109	AF065990.1
	R: TTCCGTGGTCTTCTGGTA		
LXR	F: ATTTCCAGGAGTGCCGTCTT	102	AB254406.1
	R: CTTGCCGCTTCAGTTTCTT		
FXR	F: CGGAGAAGCATTACCA	137	XM 001928800.2
	R: AAGCATTCAGCCAACA		
CYP7a1	F: TATTCCTTCCGTTACCGAGTG	262	AK230868.1
	R: ACCTGACCAGTTCCGAGAT		
CYP27a1	F: TGTGGCTCGCATCGTTC	153	EF625352.1
	R: TCACCTGGCAGCTCCTT		
GAPDH	F: TACATGGTCTACATGTTCCAGTATG	285	DQ403065
	R: CAGGAGGCATTGCTGACAATCTTG		

specificity of amplification was determined by melting curve analysis and PCR product sequence.

Tissue Protein Extraction and Western Blot Analysis

Extract total and nuclear proteins from 100 mg of frozen liver tissue, as previously described (30). Detection of protein concentration with Pierce BCA Protein Assay Kit (Thermo Scientific, USA). Western blot analysis for SREBP2 (ab30682, Abcam, UK, diluted 1:200) and HMGCR (sc-33827, Santa Cruz, USA, diluted 1:100) were follow the manufacturer's instructions. HMGCR and SREBP2 was normalized with Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (KC-5G4, Kangcheng, China, diluted 1:10,000).

Chromatin Immunoprecipitation (ChIP)

ChIP experiment was performed according to the modified based on previous reports (37, 34). Briefly, 200 mg frozen liver sample was taken in liquid nitrogen, ground, and then resuspended in phosphate buffered saline (PBS) containing a mixture of protease inhibitors cocktail (Roche Diagnostics GmbH, Mannheim, Germany) and cross-linked in 1% formaldehyde for 10 min at room temperature. Then use 2.5 M glycine to stop the cross-linking reaction.

Washing the pellets with PBS and sodium dodecyl sulfate (SDS) lysis buffer (50 M Tris-HCl pH 8.1, 10 mM EDTA, 1% SDS containing protease inhibitors. The samples after cross-linked were sonicated for 10 min on ice with 10 s on/off intervals (Sonics Vibra, USA). To remove cell debris from the crude chromatin preparations the samples were then centrifuged at 12,000 rpm for 10 min at 4°C. According to the results of 1% agarose gel measurement, the average length of ultrasonic chromatin was about 500 bp. Prepare salmon sperm DNA/G protein agarose beads (60 μl, 50% slurry, Biyuntian, Biotechnology, China) in advance, dilute the Protein-DNA complex with ChIP dilution solution, and incubate 2 μg of the corresponding antibody at 4°C overnight [histone H3 antibody, ab1791, Abcam; anti-acetyl-histone H3, 06-599, Millipore; monomethyl-Histone H3K9 (Lys9) 17-680, Millipore; trimethyl-histone H3K27 (Lys27), 17-622, Millipore; trimethyl-histone H3K4 antibody, ab1012, Abcam]. Negative control group did not add antibodies. Add G protein agarose beads (120 μl slurry 50%) to capture chromatin immunoprecipitation complex. The pellets containing the immunoprecipitation complex were washed sequentially, and the antibody/protein/DNA complex were eluted from the protein G agarose beads. Finally, reversing cross-linking DNA fragments at 65°C for 5 h to purify the DNA that released from the immunoprecipitation complex. Immunoprecipitated DNA was used as a specific primer for the RT-PCR template to amplify the genomic sequence of the promoter regions of the *HMGCR* and *CYP7a1* genes.SREBP2 has no genomic DNA sequence, so this study did not use ChIP-qPCR to detect the SREBP2 promoter.

TABLE 2 | Body weight, liver weight, liver index, and the content of cholesterol in serum and liver of the offspring piglets at birth and weaning ($n = 6$ piglets each time point per breed).

Parameters	LW		EHL	
	Birth (0 d)	Weaning (25 d)	Birth (0 d)	Weaning (25 d)
Liver weight (g)	31.76 ± 1.72	178.74 ± 15.40	17.16 ± 1.21**	119.84 ± 6.72**
Body weight (kg)	1.31 ± 0.04	8.10 ± 0.34	0.75 ± 0.03**	4.08 ± 0.25**
Liver index (g/kg)	24.27 ± 0.91	21.92 ± 1.15	23.04 ± 2.01	29.51 ± 1.10**
TG (mmol/L)	0.29 ± 0.06	0.29 ± 0.03	0.24 ± 0.05	0.62 ± 0.128**
Tch (mmol/L)	0.75 ± 0.03	1.49 ± 0.05	1.52 ± 0.14*	2.38 ± 0.13*
HDL-C (mmol/L)	0.30 ± 0.02	0.47 ± 0.02	0.43 ± 0.03**	1.16 ± 0.08**
LDL-C (mmol/L)	0.23 ± 0.02	0.47 ± 0.01	0.52 ± 0.07**	0.76 ± 0.05**

*$P < 0.05$ and **$P < 0.01$. Data are presented as mean ± SEM.

Statistical Analysis

All data are expressed as mean ± SEM and were analyzed using independent-samples test with SPSS 13.0 for windows. The method of $2^{-\Delta\Delta Ct}$ was used to analyze the real-time PCR data. $P < 0.05$ is considered statistically significant.

RESULT

Body Weight and Cholesterol in Serum and Liver

As shown in **Table 2**, the piglets born from EHL sows had significantly lower body weight and liver weight at birth and

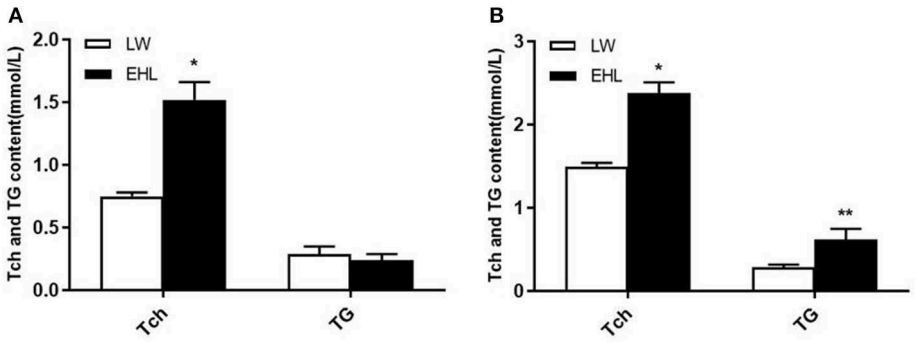

FIGURE 1 | Total cholesterol and triglyceride content of the offspring piglets at birth **(A)** and weaning **(B)** ($n = 6$ piglets each time point per breed). *$P < 0.05$ and **$P < 0.01$. Data are presented as mean ± SEM.

weaning, while the ratio of liver weight to body weight (liver index) did not change at birth. The piglets born from EHL significantly increased liver index at weaning. This growth retardation is associated with significantly increased serum concentration and liver content of Tch ($P < 0.05$; **Figure 1**), HDL-C ($P < 0.01$), and LDL-C ($P < 0.01$) at birth and weaning.

Expression of Hepatic Genes Involved in Cholesterol Metabolism

Using RT-PCR to quantitative detection mRNA abundance of 12 genes that involved in liver cholesterol metabolism. Among these genes, *ACAT2* ($P < 0.05$) and *CYP7a1* ($P < 0.01$) were found to be significantly down-regulated in the liver of EHL piglets at birth (**Figure 2A**). The levels of hepatic *SREBP2* mRNA in EHL piglets significantly higher than LW piglets at weaning ($P < 0.05$; **Figure 2B**).

Hepatic SREBP2 and HMGCR Protein Content

Western blot was used to detect the protein content of SREBP2 and HMGCR in the liver. The content of SREBP2 protein (**Figure 3A**, $P > 0.05$) at birth and HMGCR protein (**Figure 3C**, $P > 0.05$) at weaning was not affected by variety and age. However, the SREBP2 content in nuclear lysate was significantly increased (**Figure 3B**, $P < 0.05$) in the liver of EHL piglets at weaning, which was consistent with liver mRNA abundance.

DNA Methylation

MeDIP analysis showed that the HMGCR promoter was significantly hypermethylated ($P < 0.05$) in liver of EHL piglets at weaning (**Figure 4**). Because no CpG island was expected to exist in the CYP7a1 promoter, MeDIP analysis of the CYP7a1 promoter was excluded from this study.

Histone Modifications

The enrichment of four histone modification marks, namely histone H3 acetylation (H3AC), histone H3 lysine 4 trimethylation (H3K4me3), histone H3 lysine 27 trimethylation (H3K27me3), and histone H3 lysine 9 monomethylation (H3K9me1), as well as histone H3 on the promoter of *HMGCR* and *CYP7a1*, analyzed by ChIP with specific antibodies. As shown in **Figure 5A**, when expressed as a percentage, the activation of hepatic *HMGCR* gene transcription in EHL piglets at birth was associated with a 685.1% decrease in H3AC ($P < 0.01$). When expressed as a ratio relative to H3, the transcription of *HMGCR* gene was related to the decrease of H3AC ($P < 0.05$) by 593.8%, and increase of H3K9me1 ($P < 0.05$) by 77.5%. **Figure 5B** showed significant decrease in histone H3 ($P < 0.05$; −2.9%), K27H3 ($P < 0.05$; −4.13%), and H3AC ($P < 0.01$; −9.83%) at weaning, expressed as percentage of the input, yet significant increase in H3K9me1 ($P < 0.05$; 102.1%) and H3K4me3 ($P < 0.05$; 8.57%) in EHL piglets at weaning, when expressed as the ratio relative to H3.

Figure 5C shows the enrichment and the modified forms of histone H3 in the *CYP7a1* promoter. The decrease in *CYP7a1* transcription in EHL piglets at birth was related to the downward trend ($P = 0.07$) in H3K4 (−33.5%) and uptrend ($P = 0.09$) in H3K27 (75.3%) and significantly decreased ($P < 0.01$) total H3 (−52.2%), expressed as percentage of input. A significant increase ($P < 0.01$) of total H3AC (85.6%) and H3K27me3 (172.8%) and a trend of increase ($P = 0.06$) of H3K9me1 (172.4%) were seen at the CYP7a1 promoter in EHL piglets at birth when expressed as the ratio to total H3. While a trend of increase in H3AC ($P = 0.07$; 6.55%) and H3K4 ($P = 0.06$; 3.53%) and significantly decreased H3K27me3 ($P < 0.05$; −6.48%) were seen at the *CYP7a1* promoter in EHL piglets at weaning, expressed as percentage of input. But when expressed as the ratio relative to H3 (**Figure 5D**), H3K27me3 (−62.6%) significantly ($P < 0.01$) decreased but H3K4me3 ($P = 0.09$; 49.1%) had the trend of increasement at weaning. There was no significant change in H3K4me3 of the promoter *HMGCR* or *CYP7a1* when expressed as a percentage.

DISCUSSION

EHL piglets had lower body and liver weight ($P < 0.01$) compared with LW piglets in both ages, while the liver/body weight ratio was higher at weaning, associated with the variety of pigs. Large

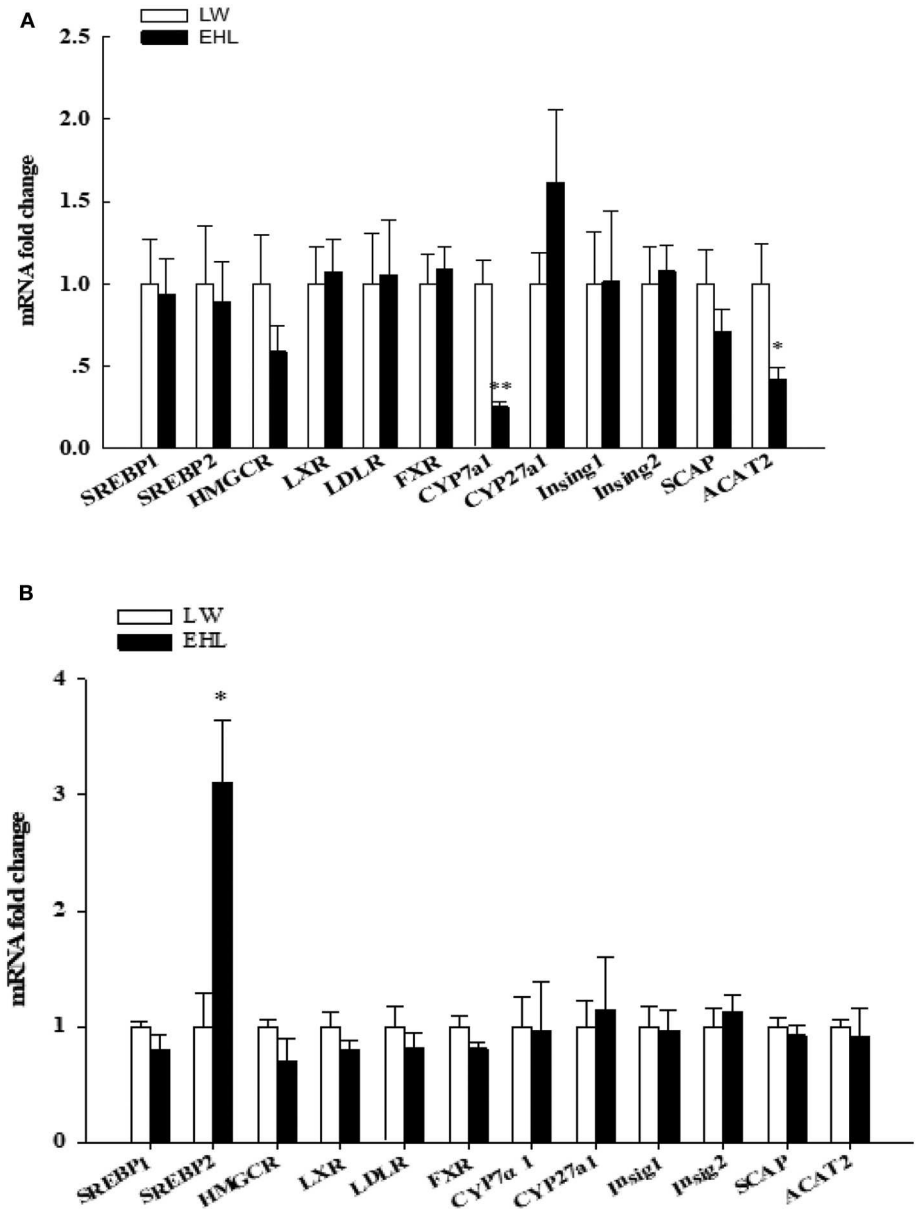

FIGURE 2 | Hepatic expression of genes involved in cholesterol metabolism of the offspring piglets at birth **(A)** and weaning **(B)** ($n = 6$ piglets each time point per breed). *$P < 0.05$, **$P < 0.01$. Data are presented as mean ± SEM.

White pig belongs to the breed with excellent growth rate, large body shape and high fecundity, but Erhualian pig is contrary. We have presented evidences that serum concentration and liver content of Tch and HDL-C were higher in EHL piglets, which was in agreement with early findings. Pond et al. found that the different content of serum cholesterol and HDL in variety was passed on from generation to generation when the serum of four-breed swine population was measured (11). We found that serum concentration of LDL-C was lower at birth but higher at weaning in EHL, but the reasons for these changes were not fully understood.

Cholesterol synthesis in pigs predominantly occurs in the liver. Among the ileum, cerebrum, kidney, heart, liver, semitendinosus muscle, longissimus muscle, and subcutaneous fat, the liver was the only tissue showing significant difference in cholesterol content among pigs with high or low serum cholesterol concentrations, indicating that liver is important to regulate cholesterol balance in pigs (25). There are four main pathways for cholesterol metabolism in liver: the first is that the *de novo* cholesterol biosynthesis mediated by HMGCR pathway; the second is that CYP7a1 and CYP27a1 catalyze the synthesis of bile acids; the third is that HDL

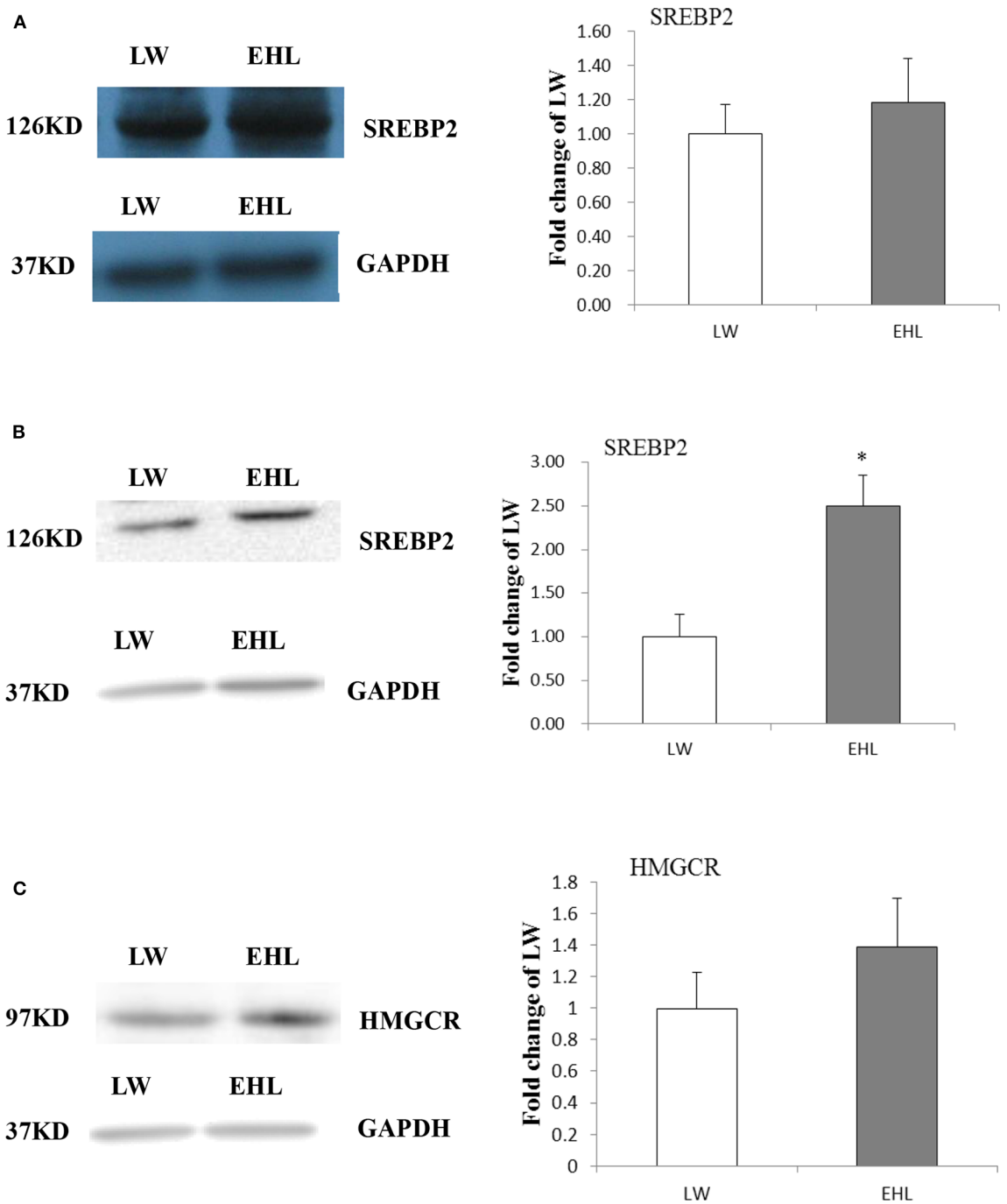

FIGURE 3 | Hepatic SREBP2 and HMGCR protein content. **(A)** Expressed SREBP2 protein content at birth; **(B)** expressed SREBP2 protein content at weaning; **(C)** expressed HMGCR protein content at weaning ($n = 6$ piglets each time point per breed). *$P < 0.05$. Data are presented as mean ± SEM.

mediate reverse cholesterol transport and LDL-R mediate endocytosis; the last is that cholesterol secrete into bloodstream via LDL-C. It has been reported that *ACAT2* participate in cholesterol absorption, esterify dissociative cholesterol in cell, and reduce the toxic effect of high free cholesterol on cell (26–28).

Similarly, we demonstrated that the expression of *CYP7a1* and *ACAT2* were decreased in EHL at birth. Serao at al. found that different pig breeds and lines present different content of intramuscular fat (16). Because no significant change in mRNA expression in liver tissue of Isigs, *Sterol-regulatory element binding protein cleavage-activating*

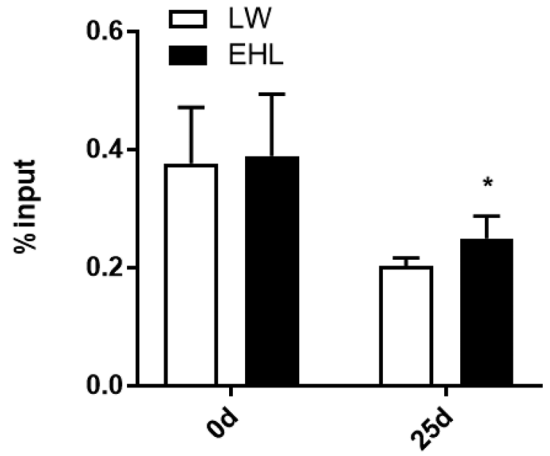

FIGURE 4 | DNA methylation at *HMGCR* promoter in the liver of weaning piglets ($n = 6$ piglets each time point per breed). *$P < 0.05$. Data are presented as mean ± SEM.

protein (SCAP), Sterol-regulatory element binding protein 1 (*SREBP1), SREBP2, HMGCR,* or liver content of SREBP1 protein and HMGCR protein, we demonstrated that the liver cholesterol biosynthesis was not increased in both pig breeds at birth. While the cholesterol esterification via *ACAT2* and transformation via *CYP7a1* were lower in EHL at birth, which is further clarified the liver cholesterol transformation to bile acids, cholesterol absorption, and dissociative cholesterol esterification were reduced. At 25-day-old, the increase of liver cholesterol content in EHL piglets is related to the significant increase of liver SREBP2 mRNA expression and the increase of SREBP2 protein in liver nuclear lysate, but no difference in CYP7a1. Thus, the liver cholesterol metabolism was balanced by reversed cholesterol transport in EHL, but the liver cholesterol content was higher in EHL compared with LW. This might be explained by higher cholesterol biosynthesis and lower cholesterol transformation in liver.

Based on our and other precious studies, liver modulates cholesterol transport, biosynthesis and transformation in pigs predominantly via epigenetic regulation (20, 21). We chose *CYP7a1* and *HMGCR* for epigenetic because the promoter sequences of these two genes can be used in pigs and both of them are key enzymes in the cholesterol metabolism (20, 29, 30). CpG island cytosine methylation located in promoter genes is associated with gene suppression (31, 32), while histone acetylation is related to the activation of transcription (20, 29, 30). According to the type of histone, the position of amino acid residues and the number of methyl groups (mono-, di- and trimethylation), histone methylation can inhibit or activate gene transcription. H3K4me3 is generally considered an activation marker, while H3K9me1 and H3K27me3 have repressive effects on transcriptional (22–24). These views are confirmed in a representative study of transcriptional suppression of *CYP7a1* in EHL is associated with increase in activation markers, H3K9me1 and H3K27me3 at birth, but the increased H3 acetylation was due to decreased histone H3, which content varies with species (33–37). Since increased histone H3K9me1 and H3K4me3, together with up-regulated *SREBP2* mRNA expression, *HMGCR* gene should be activated, but no significant alteration was observed for the mRNA expression and protein content of HMGCR in EHL and LW at weaning. While the liver content of cholesterol was higher in EHL, we speculate that *HMGCR* differ in its susceptibility to cholesterol (38–42).

These results showed that different species or breeds have different cholesterol levels and metabolic. Besides, diet and environment have influence on cholesterol metabolism. Serao et al. studied the relationship between lipid content and gene expression in the muscle of three breeds of pigs, and found that the expression of LDL-R mRNA was different in different breeds of pigs. It suggests that endocytosis of cholesterol in muscle tissue was different (16). The study showed that yolk cholesterol content was not only related to the breed of laying hens, but also to the age of the hen (43). Literature indicates that fatty acid composition varies between breeds. Due to the higher oleic acid concentration, the proportion of monounsaturated fatty acids (MUFA) in marbled Wagyu and Hanwoo beef was higher. They can lower LDL-C while increasing HDL-C, and may reduce risk for cardiovascular diseases (44). Clinical studies have shown that, low concentrations of SFA C12: 0, C14: 0 are benefit and important for longissimus lumborum muscle of Boer crossbreed goats compared with Santa Inês breed sheep. They are promoting the accumulation of low-density lipoprotein, which increases the risk factors of cardiovascular disease in humans (45). A large number of experiments indicated that the cholesterol content had variety specificity. Environmental affects cholesterol metabolism, and this effect is different in specie and tissue. Recently, methylation is very important in the development of human cardiovascular diseases. With the rapid development of high-throughput methylation technology, it has made a great breakthrough in epigenomic research, other biological and clinical fields. Studies have shown that epigenetic regulation involved in cholesterol synthesis, absorption, elimination, and storage so related to total cholesterol levels. Even be involved in the regulation of lipid concentration variability, and leading to cardiovascular disease. As methylation level increasing, the risk of Coronary heart disease (CHD), and higher TG and LDL-C levels and lower HDL-C level result in the change of DNA methylation.

In conclusion, we showed that the liver content of cholesterol was higher, and the ability of cholesterol biosynthesis was stronger in EHL compared with LW, and the molecular mechanism for this difference is enzymatic regulatory and age-dependent. These results provide a new idea for the study of lipid metabolism and meat quality difference mechanism between Erhualian pigs and large white pigs, and provide a reference for the study of the cardiovascular diseases.

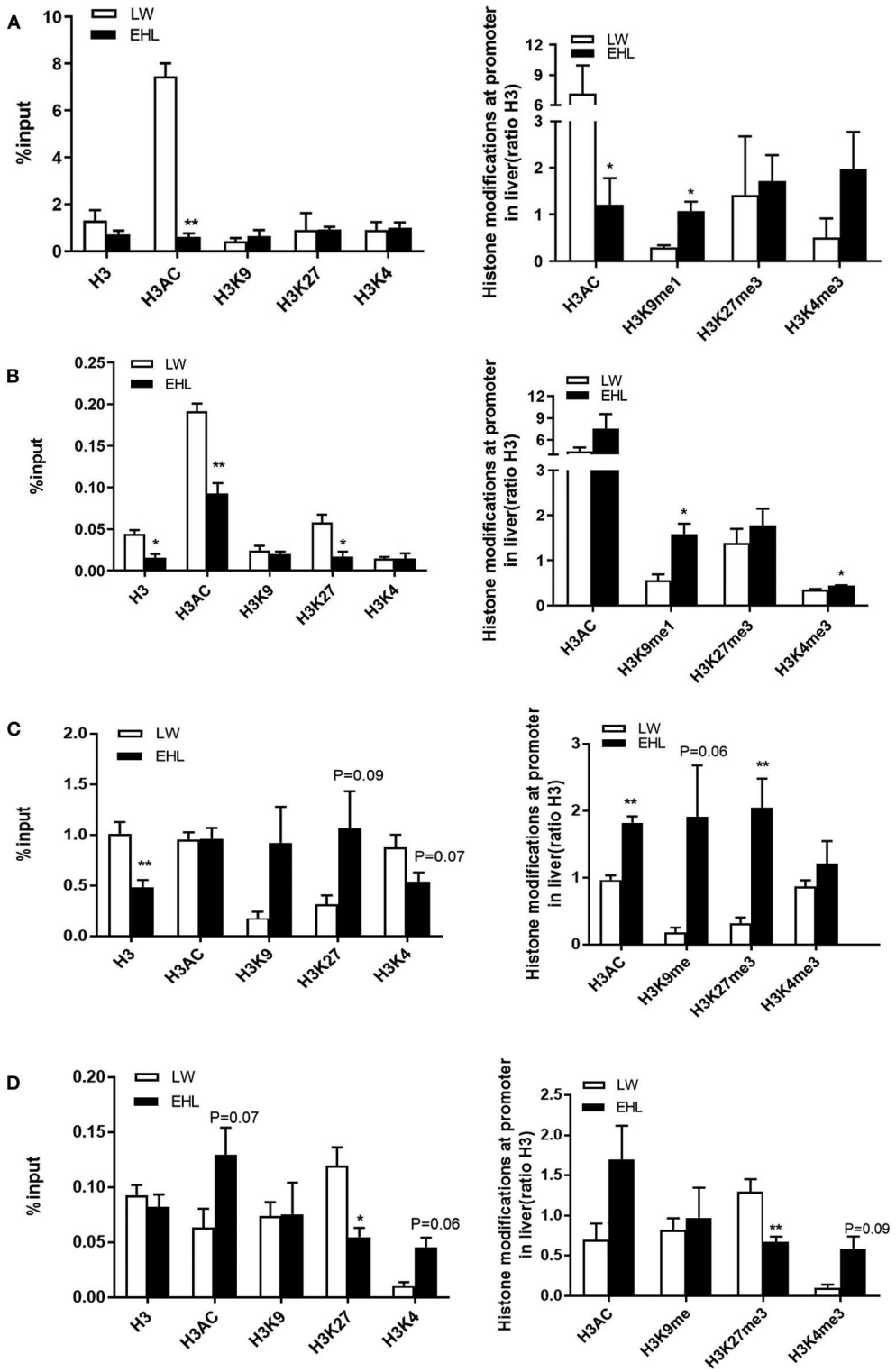

FIGURE 5 | Histone modification at *HMGCR* and *CYP7a1* promoter in the liver of weaning piglets ($n = 6$ piglets each time point per breeds). **(A)** Expressed *HMGCR* as the percentage of the input and the ratio relative to H3 at birth; **(B)** expressed *HMGCR* as the percentage of the input and the ratio relative to H3 at weaning; **(C)** expressed *CYP7a1* as the percentage of the input and the ratio relative to H3 at birth; **(D)** expressed *CYP7a1* as the percentage of the input and the ratio relative to H3 at weaning. $^*P < 0.05$ and $^{**}P < 0.01$. Data are presented as mean ± SEM.

DATA AVAILABILITY STATEMENT

All datasets generated for this study are included in the article/supplementary material.

ETHICS STATEMENT

This animal study was reviewed and approved by NWAFU.

AUTHOR CONTRIBUTIONS

XL and RC contributed to the conception of the study. HX and XZ contributed significantly to analysis and manuscript preparation. HX, XJ, and HZ performed the data analyses and wrote the manuscript. HW, YM, and SC helped perform the analysis with constructive discussions.

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frontiers in Veterinary Science

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Stress Response Simulated by Continuous Injection of ACTH Attenuates Lipopolysaccharide-Induced Inflammation in Porcine Adrenal Gland

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On modern farms, animals are at high risk of bacterial invasion due to environmental stress factors. The adrenal gland is the terminal organ of the stress response. The crosstalk between adrenal endocrine stress and innate immune response is critical for the maintenance of immune homeostasis during inflammation. Thus, it's important to explore whether stresses play a pivotal role in lipopolysaccharide (LPS)-induced inflammatory response in the porcine adrenal gland. Thirty-days-old Duroc × Landrace × Large White crossbred piglets (12 ± 0.5 kg) were randomly allocated into four groups in a 2 × 2 factorial arrangement of treatments, including ACTH pretreatment (with or without ACTH injection) and LPS challenge (with or without LPS injection). Each group consisted of six male piglets. The results showed that our LPS preparation alone induced mRNA expressions of IL-1β, IL-6, TNF-α, IL-10, COX-2, TLR2, TLR4, and GR ($P < 0.05$). ACTH pretreatment downregulated the TLR2 mRNA and IL-6 protein level induced by our LPS preparation significantly ($P < 0.05$) by one-way ANOVA analysis. Treatment with LPS alone extremely significantly decreased ssc-miR-338 levels ($P < 0.01$). Interaction of ACTH × LPS was significant for cNOS level ($P = 0.011$) and ssc-miR-338 expression ($P = 0.04$) by two-way ANOVA analysis. The LPS treatment significantly downregulated cNOS levels ($P < 0.01$), which was significantly attenuated by ACTH pretreatment ($P < 0.05$). Lipopolysaccharide alone did not affect ssc-miR-146b expression levels compared to that in the vehicle group. However, ACTH pretreatment in combination with LPS significantly increased this micro-RNA expression ($P < 0.05$). TLRs 1–10 were all expressed in adrenal tissue. The LPS challenge alone induced remarkable compensatory mitochondrial damages at the ultrastructural level, which was alleviated by ACTH pretreatment. Accordingly, ACTH pretreatment was able to block LPS-induced

secretion of local adrenal cortisol ($P < 0.05$). Taken together, our results demonstrate that ACTH pretreatment seems to attenuate LPS-induced mitochondria damage and inflammation that decreased cNOS activity in the adrenal gland and ultimately returned local adrenal cortisol to basal levels at 6 h post LPS injection.

Keywords: stress, toll-like receptor, glucocorticoid, lipopolysaccharide, adrenocorticotropine

INTRODUCTION

Issues existing in modern farm transportation and management can induce various stresses in domestic animals (1, 2), which are associated with increasing incidence of diseases (3). Effects of stress on the immune response, reported in previous studies, are conflicting. For instance, high-yield dairy cows in the transition period suffered from metabolic stress, characterized by occurrence of an inflammatory response (4). Social disruption and an acute stressor have been shown to activate an inflammatory response in mice (5, 6). Lipopolysaccharide (LPS) challenge was shown to reduce feed intake and increase plasma pro-inflammatory cytokines of pigs, which was inhibited by high-temperature stress (7). Long-term effects of social stress have been reported to inhibit antiviral immunity in pigs (8). Generally, exposure to intense acute or long-term chronic stress may compromise host immune responses (9).

The adrenal gland is the terminal organ of the stress response, which can directly respond to acute and chronic stress (10). The adrenal response to stress is crucial for the host defense against infection (11). The initial step of host defense against bacterial infections is through pattern-recognition receptors, such as toll-like receptors (TLRs). In fact, the stress response and the innate immune response is coordinated by TLRs in the adrenal gland, which is crucial for animal survival during severe inflammation (11, 12). The LPS treatment induced expression of TLR2 and TLR4 in the adrenal gland of animals (12, 13). Most TLRs are responsible for the recognition of a variety of pathogens and induce inflammatory responses. During inflammation, release of cytokines is accompanied by a high glucocorticoid (GC) output. The inflammation is normally restricted by GC as a feedback mechanism (14). The GC acts through a ligand-dependent transcription factor glucocorticoid receptor (GR) (14, 15). Previous studies on mice have shown that deletion of TLR2 or TLR4 is associated with marked cellular alterations in adrenocortical tissue and an impaired adrenal corticosterone response (11, 12). Stress can stimulate the adrenal gland to release GC (16). However, little information is available regarding effects of stresses on LPS-induced inflammatory response via TLRs and GR in the adrenal gland of pigs.

The miRNAs act as a class of endogenous non-coding RNA. They can regulate inflammation via inhibiting mRNA transcription or promoting mRNA degradation of the target gene. It was reported that miR-338 was involved in regulating inflammation (17, 18). Previous studies have shown that some miRNA families, such as let-7 and miR-146, can directly decrease TLR4 expression and inhibit inflammation and oxidative stress response (19–22). In view of the importance of the adrenal response, here we aimed to investigate, for the first time, the effect of stress on LPS-induced inflammatory response in the pig adrenal gland. Usually continuous ACTH treatment is used to mimic the stress response (23, 24). Therefore, we used a pig model exposed to LPS injection with or without continuous ACTH pretreatment to study secretions of inflammatory cytokines, enzymes regulating oxidative stress, and cortisol as well as levels of expression of TLR2, 4, GR, and all miRNAs that target TLR2 and TLR4.

Abbreviations: TLR, toll-like receptor; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GR, glucocorticoid receptor; IL, interleukin; TNF-α, tumor necrosis factor-α; COX-2, cyclooxygenase-2; T-NOS, total superoxide dismutase; iNOS, inducible nitric oxide synthase; cNOS, constitutive nitric oxide synthase; T-SOD, total superoxide dismutase; XOD, xanthineoxidase; CAT, catalase; LPS, lipopolysaccharide; miRNA, microRNA.

METHODS

Animals and Experimental Design

This study was conducted according to "Guidelines on Ethical Treatment of Experimental Animals" (2006) No. 398 set by the Ministry of Science and Technology, China, and the "Regulation Regarding the Management and Treatment of Experimental Animals" (2008) No. 45 set by the Jiangsu Provincial People's Government. All animal procedures were approved by the Institutional Animal Care and Use Committee (IACUC) of Nanjing Agricultural University. A 2 × 2 factorial design was used (i.e., LPS treatment as one factor and ACTH treatment as the other factor). Twenty-four 30-days-old *Duroc* × *Landrace* × *Large White* crossbred piglets with an average weight of 12 ± 0.5 kg were obtained from a commercial farm. The herd in the farm had been monitored regularly during the last 5 years for infectious diseases in the sows and pigs of different age groups prior to our treatment, and no clinical and pathological evidence was found. Before the experiment, all the piglets were acclimatized for a week. All the piglets were randomly allocated into four groups in a 2 × 2 factorial arrangement of treatments, including ACTH (Sigma-Aldrich, Dublin, Ireland) pretreatment (with or without ACTH injection) and LPS challenge (with or without LPS injection): (1) vehicle group (ACTH- LPS-), (2) LPS injection without ACTH pretreatment group (ACTH- LPS+), (3) ACTH alone treatment group (ACTH+ LPS-), and (4) LPS injection with ACTH pretreatment group (ACTH+ LPS+). Each group consisted of six male piglets. The pigs were randomly divided into pens in four separate rooms equipped with appropriate air filters, and the vehicle group was retained in a pen distant from the other groups. The pens had fully slatted floors with natural light conditions. All pigs were provided

with water and food *ad libitum*. They were fed a commercial feed with no antimicrobials throughout the trial, consisting of 65% corn, 24% soybean meal, 5% bran, 2% fish meal, and 4% premix, and the nutrition content included 18% crude protein and 3.4 Mcal/kg digestion energy in the diet formula, respectively. Because continuous ACTH administration to the animal was showed to be similar to stress in some studies (23, 24), all the ACTH pretreatment pigs were injected intramuscularly with ACTH (2.25 IU/kg body weight) for seven consecutive injections at 6-h intervals. The first injection started at 9 a.m. on the 1st day. As a classic ligand for TLR4, LPS stimulates the body to produce an inflammatory response (25, 26). The LPS from Escherichiacoli serotype K-235 (phenolextracted) (Sigma-Aldrich) was dissolved in 0.9% NaCl solution (27). Then all the LPS treatment pigs were injected intramuscularly with LPS (15 μg/kg weight) at 9 a.m. on the 3rd day post the first ACTH treatment. Pigs in the other two groups were mock injected with saline in the same manner. All pigs were euthanized at 6 h post-LPS treatment. The pigs were slaughtered by a head-only electric stun tong apparatus, followed by manual exsanguination. Microscopy histological analysis and levels of cortisol, cytokine protein, gene mRNA expression, and miRNA expression in the adrenal gland tissue were determined by the same person who was blinded to the experiment design.

Sample Collection

These key sampling times post LPS injection were based on previous research in our laboratory (27, 28). All blood samples were collected from the precaval vein at 6 h post LPS treatment. Blood samples were centrifuged at 1,500 × *g* for 15 min at 4°C, and the serum was stored at −20°C. The adrenal gland tissue was harvested immediately after euthanasia. Subsequently, tissues were washed with PBS to remove any blood and contaminants on their surface. The samples were snap frozen in liquid nitrogen and stored at −70°C.

Electron Microscopy

Adrenal glands were diced into small pieces at approximately 1 mm^3 and fixed in 0.1 M phosphate buffer at pH 7.2–7.4 with 2% (vol/vol) glutaraldehyde. After washing in 0.2 M phosphate buffer (pH 7.2) overnight, these specimens were post-fixed in cold 1% OsO4 (pH 7.3) for 2 h at 4°C. All tissue slices were dehydrated in different gradients of ethyl alcohols (30–100%) and then embedded in Epon 812 R (Merck, Whitehouse Station, NJ). Finally, ultrathin sections (50 nm) were stained with uranyl acetate and lead citrate and examined by an H-7650 transmission electron microscope (Hitachi High-Technologies Co., Japan) at 80 kV with an Ultrascan CCD camera (11). Ultrastructure morphometric assessments of mitochondria were conducted by using NIH ImageJ software.

Measurement of Markers for Oxidative Stress

All adrenal gland tissues were homogenized in cold radioimmunoprecipitation assay (RIPA) buffer of (50 mM Tris–HCl pH 7.4, containing 10% glycerol, 1.0% Triton-X 100, 100 mM NaCl, 50 mM NaF, 1 mM EDTA, and 1 mM EGTA) with the protease inhibitor cocktail (Roche Applied Science) then centrifuged at 1,500 × g for 15 min at 4°C, and the supernatant was extracted. The enzyme activities of total superoxide dismutase (T-SOD), catalase (CAT), inducible nitric oxide synthase (iNOS), constitutive nitric oxide synthase (cNOS), total nitric oxide synthase (TNOS), and XOD (xanthine oxidase) content in the supernatant of adrenal tissues was determined using biochemical determination kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) (29–31).

Cortisol and Cytokine Protein Levels in Adrenal Gland Tissue

Concentrations of cortisol and IL-6 in the supernatant of adrenal gland tissues at 6 h after LPS injection were measured in duplicate using a commercial 125I-RIA kit (Beijing Research Institute of Biotechnology, Beijing, China) according to the manufacturer's instructions. This kit was validated for measuring porcine samples (15, 31). The detection limits of cortisol and IL-6 were 2 ng/mL and 50 pg/mL, respectively. All samples were measured in the same assay to avoid inter-assay variations.

Gene Expression Levels in Adrenal Gland Tissue

Briefly, total RNA was extracted from adrenal gland tissues using Trizol reagent (Invitrogen, Carlsbad, CA) according to our previous description (15). From each sample, 1 μg of total RNA was converted to cDNA using the PrimeScript® RT reagent kit with gDNA Eraser (Takara, Dalian, China). The primers used are listed in **Table 1**. Also, real-time quantitative PCR (QPCR) reactions and gene expression levels of mRNA were performed using the SYBR Green QPCR Master Mix (TOYOBO Ltd., Japan) as previously described (15). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was chosen as a reference gene for normalization. All the mRNA expression levels in the adrenal gland tissues at 6 h post LPS injection were presented as the fold change relative to the average values in the Vehicle group (**Supplementary Material S1**).

miRNA Expression Level in Adrenal Gland Tissue

All miRNAs that target TLR2 and TLR4 were predicted by computer-aided algorithms from TargetScan (http://www.targetscan.org/vert_42/), and the miRNA expression level was performed according to previous publication (32). Briefly, total RNA was extracted from the adrenal gland tissues using Trizol reagent. The RNA (4 μg) was polyadenylated by poly(A) polymerase (PAP) at 37°C for 1 h in a 20-μl reaction mixture using the Poly(A) Tailing Kit (AM1350, Ambion, USA), and tailing reactions were performed. The tailing reaction solution contained 4 μg of RNA samples (1 μg/μl), 2 μl of 25 mM MnCl2, 4 μl of 5×E-PAP buffer, 0.8 μl of E-PAP, 2 μl of 10 mM ATP, and 7.2 μL of nuclease-free water in 20 μl final volume. Then, tailing RNAs (2 μg) were converted to cDNA using a gene-specific oligo dT-adapter primer (1 μg/μl). QPCR reactions were performed using the SYBR Green QPCR Master Mix (TaKaRa, Tokyo, Japan) with an Mx3000P

TABLE 1 | Nucleotide sequences of specific primers used in qPCR.

Genes	Primers
TLR1	5′–GTGTTGCCAATCGCTCAT–3′ 5′–CAGATTTACTGCGGTGCT–3′
TLR2	5′–GACACCGCCATCCTCATTCT–3′ 5′–CTTCCCGCTGCGTCTCAT–3′
TLR3	5′–TGCACTAAAACGTGAAGAACTT–3′ 5′–ATGAAAACACCCTGGAGAGAAC–3′
TLR4	5′–TCTACATCAAGTGCCCCTAC–3′ 5′–TAAATTCTCCCAAAACCAAC–3′
TLR5	5′–AGATACCCCTTGTGTGCGA–3′ 5′–TTCCTTGTGGTGTCCGCTG–3′
TLR6	5′–AGAAAGAAATCTTGAATTTGGA–3′ 5′–AATGAAGGCTTATGACAGTAGG–3′
TLR7	5′–TATGGGACCAGGAGCACACAA–3′ 5′–AAAGAGAACTGCCGATAGGGA–3′
TLR8	5′–CGGTCGCTTCCCACATC–3′ 5′–CCAGTCCCTCTCCTCCAAAC–3′
TLR9	5′–GGATGTGGGCTGAGGGAG–3′ 5′–AGGCTTTTGGGGAGGTTG–3′
TLR10	5′–TGTGGTATTGTCATGTCAGTGC –3′ 5′–AGTTGAAAAAGGAGGTTGTAGG–3′
GAPDH	5′–CGTCCCTGAGACACGATGGT–3′ 5′–CCCGATGCGGCCAAAT–3′
GR	5′–TCTGTATGAAAACCTTACTGCT–3′ 5′–TGTTCTTATCCAAAAATGTCTG–3′
IL-1β	5′–CAGGGGACTTGAAGAGAG–3′ 5′– GCTGATGTACCAGTTGGG–3′
IL-6	5′–CTACTGCCTTCCCTACCC–3′ 5′–ACCTCCTTGCTGTTTTCA–3′
TNF-α	5′–CCTCTTCTCCTTCCTCCT–3′ 5′–ATTGGCATACCCACTCTG–3′
IL-10	5′–CATCCACTTCCCAACCAG–3′ 5′–TCCTCCCCATCACTCTCT–3′
COX-2	5′–GTGTGAAAGGGAGGAAAGA–3′ 5′–AAACTGATGGGTGAAGTGC–3′
Ssc-miR-338	TCCAGCATCAGTGATTTTGTTG
Ssc-miR-146b	TGAGAACTGAATTCCATAGGC
Oligo dT-adaptor	TAGAGTGAGTGTAGCGAGCACAGAATTAATA CGACTCACTATAGGTTTTTTTTTTTTTTTVN
Exogenous reference	GTGACCCACGATGTGTATTCGC
Universal	TAGAGTGAGTGTAGCGAGCA

QPCR system (Agilent Technologies, Stratagene, USA). All special miRNA primers were designed based on mature miRNA sequences of pigs in miRbase (http://www.mirbase.org/). All primers used are listed in **Table 2**, including special miRNA primers, universal primers, exogenous reference primers, and oligo dT-adaptor primers. All the miRNA expression levels in the adrenal gland tissues at 6 h post LPS injection were presented as the fold change relative to the average values in the vehicle group.

Statistical Analysis

All data were analyzed in the general linear model (GLM) procedure of SPSS 16.0 (SPSS Inc., Chicago, IL, USA). Descriptive statistics were performed to check the normality and homogeneity of variances before using parametric analyses. IL-6 mRNA, TNF-α mRNA, IL-10 mRNA, IL-6 protein, and cNOS secretion levels were not normally distributed. Therefore, Log10 transformation was performed for these results before statistical analysis. All data were analyzed by two-way ANOVA in the general linear model (GLM) procedure of SPSS 16.0 (SPSS Inc.) with main effects of LPS and ACTH treatments and interaction of LPS × ACTH. For all data, a one-way ANOVA was performed also. Duncan's test was used as the *post-hoc* test. Two-tailed *P*-values with $P \leq 0.05$ were considered significant. Data are expressed as mean ± *SEM*.

TABLE 2 | Effects of LPS/ACTH on oxidative stress response in local porcine adrenal tissue.

Items	ACTH -		ACTH +		S.E.M.	*P*–value		
	LPS -	LPS+	LPS -	LPS+		ACTH	LPS	ACTH × LPS
T-NOS (U/mg protein)	1.70^{a}	1.12^{b}	1.38^{ab}	1.42^{ab}	0.08	0.955	0.089	0.052
iNOS (U/mg protein)	0.36	0.43	0.31	0.47	0.02	0.922	0.048	0.422
cNOS (U/mg protein)	1.34^{a}	0.69^{c}	1.07^{ab}	0.95^{b}	0.07	0.517	<0.010	0.011
T-SOD (U/mg protein)	87.94^{a}	57.94^{b}	76.28^{a}	55.08^{b}	2.59	0.215	<0.010	0.446
XOD (U/g protein)	35.58	28.63	32.91	32.02	1.77	0.901	0.187	0.304
CAT(U/mg protein)	0.90	0.88	0.80	0.90	0.03	0.515	0.55	0.328

Means with different letters (a, b, c) are significantly different (P < 0.05) from each other, n = 6. Different superscript letters indicate significant differences between the same column. Data are expressed as mean ± SEM.

RESULTS

Clinical Symptoms and Ultrastructure Change of the Adrenal Gland

LPS injection alone induced severe clinical symptoms in ACTH-LPS+ pigs. Five pigs lied down on the ground with signs of depression not drinking water within 6 h post LPS injection, and one pig was dying in the group although pigs in the ACTH+ LPS+ group showed mild clinical symptoms post ACTH pretreatment. Two pigs in the combined treatment group lied down on the ground with depressed spirits within 3 h post LPS injection but recovered quickly. The other four pigs moved and drank normally in the ACTH+ LPS+ group. All pigs in the other groups without LPS treatment did not show any clinical symptoms. At the ultrastructural level, the most pronounced effect was found in the mitochondrial architecture. All the animals without LPS treatment exhibited a rod-like or round mitochondria with characteristic tubovesicular cristae. In contrast, LPS treatment alone induced compensatory damage of the mitochondrial substructure, and mitochondrias became swollen and extremely round with a smooth outer membrane and disappeared cristae. The ACTH pretreatment obviously alleviated LPS-induced mitochondria injury and restored mitochondrial cristae to the tubovesicular structure (**Figure 1**).

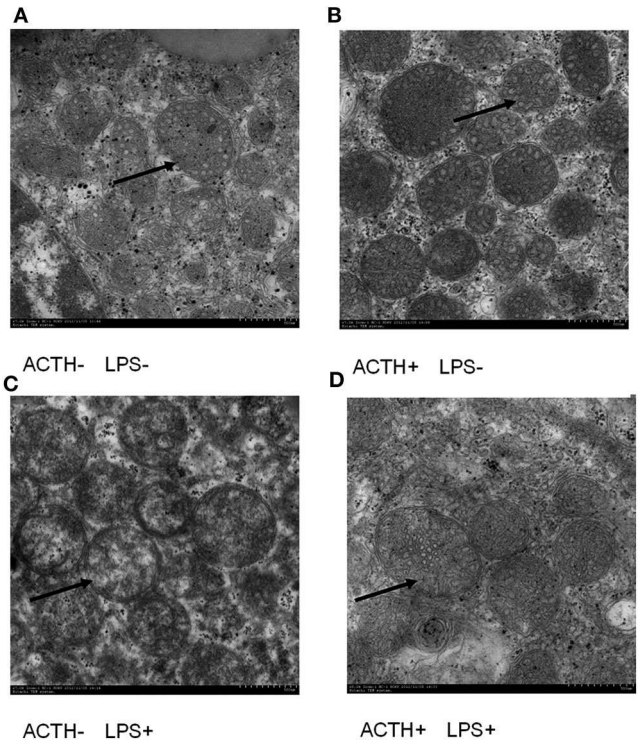

FIGURE 1 | Effect of lipopolysaccharide/ACTH treatment on ultrastructure change of pig adrenocortical cells (arrow, scale bars = 500 nm). **(A)** Vehicle group and **(B)** ACTH groups show rod-like or round mitochondrias with characteristic tubovesicular cristae (arrow). **(C)** LPS group shows extremely round mitochondrias with a smooth outer membrane and disappeared cristae (arrow). **(D)** ACTH + LPS group shows mitochondrial cristae with tubovesicular structure (arrow).

Profiles of Various Cytokines and Cortisol

As shown in **Figure 2**, a strong main effect of LPS was detected ($P < 0.01$). Treatment with LPS alone upregulated mRNA expressions of IL-1β, IL-6, TNF-α, IL-10, COX-2 ($P < 0.05$). Furthermore, ACTH pretreatment significantly inhibited LPS-induced IL-6 protein secretion (**Figure 2F**, $P < 0.05$). Consistent with this, ACTH pretreatment significantly alleviated the release of cortisol induced by LPS (**Figure 2G**, $P < 0.05$) and restored local adrenal cortisol to the basal level (**Supplementary Material S1**).

Oxidative Stress in the Adrenal Gland

As shown in **Table 2**, LPS treatment revealed a main effect on T-SOD activity ($P < 0.01$). The LPS treatment significantly decreased T-SOD activity ($P < 0.05$); however, ACTH pretreatment did not restore T-SOD activity. Treatment with LPS increased levels of iNOS protein secretion in the adrenal gland ($P = 0.048$). There were a main effect of LPS ($P < 0.01$) and a ACTH × LPS effect ($P = 0.011$) for cNOS levels by two-way ANOVA. The LPS treatment significantly downregulated cNOS level ($P < 0.01$), which was significantly attenuated by ACTH pretreatment ($P < 0.05$) (**Supplementary Table S1**).

Levels of Toll-Like Receptors and Glucocorticoid Receptor mRNA Expressions

All the 10 TLRs (TLR1 to TLR10) were found to be expressed in porcine adrenal tissue under normal physiological conditions (**Figure 3A**). The TLR3 was the most abundantly expressed TLR, which was followed by TLR1 (**Supplementary Table S2**). The statistical analysis of TLR2, 4 mRNA expressions in adrenal gland revealed a main effect of LPS treatments ($P < 0.01$, **Figures 3B,C**). Treatment with our LPS preparations alone significantly increased levels of TLR2, 4 mRNA expression ($P < 0.01$ and $P < 0.05$, respectively). Transcriptional activation of TLR2 induced by the LPS was significantly relieved by ACTH pretreatment ($P < 0.05$). Furthermore, LPS alone significantly increased levels of GR mRNA expression ($P < 0.05$, **Figure 3D**). But no significant change was shown for LPS-induced GR or TLR4 mRNA expression by ACTH stimulation (**Supplementary Table S1**).

Expressions of microRNA

All miRNAs that target TLR2 and TLR4 were predicted as shown in **Table 3**. The results showed that miR-338 and miR-146b had potential regulatory effects on TLR4. In contrast, highly reliable miRNAs that target TLR2 were not found. There were main effects of LPS ($P < 0.01$) and ACTH × LPS ($P = 0.04$) for ssc-miR-338 expression level by two-way ANOVA. Treatment with LPS alone extremely significantly decreased the ssc-miR-338 level ($P < 0.01$), yet the induction was not affected by ACTH pretreatment. In contrast, ACTH pretreatment combined with LPS promoted the expression of ssc-miR-146b significantly ($P < 0.05$) compared with the vehicle group despite no changes being detected with LPS treatment alone (**Supplementary Table S1**).

DISCUSSION

Lipopolysaccharide is known to stimulate an inflammatory response that induces production of cytokines. As expected, the body temperature was increased in all the LPS-treated pigs compared to the control group in our inflammation model of pig (27). And it was found in the present study that LPS upregulated expressions of IL-1β, IL-6, TNF-α, IL-10, and COX-2 mRNA in the adrenal gland of pigs. These results indicate that a strong inflammatory reaction may be induced locally in adrenal gland tissue. However, we found that, among all the cytokines examined, only levels of IL-1β, IL-10 mRNA were altered post LPS treatment in lung tissue (data not shown). LPS upregulated levels of pulmonary IL-1β and IL-10 gene mRNAs, but ACTH treatment has no significant effect on them. As for other genes related to inflammation, no significant differences were observed in the expression of IL-6, TNF-α, and COX-2 mRNAs post ACTH or LPS treatment. This suggests that the inflammatory response induced by LPS treatment is tissue-specific in our study. Similar results are reported in a previous publication that cytokine responses to LPS are age- and tissue-dependent in neuroendocrine tissues of neonatal pigs (33). Some previous studies on mice or rats

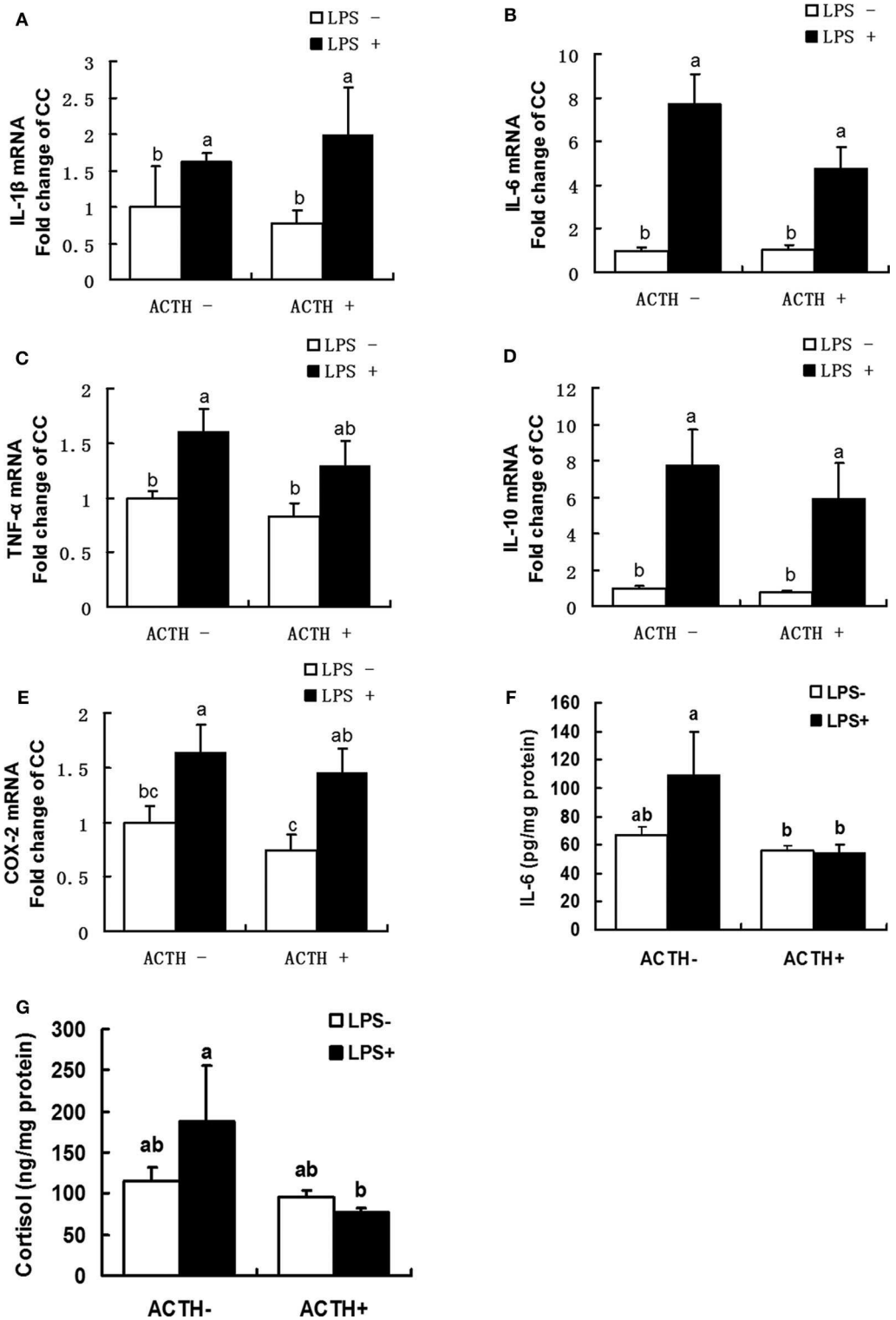

FIGURE 2 | Effects of lipopolysaccharide/ACTH on expressions of IL-1β **(A)**, IL-6 **(B)**, TNF-α **(C)**, IL-10 **(D)**, and COX-2 **(E)** mRNAs, IL-6 protein **(F)**, and cortisol **(G)** secretion levels in local porcine adrenal gland tissue. Means with different letters (a, b, c, d, e, f) are significantly different ($P < 0.05$) from each other, $n = 5–6$. Different superscript letters indicate significant differences between the same column. Data are expressed as mean ± *SEM*.

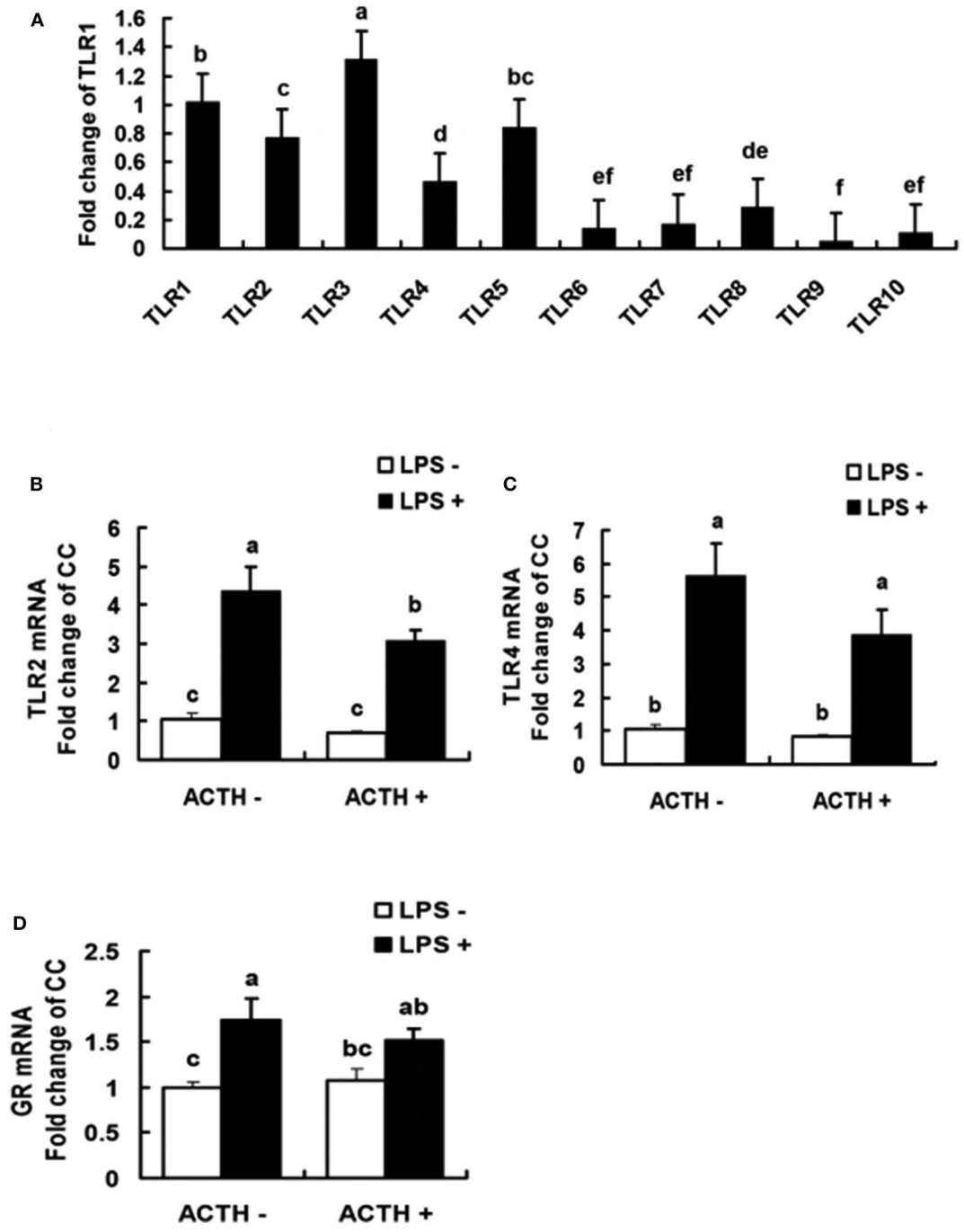

FIGURE 3 | Levels of TLR mRNA in normal porcine adrenal tissue **(A)**, and effects of lipopolysaccharide/ACTH on mRNA expressions of TLR2 **(B)**, TLR4 **(C)**, and GR **(D)** in local porcine adrenal gland tissue. Means with different letters (a, b, c, d, e, f) are significantly different ($P < 0.05$) from each other, $n = 5$–6. Different superscript letters indicate significant differences between the same column. Data are expressed as mean ± *SEM*.

have shown that ACTH attenuated LPS-induced systematic inflammatory response (34, 35). For example, it was reported that treatment with ACTH (1–24) sympathectomy attenuated LPS-induced increases of IL-1β, IL-6, and IL-10 levels in the plasma of rats. In accordance with this, we also found that ACTH pretreatment blocked intra-adrenal IL-6 protein secretion induced by LPS. Therefore, our finding provides *in vivo* evidence that the stress mimicked by continuous ACTH treatment may play a role in alleviating inflammatory reaction in the local adrenal gland.

The inflammatory response elicited by LPS mainly through activation of TLR4 has been well-documented in previous studies (36–39). It is also found that TLR2 can be upregulated by LPS stimulation in hemorrhagic shock mice (40), suggesting

TABLE 3 | Effects of LPS/ACTH on miRNA expressions targeting TLR4 gene in porcine adrenal tissue.

Target gene	miRNA	ACTH -		ACTH +		S.E.M.	*P*–value		
		LPS -	LPS+	LPS -	LPS+		ACTH	LPS	ACTH × LPS
TLR4	ssc-miR-338	1.00^{a}	0.44^{c}	0.71^{b}	0.52^{bc}	0.04	0.243	0.000	0.040
	ssc-miR-146b	1.00^{b}	1.41^{ab}	0.75^{b}	2.45^{a}	0.31	0.349	0.020	0.134

Means with different letters (a, b, c) are significantly different ($P < 0.05$) from each other, $n = 5$–6.
Different superscript letters indicate significant differences between the same column. Data are expressed as mean ± SEM.

that both TLR2 and TLR4 can be activated by their LPS preparation. Previous studies have shown that expressions of TLR2 and TLR4 were increased by LPS treatment in the mouse adrenal gland, which further promotes the secretion of IL-1, IL-6, and TNF-α (12, 13). In accordance, we found that LPS activated mRNA expression of TLR2 and TLR4 in porcine adrenal gland tissue. However, it should also take into account the contamination of commercial LPS preparations (11). Indeed, LPS is the classical ligand of TLR4, and TLR2 should not be activated by pure LPS. Actually, commercial LPS preparation was always contaminated with TLR2 ligands, such as lipopeptides, that can activate TLR2 pathways (11). In the present work, we just used LPS to stimulate the inflammatory response rather than to specifically study the TLR4 pathway. Thus, commercial LPS was used in the present work. In addition, our findings provided *in vivo* evidence that ACTH pretreatment downregulated expressions of TLR2 induced by our LPS preparations in pig model, which was coupled with changes of IL-6 protein levels. These results may suggest that stress stimulated by continuous ACTH treatment prevented the transcriptional activation of TLR2 and, thus, protected the porcine adrenal gland against excessive inflammation.

Many studies have demonstrated that activated TLRs in organ injury are associated with oxidative stress (41, 42). NO (nitric oxide) is an important inflammatory mediator derived from NOS, including induced NOS (iNOS) and constitutive NOS (cNOS) (43). Actually, the regulation of NO production by cNOS and iNOS was a complex issue, and it was reported that the activity of cNOS was regulated by LPS and some cytokines in the mouse vascular endothelial cell line (44). It was also documented that the cNOS activity showed a 4.3-fold decrease post *H. pylori* LPS treatment in rat gastric mucosal cells while ghrelin countered the LPS-induced change (45). This result is consistent with our finding. Here, we found that LPS treatment downregulated cNOS levels, which was significantly relieved by ACTH pretreatment, thus resulting in an ACTH × LPS effect for cNOS level. These results indicate that the strong inflammatory reaction induced by LPS may decrease cNOS activity in local adrenal gland tissue although ACTH pretreatment alleviates the effect of LPS. NO is predominantly produced by iNOS in the late phase of inflammation (43, 46). Here, we found LPS treatment increased levels of iNOS protein secretion in the adrenal gland but decreased T-SOD activity without an ACTH × LPS effect. These results suggest that LPS may promote local iNOS activity and damage the activity of the antioxidant enzymes, which were consistent with the result of over-activation inflammation in local adrenal gland tissue. But ACTH has no effect on activity of iNOS and antioxidant enzymes.

Studies in mouse brains indicated that miR-146a and let-7b miRNAs were upregulated, but miR-338-3p miRNA expressions were downregulated in prion disease (17). It was also reported that miR-338-3p miRNA expression was downregulated during LPS-induced inflammation in mouse lung tissue (18). In accordance, here, we found that LPS injection alone extremely significantly decreased the ssc-miR-338 level in our pig model. However, it's puzzling that the LPS-induced inhibition of ssc-miR-338 was not affected by ACTH pretreatment by one-way ANOVA although with an ACTH × LPS effect. Here, we found that transcriptional activation of TLR4 induced by LPS was not affected by ACTH pretreatment. Thus, the result of ssc-miR-338 is consistent with our findings of TLR4 expression. It has been reported in several studies that the miR-146 family targeting various pro-inflammatory molecules in the TLR4 signaling pathway plays a negative regulatory role in inflammatory response (21, 22). In line with these findings, here, we found that ACTH combined with LPS stimulation upregulated miR-146b level. But inconsistent with expectation, transcriptional activation of TLR4 induced by LPS did not changed significantly by ACTH pretreatment. In fact, the miR-146 family might be the LPS primary response gene that targeted various pro-inflammatory molecules besides TLR4 (21). It was reported that 76 miRNA were significantly upregulated, and 35 miRNAs were downregulated at different time points post LPS treatment in mice, that targeted various pro-inflammatory molecules (18). Thus, here the ACTH-stimulated miR-146 or ssc-miR-338 regulatory circuit may fine-tune LPS-induced inflammatory signaling by targeting other inflammatory molecules.

The subcellular structure of adrenal mitochondria is closely related to the release of steroid hormones (11, 12). A defined spatial and conformational arrangement of mitochondria, which maintains normal electron transfer and cytochrome P450 activity, is required for the synthesis of glucocorticoid (11). Lipopolysaccharide impaired the mitochondrial structure in the pig adrenal gland in our study, which was restored to normal tubovesicular structure by ACTH pretreatment. The morphological alterations of mitochondria suggest mitochondrial dysfunctions post LPS treatment. The change of adrenal mitochondrial structure was consistent with cortisol levels in serum and adrenal gland tissue. This phenomenon may represent a compensatory mechanism for maintaining basal corticosterone release despite impaired adrenocortical function. The altered structure of the adrenals may reflect the

enhanced synthesis and release of cortisol in the inflammatory state. We speculate that continuous ACTH treatment alleviates LPS-stimulated damage to mitochondria so that intra-adrenal cortisol can return to baseline levels.

The HPA axis is known to be activated by a variety of inflammatory insults, and innate immune-endocrine interactions and resultant GC-GR mediated action are critical for maintaining the homeostasis of immune response (33, 47). Bacterial invasion induces the secretion of cytokines, such as TNF-α, IL-1, and IL-6, which can stimulate the HPA axis to release corticotrophin-releasing hormone (CRH) and arginine vasopressin in the hypothalamus. Next, CRH stimulates the anterior pituitary to synthesize and release ACTH, which promotes the secretion of GC in the adrenal gland (48). GCs themselves exert a negative feedback regulation to suppress the HPA axis, which leads to a shift from pro-inflammatory immune responses to anti-inflammatory immune responses in circulation (49, 50). Actually, the HPA axis was stimulated directly by LPS that has been implicated in previous studies, including in the paraventricular nucleus of the hypothalamus (48), the anterior pituitary (51), or the adrenal glands (11). The activated HPA axis produced pro-inflammatory cytokines, such as TNF-α, NO, and IL-6, which involved in modulation of secretion of ACTH leading to downstream GC release. Consistent with this, here we found an exacerbated inflammatory response in local adrenal tissue and increased serum TNF-α concentration (data not shown) after LPS stimulation, accompanied by elevated cortisol levels at 6 h post LPS injection. Adrenal TLR2 and TLR4 should be tightly controlled in order to keep the balance between the adrenal endocrine and innate immune response, which are crucial for host survival (11, 12). Therefore, in the present study, activated TLR2 and TLR4 pathways by our LPS preparations may stimulate release of cytokines in local adrenal gland tissue and serum, and circulated cytokines may eventually promote cortisol release via stimulating HPA axis in a negative feedback loop.

Previous study has shown that an inflammatory transcription factor, namely NF-interleukin 6 (NF-IL-6), was activated in the anterior pituitary lobe not only by inflammatory LPS stimulation, but also by a novel environment stress that caused low-grade inflammatory responses in the brain and the anterior pituitary (51). However, in the present study, no adrenal response was observed by ACTH injection alone. This may be due to the fact that the consecutive exogenous ACTH treatment could reduce the adrenal gland sensitivity to ACTH stimulation. Interestingly, here, ACTH pretreatment relieved inflammation in adrenal gland upon exposure to our LPS preparations to a certain extent and, ultimately, restored systemic and local cortisol to basal levels at 6 h post LPS injection. Serum cortisol levels were increased at 2 h post LPS treatment and remained elevated at 6 h. However, pretreatment with ACTH significantly decreased the LPS-induced upregulation of cortisol and restored cortisol to the basal level at 6 h post LPS treatment (data not shown). It may suggest that the chronic stress stimulated by our consecutive ACTH treatment may keep the balance between the innate immune and adrenal endocrine response in the local adrenal gland post LPS injection. However, the adrenal response was in a time-dependent manner, and variations at various time points need to be investigated in a further study.

Previous studies have shown that transcriptional activation of TLR2 and TLR4 depend on CD14 and LPS-binding protein (LBP) (52). Signal transduction pathways, such as nuclear factor-kappa B (NF-κB) and mitogen-activated protein kinase (MAPK), were activated by TLR4 via a series of signal cascade reactions, which ultimately activate the inflammatory response (53). Classically, GC mediated-GR nuclear translocation plays a crucial role of anti-inflammation (54). Therefore, further investigations are needed to delineate profiles of TLR, GR protein, NF-κB, and MAPK signal transduction pathway molecules as well as the complex regulatory network of GR and TLR.

CONCLUSIONS

Taken together, our results demonstrate that ACTH pretreatment seems to attenuate LPS-induced mitochondria damage and inflammation that decreased cNOS activity in the adrenal gland and ultimately returned local adrenal cortisol to basal levels at 6 h post LPS injection although ACTH had no effect on the LPS-induced iNOS secretion and inhibition of ssc-miR-338 and T-SOD activity. This suggests that moderate stress stimulated by repeated ACTH pretreatment may be beneficial for animals to resist inflammation in the adrenal gland. This study will deepen the knowledge about the relationship between inflammation and stress and provide a theoretical basis for the control of porcine inflammation and the study of animal welfare.

DATA AVAILABILITY STATEMENT

The datasets generated for this study are available on request to the corresponding author.

ETHICS STATEMENT

All animal procedures were approved by the Institutional Animal Care and Use Committee (IACUC) of Nanjing Agricultural University. The protocol of this study was reviewed and approved specifically, with the project number 2012CB124704. The slaughter and sampling procedures strictly followed the Guidelines on Ethical Treatment of Experimental Animals (2006) No. 398 set by the Ministry of Science and Technology, China and the Regulation regarding the Management and Treatment of Experimental Animals (2008) No. 45 set by the Jiangsu Provincial People's Government.

AUTHOR CONTRIBUTIONS

ZS and XZ designed this study, guided the experiment, and analyzed data. ZS, DC, and XY have been involved in the whole experiment process and drafting and revising the manuscript. YS, CY, HZ, and XL participated all the experiments and performed the statistical analysis. QS, YJ, and YX helped for the sampling process and made contributions to acquisition of data. All

authors contributed to manuscript revision, read and approved the submitted version.

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SUPPLEMENTARY MATERIAL

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Effect of the Single and Combined Use of Curcumin and Piperine on Growth Performance, Intestinal Barrier Function, and Antioxidant Capacity of Weaned Wuzhishan Piglets

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This study was conducted to evaluate effects of the single and combined use of curcumin (CUR) and piperine (PIP) on performance, intestinal barrier function, and antioxidant capacity of weaned piglets. A total of 50 Wuzhishan piglets weaned at 35 days of age were randomly assigned to five groups receiving a corn–soybean basal diet (CON), the basal diet supplemented with 50 mg/kg piperine, 200 mg/kg curcumin (low-CUR), 200 mg/kg curcumin + 50 mg/kg piperine (PIP + CUR), and 300 mg/kg curcumin (high-CUR), respectively. The results showed that the feed/gain ratio (F/G) and plasma d-lactate and diamine oxidase activity (DAO) of the CUR + PIP and high-CUR groups were lower than those of the CON group (all $P < 0.05$), while the jejunum and ileum villus height, the villus height/crypt depth ratio, and the messenger RNA (mRNA) expression levels of *occludin*, *claudin-1*, and *zonula occluden-1* in jejunal and ileal mucosa were higher in the CUR + PIP and high-CUR groups than in the CON group (all $P < 0.05$). Moreover, the piglets in the CUR + PIP and high-CUR groups had higher serum and intestinal mucosa activity of superoxide dismutase and glutathione peroxidase and lower malonaldehyde concentration than piglets in the CON group (all $P < 0.05$). The above parameters were not significantly different between the CUR + PIP and high-CUR groups ($P > 0.05$). In conclusion, the combination of CUR and PIP seemed to be as advantageous as high-CUR to piglets, but it was more effective than the single use of CUR and PIP. These data indicated that the basal diet supplemented with CUR + PIP or high-CUR could improve the intestinal permeability and suppress oxidative stress of weaned Wuzhishan piglets.

Keywords: curcumin, piperine, growth performance, intestinal permeability, weaned piglet, antioxidant capacity

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INTRODUCTION

After weaning, piglets often suffer from intestinal barrier dysfunction, which contributes to severe diarrhea and decreased performance in piglets (1–3). Antibiotics have been widely used as animal feed additives for many years because of their efficiency in increasing the growth rate, improving feed utilization, and reducing mortality (4). However, their continuous use may lead to the

emergence of drug resistance and antibiotic residues in poultry products (5), harming human health and the environment (6). As a result, some countries have banned the addition of antibiotics to livestock as growth promoters (7, 8).

In the past two decades, a large number of materials have been investigated as alternatives to antibiotics added in piglet diets (9). Among them, plant extracts are proven to be useful in relieving the post-weaning syndrome (10). Curcumin, an active natural polyphenol derived from the curry spice turmeric, has been widely used as medicine, dietary additives, and coloring agents (11). It exhibits biological activities as diverse as antioxidant (12), antiviral (13), anticancer (14), antiproliferation (15), antidiabetic (16), and anti-inflammatory properties (17). Particularly, the protective effects of CUR on the intestinal mucosa barrier were also repeatedly demonstrated in rat (18, 19), duck (20), and human intestinal epithelial cells (21, 22). Our previous experiments have also shown that a basal diet supplemented with 300 mg/kg CUR could improve the integrity and morphology of the intestinal mucosal barrier as well as the immunity of weaned pigs challenged with enterotoxigenic *Escherichia coli* (23).

In spite of an extensive range of pharmacological potentials, the CUR has limited bioavailability when administered orally, which can be explained by its poor absorption, low stability, and fast metabolism and excretion from the body (24, 25). To increase its bioavailability, several attempts have been made. Piperine, a bioactive alkaloid in pepper, has been suggested to inhibit the hepatic and intestinal glucuronidation and improve the bioavailability of CUR (26–28). The combined use of CUR and PIP has been reported to attenuate inflammation (29–31). The addition of PIP to CUR-containing formulations increases intestinal and plasma concentrations of CUR and thus enhances its inflammation-preventing activities (32). In this paper, we hypothesized that the combined use of CUR and PIP might be more effective than the single use of CUR and PIP in preventing diarrhea and poor performance induced by weaning in pigs.

Wuzhishan pig is a miniature pig breed originating from Hainan Island of China. In the previous study, we found that weaning at the 35th day after birth was more beneficial to the intestinal barrier function of Wuzhishan piglets than weaning at the 21st day after birth (33). Therefore, the purpose of this study was to determine the effects of single and combined use of CUR and PIP on growth performance, intestinal barrier function, and antioxidant capacity of 35-day-old weaned Wuzhishan piglets.

METHODS AND METHODS

Materials

Curcumin (90.0%) was purchased from Shi jiazhuang Lv Chuan Bio Technology Co., Ltd (Hebei, China). Piperine was purchased from Shanghai Macklin Biochemical Co., Ltd. (Shanghai China). The determination kits for antioxidant indices, including superoxide dismutase (SOD), glutathione peroxidase (GSH-Px), total antioxidant capacity (T-AOC) and malonaldehyde (MDA), plasma D-lactate, and diamine oxidase (DAO) were all purchased from the Jiancheng Bioengineering Institute of Nanjing (Jiangsu, China). RNAiso Plus and PrimeScript™ RT Reagent Kit were purchased from TakaRa Biotechnology Inc. (Dalian, China).

TABLE 1 | Composition and nutrient levels of the basal diet [dry matter (DM) basis] (%).

Ingredients	Content	Nutrient levels[b]	Content
Corn	64.00	Digestible energy(MJ/kg)	13.17
Soybean meal	22.80	CP (%)	17.94
Wheat bran	5.00	Ca (%)	0.80
Fish meal	2.00	P (%)	0.60
Whey powder	3.00	Lys (%)	0.86
Limestone	1.10	Met	0.32
$CaHPO_4$	0.80	Met + Cys (%)	0.65
NaCl	0.30		
Premix[a]	1.00		
Total	100.00		

[a]*provided per kilogram of diet: vitamin A, 3,200 IU; vitamin VD_3, 480 IU; vitamin E, 25 IU; vitamin K_3, 0.5 mg; vitamin B_1, 10 mg; vitamin B_2, 4 mg; vitamin B_{12}, 0.03 mg; folic acid, 0.3 mg; nicotinic, 22 mg; pantothenate, 14 mg; biotin, 0.10 mg; chorine, 830 mg; Cu, 15.6 mg; Fe, 100 mg; Mn, 40 mg; Zn, 106 mg; Se, 0.3 mg; I, 0.2 mg.*

[b]*digestible energy was a calculated value, while the other nutrient levels were measured values.*

Animal, Diets, and Experimental Design

A total of 50 Wuzhishan piglets weaned at 35 days of age were randomly allocated to one of five treatments ($n = 10$) for 21 days, with the initial average body weight of 3.54 ± 0.28 kg. The five treatments were CON, CON + 50 mg/kg PIP, CON + 200 mg/kg curcumin (low-CUR), CON + 200 mg/kg curcumin + 50 mg/kg piperine (PIP + CUR), and CON + 300 mg/kg curcumin (high-CUR). Diets were formulated to meet the nutrient requirements suggested by the Wuzhishan pig breeding technology discipline (**Table 1**). All the piglets were housed in individual pens with room temperature maintained at 25–27°C.

During the 21-day experiment period, piglets were allowed *ad libitum* to designated diet and water. The piglets were individually weighed at the beginning and end of the trial (days 0 and 21), and feed consumption for each pig was recorded daily. Average daily gain (ADG), average daily feed intake (ADFI), and F/G ratio were calculated.

Sample Collection

On day 21, six piglets subjected to each treatment were randomly selected, and a 5-ml blood sample was harvested from the jugular vein of each piglet into a tube with anticoagulant or without anticoagulant. After 20 min standing at room temperature, plasma or serum was obtained, which were centrifuged at 3,000 *g* for 15 min at 4°C and then stored at −20°C for assays. Plasma samples were used for D-lactate and DAO detection, and serum samples were used for the analysis of antioxidant variables.

After blood sampling, the piglets were sacrificed by injection of sodium pentobarbital solution (50 mg/kg of body weight). The middle sections of the jejunum and ileum were isolated aseptically, flushed with physiological saline, and fixed in 4% paraformaldehyde for 24 h for subsequent histological assays. After mucosa samples from the jejunum and ileum were scraped with a razor, they were frozen in liquid nitrogen immediately and stored at −80°C for further assays.

Plasma D-Lactate and DAO

The levels of D-lactate and DAO in the plasma were detected by using porcine D-lactic acid ELISA kit and DAO assay kit, respectively, according to the manufacturer's instructions (Jiancheng Bioengineering Institute of Nanjing, Nanjing, China).

Intestinal Morphology Analysis

The intestinal mucosa morphology including the villus height, villus width, and crypt depth were measured as previously described (23). Briefly, the paraformaldehyde-fixed tissue was embedded in paraffin according to standard procedures. Five-micrometer-thick sections were installed on glass slides. After deparaffinization, the slides were stained with hematoxylin and eosin. The villus height, villus width, and crypt depth were measured by using the Axioskop-2 microscope (Olympus) and image processing system (Version 1, Leica Imaging Systems Ltd). Ten well-oriented and intact crypt–villus units of each intestinal cross-section were selected for measurements. The villus height/crypt depth ratio was calculated.

Relative Quantitative Real-Time PCR

Relative messenger RNA (mRNA) abundance of *occludin*, *ZO-1*, *claudin-1*, *interleukin 1β* (*IL-1β*), tumor necrosis factor a (*TNF-α*), interleukin 6 (*IL-6*), and interleukin 10 (*IL-10*) in jejunal and ileal mucosa was determined by real-time PCR as described previously by Wan (34). Briefly, total RNA was extracted using RNAiso Plus (Takara, China) following the manufacturer's guidelines. The RNA samples were reversely transcribed into complementary DNA using RT Reagents (TaKaRa, China) according to the manufacturer's instructions. The primers are listed in **Table 2**. Quantitative real-time RT-PCR was performed on a PIKO-RT 96 Real-Time PCR Detection System (Thermo Fisher Scientific, America) using SYBR Premix Ex Taq reagent (TaKaRa, China) according to the kit's instructions. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was chosen as the reference gene transcript to correct the variances in target gene transcript levels. The reaction was performed in a 10-μl system containing 5 μ SYBR® *Premix Ex* Taq^{TM}II (Tli RNaseH Plus, 2 ×), 1 μl RT products, 2 μl double distilled water (ddH_2O), and 1 μl each of forward and reverse primers at the conditions of 95°C for 7 s followed by 40 cycles of 95°C for 5 s and 60°C for 30 s and a final dissociation step from 60 to 95°C at a heating rate of 0.2°C/s. The $2^{-\Delta\Delta CT}$ method was used to analyze the relative quantification of gene expression (fold changes), calculated relative to the control group as previously described (23).

Serum and Intestinal Mucosa Antioxidant Variables Analysis

Equal amounts of jejunum and ileum mucosa from the same piglet were blended to form a single sample before testing. Antioxidant indexes, including SOD, GSH-Px, T-AOC, and MDA in serum and small intestine mucosa samples were determined using assay kits according to the manufacturer's instructions (Nanjing Jiancheng Bioengineering Institute, Nanjing, China).

Statistical Analysis

Data were analyzed mean by one-way analysis of variance (ANOVA) using SPSS for windows version 18.0 (SPSS, Inc., Chicago, IL, USA). Differences among treatments were detected by Duncan's multiple range tests. The data are presented as mean, and standard error of the mean (SEM) was given. The *P*-value for significance was set at $P < 0.05$.

RESULTS

Growth Performance

The results of growth performance are given in **Table 3**. Supplementation of the basal diet with CUR + PIP or high-CUR

TABLE 2 | Sequences of primers used for real-time PCR.

Target	Accession number	Sense and Antisense primer	PCR product (bp)
GAPDH	AF017079	F:5′GAAGGTCGGAGTGAACGGAT3′	149
		R:5′CATGGGTAGAATCATACTGGAACA3′	
Occludin	NM_001163647.1	F:5′ATGCTTTCTCAGCCAGCGTA3′	176
		R:5′AAGGTTCCATAGCCTCGGTC3′	
ZO-1	XM003353439.1	F:5′GAGGATGGTCACACCGTGGT3′	169
		R:5′GGAGGATGCTGTTGTCTCGG3′	
Claudin-1	NM_001161635.1	F:5′GGACTAATAGCCATCTTTGT3′	88
		R:5′CAGCCATCCGCATCTTCT3′	
IL-1β	NM_214055.1	F:5′ACCTGGACCTTGGTTCTC3′	124
		R:5′GGATTCTTCATCGGCTTC3′	
TNF-α	NM_214022.1	F:5′ACGCTCTTCTGCCTACTGC3′	162
		R:5′TCCCTCGGCTTTGACATT3′	
IL-10	NM_214041.1	F:5′CACTGCTCTATTGCCTGATCTTCC3′	136
		R:5′AAACTCTTCACTGGGCCGAAG3′	
IL-6	NM_214399.1	F:5′TTCAGTCCAGTCGCCTTCT3′	91
		R:5′GTGGCATCACCTTTGGCATCTTCTT3′	

TABLE 3 | Effect of curcumin (CUR) and piperine (PIP) on growth performance of weaned Wuzhishan piglets.

Items	Treatments[a]					SEM[b]	*P*
	CON	PIP	Low-CUR	PIP + CUR	High-CUR		
Initial weight (kg)	3.42	3.66	3.52	3.63	3.49	0.072	0.864
Final weight (kg)	5.01	5.31	5.16	5.62	5.57	0.124	0.535
ADG (g/day)[c]	75.7 2	78.73	78.23	94.77	98.87	3.841	0.171
ADFI (g/day)[d]	172.63	166.80	170.07	175.27	185.83	6.132	0.923
F/G[e]	2.29[f]	2.13[f]	2.18[f]	1.83[g]	1.89[g]	0.054	0.005

[a]*CON: a corn–soybean basal diet; PIP, low-CUR, PIP + CUR and high-CUR, the basal diet supplemented with 50 mg/kg piperine, 200 mg/kg curcumin, 200 mg/kg curcumin + 50 mg/kg piperine, and 300 mg/kg curcumin, respectively.*
[b]*SEM, standard error of the mean (n = 10).*
[c]*ADG, average daily body weight gain.*
[d]*ADFI, average daily feed intake.*
[e]*F/G, the ratio of feed to gain.*
[fg]*means in the same row with different letters differ significantly (P < 0.05).*

TABLE 4 | Effect of curcumin (CUR) and piperine (PIP) on intestinal mucosa morphology of weaned Wuzhishan piglets.

Items	Treatments[a]					SEM[b]	*P*
	CON	PIP	Low-CUR	PIP + CUR	High-CUR		
Jejunum							
Villus height (μm)	314.01[e]	328.50[e]	348.12[de]	377.66[cd]	395.73[c]	9.191	0.003
Villus width (μm)	155.08	140.42	132.51	131.57	133.96	5.389	0.234
Crypt depth (μm)	179.76	176.29	181.28	170.95	173.04	3.941	0.912
Villus height/crypt depth	1.75[e]	1.87[de]	1.92[d]	2.21[c]	2.30[c]	0.062	0.001
Ileum							
Villus height (μm)	298.57[d]	312.63[d]	318.46[d]	354.61[c]	367.86[c]	8.128	0.005
Villus width (μm)	158.23	152.22	159.10	145.11	150.45	3.728	0.805
Crypt depth (μm)	179.97	160.59	151.58	154.76	157.35	3.918	0.458
Villus height/crypt depth	1.71[f]	1.95[e]	2.11[de]	2.29[cd]	2.36[c]	0.069	0.001

[a]*CON: a corn–soybean basal diet; PIP, low-CUR, PIP + CUR, and high-CUR, the basal diet supplemented with 50 mg/kg piperine, 200 mg/kg curcumin, 200 mg/kg curcumin + 50 mg/kg piperine, and 300 mg/kg curcumin, respectively.*
[b]*SEM, standard error of the mean (n = 6).*
[cdef]*means in the same row with different letters differ significantly (P < 0.05).*

had no effect on the initial weight, final weight, ADFI, and ADG ($P > 0.05$), but the F/G ratio of pigs in the CUR + PIP and high-CUR groups was lower than that in the CON, PIP, and low-CUR groups ($P < 0.05$).

Intestinal Mucosa Morphology and Plasma D-Lactate and DAO

The data for intestinal morphology of piglets are shown in **Table 4**. The weaned piglets fed with CUR + PIP or high-CUR had significantly higher villus height and villus height/crypt depth ratio in jejunal and ileum mucosa than the CON and PIP pigs ($P < 0.05$). The villus height/crypt depth ratio in jejunal and ileum mucosa of pigs in the low-CUR group was higher than that in the CON group ($P < 0.05$), but the villus height was not significantly different between the two groups ($P > 0.05$). The crypt depth and villus width were not significantly different among the five treatments ($P > 0.05$).

The plasma D-lactate and DAO results are shown in **Table 5**. Compared with the control group, the plasma D-lactate and DAO in CUR + PIP and high-CUR groups were significantly lower ($P < 0.05$). Supplementation of PIP significantly reduced plasma DAO activities ($P < 0.05$), but plasma D-lactate was not affected ($P > 0.05$). Moreover, the plasma D-lactate and DAO were significantly different between low-CUR and control groups ($P > 0.05$).

mRNA Expressions of Tight Junction Proteins

Figure 1 shows the mRNA expression of *occludin*, *claudin-1*, and *ZO-1* in the jejunal and ileum mucosa of piglets. The expression levels of *occludin*, *claudin-1*, and *ZO-1* in the jejunal and ileum mucosa of piglets in the CUR + PIP and high-CUR groups were significantly higher than those in the control, PIP, and low-CUR groups ($P < 0.05$). However, there were no significant

TABLE 5 | Effect of curcumin (CUR) and piperine (PIP) on plasma d-lactate and diamine oxidase activity (DAO) in weaned Wuzhishan piglets.

Items	Treatments[a]					SEM[b]	*P*
	CON	PIP	Low-CUR	PIP + CUR	High-CUR		
Plasma DAO (U/L)	7.17^{c}	6.41^{d}	6.55^{cd}	5.58^{e}	5.29^{e}	0.199	0.001
Plasma d-lactate (mg/L)	5.91^{c}	5.22^{c}	5.34^{c}	4.23^{d}	3.67^{d}	0.240	0.001

[a]*CON: a corn–soybean basal diet; PIP, low-CUR, PIP + CUR, and high-CUR, the basal diet supplemented with 50 mg/kg piperine, 200 mg/kg curcumin, 200 mg/kg curcumin + 50 mg/kg piperine, and 300 mg/kg curcumin, respectively.*

[b]*SEM, standard error of the mean (n = 6).*

[cde]*means in the same row with different letters differ significantly (P < 0.05).*

differences in the expression of above substances between the PIP and low-CUR groups ($P > 0.05$).

mRNA Expressions of Cytokines

Figure 2 shows the mRNA expression of cytokines in the jejunal and ileum mucosa of piglets. The mRNA levels of *IL-1β*, *TNF-α*, *IL-6*, and *IL-10* were not significantly different among the five treatment groups ($P > 0.05$).

Serum and Intestinal Mucosa Antioxidant Parameters

As shown in **Table 6**, pigs in CUR + PIP and high-CUR groups had higher SOD and GSH-Px activities as well as lower MDA concentration in serum and intestinal mucosa than pigs in the CON group ($P < 0.05$). The PIP piglets had higher GSH-Px activity and lower MDA concentration in the serum and intestinal mucosa than the CON piglets ($P < 0.05$). The value of T-AOC in serum and intestinal mucosa was not significantly different among the five treatments ($P < 0.05$).

DISCUSSION

In recent years, there have been several attempts to demonstrate the use of CUR as a potential feed additive that can replace antimicrobial growth promoters. Diets supplied with 50 and 100 mg/kg CUR increased the growth performance of broilers by improving the antioxidant defense system and enhancing the mitochondrial biogenesis. Ruan et al. (20) found that CUR prevented the decrease in body weight and ADG in ducks fed with corn contaminated by ochratoxin A. Ilsley et al. (35) reported that dietary supplementation with 200 mg/kg CUR had no influence on pig growth performance. This finding was in line with that of our previous study, which suggested that no growth improvement was observed in weaned pigs fed with 200 mg/kg CUR-added diet (23). In the present experiment, supplementation of CUR + PIP or high-CUR significantly improved the growth performance of pigs by reducing F/G, indicating that high-CUR or CUR + PIP had better performance-promoting effects than PIP or low-CUR added alone in piglet diets.

As common indicators for estimating intestinal integrity, the villus height, crypt depth, villus width, and the villus height/crypt depth ratio can reveal some information on gut health in pigs. Increasing the villus height suggest an increased surface area for nutrient absorption (36). The villus crypt is considered as villus factory. The increase in crypt depth indicates fast tissue turnover and high demand for new tissue, which are generally associated with decline in nutrient digestion and absorption capacity (37, 38). Studies have confirmed that weaning is associated with villus atrophy and crypt hyperplasia (3, 39). In the present study, the increase in villus height and villus height/crypt depth ratio in jejunal and ileum mucosa caused by the addition of CUR + PIP or high-CUR was observed. Similar results were achieved in our previous study, which noticed that the addition of 400 mg/kg CUR in the diet increased the villus height and villus height/crypt depth ratio in piglets, demonstrating that both the supplementation with CUR + PIP and the addition of high-CUR could ameliorate the weaning-associated damage to small intestinal morphology, thus correspondingly improving the digestion and absorption of nutrients and promoting the growth performance (23).

The integrity of intestinal mucosa barrier is the basis for the normal function of epithelial cells and defense against the pathogenic bacteria (3). Plasma DAO and D-lactate are used as sensitive circulating indicators of the severity of mucosal injury (40, 41). DAO exists only in the villi of the upper small intestine, and a small amount is normally present in the blood. When the intestinal mucosal function is injured, mucosal permeability increases, promoting the release of more endocellular DAO into the blood (42). Therefore, plasma DAO reflects the integrity of intestinal mucosa. D-Lactate is the end product of intestinal bacterial fermentation. When the intestinal mucosal function is impaired, the D-lactate concentration in blood is increased, which is because D-lactate in mammals cannot be metabolized due to the lack of enzyme systems (43). Studies have shown that early weaning leads to impaired mucosal barrier function and increased intestinal permeability (2, 44). The present results showed that dietary supplementation of CUR + PIP or high-CUR improved intestinal barrier function by reducing plasma DAO and D-lactate. The data were supported by our previous studies, which revealed that dietary addition of 300 or 400 mg/kg CUR reduced plasma DAO and D-lactate in weaned piglets challenged with enterotoxigenic *E. coli* (23).

Tight junction proteins (*occludin*, *claudin-1*, and *ZO-1*) play an important role in the maintenance of intestinal mucosal barrier integrity. They function as the continuous intercellular barrier against the translocation of intestinal bacteria, antigens, and intraluminal toxins from the lumen into subepithelial tissue

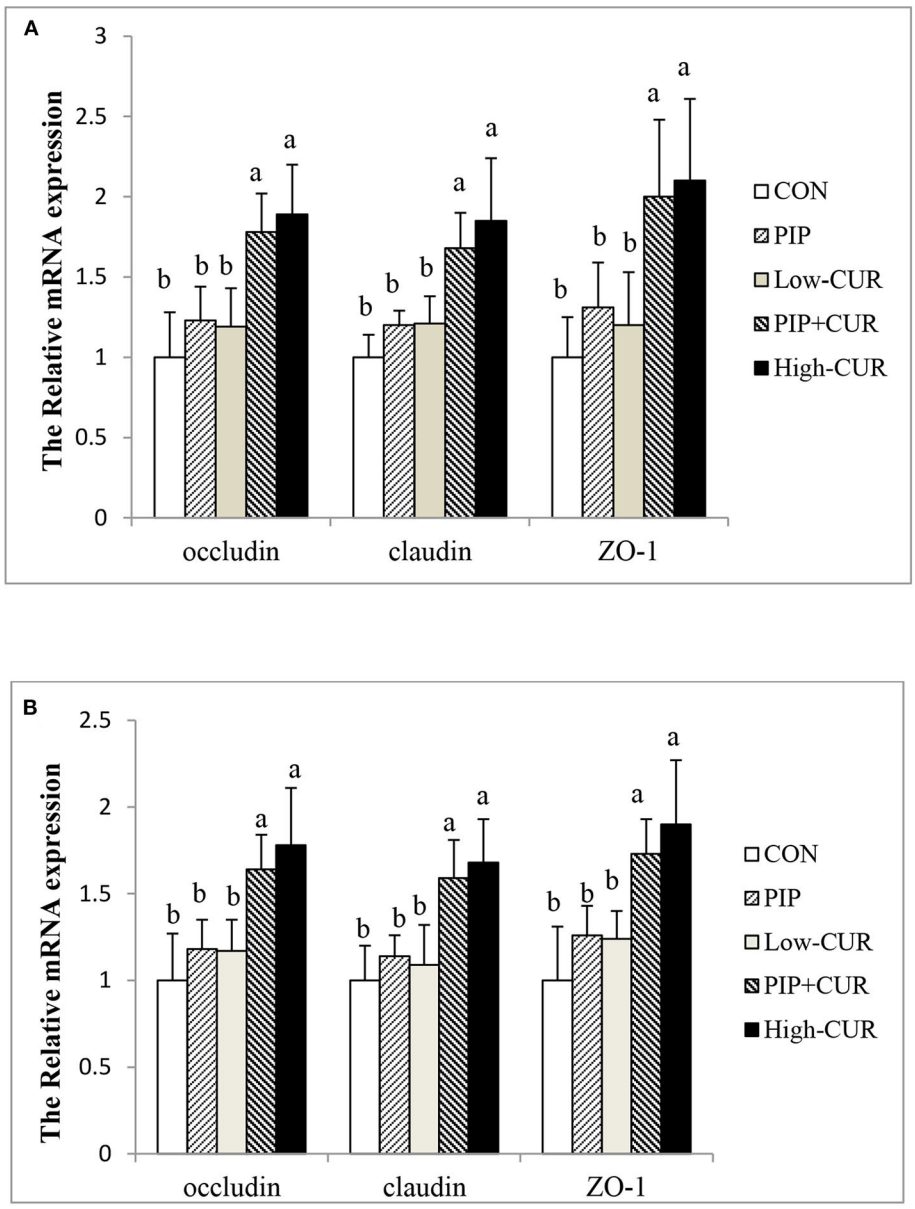

FIGURE 1 | Effect of curcumin (CUR) and piperine (PIP) on messenger RNA (mRNA) expression of tight junction protein in **(A)** jejunal mucosa and **(B)** ileal mucosa of weaned Wuzhishan piglets. Values are expressed as mean ± standard deviations ($n = 6$). ^{ab}Means values within different letters differ significantly ($P < 0.05$). CON, a corn–soybean basal diet; PIP, low-CUR, PIP + CUR, and high-CUR, the basal diet supplemented with 50 mg/kg piperine, 200 mg/kg curcumin, 200 mg/kg curcumin + 50 mg/kg piperine, and 300 mg/kg curcumin, respectively.

and systemic blood circulation (45). It has been established by several studies that CUR promotes the expression of tight junction proteins in intestinal mucosa. Tian et al. (46) found that CUR significantly upregulated the expression of *ZO-1* following the intestinal ischemia–reperfusion injury in rats, which might be partly attributed to the TNF-α related pathway. The study of Ruan et al. (20) implied that CUR increased jejunal mucosa occludin and *ZO-1* mRNA and protein levels in ducks. In the present study, the upregulation of *occludin*, *claudin-1*, and *ZO-1* mRNA expression due to the supplementation with CUR + PIP or high-CUR suggested that both treatments might improve the intestinal integrity. The results were consistent with the improved intestinal morphology and decrease in the plasma D-lactate and DAO levels. The molecular mechanism of CUR + PIP in regulation of tight junctions requires further study.

In addition to intestinal integrity, weaning-associated intestinal inflammation was also observed in weaning piglets (39, 47). Weaning causes the upregulation of proinflammatory cytokines, such as *TNF-α*, *IFN-γ*, *IL-1β*,

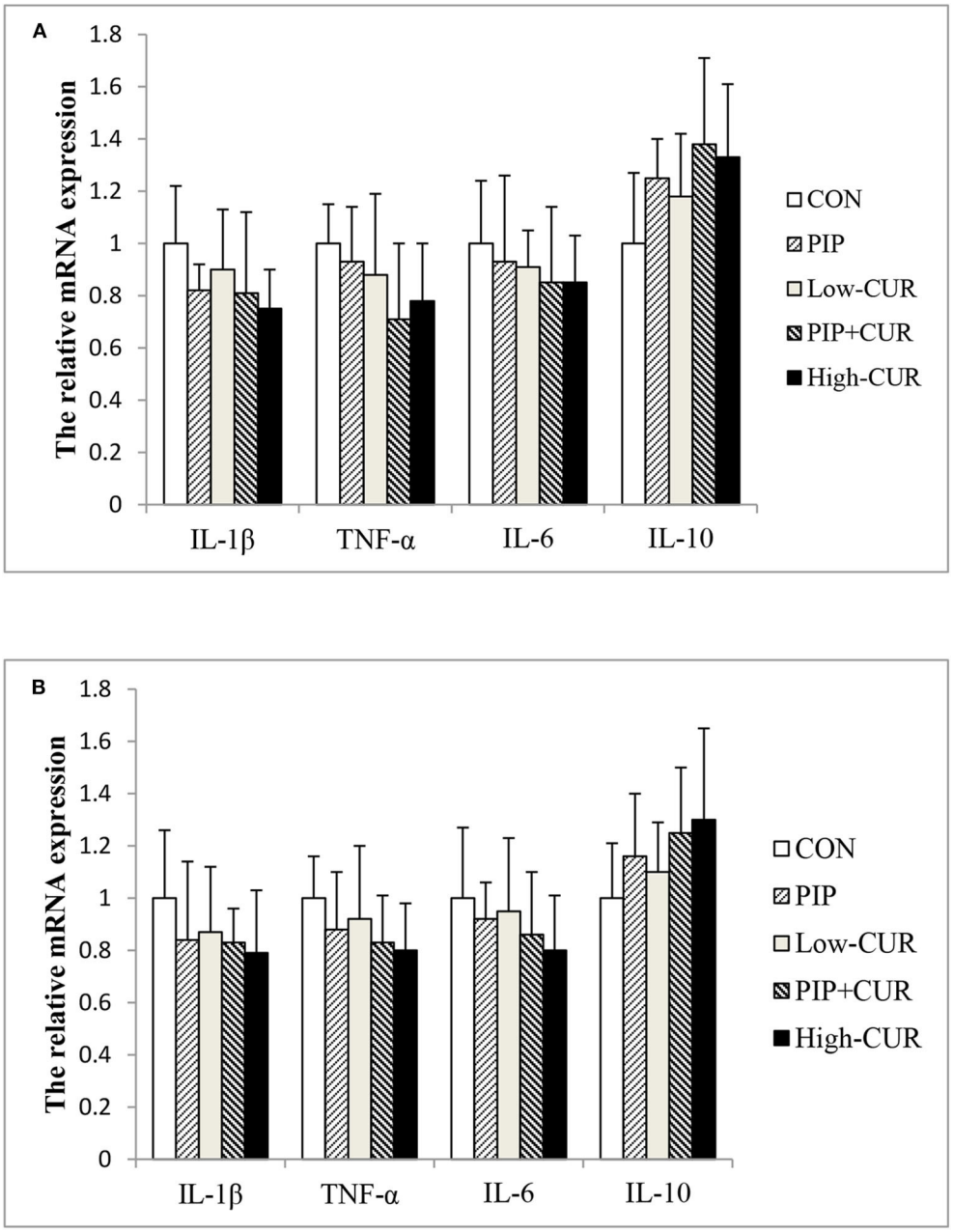

FIGURE 2 | Effect of curcumin (CUR) and piperine (PIP) on messenger RNA (mRNA) expression of cytokines in **(A)** jejunal and **(B)** ileal mucosa of weaned Wuzhishan piglets. Values are means ($n = 6$), and standard deviations represented by vertical bars. CON, a corn–soybean basal diet; PIP, low-CUR, PIP + CUR, and high-CUR, the basal diet supplemented with 50 mg/kg piperine, 200 mg/kg curcumin, 200 mg/kg curcumin + 50 mg/kg piperine, and 300 mg/kg curcumin, respectively. Dietary Rice Improves Growth Performance, Mucosal Enzyme Activities, and Plasma Urea Nitrogen in Weaning Piglets. Effects of Dietary Methionine Supplementation on Growth Performance, Intestinal Morphology, Antioxidant Capacity, and Immune Function in Intra-Uterine Growth-Retarded Suckling Piglets. Comparative Effects of Dietary Supplementations With Sodium Butyrate, Medium-Chain Fatty Acids, and n-3 Polyunsaturated Fatty Acids in Late Pregnancy, and Lactation on the Reproductive Performance of Sows and Growth Performance of Suckling Piglets. Effects of Composite Antimicrobial Peptide on Growth Performance and Health in Weaned Piglets.

and *IL-6*. Overproduction of proinflammatory cytokines induces a pathological opening of the intestinal tight junctions and increases intestinal epithelial permeability, resulting in intestinal barrier dysfunction (48, 49). Song et al. (19) noticed that CUR decreased the mRNA expression of *IL-1β* and *TNF-a* and increased the mRNA expression of *IL-10* in intestinal mucosa of rats and IEC-6 cells. Ruan et al. (20) also observed that CUR decreased the concentrations

TABLE 6 | Effect of curcumin (CUR) and piperine (PIP) on antioxidant variables in serum and intestinal mucosa of weaned Wuzhishan piglets.

Items	Treatments[a]					SEM[b]	*P*
	CON	PIP	Low-CUR	PIP + CUR	High-CUR		
Serum (U/mL)							
Superoxide dismutase	114.57^{e}	129.89^{de}	122.80^{de}	147.24^{cd}	167.24^{c}	8.40	0.019
Total antioxidant capacity	1.22	1.27	1.36	1.37	1.42	0.103	0.475
Glutathione peroxidase	160.73^{e}	234.11^{cd}	205.98^{d}	258.61^{c}	272.60^{c}	13.17	0.001
Malondialdehyde (nmol/ml)	5.57^{c}	4.70^{de}	5.16^{cd}	4.12^{e}	4.05^{e}	0.18	0.003
Intestinal mucosa (U/mg protein)							
Superoxide dismutase	4.16^{d}	4.69^{d}	4.45^{d}	5.74^{c}	5.52^{c}	0.179	0.005
Total antioxidant capacity	0.54	0.64	057	0.64	0.73	0.035	0.239
Glutathione peroxidase	20.44^{e}	36.32^{d}	29.50^{de}	44.40^{c}	48.47^{c}	2.958	0.015
Malondialdehyde (nmol/mg protein)	0.49^{c}	0.35^{d}	0.36^{d}	0.24^{e}	0.22^{e}	0.027	0.001

[a]*CON: a corn–soybean basal diet; PIP, low-CUR, PIP + CUR, and high-CUR, the basal diet supplemented with 50 mg/kg piperine, 200 mg/kg curcumin, 200 mg/kg curcumin + 50 mg/kg piperine, and 300 mg/kg curcumin, respectively.*

[b]*SEM, standard error of the mean (n = 6).*

[cde]*means in the same row with different letters differ significantly (P < 0.05).*

of *TNF-α* and *IL-1β* induced by OTA in jejunal mucosa of ducks. In our previous studies, the mRNA levels of *TNF-α*, *IL-6*, and *IL-1β* were decreased by supplementation with 400 mg/kg CUR in weaned piglets challenged with enterotoxigenic *E. coli*. However, in the present experiment, the mRNA expression levels of *IL-1β*, *TNF-α*, *IL-6*, and *IL-10* were not affected by dietary supplementation. The present study results demonstrated that the transiently upregulated inflammatory cytokines induced by weaning could rapidly return to the preweaning level 9 days after weaning, which might be the reason why dietary CUR supplementation did not affect mRNA expression of inflammatory cytokines 21 days after weaning.

Studies have confirmed that weaning induces oxidative stress and increases free radicals in tissue and blood. Antioxidants can eliminate free radicals and reduce oxidative stress. CUR has the potential *in vivo* antioxidant activity owing to its ability to scavenge reactive oxygen species (50, 51) and inhibit lipid peroxidation (52). The gastrointestinal tract can remove free radicals and prevent oxidative damage, mainly serving as antioxidant in the human body. Antioxidant enzymes include GSH-Px, SOD, CAT, and MDA (52). MDA is the end product of lipoperoxidation, and the levels of MDA in plasma and tissue are an excellent oxidative stress marker (53). In our study, both CUR + PIP and high-CUR increased the activities of serum and intestinal mucosa antioxidant enzymes (e.g., GSH-Px and SOD) and decreased the lipid peroxidation marker MDA, indicating that the oxidative stress was reduced by dietary CUR or CUR + PIP supplementation. This result was similar with that of the study of Arcaro et al. (54) who reported that the level of MDA was markedly reduced in the plasma of diabetic rats fed with 90 mg/kg curcumin or 90 mg/kg CUR + 20 mg/kg PIP. Oxidative stress caused by weaning is responsible for intestinal mucosal injury (55). Reduced oxidative stress might alleviate the damage of intestinal mucosal barrier, and high-CUR or CUR + PIP supplementation decreased the intestinal permeability subsequently.

CONCLUSIONS

In summary, the present study showed that supplementation with PIP + CUR or high-CUR could reduce intestinal permeability, enhance antioxidant capacity, and had beneficial effects on feed utilization rate of the corn–soybean basal diet. The addition of both CUR and PIP appeared to be as advantageous as high-CUR, but it was more effective than low-CUR or PIP alone.

DATA AVAILABILITY STATEMENT

All datasets presented in this study are included in the article/supplementary material.

ETHICS STATEMENT

The animal study was reviewed and approved by Committee on laboratory animal ethics of Tropical Crops Genetic Resources Institute (TCGRI). Written informed consent was obtained from the owners for the participation of their animals in this study.

AUTHOR CONTRIBUTIONS

LS and WX designed the experiments. TC, WP, and HH carried out the feeding experiments. GH analyzed the experimental results. All authors contributed to the article and approved the submitted version.

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Tea Tree Oil Prevents Mastitis-Associated Inflammation in Lipopolysaccharide-Stimulated Bovine Mammary Epithelial Cells

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The main purpose of this study was to explore the effect of tea tree oil (TTO) on lipopolysaccharide (LPS)-induced mastitis model using isolated bovine mammary epithelial cells (BMEC). This mastitis model was used to determine cellular responses to TTO and LPS on cellular cytotoxicity, mRNA abundance and cytokine production. High-throughput sequencing was used to select candidate genes, followed by functional evaluation of those genes. In the first experiment, LPS at a concentration of 200 μg/mL reduced cell proliferation, induced apoptosis and upregulated protein concentrations of tumor necrosis factor-α (TNF-α), interleukin 6 (IL-6), and signal transducer and activator of transcription 1 (STAT1). Addition of TTO led to reduced cellular apoptosis along with downregulated protein concentrations of nuclear factor kappa B, mitogen-activated protein kinase 4 (MAPK4) and caspase-3. In the second experiment, BMEC challenged with LPS had a total of 1,270 differentially expressed genes of which 787 were upregulated and 483 were downregulated. Differentially expressed genes included *TNF-α*, *IL6*, *STAT1*, and *MAPK4*. Overall, results showed that TTO (at least *in vitro*) has a protective effect against LPS-induced mastitis. Further *in vivo* research should be performed to determine strategies for using TTO for prevention and treatment of mastitis and improvement of milk quality.

Keywords: TTO, BMEC, LPS, mastitis, transcriptome sequencing

INTRODUCTION

Lipopolysaccharide (LPS) is one of the main components of the cell wall of gram-negative bacteria including *Escherichia coli* (*E. coli*) and other mastitis-inducing pathogenic bacteria such as *Staphylococcus aureus*, *Streptococcus agalactis,* and *Streptococcus lactis* (1). In dairy cows, mastitis caused by *E. coli* results in increased concentrations of acute-phase proteins in milk (2, 3), and can be treated with antibiotics (4). However, with increasing concerns about drug resistance it has become imperative to prevent usage of antibiotics and develop alternatives and treat cow mastitis using alternative therapies.

Tea tree oil (TTO; terpinen-4-ol type), also known as *M. alternifolia* oil, is an essential oil from several plants of Melaleuca, of which the main one is *M. alternifolia* (5). TTO is widely used in many over-the-counter health products and cosmetics. With the vigorous development of natural and alternative medicinals, an increasing number of people are using products containing TTO (6). TTO has a broad antibacterial spectrum and strong antibacterial activity, which explains its use to treat diseases caused by fungi, bacteria, or viruses (7). Therefore, its potential use as a natural antibacterial agent to replace antibiotics as a component of mastitis therapy is of interest.

With the development of sequencing and histochemistry technology, analysis of the complex pathogenesis of mastitis in dairy cows from multiple perspectives can be performed. More importantly, an integrative approach aids in effective biomarkers for timely and accurate prevention (8). Although numerous studies have reported alterations of mRNA abundance in the mammary gland in response to mastitis, the role of gene transcription along with the complex networks and how they respond to therapeutic agents is still unclear. For instance, microRNA expression was first confirmed during mastitis in 2007 (9). Naeem et al. detected changes in 14 miRNA in mammary tissue 12 h after infection with *Streptococcus uberis*. Compared with healthy tissue, expression of miR-15b, miR-16a, miR-21, miR-145, and miR-181a was lower, and only miR-223 was greater in infected mammary tissue. The miR-16a was decreased of some interleukins (IL-6, IL-8, and IL-10). The present study aimed to use transcriptome technology to uncover the response of bovine mammary epithelial cells (BMEC) to LPS as a way to identify key candidate genes that could be target for functional verification. Along with other assays, a combined technological approach can provide precise targets for research and development of effective therapeutic drugs, ultimately achieving positive effects in terms of prevention and treatment (10).

MATERIALS AND METHODS

Ethics Statement

The animal use protocol was approved by the Institutional Animal Care and Use Committee in the College of Animal Science and Technology, Yang Zhou University, Yang Zhou, China.

Culture of BMEC

Three peak lactation dairy cows were selected for mammary gland biopsy (11). After PBS washing, fat tissue and connective tissue were peeled off. The BMEC were separated by the tissue block method followed purification by differential digestion and cryopreservation after subculturing (11). Cells were cultured in Dulbecco's modified Eagle medium/F12 (DMEM/F12) supplemented with 10% (vol/vol) fetal bovine serum in a humidified incubator at 37°C with 5% CO_2. Medium was replaced every 48 h. The BMEC were digested with 0.25% trypsin for passaging, and the growth of cells was observed using an inverted microscope (11).

CCK-8 Detection of Cell Proliferation Activity Induced by LPS

The density of BMEC was adjusted to 1×10^4 in a 96-well plate. After 24 h incubation, the culture medium was discarded. The BMEC were treated with LPS (50, 100, 200, 500, and 1,000 μg/mL). In addition, there was a control (BMEC without LPS) and a blank group (only culture medium without cells). After 4, 8, 12, and 24 h incubation, cell proliferation activity was detected using a CCK-8 kit (Watson Technology Co., Ltd., Beijing, China) according to the manufacturer's protocols.

Detection of Apoptosis Rate Induced by LPS via Flow Cytometry

The BMEC were plated in a 6-well plate and incubated for 24 h. Cells were then washed and collected with PBS, and cell concentration adjusted with buffer to 1×10^6/100 μL/test. Then, 5 μL annexin V-FITC and 5 μL PI were added, and cell apoptosis determined in a dark room.

Effect of TTO on Apoptosis Rate During LPS Challenge via Flow Cytometry

The BMEC were plated in a 6-well plate and cultured for 24 h. LPS and various concentrations of TTO (Yuanye biology Co., Ltd., Shanghai, China) were added to the culture (0.0002, 0.0004, 0.0006, 0.0008, 0.001, 0.002, 0.004, 0.006, 0.008, and 0.01%, vol/vol). Annexin V-FITC and PI were added for detection of apoptosis.

Abundance of Inflammation- and Apoptosis-Related Proteins via ELISA

After washing with PBS, RIPA buffer was added to the cell lysate. Bovine nuclear factor kappa B (NF-κB), mitogen-activated protein kinase 4 (MAPK4), tumor necrosis factor-α (TNF-α), interleukin 6 (IL-6), signal transducer and activator of transcription 1 (STAT1), and apoptosis-related caspase-3 were determined according to protocols supplied with the ELISA kits (Qiaoshe Co., Shanghai, China).

Transcriptome Sequencing

Library Construction

Total RNA was extracted from BMEC (number of cells is 1×10^7) treated with 200 μg/mL LPS for 12 h. After total RNA was extracted and digested with DNase, eukaryotic mRNA was enriched with oligo (dT) using magnetic beads. A strand of cDNA was synthesized with random hexamers using the interrupted mRNA as template. Double-stranded cDNA was synthesized using the two-stranded synthesis system and purified followed by poly-(A) addition and sequencing. The library was inspected for quality using the Agilent 2100 Bioanalyzer, and eventually sequenced with the Illumina hiseqtm 2500 sequencer (12). The raw data generated by high-throughput sequencing was in FASTQ format. To obtain high-quality reads, we first used NGS QC Toolkit software to conduct quality control and remove joints.

Gene Quantification, Differential Gene Screening, Functional Enrichment, and Cluster Analysis

The comparison between clean reads and the reference genome were stored in a binary file (BAM file). Genes were quantified to obtain the FPKM value using cufflinks. When calculating differences in gene expression, we used Htseq-count software to determine the number of gene reads in each sample. The estimate SizeFactors function in the DESeq R package was used to standardize the data, and the nbinomTest function was used to calculate the *P*-value and fold-change values in the difference comparisons (13). The condition used to screen differentially expressed genes was a $P < 0.05$ and multiple differences was more than 2 times. Gene ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analysis of differentially expressed genes were carried out to determine main biological functions or pathways.

H&E Staining of Cells

Cell sections were deparaffinized in xylene, and slides hydrated in 95% ethanol for 5 min, 85% ethanol for 5 min; slides were then hydrated in distilled water. Hematoxylin staining was performed for 3 min, and slides rinsed with distilled water for 2 min; 1% hydrochloric acid alcohol was used for 2 s to differentiate the stain. The sections were rinsed with tap water for 15 min followed by 1–2 s of distilled water. Slides were stained with eosin for 30 s. Differentiation was determined according to the color, and 80% ethanol was used to differentiate stains. Slides were further dehydrated with 85% ethanol for 5 min, followed by 95% ethanol for 5 min. Then, the slides were dehydrated with anhydrous ethanol for 10 min. After the run off was transparent, slides were sealed by adding a drop of neutral gum. Observation and photography were performed with a microscope (Dmi4000b inverted fluorescence microscope, Leica, Germany).

Immunohistochemistry

Sections were dewaxed and hydrated followed by washing in xylene twice for 10 min each. Slides were then incubated with 100, 95, 85, and 75% ethanol for 5–10 min. The sections were soaked in distilled water for 5 min. For antigen retrieval, sections were incubated in citrate buffer (pH 6.0) and heated in a microwave at high heat for 8 min. Cells were then washed with 1× PBS (pH 7.2∼7.6) three times for 3 min each time followed by addition of 3% H_2O_2 at room temperature for 10 min to inactivate endogenous peroxidases. Slides were rinsed with 1× PBS three times, 3 min each. Slides were incubated with primary antibodies (BV20932, Qiaoshe company, Shanghai, China) followed by a secondary antibody (BV30796, Qiaoshe company, Shanghai, China) in a box at 37°C for 1.5 h. Slides were washed 3 times with 1× PBS for 5 min each. A streptavidin-HRP antibody was incubated with the slides at 37°C for 20 min. Slides were then covered with 100 μL of the previously prepared color developer DAB working solution; the reaction time was monitored under the microscope.

Statistical Analysis

Statistical analyses were performed by SPSS 19.0 (SPSS Inc., Chicago, USA). The data are presented as mean values ± s.d. from three independent experiments, duplicates. Statistical analysis was conducted using two-tailed unpaired Student's *t*-test or one-way ANOVA with Bonferroni's multiple comparisons test. $P < 0.05$ was considered significant.

RESULTS

CCK-8 Detection of Cell Proliferation Activity Induced by LPS

As shown in **Figure 1**, cell proliferation activity of BMEC began to decline to varying degrees with 100 μg/mL LPS treatment for 12 h. As the activity of cells induced by LPS of 500 μg/mL and 1,000 μg/mL was too low, we chose the challenge of LPS concentration at 200 μg/mL for 12 h as the optimal treatment condition for further analysis. Biological repeat is three times, and technical repeat is two times.

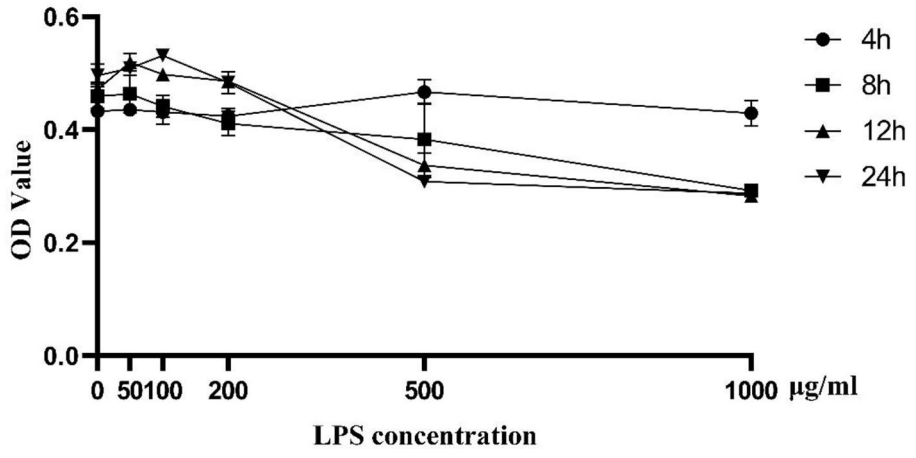

FIGURE 1 | Cell proliferation activity induced by LPS at different concentrations (0, 50, 100, 200, 500, and 1,000 μg/ml) and time points (0, 4, 8, 12, 24 h). Data were presented as means ± s.d. of at least three independent experiments.

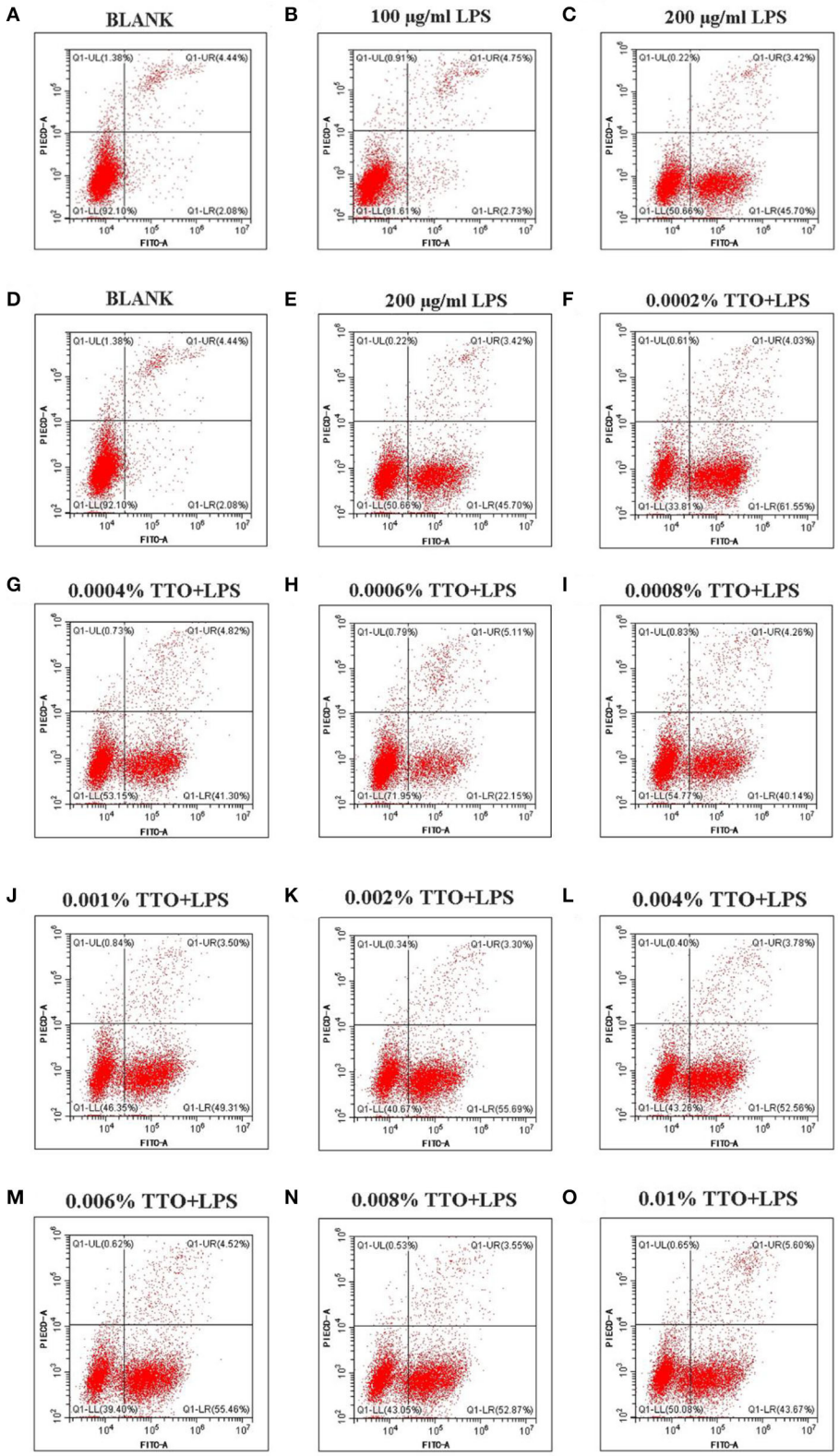

FIGURE 2 | Effect of different concentrations of TTO on apoptosis in BMEC with LPS challenge. **(A)** BLANK; **(B)** 100 μg/ml LPS; **(C)** 200 μg/ml LPS; **(D)** BLANK; **(E)** 200 μg/mL LPS; **(F)** 0.0002% TTO+LPS; **(G)** 0.0004% TTO+LPS; **(H)** 0.0006% TTO+LPS; **(I)** 0.0008% TTO+LPS; **(J)** 0.001% TTO+LPS; **(K)** 0.002% TTO+LPS; **(L)** 0.004% TTO+LPS; **(M)** 0.006% TTO+LPS; **(N)** 0.008% TTO+LPS; **(O)** 0.01% TTO+LPS. Data were presented as means ± s.d. of at least three independent experiments.

Apoptosis of LPS-Induced BMEC

Approximately 4.44% (4.44 ± 0.01) early apoptosis and late apoptosis were observed without LPS (**Figure 2A**). Upon addition of 100 μg/mL LPS, the whole image shifted to the right, and ∼7.48% (7.48 ± 0.02) [early apoptosis 2.73 (2.73 ± 0.01) +late apoptosis 4.75 (4.75 ± 0.01)] apoptosis occurred (**Figure 2B**). In contrast to those minor effects, when 200 μg/mL LPS was added to group C (**Figure 2C**), the whole image of group C showed marked clustering with ∼49.12% (49.12 ± 0.01, $P < 0.05$) of cells showing early and late apoptosis. Thus, these data confirmed this dose of LP was ideal as a "mastitis model" in the follow-up experiment (**Figure 2**).

Effect of TTO on Apoptosis of LPS-Induced BMEC

The blank control group A (**Figure 2D**, $P < 0.05$) showed apoptosis of BMEC without any treatment. The proportion of living cells was 92.10% (92.10 ± 0.03), the proportion of early apoptotic cells was 2.08% (2.08 ± 0.01) and the proportion of late apoptotic cells was 4.44% (4.44 ± 0.02). In group B, BMEC treated with 200 μg/mL LPS showed apoptosis. Among these cells, the proportion of living cells was 50.66% (50.66 ± 0.02), the proportion of early apoptotic cells was 45.70% (45.70 ± 0.01) and the proportion of late apoptotic cells was 3.42% (3.42 ± 0.01). The early withering of samples treated with 0.0002% TTO+LPS, 0.0004% TTO+LPS, 0.0006% TTO+LPS, 0.0008% TTO+LPS, 0.001% TTO+LPS, 0.002% TTO+LPS, 0.004% TTO+LPS, 0.006% TTO+LPS, 0.008% TTO+LPS, and 0.01% TTO+LPS was 61.55% (61.55 ± 0.04), 41.30%(41.30 ± 0.03, $P < 0.05$), 22.15% (22.15 ± 0.05, $P < 0.05$), 40.14% (40.14 ± 0.03, $P < 0.05$), 49.31% (49.31 ± 0.03, $P < 0.01$), 55.69% (55.69 ± 0.01, $P < 0.01$), 52.56% (52.56 ± 0.03, $P < 0.01$), 55.46% (55.46 ± 0.02, $P < 0.01$), 52.87% (52.87 ± 0.02, $P < 0.01$), and 43.67% (43.67 ± 0.02, $P < 0.01$), respectively (**Figures 2F–O**). After adding different concentrations of TTO (**Figures 2F–O**), the analysis indicated that TTO in group G (**Figure 2G**), H (**Figure 2H**), and I (**Figure 2I**) elicited protective effects, especially group H. The proportion of living cells, early apoptotic cells and late apoptotic cells was 71.95, 22.15, and 5.11%, respectively.

Effect of TTO on Inflammatory and Apoptotic Factors in the LPS-Induced Mastitis Model

Concentrations of TNF-α and IL-6 in the 200 μg/mL LPS group were more than 15-times higher than the BLANK ($P < 0.01$). Additionally, compared with the BLANK, STAT1 increased almost 6-times after addition of TTO at 0.0004% ($P < 0.01$), 0.0006% ($P < 0.01$), and 0.0008% ($P < 0.05$), respectively. Increased TTO concentrations led to decreased concentrations

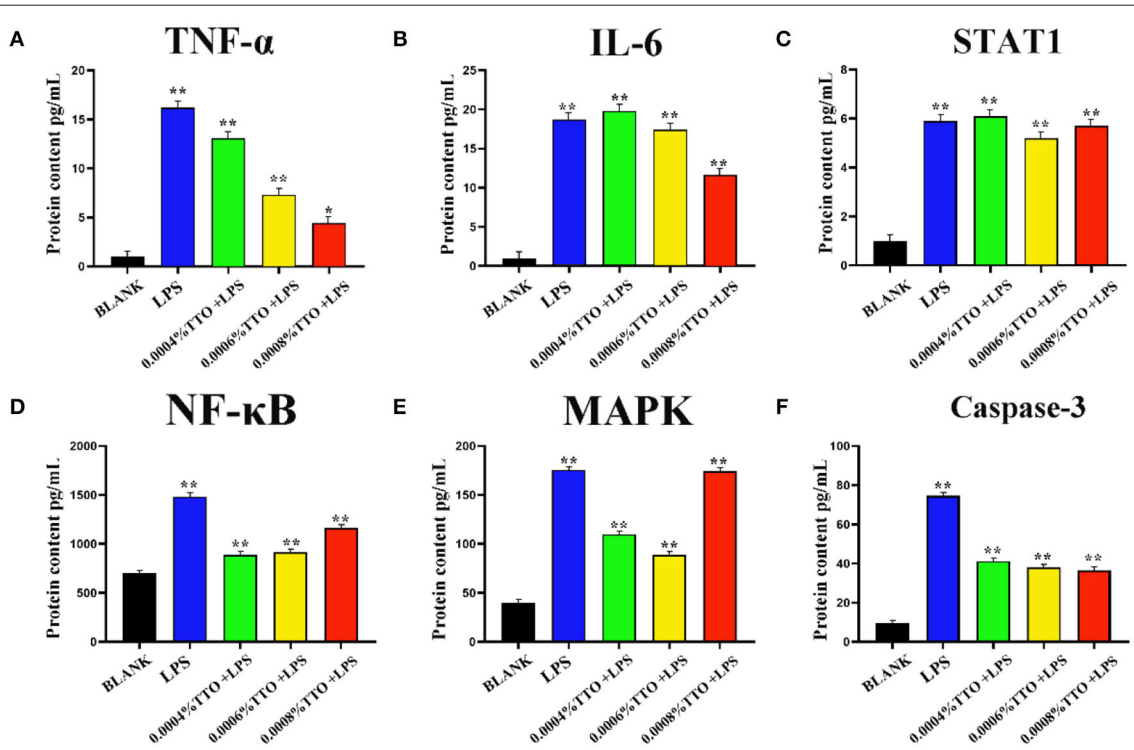

FIGURE 3 | Effect of different concentrations of TTO on protein concentration associated with inflammatory response in BEMC with LPS challenge. **(A)** Protein concentrations of TNF-α (pg/ml). **(B)** Protein concentrations of IL-6; **(C)** Protein concentrations of STAT1; **(D)** Protein concentrations of NF-κB; **(E)** Protein concentrations of MAPK4; **(F)** Protein concentrations of caspase-3 Data were presented as means ± s.d. of at least three independent experiments, $^{*}P < 0.05$, $^{**}P < 0.01$ using two tailed student *t*-test.

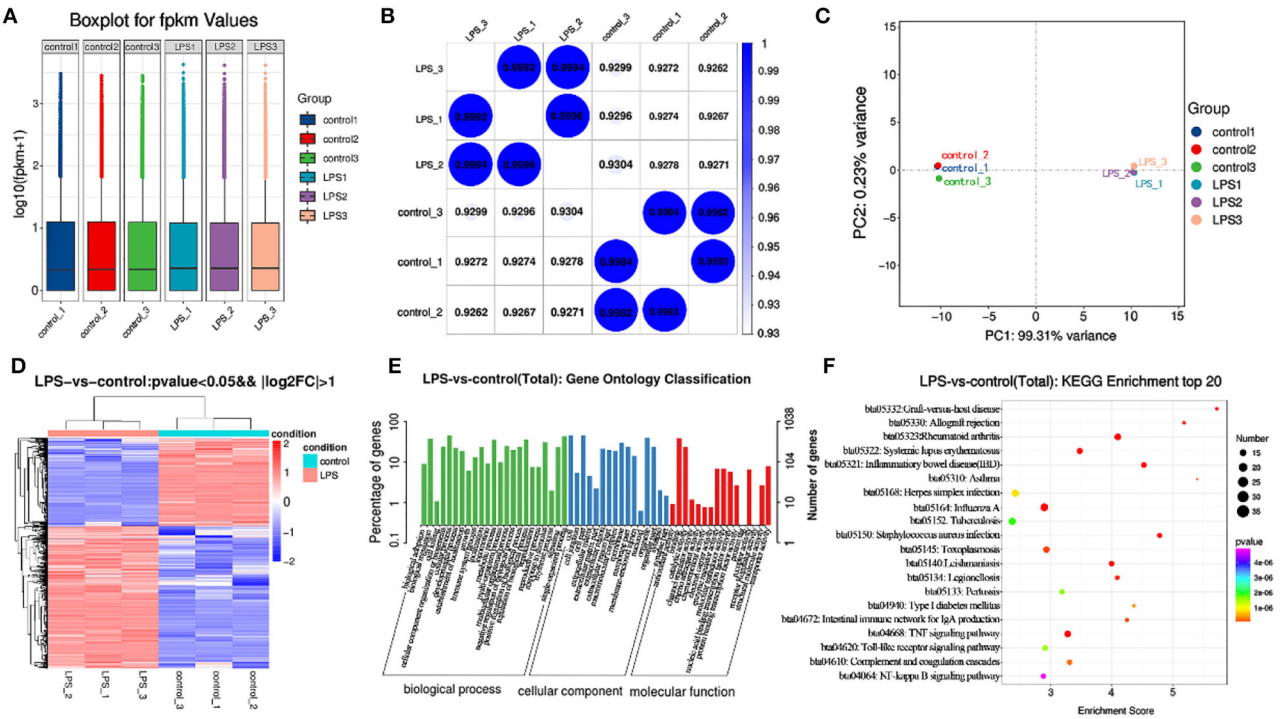

FIGURE 4 | Transcriptome analysis of control and LPS group. **(A)** Box line diagram in control and LPS group. The abscissa is the sample name and the ordinate is $\log^{10}$ (fpkm + 1). **(B)** Thermal diagram of the correlation coefficient between samples. The abscissa represents the name of the sample, and the ordinate represents the name of the corresponding sample. The color represents the size of the correlation coefficient. **(C)** PCA diagram in control and LPS group. **(D)** Screening for differentially expressed genes between control and LPS-induced BMEC. **(E)** GO enrichment. Horizontal axis is the GO entry name and the vertical axis is the $-\log^{10}$ *p*-value. **(F)** KEGG enrichment, top 20 genes.

of TNF-α ($P < 0.01$) and IL-6 ($P < 0.01$), with a more pronounced effect on TNF-α. Expression of STAT1 increased slightly upon addition of 0.0004% TTO ($P < 0.01$). Protein concentrations of TNF-α, IL-6 and STAT1 were significantly downregulated with 0.0006% ($P < 0.01$) and 0.0008% ($P < 0.01$) TTO supplementation (**Figures 3A–C**). After addition of 200 μg/mL LPS, the LPS group had a significant increase in protein concentrations of NF-κB ($P < 0.01$), MAPK4 ($P < 0.01$), and caspase-3 ($P < 0.01$) (**Figures 3D–F**). The protein expression levels of NF-κB ($P < 0.01$), MAPK4 ($P < 0.01$), and caspase-3 ($P < 0.01$) were significantly reduced in the groups treated with TTO.

Transcriptome Analysis

After building LPS induced mastitis model, we want to study its transcriptome level. Different genes were obtained by high-throughput sequencing analysis to provide data support for subsequent research. RNA-seq was used to sequence the LPS (200 μg/ml) induced model for 12 h. Considering the potential impact of the data error rate on the results, we used trimmatomatic software to preprocess the quality of the original data and to generate a statistical summary of the number of reads in the whole quality control process (**Table S1**). Fpkm is one of the most commonly-used methods to estimate expression level of protein-coding genes (**Table S2**). The degree of symmetry and dispersion also was deemed appropriate (**Figure 4A**, GEO databases: SRR11862300, SRR11862301, SRR11862299, SRR11862298, SRR11862297, SRR11862296).

The similarity of the LPS group was close to 1 (**Figure 4**), and that of the control was close to 1 (**Figure 4B**). Principal component analysis (PCA) indicated close concordance among samples in the LPS and control groups, underscoring the validity of the data generated (**Figure 4C**).

A total of 1270 mRNAs were identified as differentially expressed, of which 787 genes were upregulated and 483 downregulated. The differentially expressed genes included *TNF-α*, *IL6*, *STAT1,* and *MAPK4*. Among these genes, *TNF-α* and *IL6* were significantly upregulated. The difference multiples were 4.41 and 6.28 times, respectively (**Figure 4D**, **Table S3**).

The GO annotation results indicated that differentially expressed mRNAs participate in biological adhesion, biological regulation, cell killing, cellular component organization or biogenesis, cellular process, developmental process, growth, immune system process, negative regulation of biological process, positive regulation of biological process, and cell junction among others (**Figure 4E**).

Among the top 20 KEGG pathway entries, the differentially expressed mRNAs participate in TNF signaling, rheumatoid arthritis, inflammatory, *Staphylococcus aureus* infection, systemic lupus erythematosus, graft-vs-host disease, allograft

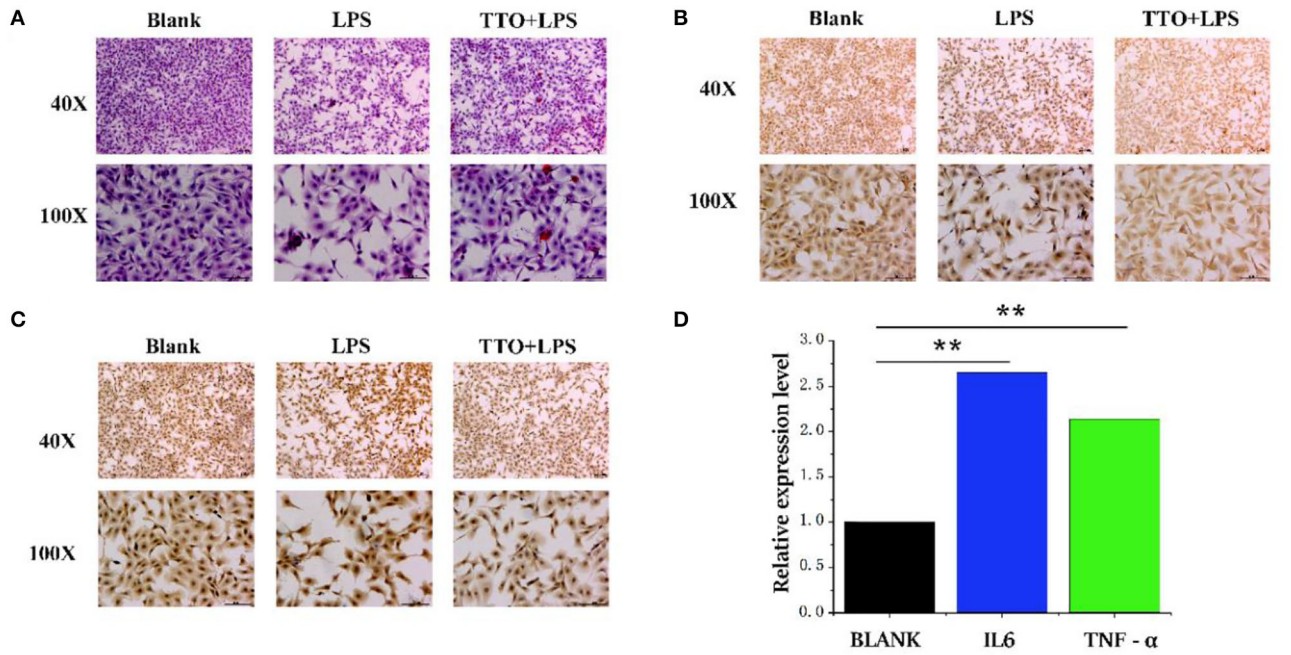

FIGURE 5 | Tea tree oil induced the expression changes of TNF-α and IL 6 in BMEC. **(A)** HE staining of BMEC in LPS (200 μg/ml) and TTO (0.008%) + LPS (200 μg/ml) of 12 h; **(B)** TNF-α immunohistochemical of BMEC in LPS (200 μg/ml) and TTO (0.008%) + LPS (200 μg/ml) of 12 h; **(C)** IL-6 immunohistochemical of BMEC in LPS (200 μg/ml) and TTO (0.008%) + LPS (200 μg/ml) of 12 h. **(D)** Sequencing results of TNF-α and IL 6 expression level. Black bar represents BLANK; blue bar represents IL6; green bar represents TNF-α. Data were presented as means ± s.d. of at least three independent experiments, $^{**}P < 0.01$ using two tailed student *t*-test.

rejection, intestinal immune network for IgA production, type I diabetes mellitus, herpes simplex infection, toll-like receptor signaling pathway, and NF-κB signaling pathway among others (**Figure 4F**).

Physiological Gene Function Evaluation

Compared with BLANK, cells treated with LPS showed a heighten degree of apoptosis. However, the TTO (0.008%) + LPS (200 μg/ml) group inhibited this state (**Figure 5A**). Immunohistochemical results showed that cells treated with LPS also had greater protein concentrations of TNF-α and IL6. The expression of TNF-α and IL6 increased significantly in the TTO + LPS group (**Figures 5B,C**). The expression of *TNF-α* ($P < 0.01$) and *IL-6* ($P < 0.01$) detected by RNA-seq was consistent with immunochemical results. In addition, sequencing results also coincided with immunohistochemical data (**Figure 5D**).

DISCUSSION

LPS, a macromolecular structural component on the outer membrane of gram-negative bacteria (14, 15), can trigger an immune response in mammalian cells leading to the release of pro-inflammatory factors. Previous research underscored that the whole process of mastitis can be simulated using an LPS-induced challenge of BMEC (15, 16). In the current study, the proliferation activity of BMEC was enhanced subsequent to LPS (50 μg/mL) challenge; however, it decreased when the concentration of LPS was >100 μg/mL, which is consistent with previous studies (17). Of particular interest was the improvement of immune system activity and increased proliferation activity of cells at the low concentration of LPS; whereas, a high concentration of LPS led to a serious inflammatory reaction followed by apoptosis. These responses suggested that there is a dose-effect of LPS on regulating BMEC homeostasis. Thus, available data support the idea that LPS might play a dual role in modulating proliferation and inflammatory response in BMEC.

Tea tree oil has significant inhibitory on *E. coli* and endotoxins (18). Gustafson et al. reported that TTO can promote autolysis of *E. coli* and induce a noticeable inhibitory effect on LPS-induced inflammation (19). Thus, we speculate that TTO might play a positive role in protection against cow mastitis. In the present study, flow cytometry results showed that the proportion of normal living BMEC stimulated by LPS increased after TTO supplementation at an appropriate concentration (<50 μ g/ml LPS). Similarly, the proportion of early apoptosis, late apoptosis and dead cells decreased. Additionally, the LPS-induced inflammation was supported by the release of pro-inflammatory cytokines. It is well-established that BMEC produce TNF-α, IL-6 and STAT1 during acute inflammation induced by LPS (20). TNF-α is a major cytokine during the early stages of infection, which in *E. coli* mastitis is closely related to endotoxin shock (21). IL-6 is a pleiotropic cytokine that mediates many immune and inflammatory reactions (22). Our results showed that TTO could attenuate the expression

of TNF-α and IL-6 induced by LPS, with a more pronounced suppression of TNF-α. STAT1 promotes apoptosis, inhibits cell growth and differentiation, and plays an important role in inhibiting the occurrence and development of tumors. Overall, our results suggest that supplementation of TTO might help alleviate inflammation at least partly due to downregulated pro-inflammatory cytokines caused by high concentrations of LPS.

Previous studies have shown that inflammatory cytokines are primarily produced by activation of the NF-κB and MAPK signaling pathways, while apoptosis-promoting factors are mainly produced by activation of the caspase-3 pathway (23, 24). To further explore the mechanism of TTO inhibition the production of inflammatory cytokines and pro-apoptotic factors, we measured protein concentrations of NF-κB, MAPK4 and caspase-3 in response to TTO. NF-κB, MAPK4, and caspase-3 were greater in LPS-infected BMEC and decreased significantly after addition of TTO, suggesting that an appropriate concentration of TTO inhibits the production of NF-κB, MAPK4, and caspase-3. Therefore, we speculate that TTO might alleviate inflammatory responses in BMEC via NF-κB, MAPK4, and caspase-3 signaling pathways. The previous study sequenced the transcriptome of BMEC infected by *Staphylococcus aureus*, *E. coli* and *Klebsiella pneumoniae* using the Solexa system, and GO analysis indicated that the differentially expressed genes in the infected and non-infected groups were enriched in cell metabolism, apoptosis and embryonic development (25). Additionally, cluster analysis of homologous proteins revealed that they participate in translation, ribosome biosynthesis and repair. Oxidative phosphorylation pathway, nod-like receptor pathway and apoptosis pathway were identified as three enriched pathways via KEGG analysis.

The acute clinical indicators caused by LPS are closely related to the enzyme activities and acute-phase proteins in milk from cows with mastitis caused by *E. coli*. LPS stimulation resulted in rapid immune response in BMEC with the most active cellular response detected at 4 h. The most active immune response pathway included the RIG-I-like receptor signaling pathway, nod like receptor signaling pathway and MAPK signaling pathway. Wang et al. sequenced the transcriptome of mammary gland infected with S56, S178, and S36 *Staphylococcus aureus* strains and screened 1720, 427, and 219 differentially expressed genes, respectively (26). GO and pathway analysis in this research showed that these genes are involved in the inflammatory response, metabolic transformation, cell proliferation and apoptosis signaling pathways. Our research showed that Interleukin1 α (IL-1α), TNF, homo sapiens ephrin-B1, IL-8, and early growth response 1 were upregulated. These data provided a reference for mastitis-related gene transcription, post-transcriptional regulation, and the host cell immune response to pathogens. Findings were consistent with the differentially expressed genes determined in this study. Overall, new genes uncovered in the present study might be potentially used as biomarkers for diagnosis and prevention of clinical mastitis in dairy cows. In addition, our preliminary identification of gene functions may help elucidate the molecular mechanism of LPS-induced mastitis at the gene network.

DATA AVAILABILITY STATEMENT

The datasets generated for this study can be found in GEO, Accession No.'s SRR11862300, SRR11862301, SRR11862299, SRR11862298, SRR11862297, SRR11862296.

ETHICS STATEMENT

The animal use protocol was approved by the Institutional Animal Care and Use Committee in the College of Animal Science and Technology, Yang Zhou University, Yang Zhou, China.

AUTHOR'S NOTE

This manuscript has been released as a pre-print at Research Square, https://www.researchsquare.com/article/rs-18655/v1 (ZC, YZ, JZ, et al.).

AUTHOR CONTRIBUTIONS

ZC and ZY conceived and designed the experiments. ZC, JZ, YZ, and LL performed the experiments. ZC, XW, YL, JL, DG, HX, and ZY analyzed the data. ZC, JL, YL, and DG wrote the paper. 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.2020.00496/full#supplementary-material

Table S1 | Screening for mRNAs with differential expression in the LPS-induced BMECs.

Table S2 | Pretreatment results of sequencing data quality.

Table S3 | Distribution statistics of fpkm value of genes.

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ORIGINAL RESEARCH
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Fine Grinding or Expanding as Pre-treatment for Pelleting in Processing Diets Varying in Dietary Rapeseed Expeller Proportions: Investigations on Performance, Visceral Organs, and Immunological Traits of Broilers

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Pelleted feed is associated with improved broiler performance but also with a higher incidence of proventricular dilatation and ascites. The present study aimed to investigate influences of expanded and pelleted (ExP) or finely ground and pelleted feeds (FgP) containing either 6% rapeseed expeller (RSE) or 12% RSE on these adverse effects by studying performance, visceral organ, and immunological traits in 36 broilers. ExP reduced daily feed intake compared to FgP when feeding a 6% RSE diet ($P < 0.05$) but did not affect the daily feed intake when feeding a 12% RSE diet, which was also reflected in the body weight gain. There were no significant differences in the size of proventriculus and gizzard between feeding groups but significant diet-by-technical feed treatment interactions in case of proventricular and gizzard weights and the proventricular length ($P < 0.05$). Proventriculi and gizzards were heavier in birds fed 6%ExP than proventriculi or gizzards of animals from all other groups except for birds of the group 12%FgP. A total of three animals (1 from 6%ExP, 1 from 6%FgP, and 1 from 12%ExP) developed ascites during the study. Pooled LsMeans of peripheral blood leucocyte proportions of $CD3^+/CD4^-/CD8^-$ cells were increased in birds fed FgP compared to birds fed ExP ($P = 0.048$). Pooled LsMeans of $CD3^+/CD4^+/CD8^+$ T cell subsets in jejunal *lamina propria* were higher in birds fed 12% RSE compared to birds fed 6% RSE ($P = 0.024$). Concluding, technical feed treatment or diet did not inhibit adverse effects of pelleting on gizzard and proventricular development. Morphometric alterations of proventriculus and gizzard might modify the local immune system of the distal digestive tract and promote the development of ascites; however, further studies are required to confirm this hypothesis since in the present study only three birds developed ascites.

Keywords: broiler performance, gastrointestinal tract, feed technology, ascites, T and B cells, rapeseed

INTRODUCTION

Pelleting is an important processing method that merges small particles to larger particles (1). Thus, this technique plays a key role for storage suitability and transportability of feeds, the inhibition of feed component separation, and avoidance of feed selection by the animals (2, 3). Further studies also give evidence that the compaction process has stimulating effects on daily feed intake and body weight (BW) gain in broilers (4, 5). In addition to the positive effects on broiler performance, negative effects of pellet feeding were reported in broiler studies such as proventricular dilatations and an inhibited development of the gizzard (4–6). In studies of Liermann et al. (5) it was indicated that these alterations also impact the down-stream digestive tract and the local immune system of broilers. Furthermore, feeding of pelleted feed is associated with a higher incidence of ascites (7, 8). Various pre-treatments of feed can contribute to a successful pelleting process (3, 9). Two common technical pre-treatments are fine grinding and expander treatment. Inter alia, they influence the pellet hardness and durability, which are important for pellet quality and texture (9, 10). Parsons et al. (11) showed that both the quality and texture of pellets influence broiler performance. Additionally, the pre-treatments allow processing of even difficult to pellet feed stuffs (2, 9). Moreover, hydrothermal processing methods such as expander treatment are hygienization-methods and able to degrade some anti-nutritive substances (10).

The current experiment studied whether the mentioned pre-treatments can possibly exacerbate or cancel nutritional disadvantages of pellet feeding in broiler fattening. Two different diet types were used that differed in their rapeseed expeller (RSE) content to study the possible interactions between technical feed treatment (TFT) and diet composition. RSE is an important protein source but also known to contain anti-nutritive substances such as glucosinolates (12). The current study also investigates the impacts of the aforementioned diet alterations on the proximal digestive tract and immunological traits in more detail to understand the impact of these modifications on the health of broilers.

MATERIALS AND METHODS

The current study was conducted in the experimental station of the Institute of Animal Nutrition, Friedrich-Loeffler-Institut, Federal Research Institute for Animal Health in Brunswick, Germany. The housing conditions and sample collection during the experimental period were in accordance with the German animal protection law guidelines and were approved by the Lower Saxony State Office for Consumer Protection and Food Safety, LAVES, Germany (registration number: 33.9-42502-04-082/09).

Abbreviations: ExP, expanded and pelleted; FgP, finely ground and pelleted; RSE, rapeseed expeller; TFT, technical feed treatment; PBL, peripheral blood leucocytes; RMPI-1640, Roswell Park Memorial Institute medium; BW, body weight; FGR, feed to gain ratio; H/L ratio, heterophilic granulocytes to lymphocyte ratio; LSMeans, least squares means.

TABLE 1 | Experimental design.

Feeding group	Percentage of rapeseed expeller [%]	Technical feed treatment
6%ExP ($n = 9$)	6	Expanded and pelleted
6%FgP ($n = 9$)	6	Finely ground and pelleted
12%ExP ($n = 9$)	12	Expanded and pelleted
12%FgP ($n = 9$)	12	Finely ground and pelleted

Experimental Design and Animals

Thirty-six male broilers (Ross 308) were allocated to four feeding groups (nine birds per group) immediately after hatching. Feeds differed both in composition and in processing as shown in **Table 1**. Briefly, two different diet types were formulated according to the requirements of the GfE (13), which contained either 6% RSE or 12% RSE (**Table 2**). After mixing of the complete feeds they were either finely ground or expanded. For fine grinding a hammer mill (Tietjen Verfahrenstechnik GmbH, Hemdingen, Germany) with a 3 mm screen size was used. Expander treatment was realized by an expander from the Amandus Kahl GmbH & Co.KG (Reinbek, Germany) at 130°C and 3% steam supply. Depending on throughput, the retention time in the expander was 5–8 s. Subsequently, all feeds were pelleted (pellet-die press, Salmatec, Gödenstorf, Germany). Finely ground feeds were pelleted at 75°C and expanded feeds at 87°C and 3% steam. The retention time in the pellet press depended on throughput and varied between 4 and 10 s. The steam supply during hydrothermal procedures was controlled by a DVHP flow measuring system (Heinrichs Messtechnik GmbH, Köln, Germany). Manufactured pellet products had a diameter of 3 mm.

Feeding Management and Husbandry

Until the 14th day after hatching birds were housed in floor pens and in groups on chopped straw. Thereafter, they were housed in individual balance cages without bedding to enable the determination of the individual daily feed intake. The floor pens and balance cages were integrated in climate controlled rooms. The illumination period lasted from 0000 h until 2300 h on the first 2 days after hatching and from day three on from 0400 h until 2000 h. Every second day the ambient temperature was decreased by 1°C from 36°C on day 1 after hatching to 22°C on day 28 after hatching. The different feeds were available *ad libitum* in dry form from the first day of life until slaughter. However, on the first 14 days after hatching the pellets were fed crumbled. Floor pens were equipped with bell drinkers (only the first 5 days after hatching) as well as nipple drinkers and balance cages with a water trough. Thus, birds had free access to water over the entire experimental period. On day 8 after hatching broilers were vaccinated against Newcastle disease virus.

Data and Sample Collection

From the 14th day after hatching broilers were weighed and the individual feed intake was recorded weekly. Furthermore, the daily water consumption was determined representatively from

TABLE 2 | Feed composition, feed particle size, and pellet abrasion.

Variable	6% rapeseed expeller		12% rapeseed expeller	
	ExP[1]	FgP[2]	ExP	FgP
Ingredients, g/kg feed				
Corn	240		240	
Wheat	364		339	
Wheat middlings	32		32	
Hi-pro soybean meal	237		202	
Soybean oil	14		11	
Rapeseed expeller	60		120	
Rapeseed oil	22		26	
Calcium carbonate	12		12	
Calcium-sodium-phosphate	4		3	
Mono-calcium-phosphate	3		3	
Premix[3]	12		12	
Calculated composition				
Metabolizable energy, MJ/kg feed	13		13	
Crude protein, g/kg feed	205		205	
Lysine, g/kg feed	11.8		11.8	
Methionine, g/kg feed	5.5		5.5	
Calcium, g/kg feed	8.5		8.5	
Phosphorus, g/kg feed	5.5		5.5	
Feed particle size before hydrothermal treatment				
> 710 μm, %	81.5	53.6	74.5	46.8
710–125 μm, %	17.7	45.9	24.5	52.9
< 125 μm, %	0.8	0.5	1.1	0.3
D50[4]	1.9	0.8	1.5	0.7
Pellet abrasion, %	6.0	3.9	4.4	4.4

[1] *Expanded and pelleted.*
[2] *Finely ground and pelleted.*
[3] *Delivers per kg feed: 12,000 IU vitamin A; 119 μg cholecalciferol; 60 mg Vitamin E; 60 mg iron; 11.3 mg copper; 67.5 mg zinc; 75 mg manganese; 1.5 mg iodine; 0.23 mg selenium; 1,220 U xylanase; 152 U glucanase; 750 U phytase.*
[4] *Cumulative particle size distribution at 50%.*

day 21 until day 25 after hatching. Therefore, the water troughs were filled with 800 ml of water in the morning. The reduction of water was measured 24 h after trough filling. To determine the evaporation loss a reference trough was positioned in the middle of the room.

On day 28/29 (18 birds per day) after hatching broilers were weighed and body temperatures were measured. Blood samples were collected for the determination of blood gases from the *Vena jugularis dexter* in a syringe containing 10 μl Heparin-Natrium Braun 25.000 I.E. solution (B. Braun Melsungen AG, Melsungen, Germany). Thereafter, birds were slaughtered by mechanical stunning and exsanguination. During exsanguination additional blood samples were collected from neck vessels in conventional EDTA and serum tubes. Heart, liver without bladder, spleen, bursa of *Fabricius* and pancreas were dissected and weighed. Furthermore, the proventriculus and the gizzard were separated, emptied, and weighed individually. Additionally, the length and width of both organs was measured by a caliper as well as the thickness of main muscles of gizzard as described by Liermann et al. (5). The jejunum was separated between the duodenum and the Meckel's diverticulum. The tissue was rinsed gently with PBS and stored in CUSTODIOL® (Dr. FRANZ KÖHLER Chemie GmbH, Bensheim, Germany) on ice. Moreover, caecal tonsils were dissected and stored in PBS on ice until further analyses.

Feed Analyses

Particle size distribution of the complete initial feeds and finely ground feeds was determined by dry sieve analyses according to DIN 66165-1:1987-04 and DIN 66165-2:1987-04 (14, 15) and by using a sieve tower corresponding to DIN ISO 3310/1 (16) before hydrothermal treatment.

The pellet abrasion was estimated by the standard method of the American Society of Agriculture Engineers (17).

Hematology, Oximetry, and Clinical Chemistry

For the determination of the hematocrit heparinized blood was collected in a micro-hematocrit tube and centrifuged at 12,000 × g by a micro-hematocrit centrifuge (Haematokrit 210; Hettich Lab Technology, Tuttlingen, Germany).

According to Pendl (18) blood smears of EDTA blood were prepared (two per bird), stained with Pappenheim solution, and counted. Leucocytes and thrombocytes were counted in 20 fields of vision (at 1,000 × magnification) and at least 200 leucocytes were differentiated according to their morphological characteristics.

Blood gas traits (pCO_2; pO_2; TCO_2; BE_{ecf}; HCO_3^-), blood pH, blood electrolytes (sodium, potassium, chloride, ionized calcium), and the metabolites glucose and lactate were determined by the analyzer GEM® Premier 4,000 (Instrumentation Laboratory, Munich, Germany) in heparinized blood. Further metabolites and liver enzymes were analyzed by an automatic clinical chemistry analyzer in blood serum (Eurolyser CCA180, Eurolab, Austria).

Isolation of Leucocytes From Different Localizations

The isolation methods of peripheral blood lymphocytes (PBL) and leucocytes from *lamina propria* and caecal tonsils were described in a previous study of Liermann et al. (5) and are briefly summarized in the following subsections.

Isolation of PBL

A total of 1 ml EDTA blood was diluted with PBS (1 ml), covered with 3 ml Biocoll separation solution (Biochrom AG, Berlin, Germany), and centrifuged at 681 × g for 12 min, without break, at room temperature. Cells were isolated from the interphase, resuspended in PBS, and centrifuged once again at 235 × g for 10 min without break at 4°C. The supernatant was discarded. The received pellet was resuspended in 500–1,000 μl HEPES buffered saline and stored on ice until further analyses.

Isolation of Leucocytes From Caecal Tonsils

Caecal tonsils were rinsed and surrounding fat tissues were cut. The rinsed caecal tonsils were transferred into a petri dish containing 5 ml PBS and cut lengthwise. Cells were released by gentle scraping using a scalpel. The received cell suspension was sieved using CellTrics® (mash size 50 μm) (Partec GmbH, Görlitz, Germany) and washed by HEPES buffered saline and centrifuged at 250 × g for 5 min at 4°C. The pellet was resuspended in 500–1,000 μl HEPES buffered saline and stored on ice until further analyses steps.

Isolation of Leucocytes From Jejunal *Lamina propria*

Firstly, the surrounding connective tissue was removed as completely as possible from the jejunum. Thereafter, the jejunum was stored on ice for 20 min in a beaker containing PBS. After the resting period the jejunum was opened and cut into small pieces, which were transferred into Erlenmeyer flasks. A total of 50 ml HEPES buffered, Hanks balanced salt solution (without Ca^{2+} and Mg^{2+}; Biochrom GmbH, Berlin, Germany) and a magnetic stir bare were added to the flasks. The samples were stirred at room temperature and after 10 min the HEPES buffered, Hanks balanced salt solution was renewed. The washing steps were repeated twice. In the meantime, 25 ml Roswell Park Memorial Institute medium (RPMI-1640; Biochrom GmbH, Berlin, Germany) was heated to 37°C. A total of 180 U collagenase type V (Sigma-Aldrich, Chemie GmbH, Munich, Germany) was added to the RPMI-1640. The resulting solution was transferred into the Erlenmeyer flasks containing the jejunal tissues after the last washing step. After 10 min incubation time the fluid fraction of the samples was collected in 50 ml tubes and the RPMI-1640-collagenase-mix was renewed. This step was repeated twice. Thereafter, samples were incubated with heated RPMI-1640 containing 260 U collagenase. After 15 min the resulting supernatant was also collected. Each collected cell suspension was stored on ice during the remaining incubation periods. Thereafter, both 50 ml tubes containing the cell suspensions were centrifuged at 191 × g for 10 min at room temperature. The pellet was resuspended in 25 ml RPMI-1640. The samples of the individual incubation steps were combined and centrifuged once again. A total of 25 ml of a 30% Percoll gradient (Sigma-Aldrich Chemie GmbH, Munich, Germany) was added to the resulting pellet. The samples were mixed carefully and centrifuged at 350 × g for 15 min at room temperature. The pellet was resuspended in 5 ml RPMI-1640 and the solution was sieved by Cell Trics®. Until further analyses the cell suspension was stored on ice.

T and B Cell Phenotyping

For T and B cell phenotyping, cells isolated from blood, *lamina propria,* and caecal tonsils were stained with antibodies for CD3 (T cells), CD4 (T helper cells), and CD8 (T cytotoxic cells) (mouse anti-chicken CD3: PE; mouse anti-chicken CD4: FITC; mouse anti-chicken CD8: Cy5) according to methods described by Liermann et al. (5). Furthermore, additional samples of PBL's and cells isolated from *lamina propria* were incubated with antibodies for Bu1 (B cells) (mouse anti-chicken Bu-1: FITC; Southern Biotech; Birmingham, USA). For corresponding isotype controls either mouse IgG1 negative control: PE; mouse IgG1 negative control: FITC, or mouse IgG1 negative control: Cy5 (Southern Biotech; Birmingham, USA) were used. T and B cell subsets were measured by FACS Canto II (BD Bioscience, San Jose, USA). Therefore, at least 10,000 cells were considered. The evaluation of results and the compensation of non-specific signals indicated by the isotype controls were conducted by using the BD FACSDiva™ Software (BD Biosciences, San Jose, USA).

Calculations and Statistical Analyses

Parameters of broiler performance and water consumption were calculated as follows:

Daily feed intake [g] = feed consumption [g]/duration of feeding period [d]

BW gain [g/d] = (final BW [g]–initial BW [g])/duration of feeding period [d]

Feed to gain ratio (FGR) [g/g] = daily feed intake [g]/BW gain [g]

Water consumption [mL/kg BW] = ((initial water volume of trough [mL]–final water volume of trough after 24 h [mL])–(initial water volume of reference-trough [mL]–final water volume of reference-trough after 24 h [mL]))/BW [kg]

Water consumption [mL/g feed intake] = ((initial water volume of trough [mL]–final water volume of trough after 24 h [mL])–(initial water volume of reference-trough [mL]–final water volume of reference-trough after 24 h [mL]))/daily feed intake [g]

Parameters of performance and water consumption of the overserved period were averaged for the observed period.

According to Pendl (18) total counts of leucocytes and thrombocytes were calculated by summation of cells counted in 20 fields of vision of a blood smear and multiplying by the factor 875. Because all measured hematocrit values were lower than 35%, leucocyte and thrombocyte counts were corrected according to the corresponding hematocrit of the bird (18).

The H/L ratio was defined as the quotient between counted heterophilic granulocytes and lymphocytes of a blood smear.

The anion gap was calculated according to the equation of Haßdenteufel and Schneider (19): Anion gap = $(Na^+ + K^+)$–$(Cl^- + HCO_3^-)$.

Statistical analyses were conducted by using the MIXED procedure of SAS Enterprise Guide 6.1. The created model included the fixed effects "diet" (RSE content) and "TFT" as well as their interactions. Least squares means (LsMeans) and standard errors were estimated for each fixed factor. The differences between LsMeans were subsequently verified by the Tukey–Kramer test. At $P < 0.05$ differences were assessed as significant. Correlation coefficients according to Pearson were estimated using the SAS Enterprise Guide 6.1 and assessed as significant at a $P < 0.05$. Principal component analyses were conducted by JMP 13.1.0 to visualize relationships between variables and cases in a two-dimensional space based on correlations. The fixed factors were included as additional variables.

TABLE 3 | Performance and water consumption (LsMeans, $n = 9$).

Variable	6% rapeseed expeller		12% rapeseed expeller		SE	*P*-value		
	ExP[1]	FgP[2]	ExP	FgP		Diet	TFT[3]	Diet x TFT
ADFI[4], g	75[b]	99[a]	84[ab]	86[ab]	4.1	0.650	0.003	0.010
BWG[5], g/d	49[b]	70[a]	60[ab]	57[b]	3.1	0.672	0.006	0.001
FGR[6], g/g	1.53	1.42	1.43	1.53	0.03	0.974	0.941	0.001
Water consumption, mL/kg BW[7]/d	205[b]	241[ab]	300[a]	251[ab]	24	0.037	0.782	0.094
Water consumption, mL/g feed intake/d	1.55[b]	1.86[ab]	2.35[a]	1.87[ab]	0.18	0.033	0.629	0.035

[1] *Expanded and pelleted.*
[2] *Finely ground and pelleted.*
[3] *Technical feed treatment.*
[4] *ADFI, average daily feed intake.*
[5] *Body weight gain.*
[6] *Feed to gain ratio.*
[7] *Body weight.*
[a,b] *Different superscripts within a row mark significant differences between feeding groups ($P < 0.05$).*

RESULTS

There were no premature animal losses during the experimental trial. The body temperature ranged between 41.0°C and 41.8°C and did not differ between feeding groups ($P > 0.05$) (data not shown). During slaughtering it was observed that three animals developed ascites. These animals belonged to the groups 6%ExP, 6%FgP, and 12%ExP.

Performance and Water Consumption of Broilers

TFT significantly affected daily feed intake and BW gain ($P < 0.01$) (**Table 3**). Moreover, a significant diet-by-TFT interaction was detected in case of daily feed intake, BW gain, and FGR ($P < 0.05$). The daily feed intake and the BW gain were significantly lower in the 6%ExP group compared to the 6%FgP group ($P < 0.01$). Additionally, the BW gain was significantly reduced by feeding finely ground and pelleted feed containing 12% RSE compared to finely ground and pelleted feed containing 6% RSE ($P = 0.025$). There were no significant differences between the FGR of different feeding groups but 6%ExP and 12%FgP tended to higher FGR compared to 6%FgP and 12%ExP ($P < 0.1$).

The water consumption per kg BW and per g feed intake was mainly affected by the diet composition ($P < 0.05$). However, there was also a significant interaction between both fixed effects considering the water consumption per g feed ($P = 0.035$). The water consumption related to BW or feed intake was significantly increased in animals fed expanded and pelleted feed containing 12% RSE compared to animals fed expanded and pelleted feed containing a lower amount of RSE ($P < 0.05$).

BW on Slaughtering Day, Visceral Organ Weights, and Morphometric Characteristics of Digestive Organs

On slaughtering day the BW of broilers fed finely ground and pelleted feed containing 6% RSE was significantly increased compared to broilers fed the other feeds ($P < 0.05$) (**Table 4**). Furthermore, the BW of birds fed 12%ExP was significantly higher compared to birds fed 6%ExP on slaughtering day ($P = 0.043$).

In general, the pooled LsMeans of heart weights were significantly higher in broilers fed expanded and pelleted feed compared to broilers fed finely ground and pelleted feed ($P = 0.002$). Moreover, heart weights were significantly increased in broilers fed expanded and pelleted feed containing 6% RSE compared to heart weights of broilers fed finely ground and pelleted feed containing 12% RSE ($P = 0.017$). There was a significant negative correlation between the daily feed intake and the relative heart weight ($P = 0.024$) (**Table 5**).

Livers of broilers fed expanded and pelleted feed containing 12% RSE were significantly heavier compared to livers of broilers belonging to the feeding groups 6%ExP and 12%FgP ($P < 0.05$) and tended to be heavier compared to the livers of animals from the feeding group 6%FgP ($P = 0.063$).

The weight of bursa of *Fabricius* was significantly reduced after feeding expanded and pelleted feed containing 12% RSE compared to the weights of bursa of *Fabricius* of birds fed expanded and pelleted feed containing smaller amounts of RSE ($P = 0.032$).

The heaviest gizzards were found in birds fed expanded and pelleted feed containing 6% RSE, which were significantly heavier compared to gizzards of birds of the feeding groups 6%FgP and 12%ExP ($P < 0.05$). The lowest gizzard weights were measured in animals fed finely ground and pelleted feed containing 6% RSE. These values differed significantly from gizzard weights of birds fed other feeds ($P < 0.05$) except for gizzard weights of birds belonging to the feeding group 12%ExP. Additionally, significantly higher proventricular weights were measured in broilers of the feeding groups 6%ExP and 12%FgP compared to broilers of the two other feeding groups ($P < 0.05$). Considering the pooled LsMeans of the gizzard/proventriculus ratio, a higher ratio was shown in birds fed expanded and pelleted feed compared to birds fed finely ground and pelleted feed ($P = 0.018$). Furthermore, the pooled LsMeans of proventricular length and width related to g organ weight tended to be higher in animals fed expanded and pelleted feed compared to their counterparts ($P < 0.1$).

TABLE 4 | Bird final body weight, organ weights and morphometric characteristics of proventriculus and gizzard (LsMeans, $n = 9$).

Variable	6% rapeseed expeller		12% rapeseed expeller		SE	*P*-value		
	ExP[1]	FgP[2]	ExP	FgP		Diet	TFT[3]	Diet x TFT
Body weight (BW), g	994[c]	1,364[a]	1,176[b]	1,123[bc]	4.6	0.531	0.002	< 0.001
OW[4], g/kg BW								
Heart	6.69[a]	6.05[ab]	6.49[ab]	5.80[b]	0.20	0.266	0.002	0.907
Liver	22.7[b]	23.4[ab]	27.8[a]	23.2[b]	1.2	0.049	0.112	0.035
Spleen	0.93	0.77	0.73	0.91	0.06	0.651	0.878	0.012
Bursa of *Fabricius*	3.08[a]	2.43[ab]	2.26[b]	2.57[ab]	0.20	0.100	0.411	0.022
Pancreas	2.71	2.48	2.45	3.00	0.15	0.402	0.280	0.015
Gizzard	15.1[a]	10.7[c]	12.5[bc]	13.4[ab]	0.6	0.903	0.008	< 0.001
Proventriculus	4.89[a]	3.95[b]	3.92[b]	4.70[a]	0.16	0.508	0.643	< 0.001
Gizzard/proventriculus, g/g	3.09	2.71	3.20	2.90	0.14	0.267	0.018	0.772
Section extent								
Proventricular length, cm	3.72	4.06	3.79	3.71	0.09	0.137	0.149	0.029
Proventricular length, cm/g OW	0.79	0.76	0.85	0.71	0.04	0.899	0.056	0.247
Proventricular width, cm	1.66	1.73	1.64	1.73	0.05	0.839	0.134	0.855
Proventricular width, cm/g OW	0.35	0.33	0.36	0.33	0.01	0.373	0.068	0.758
Gizzard length, cm	4.69	4.78	4.73	4.83	0.16	0.768	0.544	0.992
Gizzard length, cm/g OW	0.32	0.33	0.34	0.32	0.02	0.945	0.987	0.437
Gizzard width, cm	2.98	3.14	3.13	3.14	0.08	0.381	0.341	0.395
Gizzard width, cm/g OW	0.21	0.22	0.22	0.21	0.01	0.797	0.951	0.317
Main gizzard muscles[5], cm	2.25	2.21	2.21	2.05	0.12	0.437	0.426	0.635
Main gizzard muscles, cm/g OW	0.16	0.16	0.16	0.14	0.01	0.493	0.371	0.485

[1] *Expanded and pelleted.*
[2] *Finely ground and pelleted.*
[3] *Technical feed treatment.*
[4] *Organ weight.*
[5] *Main gizzard muscles = thickness of the musculus crassus cranioventralis (cm) + thickness of musculus crassus caudodorsalis (cm).*
[a–c] *Different superscripts within a row mark significant differences between feeding groups ($P < 0.05$).*

There were significant correlations between the daily feed intake and the gizzard weight as well as the length and the width of this organ (only width in cm per g organ weight) ($P < 0.05$). Moreover, the daily feed intake correlated significantly with the thickness of main gizzard muscles ($P = 0.011$). Additionally, there were significant correlations between the daily feed intake and the proventricular length and width ($P < 0.05$).

Hematological Traits

The pooled LsMeans of the hematocrit of birds fed expanded and pelleted feed were significantly higher compared the pooled LsMeans of birds fed finely ground and pelleted feed ($P = 0.007$) (**Table 6**).

The proportions of monocytes were significantly higher in animals fed finely ground and pelleted feed containing 12% RSE compared to proportions in animals of the feeding groups 6%FgP and 12%ExP ($P < 0.05$). Proportions of other leucocytes were not influenced by the fixed factors ($P > 0.05$).

Blood Gases and Electrolytes

The pooled LsMeans of the pO_2 tended to be lower in broilers fed 12% RSE compared to broilers fed a diet containing lower amounts RSE ($P = 0.055$) (**Table 7**). Furthermore, the pooled LsMeans of the pO_2 tended to be lower in birds fed expanded and pelleted feed compared to birds fed finely ground and pelleted feed ($P = 0.067$). The pooled LsMeans of TCO_2 and HCO_3^- levels tended to be higher in birds fed feeds containing 12% RSE than TCO_2 and HCO_3^- levels of birds fed lower amounts of RSE ($P < 0.1$).

The blood gas parameters TCO_2, HCO_3^-, and pCO_2 were negatively correlated with daily feed intake and the BW gain ($P < 0.05$) (**Table 5**). Furthermore, the length and the width (both in cm per g organ weight) of proventriculus correlated significantly with the blood gas parameters, pCO_2, TCO_2, and HCO_3^- ($r > 0.40$; $P < 0.05$).

The calcium concentration in blood was significantly affected by both fixed effects ($P < 0.05$). Concretely, the highest calcium concentrations were detected in broilers fed expanded and pelleted feed containing 12% RSE, which were significantly higher compared to calcium concentrations of the broilers from other feeding groups ($P < 0.05$).

Blood Metabolites and Liver Enzymes

The pooled LsMeans of cholesterol concentrations of broilers fed 12% RSE were significantly higher compared to the LsMeans of broilers fed lower amounts of RSE ($P = 0.050$) (**Table 8**). Furthermore, the pooled LsMeans of albumin concentrations

TABLE 5 | Significant correlations with performance parameters.

Performance parameters	Correlated variables	Correlations coefficient (*r*)	*P*-value
ADFI[1]	Heart weight, g/kg BW[3]	−0.377	0.024
	Gizzard weight, g/kg BW	−0.357	0.032
	Gizzard length, cm	0.454	0.005
	Gizzard length, cm/g OW[4]	−0.389	0.019
	Gizzard width, cm/g OW	−0.488	0.003
	Thickness of main muscles, cm/g OW	−0.421	0.012
	Proventricular length, cm	0.515	0.001
	Proventricular length, cm/g OW	−0.597	< 0.001
	Proventricular width, cm	0.493	0.002
	Proventricular width, cm/g OW	−0.665	< 0.001
	pCO_2[5]	−0.539	0.001
	TCO_2[6]	−0.497	0.002
	HCO_3^-[7]	−0.444	0.007
	Uric acid	−0.472	0.004
	$CD3^+/CD4^+/CD8^+$ subsets of CT[8]	0.393	0.018
BWG[2]	Gizzard weight, g/kg BW	−0.412	0.012
	Gizzard length, cm	0.386	0.020
	Gizzard length, cm/g OW	−0.380	0.022
	Gizzard width, cm/g OW	−0.451	0.006
	Thickness of main muscles, cm/g OW	−0.380	0.022
	Proventricular length, cm	0.500	0.002
	Proventricular length, cm/g OW	−0.576	< 0.001
	Proventricular width, cm	0.506	0.002
	Proventricular width, cm/g OW	−0.614	< 0.001
	pCO_2	−0.504	0.002
	TCO_2	−0.472	0.004
	HCO_3^-	−0.422	0.010
	Uric acid	−0.409	0.013
	$CD3^+/CD4^+/CD8^+$ subsets of CT	0.349	0.037

[1]*Average daily feed intake.*
[2]*Body weight gain.*
[3]*Body weight.*
[4]*Organ weight.*
[5]*Carbon dioxide partial pressure.*
[6]*Total carbon dioxide.*
[7]*Bicarbonate.*
[8]*Caecal tonsils.*

and aspartate-amino-transferase activity tended to be higher in broilers fed 12% RSE compared to LsMeans of animals fed lower amounts of RSE ($P < 0.1$).

The blood uric acid concentration in broilers fed expanded and pelleted feed containing 6% RSE was significantly higher compared to the concentration of broilers fed finely ground and pelleted feed containing similar amounts of RSE ($P = 0.041$). The uric acid concentration was significantly negatively correlated with the daily feed intake ($P = 0.004$) (**Table 5**) and significantly positively correlated with the hematocrit ($r = 0.516$; $P = 0.001$).

Subsets of T and B Cells

The pooled LsMeans of proportions of $CD3^+/CD4^-/CD8^-$ stained PBLs in birds fed expanded and pelleted feed were significantly lower compared to pooled LsMeans in birds fed finely ground and pelleted feed ($P = 0.048$) (**Table 9**). Moreover, the pooled LsMeans of proportions of $CD3^+/CD4^+/CD8^+$ stained PBLs in birds fed expanded and pelleted feed tended to be lower compared to pooled LsMeans in birds fed finely ground and pelleted feed ($P = 0.090$). The pooled LsMeans of $CD3^+/CD4^+/CD8^-$ subsets of PBLs tended to be lower in birds fed 12% RSE compared to the pooled LsMeans of birds fed lower amounts of RSE ($P = 0.070$).

The pooled LsMeans of $CD3^+/CD4^+/CD8^+$ subsets in the *lamina propria* were significantly higher in broilers fed feeds containing 12% RSE compared to the pooled LsMeans of broilers fed feeds containing lower amounts of RSE ($P = 0.024$). Furthermore, considering the pooled LsMeans the $CD3^+/CD4^-/CD8^-$ subsets tended to be less frequent in the *lamina propria* of broilers fed 12% RSE compared broilers fed 6% RSE ($P = 0.074$).

There were no significant effects of diet or TFT on Bu^+ subsets in blood and *lamina propria* ($P > 0.05$) (data not shown). No significant influences of fixed effects on the T cell subsets were detected in caecal tonsils ($P > 0.05$).

There was a significant correlation between the proventricular weight and the $CD3^+/CD4^+/CD8^+$ subsets in caecal tonsils ($r = -0.404$; $P = 0.014$) as well as between the gizzard length (cm) and the $CD3^+/CD4^+/CD8^-$ T cell subsets in *lamina propria* ($r = -0.374$; $P = 0.025$). Additionally, $CD3^+/CD4^+/CD8^+$ subsets correlated significantly with the daily feed intake ($P = 0.017$) (**Table 5**).

Principal Component Analysis

A principal component analysis was performed to visualize possible relationships between 38 variables belonging to broiler performance, organ traits, blood gases, blood electrolytes, and blood metabolites (**Figure 1**). The analysis revealed the first two principal components, which extracted ∼ 42.6% of the total variance. According to a scree plot and the eigenvalue consecutive principal components were assessed. The mean eigenvalue of 1.0 of all components corresponded to a total of nine extracted components, which explained cumulatively 86.6% of the total variance between the 38 variables.

Several variables appeared to correlate strongly to the principal component 1 and 2 indicated by their close localization to the outer circle of the graph (**Figure 1**). Only variables such as FGR, gizzard weight, gizzard weight/proventricular weight ratio, gizzard main muscle thickness, and the pO_2 value showed a closer localization to the center of the cross indicating low correlations to the considered principal component. Also the additional variables diet and TFT were closely located to the center of the cross rather than to the outer circle.

The performance parameters, daily feed intake, BW gain, and BW on slaughtering day clustered with each other and appeared to correlate highly negatively with both principal components. In contrast, blood gas parameters TCO_2 and HCO_3^- and the organ traits, proventricular and gizzard width (cm/g organ weight), as well as proventricular and gizzard length (cm/g organ weight) showed an opposite direction to the mentioned performance parameters.

TABLE 6 | Hematological traits assessed for broilers in the different feeding groups (LsMeans; $n = 9$).

Variable	6% rapeseed expeller		12% rapeseed expeller		SE	*P*-value		
	ExP[1]	FgP[2]	ExP	FgP		Diet	TFT[3]	Diet x TFT
Hematocrit, %	30.8	25.9	30.3	27.5	1.3	0.704	0.007	0.445
Leucocytes, cells/μL	23,891	26,115	20,733	19,171	3,829	0.196	0.932	0.624
Thrombocytes, cells/μL	13,878	6,264	8,393	7,104	2,642	0.386	0.102	0.240
Lymphocytes, %	47.7	54.5	54.4	53.4	3.6	0.448	0.426	0.287
Heterophilic granulocytes, %	45.2	39.8	39.8	40.6	3.1	0.473	0.468	0.324
H/L ratio[4]	1.21	1.49	1.51	1.48	0.2	0.507	0.588	0.494
Eosinophilic granulocytes, %	3.72	2.44	2.83	2.22	0.57	0.339	0.108	0.564
Basophilic granulocytes, %	3.17	2.78	2.86	2.61	0.48	0.625	0.509	0.885
Monocytes, %	0.64^{ab}	0.42^{b}	0.25^{b}	0.97^{a}	0.13	0.515	0.057	0.001

[1] *Expanded and pelleted.*
[2] *Finely ground and pelleted.*
[3] *Technical feed treatment.*
[4] *Heterophilic granulocyte/lymphocyte ratio.*
[a–b] *Different superscripts within a row mark significant differences between feeding groups ($P < 0.05$).*

TABLE 7 | Blood gas traits and electrolytes assessed for broilers in the different feeding groups (LsMeans, $n = 9$).

Variable	6% rapeseed expeller		12% rapeseed expeller		SE	*P*-value		
	ExP[1]	FgP[2]	ExP	FgP		Diet	TFT[3]	Diet x TFT
Blood gas traits[4]								
pH	7.20	7.22	7.23	7.21	0.02	0.722	0.968	0.408
pCO_2, mmHg[5]	68.9	63.9	68.3	68.6	3.8	0.596	0.538	0.491
pO_2, mmHg[6]	54.6	58.4	40.6	54.1	4.6	0.055	0.067	0.302
TCO_2, mmol/L[7]	26.9	26.4	28.1	27.6	0.6	0.071	0.407	0.997
BE_{ecf}, mmol/L[8]	−1.86	−1.99	−0.26	−1.10	0.75	0.109	0.523	0.643
HCO_3^-, mmol/L[9]	25.2	24.8	26.4	25.8	0.6	0.066	0.422	0.923
Electrolytes, mmol/L								
Na^+	147.8	146.9	146.7	148.1	0.5	0.960	0.650	0.036
K^+	4.86	4.58	4.39	4.77	0.24	0.562	0.834	0.176
Na^+/K^+ ratio	31.4	32.4	34.6	31.5	1.65	0.485	0.518	0.227
Cl^-	109.8	109.3	108.8	109.4	0.8	0.568	0.919	0.481
Ca^{2+}	1.49^{b}	1.44^{b}	1.61^{a}	1.48^{b}	0.03	0.011	0.004	0.159
Anion gap	17.7	17.4	15.9	17.6	0.90	0.398	0.443	0.278

[1] *Expanded and pelleted.*
[2] *Finely ground and pelleted.*
[3] *Technical feed treatment.*
[4] *Blood gases represented as body temperature-corrected values.*
[5] *Carbon dioxide partial pressure.*
[6] *Oxygen partial pressure.*
[7] *Total carbon dioxide.*
[8] *Base concentration in extracellular fluid.*
[9] *Bicarbonate.*
[a,b] *Different superscripts within a row mark significant differences between feeding groups ($P < 0.05$).*

DISCUSSION

The present study investigated the interactive effects between dietary rapeseed proportion and technical treatments of diets, which included expanding and pelleting or fine grinding prior to pelleting on broiler performance, morphometric traits of the proventriculus, the gizzard and other selected visceral organs, as well as immunological traits in broilers.

Broilers fed expanded and pelleted feed showed reduced daily feed intake compared to broilers fed finely ground and pelleted feed when feeding a 6% RSE diet. However, there was no difference between the daily feed intake of birds fed differently processed feeds containing higher amounts of RSE. Perhaps,

TABLE 8 | Blood metabolite concentrations and liver enzyme activities assessed for broilers in the different feeding groups (LsMeans, $n = 9$).

Variable	6% rapeseed expeller		12% rapeseed expeller		SE	*P*-value		
	ExP[1]	FgP[2]	ExP	FgP		Diet	TFT[3]	Diet x TFT
Albumin, g/L	13.5	13.1	14.5	13.8	0.4	0.057	0.223	0.669
Total protein, g/L	22.2	19.3	22.5	22.3	1.0	0.105	0.128	0.169
Glucose, mg/dL	234.1	227.0	228.0	228.3	6.7	0.726	0.619	0.585
Lactate, mmol/L	7.63	7.10	6.80	7.84	0.66	0.950	0.698	0.242
Cholesterol, mg/dL	131.4	129.4	150.3	133.8	5.7	0.050	0.116	0.211
Aspertate-amino-transferase, IU/L	154.3	145.7	170.4	159.2	7.4	0.055	0.193	0.858
γ-Glutamyltransferase, IU, L	20.9	20.7	21.8	21.0	0.9	0.526	0.607	0.698
Triglycerides, mg/dL	76.0	65.7	78.1	80.5	6.7	0.217	0.561	0.354
Urea, mg/dL	6.69	4.92	5.95	11.76	2.70	0.266	0.459	0.170
Uric acid, mg/dL	5.68[a]	4.37[b]	5.17[ab]	5.59[ab]	0.33	0.296	0.188	0.014
β-Hydroxybutyrate, mmol/L	0.63	0.63	0.62	0.71	0.06	0.552	0.447	0.478
NEFA[4], mmol/L	0.70	0.70	0.78	0.65	0.05	0.827	0.258	0.201

[1] *Expanded and pelleted.*
[2] *Finely ground and pelleted.*
[3] *TFT = technical feed treatment.*
[4] *Non-esterified fatty acids.*
[a,b] *Different superscripts mark significant differences between feeding groups ($P < 0.05$).*

TABLE 9 | T cell subsets in blood, *lamina propria,* and caecal tonsils (LsMeans ± SE, n =9).

Variable	6% rapeseed expeller		12% rapeseed expeller		*P*-value		
	ExP[1]	FgP[2]	ExP[4]	FgP	Diet	TFT[3]	Diet x TFT
Blood							
$CD3^+/CD4^+/CD8^-$, %	57.6 ± 2.2	52.8 ± 2.2	50.9 ± 2.3	51.3 ± 2.2	0.070	0.336	0.249
$CD3^+/CD4^-/CD8^+$, %	26.7 ± 2.2	29.3 ± 2.2	33.2 ± 2.4	27.8 ± 2.2	0.282	0.552	0.085
$CD3^+/CD4^+/CD8^+$, %	1.7 ± 0.5	3.3 ± 0.5	2.8 ± 0.6	3.1 ± 0.5	0.443	0.090	0.286
$CD3^+/CD4^-/CD8^-$, %	14.0 ± 1.2	14.5 ± 1.2	13.3 ± 1.3	17.8 ± 1.2	0.311	0.048	0.115
Lamina propria							
$CD3^+/CD4^+/CD8^-$, %	21.2 ± 2.9	16.0 ± 2.9	15.2 ± 2.9	13.8 ± 2.9	0.166	0.263	0.508
$CD3^+/CD4^-/CD8^+$, %	34.5 ± 4.4	40.6 ± 4.4	36.5 ± 4.4	42.4 ± 4.4	0.671	0.185	0.983
$CD3^+/CD4^+/CD8^+$, %	9.0 ± 3.3	9.1 ± 3.3	18.4 ± 3.3	15.3 ± 3.3	0.024	0.657	0.633
$CD3^+/CD4^-/CD8^-$, %	35.3 ± 3.0	34.3 ± 3.0	29.9 ± 3.0	28.5 ± 3.0	0.074	0.688	0.943
Caecal tonsils							
$CD3^+/CD4^+/CD8^-$, %	25.2 ± 3.5	25.3 ± 3.5	20.0 ± 3.5	22.1 ± 3.5	0.240	0.751	0.777
$CD3^+/CD4^-/CD8^+$, %	59.3 ± 4.4	60.0 ± 4.4	61.8 ± 4.4	60.4 ± 4.4	0.744	0.940	0.812
$CD3^+/CD4^+/CD8^+$, %	2.3 ± 0.3	2.9 ± 0.3	2.9 ± 0.3	2.6 ± 0.3	0.625	0.748	0.199
$CD3^+/CD4^-/CD8^-$, %	13.2 ± 2.4	11.8 ± 2.4	15.3 ± 2.4	14.9 ± 2.4	0.300	0.714	0.839

[1] *Expanded and pelleted.*
[2] *Finely ground and pelleted.*
[3] *Technical feed treatment.*
[4] *Blood n = 8.*

these differences were a result of the different pellet qualities, which were indicated by the pellet abrasion. It appears that a higher pellet abrasion resulted in a lower daily feed intake. A relationship between pellet quality and broiler performance was also reported in studies of Lilly et al. (20). Furthermore, the differences in pellet abrasion might be also an indication for modified pellet textures due to the various feed ingredients and processing methods. The importance of pellet texture in broiler performance was also shown in studies of Parsons et al. (11). The reason for the differences in pellet abrasion of our current feeds appeared to be related to the particle size distribution before hydrothermal treatment. The influences of feed on daily feed intake were also reflected in the BW gain, which in turn influenced the BW on slaughtering day.

The water consumption was markedly influenced by diet composition. Also in studies of Abd El-Wahab et al. (21)

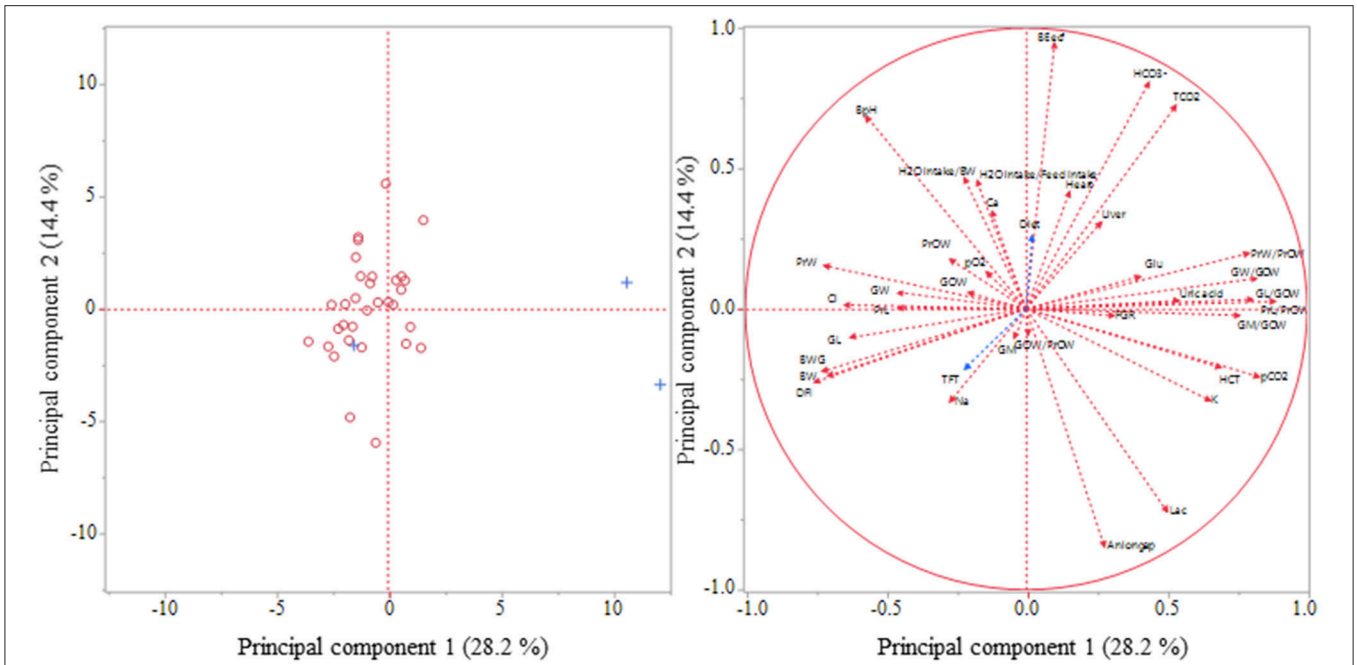

FIGURE 1 | Principal Component Analysis for visualization of the relationships between 38 selected variables. Variables for the analysis: DFI, daily feed intake; BW, body weight on slaughtering day; BWG, body weight gain; FGR, feed to gain ratio; H2O intake/BW, H_2O consumption per kg body weight; H2O intake/Feed intake, H_2O consumption per g feed intake; Heart, relative heart weight; Liver, relative liver weight; PrOW, relative organ weight of proventriculus; PrL, proventricular length (cm), PrL/PrOW, proventricular length (cm/g organ weight); PrW, proventricular width (cm); PrW/PrOW, proventricular width (cm/g organ weight); GOW, relative gizzard weight; GOW/PrOW, gizzard weight/proventricular weight ratio; GL, gizzard length (cm), GL/GOW, gizzard length (cm/g organ weight); GW, gizzard width (cm); GW/GOW, gizzard width (cm/g organ weight); GM, gizzard muscles (cm); HCT, hematocrit; BpH, blood pH; pO2, oxygen partial pressure in blood; pCO2, carbon dioxide partial pressure in blood; TCO2, total carbon dioxide concentration in blood; BEecf, base concentration in extracellular fluid; HCO3-, bicarbonate concentration in blood; Na, sodium concentration in blood; K, potassium concentration in blood; Cl, chloride concentration in blood; Ca, ionized calcium concentration in blood; Aniongap, anion gap; Glu, glucose concentration in blood; Lac, lactate concentration in blood; Uric acid, uric acid concentration in blood. Additional variables: Diet; TFT, technical feed treatment; Left: projection of cases (broilers): + with diagnosed ascites; O without diagnosed ascites; Right: projection of variables.

differences in water consumption were observed between broilers fed a soybean meal based diet and broilers fed a diet based on rapeseed meal. An effect of total protein content on water consumption as reported by Wheeler and James (22) can be excluded because the total protein content did not differ between the feeds, which was published by Liermann et al. (23). Thus, in general the water consumption seemed to depend on protein sources in the diet. The significant diet-by-TFT interaction detected in case of the water to feed ratio demonstrated that the TFT has also an impact on the water consumption. This suggestion is supported by the results of Vranjes and Wenk (24) showing an increased water consumption after feeding extruded feed components compared to only pelleted feed components. Manning et al. (25) emphasized that the water consumption is directly related with the fecal conditions. In turn, this could affect the litter quality and the incidence of foot pad dermatitis (25). In studies of Abd El-Wahab et al. (21) a higher incidence of foot pad dermatitis after feeding rapeseed meal seemed to be also associated with a higher water consumption of broilers compared to broilers fed soybean meal. However, in contrast to present results in these studies the water/feed ratio of birds fed rapeseed meal was numerically decreased compared to birds fed soybean meal.

Significantly higher liver weights and numerically enhanced cholesterol and non-esterified fatty acids concentrations as well as aspartate-amino-transferase activities indicated alterations in the liver metabolism of birds fed expanded and pelleted feed containing 12% RSE compared to birds fed the other tested feeds. The observed alterations in liver weights and blood cholesterol concentrations as well as the higher ionized calcium concentrations in these birds indicated also changes in the calcium metabolism. Cholesterol is a precursor of vitamin D_3 and both are metabolized in the liver (26, 27). In turn, vitamin D_3 and its metabolites play a key role in the absorption of calcium in the intestine and the kidneys and the incorporation in bones (28). The reasons for the apparent alterations in liver and calcium metabolism cannot be fully explained currently. In part these results appear to be diet-dependent. However, broilers fed finely ground and pelleted feed containing 12% RSE content showed no comparable alterations. Besides the diet also the TFT affected ionized calcium concentrations in blood. Moreover, there was a significant diet-by-TFT interaction in case of liver weights. Thus, also the TFT appears to play a role in the development of the effects on liver and calcium metabolism. Differences in blood calcium concentrations might be also possible by differences in vitamin D_3 supplementation. It is known, that vitamin D_3

stability varies between pelleting and expanding (29). A possible destruction of the vitamin in the premix during feed processing might result in different vitamin D_3 content in feed and therefore in differences in vitamin intake. The aspect that feeds processed by similar methods did not show similar effects on blood calcium concentrations may contradict this hypothesis. However, it has to be emphasized, that the extent of the impacts of TFT on the feed characteristics depends strongly on feed components, ingredients, and structure of feed material (30, 31). Because albumin acts as a calcium carrier it is not surprising that this serum protein was affected in a similar manner as calcium.

Different previous studies revealed that feeding compacted feed plays a key role in the development of proventricular dilatation and underdevelopment of the gizzard (4, 5, 32). Comparing gizzard weights and the proventricular size (length and width) measured in the present study with similar organ traits measured in broilers fed coarsely ground meal in a previous study of Liermann et al. (5), it is suggested that the adverse effects of pelleted feed on the development of the proventriculus or the gizzard were not markedly inhibited by any of the used pre-treatments or diets, although the gizzard and the proventricular weights differed between the feeding groups and were affected in a diet-dependent manner. The study of Liermann et al. (5) and the current study demonstrated that the proventricular extent appears to be a more reliable indicator for proventricular dilatation than the weight of this organ, because the increase in organ size is not associated with an increase in organ muscle or tissue growth but by stretching of this organ. Significant correlations between the daily feed intake and some morphometric traits of the digestive organs indicated that differences in the development of the proventriculus and the gizzard are strongly related with the daily feed intake. This aspect was also supported by findings of Liermann et al. (5) and in turn explains the differences in gizzard and proventricular traits between the current feeding groups. As discussed by Svihus (33) one main function of the gizzard is the feed intake regulation. Because of the underdevelopment of the gizzard by feeding pellets and by covering the satiety signal by the appetite in modern broiler lines (33), an overconsumption will be supported, which leads to proventricular dilatation.

In the present study the risk for pulmonary hypertension and ascites seemed to be neither increased nor markedly decreased in the different feeding groups, although the feeding groups differed markedly in animal performance. In the literature, fast growing by a high nutrient intake and overconsumption of feed was associated with a higher incidence of ascites (7, 34, 35). So, Baghbanzadeh and Decuypere (7) suggested feed restriction as a successful method to reduce ascites. Contrary to the expectations, birds of the 6%FgP group showing the highest daily feed intake and BW gain were not associated with a higher occurrence of this metabolic disease at an age of 28 days after hatching. Additionally, one animal showing low fattening performance and belonging to feeding group 6%ExP suffered from ascites. Indeed, ascites is based on multifactorial causes as also reported by Baghbanzadeh and Decuypere (7). Ascites is characterized by hypoxemia, hypercapnia, right ventricular hypertrophy, and an elevated hematocrit (7, 35). Therefore, it was assumed that fast growing broilers might show similar alterations. However, pCO_2 and TCO_2 levels, heart weights, as well as the hematocrit were negatively correlated to the daily feed intake and in part to the BW gain. Previous studies also reported relationships between performance parameters and, for example, hematocrit values or blood gas values, although these parameters were in part affected in an opposite direction (35, 36). Ascites was only diagnosed in three animals during slaughtering. However, the principal component analysis shows that birds suffering from ascites markedly differed in variables considered in the principal component analysis except for one animal. In contrast, all other animals clustered with each other. It cannot be excluded that some further broilers would develop ascites at a later time. Using the hematocrit as a selection criteria as reported by Baghbanzadeh and Decuypere (7), birds fed expanded and pelleted feed might have had a higher risk for ascites, and this risk is more related on the TFT than on the diet.

Ascites is associated with oxidative stress in birds (7). Uric acid is a potent scavenger of reactive oxygen species, and it was shown in studies of Simoyi et al. (37) that a decrease in uric acid concentrations in plasma is associated with a marked increase of oxidative stress. In contrast in studies of Enkvetchakul et al. (38) ascites increased uric acid concentrations in blood, lung, and liver. In the present study broilers suffering from ascites showed higher uric acid concentrations compared to broilers without ascites (data not shown). Furthermore, the uric acid concentration was positively correlated with the hematocrit. According to these findings possibly a higher uric acid concentration might be also a distinguishing feature for birds prone to ascites. Using both the hematocrit and the uric acid concentration as distinguishing feature, especially animals from the 6%ExP group might have a higher risk for ascites in the present study. This hypothesis should be tested in further studies with a higher number of animals.

Already, in studies of Liermann et al. (5) it was suggested that the alterations in proventriculus and gizzard could also increase the incidence of pulmonary hypertension and ascites. Both the correlations between the traits of the digestive organs and the blood gases as well as the principal component analysis clearly showed that there are some relationships between these factors indicating a possible impact of the development of the digestive tract and functions of pulmonary gas exchange.

Interestingly, PBL proportions of CD4/CD8 double positive and double negative T cells were significantly affected or tended to be affected by the TFT. In contrast, these cell types were significantly influenced or tended to be influenced by the diet type in *lamina propria*. It was assumed that the effects on the peripheral system are a result of the nutrient absorption and possible changes in blood metabolites. However, the mentioned subsets correlated neither with the daily feed intake nor with blood metabolites. However, there was a significant correlation between the digestibility of crude fiber [as presented in Liermann et al. (23)] and the PBL proportions of CD4/CD8 double negative stained T cells ($r = -0.371$; $P = 0.028$). The fiber digestibility was also affected by the TFT (23). During the digestion of fiber in the caeca by microbes different metabolites and especially short chain fatty acids are produced. These metabolites are often

discussed as immune-modulators (39). The effects of the diet on T cell subsets in the *lamina propria* might be a local effect possibly based on changes on physico-chemical conditions in the intestinal content. The formulated diets showed no marked differences in crude nutrients and were free from glucosinolates as published by Liermann et al. (23). Therefore, these aspects can be excluded as a potential impact factor. This applies also to the trend to affect PBL $CD3^+/CD4^+/CD8^-$ T cell subsets by diet. The biological importance of these findings needs to be proved in further studies. In general, both the CD4/CD8 double positive and the double negative T cells are associated with immune-regulatory activities (40, 41); however, the role of these cells has not been fully explored, especially in poultry. The found correlations between morphometric traits of gizzard and proventriculus and T cell subsets in *lamina propria* and caecal tonsils in studies of Liermann et al. (5) and in the current study let assume that alterations in the development of organs of the proximal digestive tract are associated with modifications in the local immune system of the distal digestive tract. Similar observations have been made in studies with pigs of Liermann et al. (42). Interestingly and similar to studies of Liermann et al. (5), the T cell subsets that show relationships to gizzard or proventricular weight show also correlations to the daily feed intake.

The differences in the relative weights of bursa of *Fabricius* between the feeding groups 6%ExP and 12%ExP cannot be explained at the moment. In previous studies decreasing weights of this organ was associated with an immunosuppression and as an indicator for stress. However, as also reported in studies of Liermann et al. (5) this parameter did not correspond to the H/L ratio, which was not different between the feeding groups and is also accepted as a stress indicator in chickens (43).

Because of the low frequency of monocytes in blood in the current study, the biological relevance of the observed differences between feeding groups seemed to be unlikely.

CONCLUSION

In conclusion, the pellet quality and texture that are mainly influenced by the feed processing methods seemed to play a key role in the development of the gizzard and the proventriculus because of their influences on the daily feed intake. Alterations in morphometric traits of proventriculus and gizzard modify the local immune system of the downstream digestive tract and might have influences on the development of ascites. However, further studies are required to confirm this hypothesis since in the present study only three birds developed ascites.

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 Lower Saxony State Office for Consumer Protection and Food Safety, LAVES, Germany (registration number: 33.9-42502-04-082/09).

AUTHOR CONTRIBUTIONS

WL, AB, VB, and SD: conceptualization. WL and JF: methodology. WL, JF, and AB: investigation. VB, AB, and SD: resources. WL: writing-original draft preparation. AB, JF, and SD: writing-review and editing. SD: supervision. AB and VB: project administration and funding acquisition. All authors contributed to the article and approved the submitted version.

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Effect of Egyptian Leek Leaf Extract Supplementation on Productive and Economic Performance of Broilers

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Antibiotic growth promoters have been used to improve growth and feed conversion in the poultry industry for a long time; however, they were banned because of several life-threatening side effects in animals, poultry, and humans. This work was carried out to investigate the effect of leek (*Allium ampeloprasum var. kurrat*) leaf extract (LLE) as a non-traditional growth promoter and feed additive on growth performance, carcass characteristics, serum biochemical parameters, and economic efficiency of broilers. Hubbard unsexed 1-day-old broilers ($n = 250$) were fed with diets supplemented with LLE for 42 days. The experimental chicks were randomly assigned to one of the five treatment groups varying in LLE quantity in diets: 0% (control), 0.05, 0.1, 0.15, and 0.2%, with five replicates per treatment (50 chicks/treatment or 10 chicks/replicate). Results showed that LLE supplementation improved ($P < 0.05$) different growth performance parameters. Furthermore, dietary LLE not only decreased serum total cholesterol, triglyceride, low-density lipoprotein, and glucose levels but also increased serum high-density lipoprotein level compared to the control diet. The weight percentages of dressing ($P = 0.022$) and liver ($P = 0.041$) showed a marked increase after the addition of LLE. Return, net profit, and collective efficiency measures were increased ($P = 0.001$) in all LLE groups compared with the control group. Broilers that fed on diets containing 0.2% LLE showed the highest growth and economic efficiency. It could be concluded that supplementation with LLE in broilers has growth-promoting effects, improved biochemical parameters, carcass quality, and promoted economic efficiency through maximizing both return and net profit.

Keywords: broiler, leek leaf extract, performance, biochemical parameter, economic

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INTRODUCTION

The use of growth promoters in the poultry industry leads to the net increase in the weight of broilers without the use of any additional dietary supplements. Recently, the use of medicinal plants and their extracts in controlling poultry diseases and growth promotion has gained attention because of their cost-effective applications and minimal side effects (1). Although antibiotic growth promoters were used to improve growth and feed conversion for a large sum of time, they were banned because of several side effects reported in animals, poultry, and humans (2). Finding a

cheap, safe, and acceptable alternative to replace antibiotics is desirable. Therefore, vegetables, herbs, and edible plants and/or their extracts have been researched as valid non-traditional growth promoters, feed additives, and immunostimulants. Leek (*Allium ampeloprasum var. kurrat*) is a biennial plant related to the genus Allium (family Alliaceae or Liliaceae), including onion and garlic (3). They are considered one of the oldest cultivated plants in Egypt and are consumed for food and/or medical purposes. Also, leek and its leaves are considered a potential source of several bioactive or health-promoting compounds, including a high number of phytonutrients. Their extracts have been studied for antimicrobial, antioxidant, cytotoxic (4), hypoglycemic (5), hypolipidemic (6), and hypercholesterolemic (7) effects.

Although the use of various herbal extracts as dietary supplements has been reported to improve the growth and protection of broilers against several diseases (8), the potential use of leek leaf extract has remained elusive. The current trial was delineated to investigate the improvement of productive performance, carcass characteristics, some serum biochemistry, and economic efficiency of broiler chickens by leek (*Allium ampeloprasum var. kurrat*) leaf extract (LLE) dietary supplementation.

MATERIALS AND METHODS

Preparation and Analysis of Leek Leaf (LLE) Ethanolic Extract

Fresh leek leaves free from physical defects were collected from a local market. The collected sample was then dried at 40°C for 72 h in an air convection oven, ground, and passed through a 150-μm mesh sieve. The dried material (500 g) was then extracted with 2,000 mL of solvent (70% ethanol) at room temperature for 72 h. The extract was filtered through a filter paper (Whatman no. 1), and the extraction and filtration processes were repeated three times. The solvent was separated under vacuum at 40°C using a rotary evaporator (BUCHI-water bath-480, Germany). The extract was then freeze-dried by Thermo-Electron Corporation-Heto power dry LL300 Freeze Dryer and kept in dark-colored containers away from the light at −20°C until use, according to the previous reports (9). The total phenols, flavonoids, and radical scavenging activity of LLE were assessed by following the previously reported procedures (10).

Total phenols were estimated as gallic acid equivalents (mg GA/g dry extract) by using the Folin–Ciocalteu reagent. An aliquot of 0.5 mL LLE was mixed with 2.5 mL of Folin–Ciocalteu reagent (previously diluted with water 1:10, V/V) and 2 mL of $NaHCO_3$ (7.5%). The absorbance was measured by using a spectrophotometer at 765 nm after incubation at 40°C for 15 min.

Flavonoids were evaluated as mg rutin equivalents. Aluminum chloride (0.5 mL, 2%) in methanol was mixed with the same volume of LLE. The absorbance was measured by using a spectrophotometer at 415 nm after 1-h incubation at room temperature.

Animals and Experimental Design

This study was conducted at the Poultry Research Farm and was approved by the Committee of Animal Welfare and Research Ethics, Faculty of Veterinary Medicine, Zagazig University, Egypt. Unsexed 1-day-old Hubbard chicks ($n = 250$) weighing 40 ± 1 g were selected from a local hatchery. The chicks were randomly assigned to five experimental treatment groups: 0% (control treatment 1), 0.05% (treatment 2), 0.1% (treatment 3), 0.15% (treatment 4), and 0.2% (treatment 5) for 42 days with five replicates/treatment (50 chicks/treatment; 10 chicks/replicate). Each replica contained equal numbers of males and females. First, LLE was mixed well with other dietary feed additives (as mineral, vitamin mixture, and amino acids) and then mixed with other main dietary ingredients (as corn and SBM). Broilers were vaccinated against Gamboro and Newcastle diseases. Broilers were observed on a daily basis and checked for any syndromes without mortalities during the whole experiment. Broilers were kept in separate pens with a stocking density of 10 birds/m^2 under suitable temperature and proper lighting conditions for 6 weeks of feeding. The isocaloric and isonitrogenous diets were prepared to fulfill the nutrient requirements of the Hubbards (11) and were given in a mash form. In brief, diet in the starter period (0–10 days) contained 23.02% crude protein (CP) and 3,035.75 kcal/kg diet metabolizable energy (ME); the grower period (11–22 days) contained 20.58% CP and 3,110.69 kcal/kg diet ME; and the finisher period (23–42 days) contained 19.07% CP and 3,184.79 kcal/kg diet ME, as shown in **Table 1**. Broilers were fed with water and diet *ad-libitum*. Diets were examined for dry matter (hot air oven at 105°C), CP (Kjeldahl method), crude fiber (Weende method), ash (muffle furnace at 600°C), and ether extract (Soxhlet apparatus) according to Official Methods of Analysis by AOAC (12). ME was estimated based on the NRC (13) prediction equation.

Growth Performance Parameters

The individual chicks were weighed at the beginning and after 6-week feeding to obtain the live body weight (LBW). In a similar manner, body weight gain (BWG) was calculated as the final minus initial body weight. An average feed intake (FI, g/bird) was calculated as the difference between weights of the feed offered, and residues left after consumption by broilers and divided by the number of birds. The feed conversion ratio (FCR) was calculated according to previous reports (14).

Carcass Traits

After 42 days of feeding, five broilers from each experimental treatment group (one from each replicate) were randomly selected, fasted overnight, weighed, and then slaughtered using a sharp knife to until complete bleeding. The dressing percentage in which the head, neck, feet, and lower wings were removed was estimated by final weighing, followed by the plucking of the feathers and evisceration. The liver, heart, stomach, intestine, and spleen were also weighed and expressed as a percent of LBW.

Biochemical Analysis

The blood samples from five broilers per treatment group, one from each replicate, were collected after slaughtering in

TABLE 1 | Ingredients and composition of the experimental diets.

Ingredients	Experimental diets		
	Starter diets (1–10 days)	Grower diets (11–22 days)	Finisher diets (23–42 days)
Yellow corn	56.055	61.405	63.405
Soybean meal, 48%	32.60	28.64	29.00
Corn gluten, 60%	4.60	3.00	0.00
Soybean oil	1.92	2.5	3.70
Calcium carbonate	0.59	0.56	0.48
Ca. dibasic phosphate	2.73	2.49	2.08
Common salt	0.35	0.25	0.27
Sodium bicarbonate	0.25	0.25	0.20
Mineral mixture**	0.072	0.074	0.074
Vitamin mixture*	0.033	0.031	0.031
Choline chloride, 60%	0.07	0.06	0.05
L-Lysine, Hcl, 79.80%	0.35	0.33	0.26
L-Methionine, 100%	0.29	0.27	0.30
L-Threonine, 99%	0.09	0.09	0.10
Mycofix select[a]	0.05	0.05	0.05
Calculated composition			
ME, kcal/kg	3035.75	3110.69	3184.79
CP, %	23.02	20.58	19.07
EE, %	4.62	5.29	6.47
CF, %	2.56	2.30	2.53
Ca, %	0.96	0.88	0.76
AP, %	0.48	0.44	0.38
Lysine, %	1.41	1.27	1.20
Methionine, %	0.68	0.62	0.61
Analyzed composition			
DM, %	90.60	90.30	90.70
CP, %	22.25	19.30	18.15
EE, %	4.50	5.00	6.25
CF,%	2.36	2.17	2.30
Ash,%	3.60	3.55	3.57

Premix per kg of diet.*
**Vitamin mixture: vitamin A, 1 500 IU; vitamin D3, 200 IU; vitamin E, 10 mg; vitamin K3, 0.5 mg; thiamine, 1.8 mg; riboflavin, 3.6 mg; D pantothenic acid, 10 mg; folic acid, 0.55 mg; pyridoxine, 3.5 mg; niacin, 35 mg; cobalamin, 0.01 mg; biotin, 0.15 mg.*
***Mineral mixture: Fe, 80 mg; Cu, 8 mg; Mn, 60 mg; Zn, 40 mg; I, 0.35 mg; Se, 0.15 mg.*
[a]Mycofix select is anti-mycotoxin from Anani Company, Egypt.
ME, metabolizable energy; DM, dry matter; CP, crude protein; EE, ether extract; CF, crude fiber; Ca, calcium; AP, available phosphorus.

TABLE 2 | Analyses of leek leaf extract.

Parameters	Leek leaf extract (LLE)
Quantity of extract yield, %	24.33
Total phenolic content	223 mg GAE*/g extract powder
Total flavonoid content	65 mg RE**/g extract powder
Radical scavenging activity (Inhibition percent, %) at concentration 10 μg/mL	15.00

**GAE–mg gallic acid equivalents.*
***RE–mg rutin equivalents.*

sterile glass tubes without anticoagulant and placed in a slanted position for 20 min at room temperature, followed by 10-min centrifugation at 3,000 rpm. The serum was then removed and stored at −20°C until further use for biochemical study by using various diagnostic kits (Roche Diagnostics, GmbH, USA). The total protein and albumin were estimated by using diagnostic kits described in the previous report (15), while the globulin was estimated by subtracting the albumin value from the total protein value. Alanine aminotransferase (ALT) (16) and serum aspartate-aminotransferase (AST) (17) were estimated according to the previously described methods. The Fossati (18) procedure was used to estimate the blood creatinine, urea nitrogen, and uric acids. Glucose (19), total cholesterol (20), triglyceride (21), HDL-cholesterol (22), and LDL cholesterol (23) were estimated according to the previously described methods.

Determination of Economic Efficiency

Cost parameters were categorized into the total fixed costs (TFC), total variable costs (TVC), and total costs (TC) as described in previous reports (24, 25). On the other hand, return parameters, including total returns (TR) from chick sale equals kg price (20 LE in May 2017) × body weight and net profit (total returns minus total costs), were calculated. Furthermore, the efficiency of feed additives in the form of collective measures of efficiency was measured according to previous studies (26, 27).

Statistical Analysis

Data were analyzed by the one-way analysis of variance by the GLM procedure in the SPSS (version 25; IBM Corp., Armonk, NY) statistical software package. Shapiro–Wilk's test was used to calculate the normality, and Hortly's test was used to calculate the homogeneity. The least significance difference test was used to separate significant means (28). The group means were compared using Duncan's multiple range tests (29). The results were reported as mean ± SE (standard error). A P-value of <0.05 was considered statistically significant.

RESULTS AND DISCUSSION

The total phenols, flavonoids, and radical scavenging activity of LLE are shown in **Table 2**. The quantity of extract yield (%) means that every 100 g of raw LLE produces about 24.33 g of dried leek leaf extract. The total phenols and flavonoids of LLE are as high as other plants, and radical scavenging activity is also high. This result helped prompt manner and strong improvement in the poultry growth and serum biochemical parameters.

Few studies have reported the beneficial effects of LLE as growth promoters in poultry feed. Leek is closely related to garlic and onion, belonging to the same family. LLE has higher total phenolic compounds in the form of organo-sulfur compounds with antioxidant capacity compared with that of garlic and green onion, considering the scavenging or preventive capacity against superoxide anion, hydroxyl, and peroxyl radicals that exert diverse pharmacological functions (4, 30). Also, there is a significant correlation between the total antioxidant capacity

and phenolic contents, indicating that the phenolic contents are the dominant antioxidant constituents of LLE (31). LLE contains several biologically active compounds such as ellagic acid, phenols, flavonoids, and tannins that have high antioxidant and anti-inflammatory effects on the intestinal tract (32).

The effect of LLE supplementation in Hubbard diets on productive performance is given in **Table 3**. LLE-supplemented diets had a significant ($P = 0.001$) effect on the productive performance parameters (FBW, BWG, and total FI) compared with the control. Broilers that fed on the diet containing 0.2% LLE had the heaviest final BW and highest BWG compared with the broilers fed on other LLE treatments. Broilers fed on LLE-supplemented diets showed a significant ($P = 0.001$) increase in the final body weight, BWG, and total FI, while FCR was significantly ($P = 0.038$) improved in the 0.2% LLE supplementation group compared with the control group. Broilers that were fed on a diet contained 0.20% LLE had the heaviest final BW, highest BWG, and best FCR.

The diets containing LLE showed a positive effect on the growth performance of the broilers and are in accordance with the previous reports. The performance of broilers was positively affected by the dietary supplementation of 0.75% and 1% of leek powder for 5 weeks according to previous reports (33), and Egyptian leek leaf powder as an unconventional feed at 4, 6, 8, and 10% significantly increased the final body weight, BWG, and total FI of broilers compared with the control group as described earlier (34). Jo et al. (35) stated that broilers fed on diets containing 100 ppm garlic extract showed an improved growth rate during the period of 1–35 days; additionally, the aqueous extract of 1 and 2% of garlic significantly increased the body weight of broilers from 7 to 35 days of age compared with the negative and positive ciprofloxacin controls, and 1% supplementation level showed the highest weight gain and FCR at day 35 as described earlier (36). Goodarzi et al. (37) reported that broilers fed on diets supplemented with 30 g onion/kg diet had a significant increase in the body weights on day 42. Chickens showed a significant increase ($P < 0.05$) in average daily feed intake during the grower period and an experimental period of 42 days compared with the control group and a group fed on diet supplemented with an antibiotic. Similarly, chicks fed on diets supplemented with 0.3 and 0.5% onion extract for 35 days showed an increase in BW and BWG compared with those fed on an un-supplemented diet, as reported before (38). In contrast to our results, FI was not affected by the supplementation of garlic extract (36) or onion extract (38) in broiler chicks, as reported previously. The beneficial effects of LLE on the growth performance and FI could be attributed to its high content of polyphenols which increased food and calorie intake (39), high

TABLE 3 | Effect of diets supplemented with leek leaf extract on the overall performance of broiler chicks (means ± SE).

Trait studied	Experimental diets					*P*-value
	Control	Leek (kurrat) leaf extract in diets				
		0.05%	0.10%	0.15%	0.20%	
IBW, g	40.20 ± 0.11	40.20 ± 0.05	40.40 ± 0.08	40.37 ± 0.08	40.27 ± 0.04	0.227
FBW, g	1950.20 ± 3.04^{d}	2199.99 ± 9.87^{c}	2286.50 ± 16.36^{b}	2300.50 ± 1.69^{b}	2350.46 ± 6.26^{a}	0.001
BWG, g	1910.00 ± 3.01^{d}	2159.79 ± 9.87^{c}	2246.10 ± 16.42^{b}	2260.13 ± 1.75^{b}	2310.20 ± 6.23^{a}	0.001
Total FI, g	3413.07 ± 7.57^{c}	3790.67 ± 5.92^{b}	3959.90 ± 4.25^{a}	3959.53 ± 3.70^{a}	3973.80 ± 5.97^{a}	0.001
FCR	1.79 ± 0.004^{a}	1.76 ± 0.024^{ab}	1.76 ± 0.011^{ab}	1.75 ± 0.002^{ab}	1.72 ± 0.003^{b}	0.038

^{abcd}Means within the same row carrying different superscripts were significantly different at ($P \leq 0.05$). IBW, initial body weight; FBW, final body weight; BWG, body weight gain; FI, feed intake; FCR, feed conversion ratio.
Number of observation (n) = 50 in each group.

TABLE 4 | Effect of diets supplemented with leek leaf extract on carcass traits relative to the live weight of broiler chickens (means ± SE).

Parameters	Experimental diets					*P*-value
	Control	Leek leaf extract in diets				
		0.05%	0.10%	0.15%	0.20%	
Dressing %	66.82 ± 0.71^{b}	70.56 ± 0.22^{ab}	72.52 ± 0.52^{a}	72.07 ± 0.96^{a}	73.32 ± 1.55^{a}	0.022
Liver %	2.88 ± 0.16^{b}	3.19 ± 0.24^{ab}	3.30 ± 0.08^{ab}	3.29 ± 0.11^{ab}	3.52 ± 0.05^{a}	0.041
Heart %	0.74 ± 0.19	0.76 ± 0.06	0.74 ± 0.01	0.68 ± 0.08	0.80 ± 0.09	0.943
Stomach %	3.95 ± 0.48	4.20 ± 0.38	4.34 ± 0.05	4.32 ± 0.14	4.32 ± 0.18	0.637
Intestine %	5.88 ± 0.29	5.77 ± 0.11	5.74 ± 0.13	5.43 ± 0.14	5.68 ± 0.04	0.504
Spleen %	0.37 ± 0.013	0.38 ± 0.012	0.35 ± 0.007	0.38 ± 0.007	0.38 ± 0.010	0.065

^{ab}Means within the same row carrying different superscripts were significantly different at ($P \leq 0.05$).
Number of observation (n) = 5 in each group.

TABLE 5 | Effect of diets supplemented with leek leaf extract on some serum biochemical analysis of broiler chickens (means ± SE).

Parameters	Experimental diets				*P*-value
	Control	Leek leaf extract in diets			
		0.05%	0.10%	0.15%	0.20%	
Effect leek leaf extract on liver function						
Total protein, g/dl	4.27 ± 0.92	4.30 ± 0.58	4.17 ± 0.20	4.20 ± 0.05	4.19 ± 0.20	0.692
Albumin, g/dl	1.70 ± 0.02	1.69 ± 0.02	1.71 ± 0.01	1.70 ± 0.01	1.70 ± 0.01	0.748
Globulin, g/dl	2.57 ± 0.01	2.61 ± 0.12	2.46 ± 0.05	2.50 ± 0.08	2.49 ± 0.06	0.163
AST, IU/dl	39.00 ± 1.52	39.33 ± 2.40	37.33 ± 1.45	40.33 ± 2.28	36.67 ± 0.33	0.059
ALT, IU/dl	23.67 ± 1.33	24.33 ± 0.88	22.00 ± 1.73	23.67 ± 1.76	19.33 ± 1.85	0.059
Effect leek leaf extract on kidney function						
Creatinine, mg/dl	0.37 ± 0.03	0.39 ± 0.01	0.40 ± 0.02	0.41 ± 0.02	0.41 ± 0.02	0.547
Urea, mg/dl	2.65 ± 0.003	2.66 ± 0.005	2.65 ± 0.006	2.64 ± 0.006	2.66 ± 0.008	0.609
Uric acid, mg/dl	3.21 ± 0.044	3.20 ± 0.011	3.22 ± 0.012	3.23 ± 0.120	3.22 ± 0.017	0.999
Effect leek leaf extract on glucose						
Glucose, mg/dl	119.93 ± 1.35^{a}	89.17 ± 1.68^{b}	88.93 ± 1.17^{b}	80.47 ± 0.83^{c}	80.63 ± 1.16^{c}	0.001
Effect leek leaf extract on lipid profile						
Cholesterol, mg/dl	153.33 ± 2.09^{a}	113.07 ± 1.62^{b}	105.97 ± 1.95^{bc}	101.43 ± 0.32^{c}	102.23 ± 0.89^{c}	0.001
Triglyceride, mg/dl	130.37 ± 1.13^{a}	102.90 ± 1.30^{b}	102.27 ± 0.66^{b}	94.93 ± 0.37^{c}	94.17 ± 0.84^{c}	0.001
HDL, mg/dl	68.40 ± 0.77^{c}	88.00 ± 1.30^{b}	87.20 ± 0.34^{b}	104.47 ± 2.48^{a}	103.00 ± 1.37^{a}	0.001
LDL, mg/dl	195.23 ± 0.48^{a}	114.70 ± 3.25^{b}	111.50 ± 4.05^{b}	107.70 ± 1.27^{b}	110.23 ± 1.50^{b}	0.001

^{abc}*Means within the same row carrying different superscripts were significantly different at (P ≤ 0.05).*

Number of observation (n) = 5 in each group. AST, Serum aspartate-aminotransferase; ALT, Alanine aminotransferase; HDL, High density lipoprotein; LDL, Low density lipoprotein.

antibacterial activity due to dialkylpolysulfide (40), and increased villi height (33).

Carcass dressing was significantly ($P = 0.022$) increased in LLE-fed groups compared with the control group. The liver percentage was significantly ($P = 0.041$) increased in the group fed on the diet supplemented with 0.2% LLE, and a non-significant increase was observed in the other LLE-supplemented groups compared with the control group (**Table 4**). No significant difference in the heart ($P = 0.943$), intestine ($P = 0.504$), spleen ($P = 0.065$), and stomach ($P = 0.637$) was observed among different experimental groups compared with the control group.

Also, the broilers that fed on garlic extract 40 or 60 ppm/kg diet from day 13 to 47 (41) and on 0.3 or 0.5% of onion extract for 5 weeks (38) showed an increase in carcass dressing % similar to our results. In contrast to our result, others reported that broilers that fed on diets containing 25, 50, and 100 mg of garlic or onion for 21 days had a non-significant effect on the carcass dressing (42). Diets containing garlic (36, 43) or onion extracts (38) showed a non-significant increase in the weights of different visceral organs of broilers, similar to the present study. An increase in dressing %, especially in the LLE fed group, could be attributed to the effect of LLE on final body weight that may have reflected in the dressing weight.

Biochemical parameters of blood are the valid indicators of health (physiological and nutritional) status of the broilers. The effect of LLE-containing diets on the serum metabolites of the broilers is given in **Table 5**. There were no significant changes in total protein ($P = 0.692$), albumin ($P = 0.748$), globulin ($P = 0.163$), and liver enzymatic activity (ALT and AST) ($P = 0.059$). Also, no significant changes in serum creatinine ($P = 0.547$), urea ($P = 0.609$), and uric acid ($P = 0.999$) were observed in the broilers fed on LLE compared with the control group. These results are similar to previous reports (34) on liver enzymes (ALT and AST), creatinine, urea, total protein, albumin, and globulin of broilers fed on diets supplemented with 2, 4, 6, 8, and 10% of leek leaf powder. We found that the effect of diets supplemented with LLE was non-significant on the serum total protein and albumin levels and is in accordance with other studies performed on the dietary supplementation of allicin (44). Serum AST and ALT are considered important parameters to evaluate the effect of unconventional feed stuff or new feed additives on broilers (45, 46). We found no significant changes in the serum AST and ALT levels of broilers that fed on LLE-containing diets. Similarly, broilers fed on diets containing garlic extracts (36) and onion extracts (38) showed no significant effect on AST and ALT levels, as reported previously. Supplementation of onion juice of various concentrations had a non-significant effect on the levels of serum urea, uric acid, and creatinine in rats, as described previously (47).

Interestingly, broilers fed on LLE-supplemented diets had a significant ($P = 0.001$) reduction in serum glucose level compared with the control group (**Table 5**). Our results are in accordance with other studies (33, 34) experimented on the blood glucose levels of broilers. Also, a reduction in blood glucose level in diabetic rats fed diets containing leek (*Allium Ampeloprasum*) for 1 month (5) and diabetic rabbits fed diets containing an aqueous extract of onions has been reported (48). The hypoglycemic effects of leek may be attributed to

TABLE 6 | Effect of diets supplemented with leek leaf extract on economic measures of broiler chickens (means ± SEM).

Trait studied	Experimental diets					*P*-value
	Control	Leek leaf extract in diets				
		0.05%	0.10%	0.15%	0.20%	
Cost parameters						
Feed costs	21.28 ± 0.05^{d}	23.95 ± 0.23^{c}	25.46 ± 0.03^{b}	25.54 ± 0.02^{ab}	25.86 ± 0.04^{a}	0.001
Total variable costs	21.48 ± 0.05^{d}	24.16 ± 0.23^{c}	25.47 ± 0.03^{b}	25.74 ± 0.02^{b}	26.07 ± 0.04^{a}	0.001
Total cost	44.40 ± 0.05^{d}	47.08 ± 0.23^{c}	48.39 ± 0.02^{b}	48.66 ± 0.03^{b}	48.99 ± 0.04^{a}	0.001
Return parameters						
Total returns	44.85 ± 0.07^{d}	50.60 ± 0.23^{c}	52.59 ± 0.37^{b}	52.91 ± 0.38^{b}	54.06 ± 0.14^{a}	0.001
Net profit	0.45 ± 0.10^{c}	3.52 ± 0.45^{b}	4.20 ± 0.35^{ab}	4.25 ± 0.05^{ab}	5.07 ± 0.11^{a}	0.001
Collective measures of feed additive efficiency						
Net profit / total cost %.	1.01 ± 0.22^{c}	7.49 ± 1.00^{b}	8.69 ± 0.73^{ab}	8.73 ± 0.08^{ab}	10.35 ± 0.22^{a}	0.001

^{abcd}*Means within the same row carrying different superscripts were significantly different at ($P \leq 0.05$).*

allyl-propyl-disulfide compounds present in it that compete with the insulin for metabolism, thus increasing the free insulin. Other mechanisms postulated are sulfur-containing compounds, including dialkyl disulfides, and their oxidized thiols can trap electrons from the body to act as antioxidants or phenolic acids produced antioxidant activity (48, 49).

Furthermore, broilers fed on the diets containing LLE showed a significant ($P = 0.001$) decrease in serum levels of total cholesterol, triglyceride, and LDL and a significant ($P = 0.001$) increase in HDL levels compared with the control group (**Table 5**). Similarly, serum levels of total cholesterol, triglyceride, and LDL cholesterol were significantly decreased in rabbits fed diets containing leek extract (6) and broilers that fed on diets containing leek powder as reported previously by Kamali et al. and Mahmoud et al. (33, 34) and broilers that fed on diets containing onion extract (38). The hypocholesterolemic and hypolipidemic effects of leek may be because of high levels of sulfur-containing compounds such as S-methylcysteine sulfoxide (50). Contrary to our results, Adjei et al. (44) showed no significant reduction in LDL, VLDL, total cholesterol, and triglycerides levels by dietary supplementation with varying levels of allicin in broilers.

The effect of diets containing LLE on economic measures of broilers is given in **Table 6**. We found a significant ($P = 0.001$) increase in feed costs, TVC, TC, return and net profit, and collective economic efficiency measures in all the groups fed on the diets containing LLE compared with the control group. Broilers fed on the diets containing 0.2% LLE showed the highest return, net profit values, and net profit/total cost with the lowest values for the control group.

CONCLUSION

Dietary supplementation with leek (*Allium ampeloprasum var. kurrat*) leaf extract in broilers provided a safe growth-promoting effect without any adverse effects in terms of biochemical parameters and carcass quality. Furthermore, the diets containing LLE can improve economic efficiency by increasing the return and net profit. Finally, LLE can act as an eco-friendly alternative for antibiotic growth promoters in the poultry industry.

DATA AVAILABILITY STATEMENT

The original contributions generated for this study are included in the article/supplementary materials, further inquiries can be directed to the corresponding author/s.

ETHICS STATEMENT

The animal study was reviewed and approved by Institutional Animal Care and Use Committee of Zagazig University, Egypt (ZUIACUC−2019), and all animal experiments were performed following recommendations described in The Guide for the Care and Use of Laboratory Animals in scientific investigations.

AUTHOR CONTRIBUTIONS

MB, AO, and HA-k: design of the experiment. MB, AO, RA, and MA: methodology, data collection, and analysis. HA-k, MB, AO, RA, and MA: writing of the manuscript. All authors have read and approved the manuscript.

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Effects of Phenolic-Rich Onion (*Allium cepa* L.) Extract on the Growth Performance, Behavior, Intestinal Histology, Amino Acid Digestibility, Antioxidant Activity, and the Immune Status of Broiler Chickens

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The effect of phenolic-rich onion extract (PROE), as a feed additive, was evaluated on the growth, carcass traits, behavior, welfare, intestinal histology, amino acid ileal digestibility "AID%," and the immune status of broiler chicks for 35 days. A total number of 400, 1-day-old broiler chicks (45.38 g ± 1.35) were allocated to four different treatments with 10 replicates each (100 chicks/treatment) consisting of: T1, basal diet without additives (control treatment) (PROE0); T2, basal diet + phenolic-rich onion extract (1 g/kg diet) (PROE1); T3, basal diet + phenolic-rich onion extract (2 g/kg diet) (PROE2); and T4, basal diet + phenolic-rich onion extract (3 g/kg diet) (PROE3). An increase in the final body weight "FBW," bodyweight gain "BWG," and feed consumption was observed ($P < 0.05$) at different PROE levels. Also, the thymus and bursa percentages were increased in the PROE2 and PROE3 treatments ($P < 0.05$). The chicks fed on PROE supplemented diets had increased frequency of feeding and drinking and showed comfortable behavior ($P < 0.05$) with lesser aggression ($P < 0.05$). Additionally, an increase was observed in the antioxidant enzyme activity, phagocytic %, phagocytic index, and serum lysozyme activity in PROE supplemented treatments, with the best outcome reported in the PROE3 treatment ($P < 0.01$). IgM was increased in the birds fed with PROE2 and PROE3 diets ($P < 0.01$). PROE supplementation increased the AID% of lysine and methionine ($P < 0.01$), PROE3 treatment increased the AID% of threonine ($P < 0.05$), and PROE2 and PROE3 treatments increased the AID% of leucine and isoleucine ($P < 0.05$). Besides, PROE2, and PROE3 treatments increased the villus height and width, mucosal thickness, and goblet cell count from the duodena, jejuna, and ilea ($P < 0.05$) compared to control treatment. Based on these results, we concluded

that the dietary addition of phenolic-rich onion extracts can improve the growth rate of broiler chicken by improving the AID% of amino acids and intestinal histology. Also, it can improve the welfare, antioxidant enzymes activity, and immune status of the birds. Phenolic-rich onion extracts can be used as a natural growth promoter in the poultry feed for good health and improved performance.

Keywords: broiler—chicken, onion extract, growth performance, behavior, immunity, gut histomorphology

INTRODUCTION

Feed additives are widely used in the poultry industry to support animal production traits and maintain their good health (1–8). Sub-therapeutic uses of antibiotics as growth promoters in poultry feed have caused controversies, such as drug resistance and residues in the meat (9). These side effects restricted their use in many countries, and on January 1, 2006, antibiotics were strictly prohibited in the poultry feed by the European Union. As a result, efforts are made to find other alternatives to improve broiler chickens' performance and increase their immunity. One of these alternatives was to use onion extract as a promoter of natural growth in poultry production to enhance productivity, control diseases, and improve their immunity. Onions (*Allium cepa* L.) are bulb vegetables that belong to the Liliaceae family. They are widely cultivated in many large-producing countries, such as India, the USA, and China and also used as a common medicinal plant and human food (10).

Onions have numerous biological activities, i.e., as an antioxidant, antimutagenic, and antibacterial (11–13). They have a high content of lectins (the most abundant proteins) along with amino acids cysteine and methionine (14). Dehydrated onions could significantly reduce the serum cholesterol in experimental mice with hypercholesterolemia (15). Similarly, diets supplemented with onions also reduced the serum cholesterol of male albino rats (16). The hypocholesterolemic effect of onion is due to the reduced cholesterol secretion or improved absorption of high-density lipoprotein (HDL) by the liver (17). Onions are a rich source of flavonoids, polyphenols, glycosides, anthocyanins, allicin, and quercetin (18). They are used as a natural antioxidant to prevent oxidation of meat. This antioxidant activity is due to their capacity to scavenge for free radicals and give out electrons or hydrogen atoms (19). The antioxidant activity has been associated with the total phenol content, which is high in red onions, and the flavonoids, mainly quercetin, which is high in yellow onions (347 mg/kg quercetin) (20). Quercetin has powerful antioxidant activity that can protect against diseases caused by oxidative stress (21, 22). One of the studies (23–25) has shown the positive effect of synthetic quercetin on the egg quality and laying performance of hens. Further, a diet supplemented with quercetin reduced the thiobarbituric acid-reactive substance (TBARS) value in broiler meat (26) and pork pie (27). In ruminant nutrition, onion extract improved the feed intake and had a beneficial effect on the leptin and ghrelin concentrations, total antioxidant capacity, and the lambs performance (28).

Despite these results, there was a lack of data on the effect of phenolic-rich onion extract supplementation on chickens' growth, behavior, intestinal tissues, amino acid digestion, and immunity. Thus, the current study was planned to highlight the effect of using different levels, including 1, 2, or 3 g/kg diet of phenolic-rich onion extracts as a natural feed additive, on the broiler chickens' growth performance, carcass traits, behavior, tonic immobility, intestinal histology, amino acids, amino acids' apparent ileal digestibility%, antioxidant enzymes, and their immunity.

MATERIALS AND METHODS

Preparation and Description of the Phenolic-Rich Onion Extracts

The phenolic-rich onion extract was obtained by the technique (29) described previously (30). Fresh red onions (*Allium cepa* L.) were obtained from a native market of Zagazig City, Egypt. The leaves plus the outer skin of fresh onions were closely secluded, and 100 g of onion bulb was mixed and homogenized in 70% methanol (250 mL); this homogenate was stirred for 2 h and then filtered through Whatman No. 2 filter paper. Methanol was removed from the extract using vacuum in a BüCHI-water bath-B-480 evaporator at 45°C, followed by lyophilization in a freeze-Dryer (Thermo-electron Corporation–Heto power dry LL 300 Freeze dryer). Resulting fractions were denoted as phenolic-rich onion extracts (PROE).

The total phenolic compounds (TPCs) were determined in the PROE (1 mg/mL) as described previously (31), using a reagent of Feline-Ciocalteu (diluted with water 1:10, V/V). The calibration equation for gallic acid was $y = 0.001x + 0.0563$ ($R^2 = 0.9792$), where y and x were the absorbance and concentration of gallic acid in μg/mL, respectively.

Total flavonoids (TFs) were evaluated following the protocol stated previously (32). Quercetin was used to obtain the standard curve (10–500 μg/mL) with total flavonoid contents stated as quercetin equivalent (QE) and calculated based on the calibration curve: $y = 0.0012x + 0.008$ ($R^2 = 0.944$), where y was the absorbance and x was the concentration of quercetin in μg/mL.

Birds

The experiment was done in the poultry research unit of the veterinary medicine faculty, Zagazig University, Egypt, to show the influence of onion extract as a feed additive on the productive parameters, carcass traits, behavior, small intestine histology, amino acids AID%, antioxidant enzymes, and immune status of broiler chickens. The experimental protocol was approved by

the Ethics of the Institutional Animal Care and Use Committee of Zagazig University, Egypt (ZUIACUC−2019), and all animal experiments were performed following the recommendations described in "The Guide for the Care and Use of Laboratory Animals in scientific investigations."

A total number of 400 Ross-308 broiler chicks, aged 1 day, were obtained from a hatchery (Dakahlia Poultry, Mansoura, Egypt) and used in this experiment. They were weighed on arrival (45.38 ± 1.35 g) and further reared using dehydrated solution and neomycin broad-spectrum antibiotic for 3 days until they attained an average weight of 57.01 ± 1.77 g. Birds were raised in a naturally ventilated open house with sawdust as litter. The room temperature was controlled and regulated thermo-statically by two heaters. Room temperature during the first week was established at 34°C and gradually was reduced by 3°C every week until it reached 24°C. The lighting program for the first week was 24 h a day and then was changed to 16 h of light and 8 h of darkness, within 7–35 days. Standard health and vaccination programs against New Castle and Gumboro diseases were conducted. Chicks were daily observed and checked for any syndromes without the mortalities, during the entire experiment. After the study was completed, the remaining birds were released.

Experimental Design and Diets

Birds were randomly allocated to four treatments (100 chicks for each treatment), with 10 replicates each. The experimental treatments consisted of: T1, basal diet without additives (control treatment) (PROE0); T2, basal diet + phenolic-rich onion extract (1 g/kg diet) (PROE1); T3, basal diet + phenolic-rich onion extract (2 g/kg diet) (PROE2); and T4, basal diet + phenolic-rich onion extract (3 g/kg diet) (PROE3). The experiment was extended to 35 days with free access to feed and water. These diets were given in the mashed form and formulated according to the Ross Manual Guide (33), as presented in **Table 1**. Different nutrients (DM, CP, and EE) were determined in the experimental feedstuffs and the diets as described by AOAC (34).

TABLE 1 | Proximate and chemical composition of the basal diets (%).

Ingredient	Starter stage (4–10 day)	Grower stage (11–23 day)	Finisher stage (24–35 day)
Yellow corn	55.64	59.43	62.43
Soybean meal, 48%	31.9	27.9	23.03
Corn gluten, 60%	5.98	5.57	6.57
Soybean oil	2	3	4
Calcium carbonate	1.3	1.2	1.05
Calcium dibasic phosphate	1.5	1.3	1.3
Common salt	0.15	0.15	0.15
Premix*	0.3	0.3	0.3
DL- Methionine, 98%	0.23	0.2	0.18
Lysine, HCl, 78%	0.47	0.42	0.46
Choline	0.07	0.07	0.07
Threonine	0.1	0.1	0.1
Phytase	0.01	0.01	0.01
Antimycotoxin	0.1	0.1	0.1
NaCo3	0.25	0.25	0.25
Chemical analysis (%)			
ME kcal/kg diet	3008.93	3104.47	3210.19
CP	23.43	21.52	20.09
Ca	0.97	0.87	0.81
Available P	0.48	0.43	0.41
Lysine	1.44	1.29	1.19
Methionine	0.56	0.51	0.48
Threonine	0.97	0.88	0.81

**premix per kg of diet: vitamin A, 1 500 IU; vitamin D3, 200 IU; vitamin E, 10 mg; vitamin K3, 0.5 mg; thiamine, 1.8 mg; riboflavin, 3.6 mg; pantothenic cid, 10 mg; folic acid, 0.55 mg; pyridoxine, 3.5 mg; niacin, 35 mg; cobalamin, 0.01 mg; biotin, 0.15 mg; Fe, 80 mg; Cu, 8 mg; Mn, 60 mg; Zn, 40 mg; I, 0.35 mg; Se, 0.15 mg.*

ME, metabolizable energy; CP, crude protein; EE, ether extract; Ca, calcium; P, phosphorus.

Growth Performance

When the birds were 4 days old, their initial weights were recorded individually, and the average body weight from each treatment was determined at 10, 23, and 35 days. The body weight gain at each time interval was calculated as the difference between the final and the initial body weight. The average feed intake per bird in each replicate was estimated as the difference between the amount of given food and the residue left, which was then divided by the number of birds in each replicate.

Average daily gain (ADG) (g/bird/day) = final weight gain-initial weight/(number of birds × number of days).

Average daily feed intake (ADFI) (g/bird/day) = cumulative feed intake/(number of birds × number of days).

The feed conversion ratio was assessed as stated by (35) and was calculated as follows; FCR = feed intake (g)/weight gain (g).

Behavioral Data

Behavioral Observation

The behavioral observations were commenced once the birds attained the age of 2 weeks. Video cameras were installed in the pens using scan sampling techniques, and the behavioral observation was performed as follows: each treatment was observed weekly, twice a day (30 min/each time), for 6 days and 6 h for each week. The behavioral patterns were recorded from 7–9 a.m. to 2–4 p.m. The recorded behavioral patterns were feeding, drinking, foraging, sitting, walking, standing, feather preening, and other comfort behaviors, including wing/leg stretching and/or wing flapping, head shaking or body shaking, and aggression. A comprehensive explanation for those patterns was presented previously (36).

For each examination, the number of chicks detected for each behavioral activity was further recorded every 5 min, and results were calculated as the percentage of birds performing the behavior/total observed birds (37).

Tonic Immobility (TI)

For each treatment, 15 birds were examined for tonic immobility on the 33rd and 34th day (38). TI was forced by putting the broiler on its back with its head hanging in a wooden cradle, resembling the letter U (39), and the broiler was gently reined for 10 s. The examiner took a seat at a distance of about 1 m from

the bird without doing unnecessary noise and movements and commenced a stopwatch to register the latencies until the bird adjusted him/herself after the removal of restraint by hand. If they corrected their position in < 10 s, the restraining step was repeated, since there was no induction of tonic immobility. If TI was not achieved after three trials, the time of TI was recorded as 0 s. On the contrary, if the chick failed to correct him/herself after 600 s, the test was ended, and the TI duration was recorded as 600 s (3, 40).

Carcass Traits

On day 35, ten birds from each treatment, with an average body weight that corresponded to the respective treatment, were selected for the carcass evaluation. They were allowed to fast for 12 h and then weighed, slaughtered until they completely bled out, de-feathered, eviscerated, and finally weighed to determine the dressing percentage. The weights of the dressed carcass, viscera, intestine, liver, heart, and spleen were determined and expressed as a percentage of live body weight.

Amino Acids Ileal Digestibility

In order to determine the amino acids ileal digestibility, titanium dioxide was used, which is an indigestible indicator substance that does not affect the digestibility of nutrients and has a recovery rate of almost 100%. It was added to the feed at 0.5% dosage (5 kg/t of feed) and used for 5 days. Every assay diet was offered freely to four replicates (five chickens per replicate) between 35 and 40 days of their age. All birds were slaughtered at the end of the experiment, and the contents of the lower half of the ileum were collected in a plastic vessel by gentle flushing with distilled water. Ileal digesta of the chickens within a pen was collected, assembled, and dried by freezing. Dried ileal digesta samples were ground and passed through a 0.5 mm sieve and stored in airtight vessels at -20°C, until further chemical analysis.

The amino acid concentration in the diet and ileal digesta samples were assessed according to Li et al. (41) and Siriwan et al. (42). Tryptophan was determined separately according to Ravindran and Bryden (43). Titanium dioxide was estimated following the procedures described by Fenton and Fenton (44). AID% of amino acids was estimated by the following equation: $\text{AID}\ (\%) = 100 - [(\text{Ti}_{(\text{diet})} \times \text{AA}_{(\text{ileum})})/(\text{TI}_{(\text{ileum})} \times \text{AA}_{(\text{diet})}) \times 100]$.

Where Ti (diet) was the concentration of titanium dioxide in the diet, Ti (ileal) was the concentration of titanium dioxide in ileal digesta, AA (ileal) was the concentration of the test AA in ileal digesta sample, and AA (diet) was the concentration of the test AA in the diet.

Sample Collection

At the end of the experiment, birds were made to fast for 12 h and then euthanized by cervical dislocation (45); later blood samples were collected from five birds randomly selected from each experimental treatment. The first sample was collected for hematological analysis (Leukogram) in an EDTA tube; the second sample was collected in heparinized tubes for phagocytosis; meanwhile, the third sample was drawn into a clean, dry centrifuge tube without anticoagulant, which was left to clot at room temperature and then centrifuged for 5 min at 3000 rpm to separate the serum for further clinicobiochemical analysis, including the antioxidant defense system, and selective immunological parameters, such as IgM and lysozyme concentrations. Samples were taken from different parts of the gut for histological examination.

Hematological Studies

The leukocytic count for chicken's blood was done using an improved Neubauer hemocytometer and a special diluent, namely, Natt and Herrick solution (46). To count the differential leukocytes and detect the abnormalities in RBCs morphology, blood films were prepared, fixed by methyl alcohol, and then stained with Giemsa stain as described previously (47).

Clinicobiochemical Analysis

Antioxidant defense systems, such as serum levels of CAT and SOD were measured, as stated by Aebi (48) and Nishikimi et al. (49), respectively. The serum level of reduced glutathione (GSH) was measured by the method of Beutler (50). The concentration of serum lysozyme was determined according to Lie et al. (51). The concentration of IgM was determined using chicken ELISA kits of ABCAM Co. with CAT.NO. AB157692, according to the manufacturer's instruction.

Phagocytic Activity

Phagocytic activity was measured by separating white blood cells (WBCs) from peripheral blood using Ficoll–Histopaque density gradient centrifugation, as described by Hampton et al. (52). We used heat-inactivated *Candida albicans* (*C. Albicans*) in 24-well gelatin/plasma-coated plates to determine the phagocytic capacity of leukocytes, according to the method of Elmowalid (53). A minimum of three slides/bird was assessed, and the cells containing (>10, >20, and >30) FITC-labeled yeast in at least 20 microscopic fields (containing at least 200 cells) were calculated. *C. Albicans* numbers/100 phagocytes were evaluated to yield the phagocytic index of each bird.

Histopathological Examination of the Small Intestine

The specimens from the intestine were stored in 10% neutral buffered formalin (fixation) and managed until further histological analysis. The specimens were dehydrated with an ascending grade of ethanol (75–100%), then treated with xylol I, II, and later embedded in paraffin and finally sliced into 4 μm longitudinal and cross-sections using a microtome (Leica RM 2155, England). Slides were stained using hematoxylin and eosin by following the method of Bancroft et al. (54). Camera microscope AmScope® software (AmScope digital camera-attached Ceti England microscope) was used for morphometric analysis as follows: villus height was measured (μm) from the tip to the base of villus and diameter. Also, muscular thickness, the thickness of the submucosa layer, the goblet cell numbers per area of the epithelium layer, and the intraepithelial leucocytes were considered as well.

Statistical Analysis

Data were analyzed with a one-way analysis of variance (ANOVA) using the GLM procedure in SPSS (SPSS Inc., Chicago, IL, USA) after Shapiro-Wilk's test was used to verify the normality and Levene's test was used to verify homogeneity of variance components between experimental treatments. The replicate, or the individual bird, served as an experimental unit for all statistical analyses. The significant difference between the mean values was tested using Duncan's multiple range test (55), and the variation in the data was expressed as pooled SEM, and the significance level was set at $P < 0.05$.

RESULTS

Description of the Phenolic-Rich Onion Extract

Onion bulbs contain a high percentage of TPCs. The total phenolic compounds in the onion were 70.55 mg GAE g-1 DW with flavonoids being the main series of these phenolic compounds, representing a high proportion of TPCs in onion (11.8 mg QE g^{-1} DW) (data not shown).

Growth Performance

The growth performance of Broilers was presented in **Table 2**. During the starter period, different levels of PROE could increase ($P < 0.05$) the BW, ADG, and ADFI with no effect on FCR when compared to PROE0 treatment. Throughout the grower period, broilers fed with PROE2, and PROE3 diets showed an increase ($P < 0.05$) in the BW and ADG, while broilers fed on the PROE1 diet showed no significant difference compared to the PROE0 treatment. PROE supplemented treatments significantly improved the ADFI and FCR ($P < 0.05$). The finisher period and overall performance results showed that different levels of PROE increased ($P < 0.05$), the BW, ADG, and ADFI. Also, during the finisher period, the FCR was decreased ($P = 0.04$) in PROE1 and PROE2 treatments; however, the overall FCR was not significantly affected ($P = 0.31$). The final body weight was highest in the PROE3 treatment, while the least final body weight was observed in the PROE0 treatment group.

Behavioral Data

The impact of PROE supplementation on the broiler's behavior is presented in **Table 3**. The frequency of ingestive behavior (feeding and drinking) was significantly increased by PROE supplementation. However, the impact of PROE supplementation was not significant on other behaviors, including foraging, sitting, walking, and standing compared to the control treatment. Feather preening and other comfort behaviors were improved ($P < 0.05$) in PROE-supplemented treatments, while abnormal behavior (feather pecking and aggression) was increased ($P < 0.05$) in the PROE0 control. Regarding the tonic immobility (TI), TI duration was not significantly decreased ($P > 0.05$) with PROE supplementation, and TI attempts were not significantly different between the treatments ($P > 0.05$).

Carcass Traits

The effect of PROE supplementation on the carcass trait percentage is shown in **Table 4**. Dressing percentage was not affected ($P > 0.05$), while the percentage of thymus and bursa was increased ($P < 0.05$) in PROE2 and PROE3 treatments but was not significantly ($P > 0.05$) increased in the PROE1 treatment compared to the PROE0 control. Also, no significant ($P > 0.05$) changes in the percentage of the intestine, gizzard, liver, spleen, and heart were observed between PROE supplemented and the PROE0 treatment.

Ileal Digestibility of Amino Acids

The impact of different onion extract levels on the AID% of amino acids is highlighted in **Table 5**. The results revealed that different levels of PROE supplementation statistically improved ($P < 0.01$) the AID% of lysine and methionine but did not have a significant influence ($P > 0.05$) on the AID% of tryptophan and valine when compared to the PROE0 treatment. The AID% of threonine was improved ($P < 0.01$) in PROE3 treatment, while it did not differ ($P > 0.05$) in PROE1 and PROE2 treatments when compared to the control. Also, a significant improvement in AID% of leucine and isoleucine ($P < 0.01$) and a significant increase in AID% of arginine ($P = 0.03$) was observed in PROE2 and PROE3 diets compared to the PROE0 control.

Serum Antioxidant Activity

The effect of PROE on the serum antioxidant activity of broiler chickens is represented in **Table 6**. Compared to the control treatment, a significant increase was observed in the CAT, SOD activity, and GSH level in all PROE supplemented treatments ($P < 0.01$).

Immunological Parameters

The effect of PROE on the selective immunological parameters and leukogram data of broiler chickens is highlighted in **Table 6**. Different levels of onion extract supplementation increased phagocytic%, phagocytic index, IgM level, and lysozyme activity ($P < 0.01$). The phagocytic activity was normal in the control treatment while it was mild, moderate, and marked in PROE1, PROE2, and PROE3 treatments, respectively (**Figure 1**). These changes were greater in the PROE3 treatment, followed by the PROE2 treatment. Meanwhile, in all the PROE supplemented treatments, the total leucocytic count (TLC), neutrophils, eosinophil, and lymphocytes were not significantly changed ($P > 0.05$), but the monocytes were increased ($P < 0.01$).

Histological Finding

A representative photomicrograph of H&E-stained small intestine sections of broiler chickens in 40 × magnification is shown in **Figures 2–4**. Sections of the duodenal segments from PROE0 to PROE1 treatment showed separated tall and arranged intestinal villi with a free lumen (**Figures 2A,B**), whereas duodenal segments from PROE2 treatment revealed markedly active intestinal villi and crypts, characterized by limited goblet cells metaplasia, increased sizes and rows of enterocytes with arranged lamina propria besides some desquamated villi (**Figure 2C**) while the chickens fed with the PROE3 diet revealed

TABLE 2 | The effect of PROE supplementation on the growth performance parameters of broiler chickens.

Item	PROE0	PROE1	PROE2	PROE3	SEM	*P-value*
Initial wt. (g)	57.01	58.05	58.06	58.61	0.770	0.302
Starter Period (4–10 day)						
BW (g)	181.13^{b}	218.06^{a}	222.50^{a}	221.11^{a}	5.430	0.001
ADG (g/bird/d)	17.73^{b}	22.85^{a}	23.49^{a}	23.21^{a}	0.731	0.001
ADFI (g/bird/d)	26.89^{b}	33.08^{a}	33.51^{a}	32.82^{a}	0.872	0.002
FCR	1.52	1.45	1.43	1.42	0.045	0.195
Grower Period (11–23 day)						
BW (g)	718.89^{b}	754.44^{b}	806.11^{a}	797.22^{a}	17.623	0.001
ADG (g/bird/d)	41.36^{b}	41.26^{b}	44.89^{a}	44.31^{a}	0.599	0.015
ADFI (g/bird/d)	64.25^{b}	72.78^{a}	73.49^{a}	73.07^{a}	1.447	0.033
FCR	1.55^{b}	1.77^{a}	1.64^{a,b}	1.65^{a,b}	0.055	0.074
Finisher Period (24–35 day)						
BW (g)	1586.22^{b}	1868.03^{a}	1870.43^{a}	1906.27^{a}	40.531	0.003
ADG (g/bird/d)	72.27^{b}	92.79^{a}	88.69^{a}	92.42^{a}	2.671	0.002
ADFI (g/bird/d)	140.97^{b}	152.68^{a,b}	150.74^{a,b}	166.43^{a}	3.375	0.025
FCR	1.95^{a}	1.64^{b}	1.70^{b}	1.80^{a,b}	0.050	0.055
Overall performance						
FBW(g)	1586.22^{b}	1868.03^{a}	1870.43^{a}	1906.27^{a}	40.532	0.001
ADG (g/bird/d)	47.78^{b}	56.56^{a}	56.63^{a}	57.73^{a}	3.564	0.001
ADFI (g/bird/d)	84.85^{b}	94.06^{a}	93.72^{a}	99.28^{a}	4.696	0.029
FCR	1.78	1.66	1.65	1.72	0.039	0.318

Means within the same row carrying different superscripts are significantly different at ($P < 0.05$).
PROE0, basal diet without additives; PROE1, basal diet + phenolic rich onion extract (1 g/kg diet); PROE2, basal diet + phenolic rich onion extract (2 g/kg diet); PROE3, basal diet + phenolic rich onion extract (3 g/kg diet).
BW, body weight; ADG, Average daily gain; ADFI, Average daily feed intake; FCR, feed conversion ratio.
SEM, standard error of mean.

markedly thin, tall, and separate villi with mild goblet cell proliferation (**Figure 2D**).

Sections from the jejunal segments of PROE0 treatment showed free lumen with nearly normal villus structures (**Figure 3A**), while sections of the jejunal segments from the PROE1 treatment group showed free lumen and a few denuded villi tips with increased intestinal crypt layer depth besides partial fusion (**Figure 3B**). The jejunal segments from PROE2 treatment showed separate tall villi and markedly serrated surfaces with goblet cell metaplasia besides a partial fusion of some villus (**Figure 3C**), whereas in the PROE3 treatment, an increase in intestinal gland layer was observed besides partially destructed villi with some fused villi (**Figure 3D**).

Sections of the ileal segments from PROE0 treatment showed free lumen and nearly a normal limit gut-associated lymphoid follicles with a few denuded villi (**Figure 4A**), while the ileal sections from the PROE1 treatment showed thickened and serrated villi due to the enterocytes and goblet cell metaplasia along with narrowing of the lamina propria (**Figure 4B**), the sections from the PROE2 treatment showed markedly hyperplastic intestinal gut-associated lymphoid follicles (**Figure 4C**), and the sections from the PROE3 group showed marked fusion villi due to an increase in enterocytes proliferation and goblet cell metaplasia (**Figure 4D**).

Small Intestine Morphometric Measures

The results of morphometric intestinal measures are shown in **Table 7**. Birds fed with PROE2 and PROE3 diets showed ($P < 0.05$) an increase in the villus height and width, mucosal thickness, and goblet cell numbers in different sections of the small intestine, while different levels of onion extract supplementation significantly increased the duodenal and ileal crypt depth. In the PROE3 treatment group, an improvement ($P < 0.05$) was observed in the duodenal, jejunal, and ileal intra-epithelium lymphocytic linked-cells infiltration and the ileal crypt depth, compared to PROE0 control.

DISCUSSION

Our study assessed the effects of using phenolic-rich onion extract, containing high amounts of TPCs and TFCs, as a natural feed additive on the growth performance, behavior, TI, carcass traits, AID% of amino acids, intestinal tissues, antioxidant enzymes, and immunity of broiler chickens. The total phenolic compounds and total flavonoids are recorded as 70.55 mg GAE g-1 DW and 11.8 mg QE g-1 DW, respectively. The total phenolic compounds were previously recorded in different types of onions (56), which ranged from 4.6 to 74.1 mg/g GAE for red, purple,

TABLE 3 | The effect of PROE supplementation on various behaviors (% means of broilers/5 min) and tonic immobility of broiler chickens.

Behavioral patterns	PROE0	PROE1	PROE2	PROE3	SEM	*P*-Value
Feeding	28.39^{b}	39.19^{a,b}	46.98^{a}	51.79^{a}	10.623	0.016
Drinking	16.09^{b}	25.65^{a}	28.58^{a}	28.84^{a}	9.512	0.085
Foraging	21.45	19.40	17.37	15.43	3.550	0.645
Resting	59.57	62.49	68.05	71.66	3.044	0.529
Standing	25.65	22.78	18.85	22.54	10.232	0.609
Walking	24.56	23.56	18.10	20.33	8.225	0.415
Feather preening	25.59^{b}	27.72^{a,b}	21.07^{b}	34.76^{a}	6.914	0.077
Other comfort*	11.07^{b}	16.97^{b}	18.85^{a,b}	28.46^{a}	4.581	0.019
Aggression	2.31^{a}	0.68^{b}	0.36^{b}	0.00^{b}	0.750	0.038
Tonic immobility						
TI attempts**	2.16	2.37	2.33	2.66	0.041	0.890
TI duration (sec)	122.33	100.5	84.83	72.16	11.635	0.410

Means within the same row carrying different superscripts are significantly different at (P < 0.05).
**others comfort behavior included: wing flapping, wing/Leg stretch, body shaking, and head shaking.*
***TI, Tonic immobility.*
PROE0, basal diet without additives; PROE1, basal diet + phenolic rich onion extract (1 g/kg diet); PROE2, basal diet + phenolic rich onion extract (2 g/kg diet); PROE3, basal diet + phenolic rich onion extract (3 g/kg diet).

TABLE 4 | The effect of PROE supplementation on the carcass traits percentages relative to the live body weight.

Item	PROE0	PROE1	PROE2	PROE3	SEM	*P-value*
Intestine	5.14	6.36	6.05	6.49	0.171	0.335
Spleen	0.09	0.08	0.09	0.09	0.009	0.881
Bursa	0.09^{b}	0.14^{a,b}	0.17^{a}	0.18^{a}	0.022	0.026
Thymus	0.33^{b}	0.41^{a,b}	0.48^{a}	0.53^{a}	0.035	0.018
Gizzard	2.47^{a,b}	2.97^{a}	2.79^{a,b}	2.35^{b}	0.081	0.074
Liver	2.02	2.10	2.21	1.92	0.077	0.708
Carcass	64.81	63.46	63.63	64.02	0.425	0.725
Heart	0.41	0.44	0.39	0.39	0.023	0.841

Means within the same row carrying different superscripts are significantly different at (P < 0.05).
PROE0, basal diet without additives; PROE1, basal diet + phenolic rich onion extract (1 g/kg diet); PROE2, basal diet + phenolic rich onion extract (2 g/kg diet); PROE3, basal diet + phenolic rich onion extract (3 g/kg diet).

white, and green onion cultivars (57). Flavonoids' data was also consistent with the other publications on the TPCs (58).

Growth Performance and Digestibility

Our results showed a positive effect of PROE as a dietary supplement on BW, ADG, and ADFI of broiler chickens, and the best results were with the highest level of supplementation. This overall improvement in the growth criteria may be due to the improvement in birds' health, amino acid digestibility, intestinal health, and an increase in their absorptive surface. Herbal products control the growth of many pathogenic and non-pathogenic microbes in the broiler intestine, increasing the efficiency of feed use, and improving the growth rate (59). Onion extract also contains active compounds, including phenols, polyphenols, terpenoid, polypeptides, lectin, alkalis, and essential oils that stimulate digestion (60, 61) and promote growth. Onion also stimulates the synthesis of bile acid and pancreatic enzyme activity, mainly lipase and amylase, ultimately improving fat digestion (62). Moreover, onions contain non-digestible prebiotic and fructooligosaccharide (FOS) components, which are fermented by bifidobacteria to further maintain intestinal and colon health (63). Another reason for the improved AID ratio of amino acids (26) and increased feed consumption (17, 64) could be the increased retention of the total pathway of energy and ether extract. The increased feed consumption is due to the favorable taste and flavor of onion extract (65). Onion has sulfur-containing compounds, including *S-Methylcysteine sulfoxide* (SMCS) and *S-allyl cysteine sulfoxide* (SACS) that reduces the blood sugar levels and stimulates growth by accelerating the glucose flow into the tissues and increasing the thyroid activity (17).

Goodarzi and Nanekarani (66) assessed the addition of 1 and 2% onion extract in the drinking water of broiler chickens and reported an increased ADFI in 1% onion extract supplementation during the grower and total period. Farahani et al. (63) assessed 1% onion extract supplementation in the drinking water of two strains of broiler chickens

TABLE 5 | The effect of PROE supplementation on the apparent ileal digestibility coefficient (AID%) of amino acids.

Item	PROE0	PROE1	PROE2	PROE3	SEM	*P-value*
Lysine	83.54^{c}	83.95^{b}	85.00^{a}	85.00^{a}	0.180	0.001
Methionine	88.40^{d}	88.91^{c}	89.07^{b}	89.83^{a}	0.074	0.003
Threonine	85.18^{b}	84.44^{c}	84.32^{c}	85.68^{a}	0.115	0.001
Tryptophan	87.02	86.54	86.54	86.71	0.244	0.528
Arginine	89.08^{a,b}	88.99^{b}	89.25^{a}	89.22^{a}	0.058	0.035
Valine	84.83	84.91	84.83	84.83	0.077	0.904
Leucine	90.05^{c}	90.05^{c}	90.21^{b}	90.63^{a}	0.046	0.004
Isoleucine	84.91^{b}	84.79^{b}	85.15^{a}	85.27^{a}	0.093	0.001

Means within the same row carrying different superscripts are significantly different at ($P < 0.05$).
PROE0, basal diet without additives; PROE1, basal diet + phenolic rich onion extract (1 g/kg diet); PROE2, basal diet + phenolic rich onion extract (2 g/kg diet); PROE3, basal diet + phenolic rich onion extract (3 g/kg diet).

TABLE 6 | The effect of PROE supplementation on the antioxidant activity, selective immunological parameters, and Leukogram of broiler chickens.

Item	PROE0	PROE1	PROE2	PROE3	SEM	*P-Value*
Antioxidant indices						
Serum CAT (U/L)	556.67^{b}	582.33^{a}	593.33^{a}	593.67^{a}	4.175	0.001
Serum SOD (U/mL)	23.40^{c}	29.80^{b}	37.90^{a}	40.87^{a}	2.496	0.001
Serum GSH(mmol/L)	3.40^{c}	6.93^{b}	8.43^{a}	9.03^{a}	0.654	0.002
Immunological parameters						1.81
Phagocytic%	54.00^{d}	60.00^{c}	64.00^{b}	70.00^{a}	3.149	0.004
Phagocytic index	2.73^{d}	3.60^{c}	4.43^{b}	5.23^{a}	0.238	0.001
IgM (ng/mL)	38.33^{c}	44.00^{b,c}	49.00^{b}	58.67^{a}	1.047	0.001
Lysozyme (mg/L)	1.18^{d}	1.46^{c}	1.81^{b}	2.06^{a}	0.054	0.002
Leukogram						
TLC ($\times 10^3/\mu L$)	2.38	2.46	2.62	2.71	0.715	0.715
Neutrophils ($\times 10^3/\mu L$)	0.23	0.23	0.23	0.22	0.024	0.980
Eosinophils ($\times 10^3/\mu L$)	0.03	0.04	0.04	0.03	0.002	0.970
Lymphocytes ($\times 10^3/\mu L$)	1.98	1.97	2.08	2.13	0.361	0.945
Monocytes ($\times 10^3/\mu L$)	0.12^{c}	0.21^{b}	0.26^{a}	0.31^{a}	0.012	0.001

Means within the same row carrying different superscripts are significantly different at ($P < 0.05$).
PROE0, basal diet without additives; PROE1, basal diet + phenolic rich onion extract (1 g/kg diet); PROE2, basal diet + phenolic rich onion extract (2 g/kg diet); PROE3, basal diet + phenolic rich onion extract (3 g/kg diet).

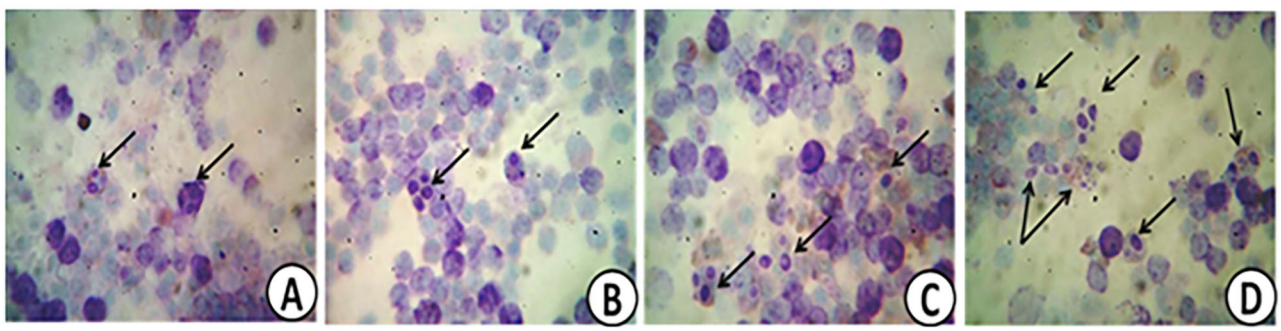

FIGURE 1 | Representative images showing normal **(A)**, mild **(B)**, moderate **(C)**, and marked **(D)** phagocytic activities in PROE0, PROE1, PROE2, and PROE3 treatments, respectively.

(Ross and Cobb) and confirmed the positive effects of onion extract on the growth and blood parameters of both strains.

Aditya et al. (26) demonstrated that BWG is generally higher in broiler chickens fed with 7.5 g/kg of onion extract, and the feed intake was increased by supplementing 5, 7.5, and 10 g of onion

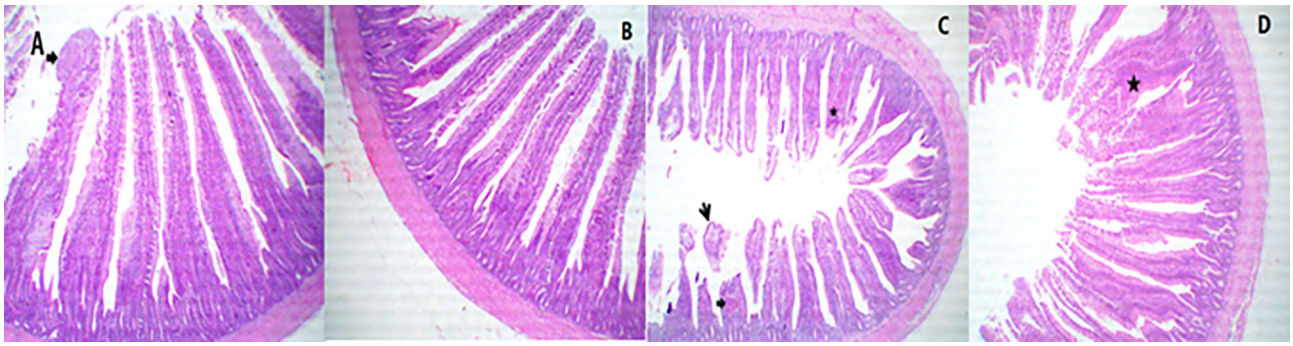

FIGURE 2 | A representative photomicrograph of 40× magnification H&E stained small intestine sections of the broiler chickens. The sections from the duodenal segments from PROE0group showed separate tall and arranged intestinal villi with (arrow) free lumen **(A)**, the duodenal segments from PROE1 group showed apparently separated tall and arranged intestinal villi with free lumen **(B)**, the duodenal segments from PROE2 group revealed marked active intestinal villi and crypts which characterized by limited goblet cells metaplasia (star)' increased sizes and rows of enterocytes (thick arrow) with arranged lamina propria beside some desquamated villi (small arrow) **(C)**. Chicken fed on PROE3 diet revealed marked thin, tall, and separate villi with mild goblet cell proliferations (star) **(D)**.

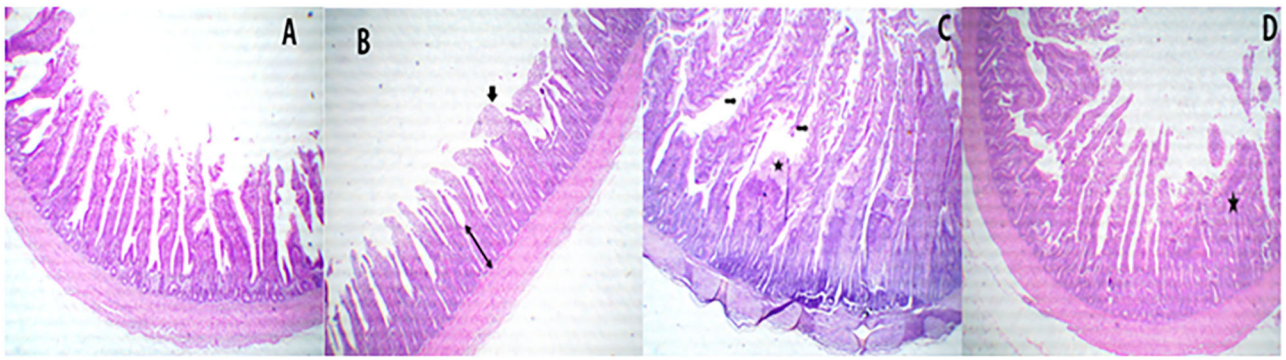

FIGURE 3 | A representative photomicrograph of 40× magnification H&E stained small intestine sections of the broiler chickens. The sections from the jejunal segments of PROE0 group showed free lumen with nearly normal villus structures **(A)**, the sections from the jejunal segments of PROE1 group showed free lumen, a few denuded villi tips with increase intestinal crypt layer depth (towheads arrow) besides partial fusion (arrow) **(B)**, and the jejunal segments from PROE2 group showed separate tall villi and marked serrated surfaces (arrows) with goblet cell metaplasia (star) beside partial fusion some villus **(C)**. In addition to, increased intestinal glands layer besides partial destructed villi with some fusion's villi (star) were observed in PROE3 group **(D)**.

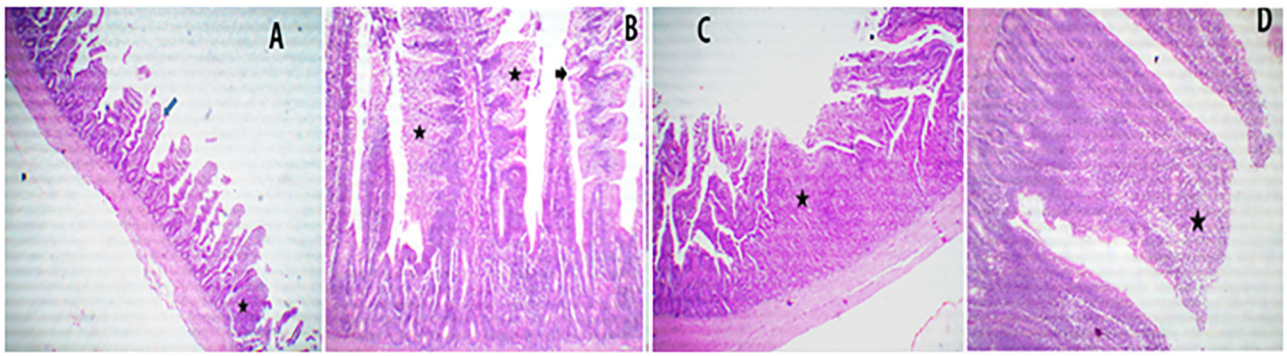

FIGURE 4 | A representative photomicrograph of 40× magnification H&E stained small intestine sections of the broiler chickens. The sections from the ileal segments of PROE0 group showed free lumen and nearly normal limit gut associated lymphoid follicles (star) with a few denuded villi (arrow) **(A)**, the sections from ileal segments of PROE1 group showed thickened and serrated (arrow) of villi due to enterocytes and goblet cell metaplasia (stars) with narrowing of the lamina propria **(B)**. The sections from PROE2 group showed marked hyperplastic intestinal gut associated lymphoid follicles (star) **(C)**. The sections from PROE3 showed marked fusion villi due to increase enterocytes proliferations and goblet cells metaplasia (star) **(D)**.

TABLE 7 | The effect of PROE supplementation on the morphometric measures of the small intestine of broiler chickens.

Item	PROE0	PROE1	PROE2	PROE3	SEM	*P-Value*
Duodenum						
Villous height	966.10^{b}	966.10^{b}	1366.10^{a}	1399.44^{a}	70.750	0.002
Villous width	187.19^{c}	203.86^{b,c}	270.53^{a,b}	337.19^{a}	27.194	0.001
Crypt depth	120.70^{c}	187.37^{b}	220.70^{a}	220.70^{a}	47.771	0.001
Mucosal depth	137.37^{c}	150.70^{b,c}	197.37^{a,b}	237.37^{a}	22.484	0.004
Goblet cells count	25.00^{b}	29.33^{b}	49.33^{a}	52.67^{a}	6.223	0.028
IELI	175.67^{b}	309.00^{a,b}	375.67^{a,b}	509.00^{a}	27.594	0.025
Jejunum						
Villous height	1127.63^{b}	1127.63^{b}	1427.63^{a}	1560.96^{a}	39.961	0.001
Villous width	171.30^{b}	211.30^{b}	264.64^{a}	277.97^{a}	21.712	0.004
Crypt depth	115.29^{c}	171.96^{b}	198.62^{a,b}	221.96^{a}	25.005	0.001
Mucosal depth	188.62^{c}	195.29^{b,c}	238.62^{b}	288.62^{a}	14.130	0.001
Goblet cells count	17.33^{c}	20.67^{c}	27.33^{b}	37.33^{a}	10.890	0.001
IELI	156.33^{b}	173.00^{b}	199.67^{b}	256.33^{a}	28.321	0.003
Ileum						
Villous height	937.85^{b}	971.19^{b}	1404.52^{a}	1504.52^{a}	35.066	0.001
Villous width	153.48^{b}	203.48^{a,b}	253.48^{a}	253.48^{a}	25.505	0.011
Crypt depth	138.78^{b}	172.11^{a,b}	188.78^{a,b}	205.45^{a}	17.406	0.071
Mucosal depth	172.11^{b}	188.78^{b}	242.11^{a}	272.11^{a}	19.891	0.001
Goblet cells count	26.00^{b}	29.33^{b}	39.33^{a,b}	52.67^{a}	6.184	0.082
IELI	323.00^{b}	423.00^{a,b}	556.33^{a,b}	656.33^{a}	51.152	0.065

Means within the same row carrying different superscripts are significantly different at ($P < 0.05$).

IELI, Intra-epithelium lymphocytic lick cells infiltrations.

PROE0, basal diet without additives; PROE1, basal diet + phenolic rich onion extract (1 g/kg diet); PROE2, basal diet + phenolic rich onion extract (2 g/kg diet); PROE3, basal diet + phenolic rich onion extract (3 g/kg diet).

extract/kg to the diet. An increase in the body weight, BWG, and feed consumption were observed in both the broiler strains "Cobb and Ross" by supplementing liquid onion extract in the drinking water (63). Goodarzi et al. (67) reported a positive effect of 3% onion supplementation on broiler growth parameters. Aji et al. (64) found that the BW, BWG, and feed intake were higher in chicks that were fed with onions and garlic diets. An et al. (68) reported that chicks fed with 0.3% or 0.5% onion extract-supplemented diets showed a slight increase in final body weight and BWG compared to the non-supplemented ones, with no effect on feed consumption during the start-up and farm stages. However, Al-Homidan (69) observed no effect on the feed intake of the broilers by 2% supplementation of dried onion to the feed but observed a reduced feed intake by supplementing 6% dried onion.

Behavior and Welfare

Our results showed a significant effect of PROE supplementation on feeding and drinking behavior of broiler chickens, which may be due to an increase in the FI of the supplemented treatment group compared to the non-supplemented one (26, 64). However, Ramamneh (70) found no effect of liquid onions on the behavior of broiler chickens. Our study indicated that comforting behavior (feather preening, wing/Leg stretching, wing flapping, head shaking, or body shaking) was improved by adding onion extract to the basic diet. Also, there was a decrease in the aggression in the treatment groups fed with enriched onion diets compared to the non-supplemented diet. These positive changes in the behavior of the broiler chickens fed with onion extract supplemented diet may be attributed to the fact that onions act as an antioxidant and anti-stress agent.

Duration of TI is a method for assessing the level of fear (71, 72) where longer duration of TI indicates a high level of fear and vice versa (73). A high level of fear can negatively affect the well-being and performance of the birds (74). Our results showed that the duration of TI was non-significantly decreased by adding onion extract to the diet, which may be attributed to a decrease in the stress level of the birds, improving their welfare. These results coincided with Mohamed et al. (75), who stated that supplementing onion and garlic in the diet of birds, provides a feasible way to improve their welfare by mitigating the clinicopathological changes.

Carcass Traits

Our study revealed the insignificant effect of PROE supplementation on the percentages of carcass dressing, intestine, viscera, liver, spleen, and heart weights. Similarly, in other reports, carcass dressing, abdominal fat percentage, and relative weights of the heart, liver, and spleen were not also affected by supplementing onion extract in broiler chicken meals (26, 68). Also, Aji et al. (64) found no effect on the carcass yield of broiler chickens by supplementing onion and garlic in the feed.

Antioxidant Activity

Onion extract is a rich source of phenolic compounds and flavonoids such as allicin, quercetin, campherol, caffeic acid, gallic acid, para coumaric acid, vanillic acid, and salicylic acid, all of which have potent antioxidant properties. Our results showed increased serum antioxidant enzymes, CAT, SOD, and GSH as a result of PROE supplementation, which can be attributed to the onion's total phenolic and flavonoid contents that were reported to be 70.55 mg GAE g-1 DW and 11.8 mg QE g-1 DW, respectively. These results are consistent with the results of Chang et al. (76), who revealed the positive effects of onion extract on the antioxidant activity. Aditya et al. (26) reported that onion extract supplementation improved the antioxidant capacity and quality of the meat and attributed these results to the total polyphenol content of onion extract which was recorded as 0.39 g/kg with a quercetin concentration of 0.36 g/kg OE, which represented 92.3% of the total polyphenol.

Immunological Parameters

In our study, PROE supplementation significantly improved the immune response of birds represented by increased IgM concentration, phagocytic%, and phagocytic index and increasing weights of thymus and bursa fabrics. The excess weight of lymph organs in PROE treatments can be attributed to the active compounds in onions along with the antibacterial, antiviral, antioxidant, and anti-inflammatory activities that stimulate positive effects on these organs (77). The oversized follicles lead to an increased immune globulin synthesis (78). Our results were consistent with Hanieh et al. (79) who reported that humoral immune function was improved by supplementing garlic and onions in the chicken diet, after vaccinating the birds with the Newcastle Virus (NDV), sheep red blood cells (SRBC), and *Brucella abortus* (BA). Also, Goodarzi et al. (17) reported an improvement in follicle and spleen weight by supplementing 30 g/kg of fresh onion in fattening meals. Further, the IgG level in onion extract treatments was also increased against the control treatment (26). Also, no effect was observed on the antibody titer against the Newcastle disease virus by using garlic (80) and onions (17) in broiler diets. The data of the blood parameters were found to be consistent with the result of El-Katcha et al. (81) where dietary supplementation with 25, 50, 75, or 100 mg of allicin/kg did not affect the white blood cell, lymphocytes, neutrophils, eosinophils, and basophil percentages of broiler chickens. However, phagocytosis showed significant improvement.

Histological Finding

The positive effect of phenolic-rich onion extract on villus height and width, crypt depth, mucous thickness, and goblet cell count of duodenum, jejunum, and ileum may be attributed to the fact that including herbs in poultry diets promotes the development and enzymatic activity of the intestinal structure. Our results were consistent with different researchers who reported positive effects of onion powder on villus height and width, crypt depth, and small intestine absorptive surface (82). Moreover, Mahmood et al. (83) reported that the height of villi, the depth of the crypt, and the surface area of jejuna increased significantly by including onions in broiler chicken feed.

Based on our results, we conclude that using phenolic-rich onion extract as a feed additive in broiler chicken diets can improve their growth performance represented by increased body weight, average daily gain, and average daily feed intake in a dose-dependent manner by improving AID% of amino acids and integrating the intestinal histology. It can also improve the birds' behavior and tonic immobility, along with enhancing the antioxidant activity which is represented by increased CAT, SOD activity, and GSH level and act as an immunomodulatory substance by improving the immune status of birds through an increased IgM level, phagocytic percentage, phagocytic index, and increased weights of thymus and bursa fabrics. Therefore, the PROE could be used as an alternative natural growth promoter, immune stimulant, and an antioxidant for broiler production.

DATA AVAILABILITY STATEMENT

The datasets generated for this study are available on request to the corresponding author. Requests to access these datasets should be directed to Shimaa A. Amer, shimaa.amer@zu.edu.eg.

ETHICS STATEMENT

The animal study was reviewed and approved by the Ethics of the Institutional Animal Care and Use Committee of Zagazig University, Egypt (ZUIACUC−2019), and all animal experiments were performed following recommendations described in The Guide for the Care and Use of Laboratory Animals in scientific investigations.

AUTHOR CONTRIBUTIONS

AOm and SA: design of the experiment. AOm, SA, HA-K, WM, HG, AOs, and NA-G: methodology. AOm, SA, WM, and HG: data collection and analysis. AOm, SA, and HG: writing of the manuscript. All authors have read and approved the manuscript.

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In ovo Administration of Nucleosides Improved the Performance, Apparent Metabolizable Energy and Gut Development in Broiler Chickens

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An *in ovo* study on the effect of the administration of a combination of nucleosides (25, 50, and 100 mg/egg) on hatchability, growth performance, energy metabolizability, and intestinal morphology in broilers was carried out. Four hundred eighty (480) fertile eggs were divided into four groups (in four replicates each having 30 eggs). On the 18th days of incubation of the eggs, candling was carried out and the fertile eggs were selected and given one of the four *in ovo* administrations. Group one served as control and was injected with phosphate-buffered saline (PBS). The other groups were given *in ovo* administration of nucleosides (25, 50, and 100 mg/egg) at 100 μl through the yolk sac route, and chicks of respective groups were hatched out. Among the experimental groups, the hatchability was comparable; however, the hatchability was affected in the group injected with a higher level of nucleosides at 100 mg/egg. The hatched out chicks from higher doses of nucleosides (50 and 100 mg) had higher body weight (BW) ($P < 0.05$) than the control. Higher energy metabolizability (%) was observed in nucleoside-injected groups. Plasma protein concentration was higher in groups administered with nucleosides (50 and 100 mg). Histologically, the intestinal villi length was maximum in 100 mg-injected group followed by 50 and 25 mg. Relative expression of homeobox (Cdx) in the jejunum was significantly ($P < 0.05$) upregulated in all the injected groups at 3, 7, and 14 days of age. Nucleoside-administered groups had better performance, energy metabolizability, and intestinal morphology. Among the experimental groups, the administration of nucleosides at 50 mg/egg resulted in higher growth performance, plasma protein, intestinal surface, and villi development in broiler chickens.

Keywords: nucleosides, *in ovo*, gut development, performance, broiler

INTRODUCTION

Genetic improvement paved the way for heavier birds with improved feed utilization efficiency under reduced rearing periods. As the productivity of birds increases, the demand of embryos for nutrients does changes. Early functions of the digestive tract are vital for chicken's growth and optimum muscle development. The small intestine undergoes both morphological and molecular changes during incubation. These changes are essential for the birds to adapt to rapid transition

from yolk to physical nutrient sources. The changes were quite evident as the weight of the intestine reaches about 3.5% of embryonic weight at hatch from 1% at 17 days of incubation (1). From the 19th day, the yolk sac's internalization into the body cavity takes place and serves as source of energy following hatching until the exposure to feed (2–4).

In broilers of 35 days growing period, 37% of their life span is spent in incubation (21 days) at hatchery (5). Any interventions during incubation that promotes/accelerates growth would have a significant impact on post-hatch performance. The use of *in ovo* technique (administration of nutrients or vaccines) had resulted in beneficial effects during their post-hatch performance. Moreover, this prenatal feeding triggers the intestinal development by enhancing villi development and intestinal capacity to digest and absorb nutrients and provides a basis for muscle growth (6–8). Similarly, the *in ovo* administration of nanoparticles of calcium carbonate accelerated the bone development in broilers (9). For *in ovo* injection, with more area, the yolk sac route is an ideal site for administration (5). A recent study indicated that the use of prebiotic (galactooligosaccharides) through *in ovo* route mitigated the negative effects of heat stress in broiler chickens (10). It is quite evident that any interventions during embryonic stage has a positive effect of post-hatch performance in broiler chickens.

Nucleotides (low molecular weight compound) are the materials for nucleic acids. Nucleotides consist of a pentose sugar, nitrogenous base, and phosphate group, whereas nucleosides consist of all the above except phosphate group (11). Nucleotides play critical roles in many biological processes in the body. Rapidly proliferating tissues such as immune system and intestinal mucosal cells during stress and early growth periods require more amount of nucleotides which cannot be supplied merely by *de novo* synthesis. The salvage pathway, which harvests nucleobases from blood and diet, could support their demands (12). Nucleotides are involved in gastrointestinal tract and skeletal muscle development, and immune response (13).

The *in ovo* supplementation of nucleotides will play an important role in developing various systems, especially the gastrointestinal and immune systems. During the embryonic stage, the intestinal mucosal barrier and enzyme system is immature and hence the administered quantity will enter into the bird's body without any loss (14, 15). Since nucleotides are converted to nucleosides after absorption, this study was undertaken to assess the their effect following *in ovo* administration at different dosages on growth performance, energy metabolizability, plasma total protein, uric acid, villi length, and expression of gut development genes.

MATERIALS AND METHODS

Ethical Approval

All experimental procedures involved in the study, such as the rearing of experimental birds and sampling, were approved by the institute animal ethics committee and members of CPCSEA nominees. The IAEC approval number is CARI/CPCSEA/2017/07.

Birds and Sampling

A total of 480 fertile eggs of white commercial broiler chicken (CARIBRO Vishal) were incubated in an incubator. All the eggs were incubated at normal incubation temperature (99.5°F—99.75°F) and relative humidity (50–60%) for initial 17 days. After 17 days of incubation, the eggs were divided into four treatments, each consisting of 120 eggs for i*n ovo* injection. Each group was consisted of four replicates having 30 eggs in each. On the 18th day, the eggs were candled, and the fertile eggs were reweighed and transferred to the laminar airflow for *in ovo* administration of nucleosides. Out of the four groups, eggs in the first group served as control which was injected with phosphate-buffered saline (PBS). The other three were treatment groups and were given *in ovo a*dministration of nucleosides (25, 50, and 100 mg/egg) at the rate of 100 μl through the chorioallantoic membrane using tuberculin needles and deposited into the yolk sac as per the protocol of Bhanja et al. (16). The doses were based on our earlier *in vivo* experiments (17). Commercial nucleosides (adenosine, guanosine, cytosine, and uridine—100% purity) were used in the study (HiMedia India Pvt. Ltd., Mumbai, India), mixed in equal proportion, and suspended in autoclaved PBS. Immediately after injection, the site was sealed with sterile paraffin. All the eggs were transferred to a hatcher, and the chicks were hatched out.

Hatchability Percentage and Experiment Diets

The hatchability percentage was calculated on fertile egg basis, and break out analyses was performed to study the causes for embryonic mortality. Following hatching, the chicks from respective groups were pulled out, wing banded, weighed, and grouped into respective groups and labeled. The hatched out chicks of respective groups (five replicates) were reared with common broiler feed for 42 days. The birds were fed with pre-starter (1–14 days), starter (15–28 days), and finisher (29–42 days) diets as per the nutritional recommendations of the Indian Council of Agricultural Research (18). The physical and chemical composition of the experimental diet is presented in **Table 1.**

Energy Metabolizability and Production Performance

A metabolism trial was conducted after 14 days of age, with 4 days of collection period and energy metabolizability was studied. The birds were kept individually in metabolic cages. The feed was offered to the birds in the cages in a separate feeder. Simultaneously, the residue was collected next day. The excreta were collected daily at the morning (10 a.m.), weighed, and stored appropriately for further analyses. Samples of experimental diets together with droppings were chemically analyzed for gross energy (GE) estimation using an adiabatic bomb calorimeter. The apparent metabolizable energy (AME) was determined by utilizing the balance data and the GE content of diets and droppings (19). The replicate feed consumption at different phases (14th, 28th, and 42nd day of age) and body weight (BW)

TABLE 1 | Feed ingredients and chemical composition of experimental diet (as-fed basis).

Ingredients (%)	Pre-starter	Starter	Finisher
Corn	546.0	542.0	576.2
Soybean meal (48% CP)	395.8	378.0	325.8
Rice bran oil	21.20	42.40	58.60
Calcite	15.40	15.20	17.30
Di-calcium phosphate	9.00	9.50	11.00
Salt	1.80	1.80	1.80
L-Lysine	3.00	1.50	1.70
DL-Methionine	3.00	2.80	2.70
Phytase	0.15	0.15	0.15
Mineral premix[a]	0.15	0.15	0.15
Vitamin premix[b]	0.14	0.14	0.14
Coccidiostat[c]	0.10	0.10	0.10
Toxin binder[d]	0.50	0.50	0.50
ANALYZED VALUES			
Crude protein	226.5	216.5	197.0
Metabolizable energy (MJ/kg)	12.55	13.08	13.60
Calcium	9.60	9.50	9.00
Available phosphorus*	4.50	4.60	4.60
Lysine*	14.20	12.50	11.40
Methionine*	6.20	5.90	5.50

**Calculated values.*

[a]Mineral premix composition: 91 mg manganese, 91 mg zinc, 85 mg iron, 1.82 mg iodine, 30.24 mg copper, and 0.365 mg cobalt/kg.

[b]Vitamin premix composition: 16,500 IU retinol, 3,200 IU cholecalciferol, 2 mg menadione, 5 mg thiamine, 13 mg riboflavin, 8 mg pyridoxine, 320 mg niacin, 0.05 mg cyanocobalamin, 95 mg DL-α-tocopherol, 27.5 mg calcium D pantothenate, 14 mg folic acid/kg.

[c]Coccidiostat supplied 125 mg Dinitro-ortho-toluamide/kg.

[d]Toxin binder composition: blend of organic acids, hydrated sodium calcium aluminum silicate, mannan oligosaccharides, and oxine copper (Check-O-Tox, Zoetis, India).

at similar stages were recorded.

$$\text{AME (kcal/kg)} = \frac{(\text{FC} \times \text{GE}_\text{f}) - (\text{E}_\text{w} \times \text{GE}_\text{e})}{\text{FC}}$$

Where,

FC, feed consumption (g/bird/day)

E_w, dried excreta weight (g/bird/day)

GE_f, gross energy of feed (kcal/kg)

GE_e, gross energy of excreta (kcal/kg).

Plasma Total Protein and Uric Acid Content

On 0, 3, 7, 14, 21, and 42 days of age, 10 birds (one male and one female per replicate) from each group were sacrificed in the morning before feeding, and blood samples were collected from the jugular vein in anticoagulant-coated tubes. The tubes were centrifuged at 2,500 rpm for 20 min to separate the plasma, and it was stored at −20°C until analysis. Plasma total protein and uric acid contents were quantified using Coral Clinical Systems, Tulip Diagnostics (P) Ltd., Goa. Plasma total protein and uric acid concentration were estimated by biuret and Uricase/PAP method, respectively.

TABLE 2 | Oligonucleotide sequences for Cdx gene expression studies.

Gene	Primer sequence	Annealing temperature	Accession no.
Cdx	F-CTCGGACTTCGCCAGCTACC	56.0°C	AB046532
	R-TGCGCCTCATCCATTCGTAC		
GAPDH	F-GTGTGCCAACCCCCAATGTCTCT	58.2°C	K01458
	R-GCAGCAGCCTTCACTACCCTCT		

Intestinal Gross and Histomorphology

Ten birds from each group were randomly selected and sacrificed (days 0, 7, and 14) by cervical dislocation and the whole intestinal segment was removed from the birds. Both the weight and length were measured and expressed in percent BW and centimeter per kilogram BW, respectively. The midpoint from the pancreatic loop to the Meckel's diverticulum about 1 cm of the jejunum part was excised, flushed with normal saline to remove the contents, and fixed in 10% neutral-buffered formal saline for histological analysis. For each sample, both longitudinal and cross sectional segments were made and analyzed under light microscope. Intestinal villi length was measured from tip of the villi to the villus-crypt junction using Zeiss microscope blue core software, and for each sample, six measurements were recorded.

Relative Expression of Intestinal Development Gene (Cdx)

The relative expression and quantification of Cdx gene in the jejunum was quantified by real-time PCR. The jejunum samples ($N = 40$; 10 per treatment) were collected in RNA*later* at 3, 7, and 14 days of age and stored at −20°C until further processing. The total RNA was isolated from the jejunum by TRIzol (Invitrogen, USA) extraction method. The quality of isolated RNAs was assessed by the absorbance ratio at 260–280 nm using a microvolume spectrophotometer (NanoDrop®, Thermo Scientific Fischer, USA). The RNAs having a ratio value of 1.8–2 were taken for further processing. The first strand cDNA were synthesized using RevertAid cDNA Synthesis Kit (MBI Fermentas, USA). PCR and qPCR were carried out in thermal cycler and real-time cycler (Bio-Rad Laboratories, USA) using standard conditions. All the samples were run in triplicate with non-template control (NTC) included in each PCR reaction to check DNA contamination. Oligo-nucleotide sequence of gene primers forward and reverse are provided in **Table 2**. GAPDH was used as a reference gene.

Statistical Analysis

Data collected on various parameters were subjected to analysis of variance using Statistical Package for Social Sciences version 16.0. The observations on hatchability, body weight, and feed intake were subjected to single-factor analysis. The observations on plasma total protein, plasma uric acid, gross intestinal morphology, and jejunal villi length were subjected to two-factor analysis to interpret the effect of treatment, duration, and its interaction. The means were compared for significance using Tukey's range test.

TABLE 3 | Effect of *in ovo* nucleoside administration on the hatchability (%) and embryonic mortality (%) in broiler chickens.

Group	Egg weight (g)		Hatchability (%)	Embryonic mortality (%)			
	Day 1	Day 18		Dead in shell	Infected	Dead after injection	Live pipping
Control	60.78	52.35	75.97	12.00	3.00	7.92	1.11
25 mg	60.20	53.48	78.55	10.89	3.22	7.34	0.00
50 mg	59.46	53.43	78.33	11.54	3.45	8.23	1.51
100 mg	59.56	53.48	74.76	8.78	3.33	10.69	2.44

TABLE 4 | Effect of *in ovo* nucleoside administration on the feed consumption (g/bird) and energy metabolizability (%) in broiler chickens.

Group	2nd week	4th week	6th week	Overall	Energy metabolizability (%)
Control	539	1,038^{c}	2,053	3,630	77.13^{b}
25 mg	537	1,093^{b}	2,026	3,656	83.79^{a}
50 mg	557	1,096^{b}	2,037	3,690	86.65^{a}
100 mg	573	1,143^{a}	2,101	3,717	84.46^{a}
SEM	1.24	4.70	10.88	16.87	0.02
P-value	0.241	0.031	0.094	0.134	0.001

Means within column bearing different superscripts differ significantly (P < 0.05).

TABLE 5 | Effect of *in ovo* nucleoside administration on the body weight (g) in broiler chickens.

Group	Hatch weight	2nd week	4th week	6th week
Control	46.2	449.1^{b}	1,091.7^{b}	2,026.3^{b}
25 mg	46.3	447.1^{b}	1,134.8^{ab}	2,072.9^{ab}
50 mg	46.9	463.8^{a}	1,188.5^{a}	2,123.5^{a}
100 mg	48.41	477.6^{a}	1,189.9^{a}	2,162.4^{a}
SEM	0.36	3.25	12.19	14.03
P-value	0.232	0.036	0.043	0.031

Means within column bearing different superscripts differ significantly (P < 0.05).

RESULTS

Hatchability

The effect of *in ovo* nucleoside administration on egg weight during incubation, the percentage of hatchability, and embryonic mortality is presented in **Table 3**. There was a reduction in egg weight during incubation on the 18th day compared to day 0. The hatchability was comparable among the injected groups; however, the hatchability was affected in groups injected with a higher level of nucleosides at 100 mg/egg. The unhatched eggs were broken out, and the cause of embryonic mortality was found out. The results of egg break analysis of unhatched eggs did not show any group-specific lesions, and the pattern of mortalities were uniform among the groups.

Energy Metabolizability and Production Performance

No significant difference among the groups was observed at fortnight and overall feed intake, except during the fourth week of experiment (**Table 4**). At the fourth week, the birds under higher administered dose (100 mg) had consumed more ($P < 0.05$) feed than the other three groups. The birds hatched out from the groups that are injected with higher levels of nucleosides (50 and 100 mg) had higher BW ($P < 0.05$) than the control (**Table 5**). The birds injected with lower dose (25 mg) exhibited intermediate response. The energy metabolizability (%) among the different groups showed a significant ($P < 0.01$) difference at 14 days of age (**Table 6**). Higher energy metabolizability (%) was observed in the *in ovo*-injected birds (25, 50, and 100 mg) when compared to the control group (83.79%, 86.65%, and 84.46 vs. 77.13%).

Plasma Total Protein and Uric Acid

The plasma concentration of total protein showed significant difference ($P < 0.05$) due to treatment, period, and its interaction (**Table 6**). The results revealed higher protein concentration in groups administered with higher nucleosides (50 and 100 mg) when compared to the control group. Similarly, the protein content showed age-dependent linearity and reached a higher concentration at 42 days of age. The interaction effect revealed a lower protein concentration at initial stages (days 0 and 3), and then, the values showed a steady increase with higher levels in supplemented groups than the control. The result for the concentration of plasma uric acid (mg/dl) is furnished in **Table 7**. Group mean showed no significant difference ($P > 0.05$), while the period mean exhibited a significant ($P < 0.05$) reduction in their concentration as the age increases. The interaction did not reveal any treatment × period association in the levels of serum uric acid.

Intestinal Morphology and Development

The intestinal length and weight were comparable ($P > 0.05$) among the groups, but their weight showed period mean significant difference ($P < 0.01$) (**Table 8**). The results showed that intestinal length at days 0 and 7 differed significantly ($P < 0.01$) from day 14 and intestinal weight at day 0 significantly differed ($P < 0.01$) from other periods. Reduction in the intestinal length has been observed on day 14 when compared to other periods. Among all periods, day 7 had the highest intestinal length and weight. The least length and weight were noticed on day 14 and day 0, respectively. Interaction study of intestinal morphology revealed that the control group had higher intestinal length and weight. The least intestinal length

TABLE 6 | Effect of *in ovo* nucleoside administration on the plasma total protein (g/dl) concentration in broiler chickens.

Group	Period (days)						Group mean	*P*-value			SEM
	0	3	7	14	21	42		*G*	*P*	*G* × *P*	
Control	3.36^{h}	3.66^{gh}	5.14^{e}	5.90^{b}	5.72^{c}	5.18^{e}	4.83^{z}	0.033	0.001	0.016	0.17
25 mg	4.02^{fg}	4.30^{f}	5.86^{bc}	5.30^{de}	6.02^{b}	6.35^{a}	5.14^{xy}				
50 mg	3.55^{gh}	4.49^{f}	5.74^{c}	5.75^{c}	6.11^{ab}	6.43^{a}	5.35^{x}				
100 mg	3.74^{gh}	4.28^{f}	5.47^{d}	5.39^{d}	6.14^{ab}	6.64^{a}	5.28^{x}				
Period mean	3.67^{C}	4.18^{C}	5.55^{AB}	5.59^{AB}	6.00^{A}	6.15^{A}					

Means bearing different superscript within column (x–z), row (A–C), and interaction (a–h) differ significantly ($P < 0.05$).
G × P, group × period interaction.

TABLE 7 | Effect of *in ovo* nucleoside administration on the plasma uric acid (mg/dl) content in broiler chickens.

Group	Period (days)						Group mean	*P*-value			SEM
	0	3	7	14	21	42		*G*	*P*	*G* × *P*	
Control	5.89	5.59	5.44	5.32	5.41	5.17	5.47	0.511	0.001	0.316	0.325
25 mg	6.07	5.95	5.56	5.46	5.39	5.29	5.62				
50 mg	6.18	5.98	5.68	5.48	5.23	5.16	5.62				
100 mg	6.22	6.02	5.76	5.55	5.47	5.24	5.71				
Period mean	6.09^{A}	5.89^{A}	5.61^{B}	5.45^{BC}	5.38^{BC}	5.22^{C}					

Means bearing different superscript differ significantly ($P < 0.01$).
G × P, group × period interaction.

TABLE 8 | Effect of *in ovo* nucleoside administration on the intestinal gross morphology in broiler chickens.

Group	Period (days)			Group mean	*P* value			SEM
	0	7	14		*G*	*P*	*G* × *P*	
Intestinal length (cm/kg body weight)								
Control	815.56^{abc}	910.47^{a}	529.92^{bcd}	751.98	0.446	0.001	0.005	28.991
25 mg	902.55^{a}	963.75^{a}	488.27^{cd}	784.86				
50 mg	822.03^{abc}	789.39^{abcd}	487.74^{cd}	699.72				
100 mg	808.97^{abcd}	891.76^{a}	634.77^{abcd}	778.50				
Period mean	837.28^{A}	888.84^{A}	535.18^{B}					
Intestinal weight (% body weight)								
Control	38.52^{c}	149.25^{a}	139.01^{a}	108.93	0.286	0.001	0.001	7.267
25 mg	52.09^{bc}	153.44^{a}	128.39^{a}	111.31				
50 mg	39.96^{c}	137.33^{a}	126.89^{a}	101.39				
100 mg	34.01^{c}	133.79^{a}	131.89^{a}	99.90				
Period mean	41.15^{B}	143.45^{A}	131.55^{A}					

Means bearing different superscript within row (A–B) and interaction (a–d) differ significantly ($P < 0.01$).
G × P, group × period interaction.

was observed in the control group at day 14 and the least weight was observed in the 50-mg group at day 0. Other groups exhibited an intermediate response. All the groups showed a reduction in intestinal length and weight per unit from day 7–14.

Histomorphological results showed that both group mean and period mean were significantly different ($P < 0.01$). The highest intestinal villi length was observed in the 100-mg-dose-injected group, followed by 50 and 25 mg (**Table 9**). The period means exhibited a significant increase in the pattern of villi length up to 42 days. Interaction study revealed that the 100-mg group had the highest villi length on day 42, and the lowest length was observed in the control group at day 0. The relative expression of homeobox (Cdx) gene in the jejunum showed significant ($P < 0.05$) upregulation in the injected groups at 3, 7, and 14 days of age (**Figure 1**). The magnitude of expression is higher during 7 days of age after hatch, whereas the expression is lower at days 3 and 14.

TABLE 9 | Effect of *in ovo* nucleoside administration on the jejunal villi length (μm) in broiler chickens.

Group	Period (days)					Group mean	*P*-value			SEM
	0	3	7	14	42		*G*	*P*	*G* × *P*	
Control	1,255.53^{j}	1,658.64^{i}	2,245.79^{g}	2,849.17^{ef}	5,326.72^{c}	2,667.17^{z}	0.001	0.001	0.001	19.80
25 mg	1,340.87^{j}	1,839.45^{i}	2,374.16^{g}	3,074.81^{de}	5,653.19^{b}	2,856.50^{y}				
50 mg	1,419.50^{j}	1,894.52^{hi}	2,749.47^{f}	3,174.10^{d}	5,880.21^{ab}	3,023.56^{x}				
100 mg	1,408.64^{j}	2,119.41^{gh}	2,918.21^{e}	3,248.62^{d}	5,917.50^{a}	3,122.48^{x}				
Period mean	1,356.14^{E}	1,878.01^{D}	2,571.91^{C}	3,086.68^{B}	5,694.41^{A}					

Means bearing different superscript within column (x–z), row (A–E), and interaction (a–k) differ significantly ($P < 0.01$).
G × P, group × period interaction.

DISCUSSION

Hatchability (%)

The egg weights were comparable among the groups at both during the day of setting and after 18 days of incubation. About 10–12% of embryonic mortality was encountered after *in ovo* injection across the injected groups. Among the experimental groups, better hatchability was observed in 25 and 50-mg group. It indicated that the administration of nucleosides had an impact in the hatchability percentage of eggs. The hatchability percentage was negatively related to the concentration of nucleosides. Eggs with lower concentration at 25 mg/egg showed better hatchability than other eggs which had higher concentration of nucleosides. The hatchability percentage reduction might be due to alteration in the osmolarity inside the egg as the nucleosides were administered as suspension. The nucleosides are sparingly soluble in aqueous solvents, and the administration of nucleosides as a solution instead of suspension would have resulted in better hatchability percentage following *in ovo* administration. Further studies could be directed toward improving the solubility of these nucleosides by manipulating the pH of the solvents which could result in better hatchability.

Growth Performance and Energy Metabolizability

There was a non-significant increase in feed intake of T3 group birds observed in all other three phases (pre-starter, finisher, and overall growth phase). Post-hatch feeding of nucleotides at 0.04, 0.05, 0.06, and 0.07% resulted in significantly higher feed consumption than the control group (20). Hatch weight did not show any significant difference among the groups, which indicated that the administration of nucleosides at the 18th day of incubation did not influence the weight at hatch. Post-hatch bi-weekly BW measurements revealed significant difference ($P < 0.05$) at all the growth phases and overall BW. Supplementation of 0.05% of commercial nucleotide products (Nucleoforce, containing 26.4% of balanced total nucleotides) in broiler diets resulted a significant increase in BW from 0 to 21 days of age (21). Similarly, in swine, supplementation of 4.0% of yeast extract product (NuPro^{TM} containing 7% of total nucleic acids) increased the BW (22). Incorporation of 2% nucleotide and other related compounds (NuPro) increased BW in broiler chickens when compared to control (23). This increase has been attributed to an increase in the digestibility and absorption of nutrients by the pancreatic and brush border digestive enzymes activity. A similar effect was also observed in the present study. The results further justified our earlier findings, where the dietary supplementation of nucleosides at 0.1% resulted in significantly higher BW and gain in broilers. Energy metabolizability results revealed that all the nucleoside-injected groups have higher energy metabolizability than other groups. It indicated that the administration of nucleosides had improved energy metabolizability in broilers. It might be due to the intestine's rapid cell turnover, which increases enzyme activity and energy metabolizability. A study carried out in broilers with supplementation of 2% nucleotides, NuPro, resulted in higher nutrient digestibility (23).

Plasma Concentration of Total Protein and Uric Acid

There was an increasing pattern of concentration of total protein, albumin, and globulin observed up to 42 days. These results revealed that nucleoside-injected groups showed a higher protein concentration than the control group in all the periods. Intraperitoneal administration of nucleoside–nucleotide mixture increased total protein concentration in mice (24). This indicates that nucleotides are essential to increase body protein turnover rate, specific protein synthesis, and enterocyte proliferation. Nucleotides facilitate protein formation through messenger RNA synthesis (25). An age-related inverse relation was observed for uric acid due to the degradation of purine bases (26). The administration of nucleosides elevated the concentration of uric acid except at day 42. The higher values in the supplemented groups might be due to higher breakdown of nucleosides, which are then metabolized and excreted as uric acid.

Intestinal Morphology and Development Studies

Intestinal length and weight per unit weight were reduced ($P < 0.01$) as the bird's age increases from 7 to 14 days. This is due to an increase in the proportion of BW higher than the length and weight of the intestine from day 7–14. An interaction study revealed that up to day 7, a lower dose-injected group (25 mg) had the highest intestinal length and weight among all groups. Nucleotides appear to stimulate the development of the

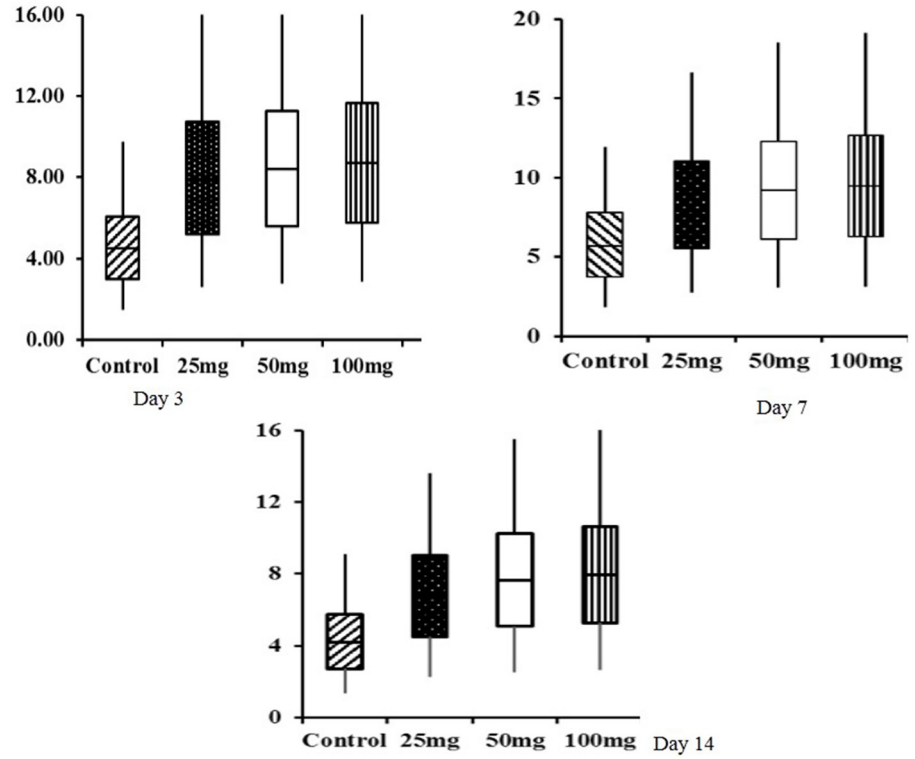

FIGURE 1 | Box plot analysis of Cdx gene in jejunum tissue of *in ovo* injected nucleosides broiler chickens. Box plots, box shows the lower quartile, median (dark line), mean (+ symbol), and upper quartile values and the whisker's show the range of relative expression of Cdx gene in jejunum tissue at 3, 7, and 14 days of age.

intestinal lining and intestinal enzyme concentrations in animals. They also have beneficial effects on recovery from intestinal injuries caused by malnutrition or chronic diarrhea (27, 28). This positive effect on the gastrointestinal tract development might be due to enhanced DNA and RNA synthesis because of increased nucleotide pools following intake. This increased DNA and RNA synthesis enhances growth and differentiation of the enterocytes after injuries or malnutrition. Therefore, the administration of nucleosides, which can be converted to nucleotides, may help optimize tissue function in the gastrointestinal tract and stimulate the activity of brush border enzymes. These entire factors may be contributing in increasing intestinal weight.

The intestinal villi length results showed that nucleoside-administered groups were significantly different ($P < 0.01$) from other groups that were non-injected. In all the periods of measurements, the intestinal villi's length was increased in nucleoside-injected groups of broilers. The administration of nucleosides plays an essential role in developing the villi length in the small intestine of broilers. The increased villi length in all nucleoside-administered groups might be due to increased Cdx gene expression, which is the indicator of intestine development. Thus, the mucosal villi's rapid development allows chicks to utilize nutrients more efficiently in their early life and improve growth performance (29). The intestinal epithelium is a rapidly proliferating tissue with a high cell turnover rate, and dietary nucleotides are reported to play a role in the growth and differentiation of the gastrointestinal tract (30). Uauy et al. (27) observed an increased tissue protein, DNA content, as well as the activities of disaccharidase in the intestine of weanling rats fed with 0.8% *w*/*w* dietary nucleotide than control. Jung and Batal (23) also reported that birds fed on a diet supplemented with 0.25% torula yeast RNA and 2% NuPro, a commercial nucleotide product, had significantly ($P < 0.05$) higher villus heights as compared with the birds fed on the control.

Early weaning in piglets is the most stressful condition which adversely affects the intestine. The consistent occurrence of piglet diarrhea is the most common problem with early weaned piglets. Under this condition, the diet supplemented with 0.1% or 0.2% commercial nucleotide-rich yeast extract product (containing 25% nucleotides) had higher villus heights than the control group (31, 32). As nucleosides are the preferred form for absorption by enterocytes, nucleosides were administered in this study. All these effects were due to the administration of nucleosides, which were converted to nucleotides after absorption. Nucleosides also increase the villi length of the small intestine through rapid cell turnover.

The box-plot analysis of Cdx gene expression revealed upregulation in all the three doses of *in ovo* injection compared to both the controls. Intraperitoneal administration of nucleoside–nucleotide mixture increased small intestinal RNA levels in mice, which indicated an increased proliferation of enterocytes compared to the control group

(24). Supplementation of nucleotides provides benefits to enterocyte function during normal periods of growth and development characterized by high demand for DNA and RNA synthesis (33).

CONCLUSION

From the experiment, it could be concluded that nucleoside administration at the rate of 50 mg/egg resulted in higher growth performance, intestine surface, and villi development in broiler chickens. Further improvement can be made to overcome the reduction in hatchability percentage by improving the solubility of nucleosides.

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 Chairman, Institute Animal Ethics Committee, CARI. All experimental procedures involved in the study, such as the rearing of experimental birds and sampling, were approved by the institute animal ethics committee and members of CPCSEA nominees. The IAEC approval number is CARI/CPCSEA/2017/07.

AUTHOR CONTRIBUTIONS

MG, JR, and AV: designed the experimental design and analyzed the collected data. PS and VMad: prepared the manuscript. VMan, MM, and MS: carried out the *in ovo* administration, biological experiment, and performed sample collection. BP and TT: carried out the laboratory analysis of biological samples. All authors read and approved the manuscript.

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Longissimus Dorsi Muscle Transcriptomic Analysis of Simmental and Chinese Native Cattle Differing in Meat Quality

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With the rapid development of economy, the demand for beef, with regard to quantity and quality, by consumers has been increasing in China. Chinese native cattle are characterized by their abundant genetic resources, unique origins, large breeding stocks, and robust environmental adaptability. Thus, to explore the genetic mechanisms on regulating meat quality in Chinese native cattle is of great importance to satisfy increased requirements for beef production. In this study, we investigated three breeds of cattle, namely Yunling, Wenshan, and Simmental, at the age of 12 months. Animals were classified into three groups ($n = 5$/breed). Growth traits including body weight and body size and plasma hormone levels were measured. Body weight of Wenshan cattle was significantly lower than that of Yunling and Simmental cattle ($P < 0.05$). Again, body size indexes, such as withers height, body slanting length, chest circumference, and hip and rump length, were significantly lower in Wenshan cattle than those in Yunling and Simmental cattle ($P < 0.05$). However, there were no significant differences in those indexes between Yunling and Simmental cattle ($P > 0.05$). Cattle were slaughtered at the age of 18 months and then meat color, pH, pressing losses, muscle tenderness, and cooking losses were measured at 0, 1, 2, 3, 5, and 7 days. Data revealed differences in meat quality among the three breeds analyzed. Based on transcriptomic sequencing and bioinformatic analysis, we observed 3,198 differentially expressed genes related to meat quality, of which 1,750 genes were upregulated. Moreover, we found two important signaling pathways closely linked to meat quality, namely adipocytokine signaling pathway [e.g., *Leptin receptor* (*LEPR*)] and protein processing in the endoplasmic reticulum [e.g., *signal transducer and activator of transcription 3* (*STAT3*), *heat shock protein* (*HSPA12A*), and *calpain 1* (*CAPN1*)]. The results of transcripts were further verified by qRT-PCR. Using correlation analysis between gene expression levels and shear force, we also identified two functional genes (e.g., *HSPA12A* and *CAPN1*) associated with meat quality. Overall, this study provides new sights into novel targets and underlying mechanisms to modulate meat quality in Chinese native cattle.

Keywords: Chinese native cattle, Simmental, ripening, meat quality, cooked degree

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INTRODUCTION

With the development of economy, the dietary structure of Chinese consumers has been altered. During this process, beef has gained prominence in China's meat market (1). The rapid increase in consumers' demand for livestock products, especially beef, has led to an insufficient supply of beef (2–4). Therefore, the development of native beef cattle production is of great significance for promoting the improvement of dietary structure and people's living standards in China. According to the statistics of beef cattle industrialization technology system, the annual demand and supply gap of beef in China is approximately 1.6 million tons. China's native cattle genetic resources are rich, with a large number, various breeds, strong feed tolerance, and stress resistance capacity. However, compared with widely used breeds, native breeds usually have smaller size and lower growth rate, along with poorer meat production performance, which hinder the development of local beef production in China. In this study, local Chinese breeds of Yunling cattle and Wenshan cattle were investigated, in comparison with the conventional breed, Simmental cattle. First, body weight, body size, and growth hormone levels in plasma were evaluated. Second, meat quality indicators of the longissimus dorsi muscle were measured including meat color, pH, pressing losses, tenderness, and cooking losses. Lastly, cooking differences using different cooked systems were measured to reveal the effect of tenderness on steak quality under different acidic conditions. Overall, our study potentially contributes to promoting the development of native beef cattle industry in China.

To date, genomic sequencing in most livestock has been completed (5, 6). This study may help elucidate molecular mechanisms governing meat quality in specific cattle breeds. Previous studies have used transcriptomic sequencing to evaluate molecular mechanisms controlling species-specific traits. Huang et al. obtained 19,043 known genes and 1,785 novel genes in bovine early embryos through transcriptomic sequencing as a reproduction-related database (7). Wickramasinghe et al. used Holstein cow somatic cells as research objects and analyzed them by transcriptomic sequencing to determine gene expression across different lactation stages (8). Of particular interest, there were 16,892 differentially expressed genes during prelactation, 19,094 differentially expressed genes during mid-lactation, and 18,070 differentially expressed genes during late lactation. Pathways include whey protein and casein metabolism and fat metabolism. However, the former mainly displayed during prelactation. Zhang et al. screened 1,300 differentially expressed genes in muscle tissue between small-tailed Han sheep and Dubo sheep *via* transcriptomic sequencing (9). He et al. analyzed the longissimus muscle in Qinchuan cattle at different periods by RNA-Seq technology. A total of 6,800 differentially expressed genes were screened using the $P < 0.05$ principle, of which 1,893 genes were upregulated in the fetal calf group (10). The reliability of the sequencing results was verified by qPCR, and 47 differentially expressed genes were selected from the sequencing results. Quantitative PCR detection revealed that the expression levels of 47 genes in the latissimus dorsal muscle were in line with the sequencing results. Therefore, RNA-Seq transcriptomic sequencing serves as an important strategy for exploring genetic potentials in livestock. In the current study, samples with significant differences in meat quality were used for RNA-Seq transcriptomic sequencing to screen and identify pathways and target genes associated with growth and development in Chinese native cattle breeds.

MATERIALS AND METHODS

Experimental Animals

Animals ($n = 5$/breed) selected from 1-year-old Yunling cattle, Wenshan cattle, and Simmental bulls were raised under the same breeding environment. After growth and development measurement and the determination of meat quality, three Yunling cattle and three Simmental cattle were selected. The cattle were fed twice a day with adequate water and the manure was cleaned daily. Approximately 350 g of longissimus dorsi (LD) samples between the 12th to 13th ribs of each cattle were taken. Longissimus dorsi muscle tissue was obtained and immediately placed into liquid nitrogen and subsequently brought to the laboratory for storage at −70°C.

Weight and Body Size

Growth traits were measured every month. Body weight: body weight was measured using a land scale before morning feeding; body height: the vertical distance from the highest point of the bun nail to the ground; body slant length: the distance from the shoulder end to the ischial end; body straight length: the horizontal distance from the shoulder end to the vertical line of the posterior edge of the ischial end; chest circumference: the circumference around the chest by the vertical axis of the posterior angle of the scapula; tube circumference: the circumference at the thinnest point of the tube bone, generally in the tibia of the left front leg measure from the bottom to the top third.

Hormone Determination

Blood was collected from Yunling cattle, Wenshan cattle, and Simmental cattle from the jugular vein and stored at −20°C. After centrifugation, plasma was collected and determined by enzyme-linked immunosorbent assay (ELISA) kits (Promega Corp, Beijing, China) for thyroid-stimulating hormone (TSH), triiodothyronine (T3), thyroxine (T4), growth hormone (GH), insulin, and insulin-like growth factor-1 (IGF-1). The absorbance (OD value) was measured at 450 nm using an enzyme standard instrument, and concentrations of hormones were calculated by standard curves.

Chemical Composition

Approximately 350 g of LD samples between the 12th to 13th ribs of each cattle were taken and experienced a 7-day aging under vacuum conditions at 4°C to evaluate meat quality (11). Sampling was performed on days 0, 1, 2, 3, 5, and 7 (12). The composition of beef was measured using a Foss Lab Meat/Food Composition fast analyzer (FOSS Ltd., Hillerød, Denmark). The 200-g samples on days 0, 3, and 7 were separated from the LD sample and extended into the analysis plate. The results were the average of

16 technical replicates per sample. The equipment was calibrated to the Soxhlet method (13). Protein, collagen, moisture, and fat content were recorded.

Color Measurement

Samples collected on days 0, 1, 2, 3, 5, and 7 were exposed to the air for 30 min (blooming) at 4°C to measure their color, and color was measured with a Chroma Meter CR-400 colorimeter (Konica Minolta, Inc., Tokyo, Japan) based on luminance (L^*), redness (a^*), and yellowness (b^*) in the CIELab color space. The color values were the average of six scans for each LD. The C^* and H^* values were calculated from the a^* and b^* values using the following respective formulas: $C^* = (a^*2 + b^*2)^{0.5}$ and $H^* =$ arctan (b^*/a^*) (14, 15).

pH Value

The pH of each sample collected on days 0, 1, 2, 3, 5, and 7 was measured by a pH meter (Eutech Instruments, pH Spear, USA) (16). Calibration of the pH electrode was performed with standardized buffers (pH 4.0 and 7.0). Each LD sample was measured three times.

Cooking Loss and Shear Force

The cooking loss rate was measured by the direct weight method (17). Thirty grams of LD was taken from the LD samples collected on days 0, 1, 2, 3, 5, and 7. Next, it was wrapped and sealed in a cooking bag. Then, it was cooked in a water bath at 80°C. When the central temperature reached 70°C, the sample was unwrapped and the surface moisture was dried. The cooking loss rate was calculated according to the formula: cooking loss (%) = (Wc1–Wc2)/Wc1 × 100%. Wc1 represented the weight of the sample before cooking and Wc2 represented the weight of the sample after cooking. Then, to measure the Warner–Bratzler shear force (WBSF), approximately 3-cm-thick LD sample was removed from the cooked LD sample, and the WBSF was measured using the method of Luo et al. (18). Each LD sample was measured three times.

Water Loss Rate (Muscle Tenderness)

A 2 × 2 × 1 cm meat piece was cut from the LD samples collected on days 0, 1, 2, 3, 5, and 7. Then, a pressure of 343 N (35 kg) was applied to it and maintained for 5 min. The water loss rate was calculated according to the following formula: water loss rate (%) = (Ww1–Ww2)/Ww1 × 100%. Ww1 represented the weight of the sample before water loss and Ww2 represented the weight of the sample after water loss. Each LD sample was measured three times.

Transcriptomic Sequencing

Total RNA was extracted from tissue using the TRIzol reagent (catalog number: 15596026, Invitrogen, Carlsbad, CA, USA). The RNA integrity number (RIN) method was used to detect RNA quality, and RIN values are above 7.1. Only good-quality RNA was used for this experimental study. Total RNA was extracted from muscle tissue, and then DNA was digested with DNase (Tiangen, Shanghai, China). Subsequently, mRNA was broken into short segments by adding an interruption reagent, and a strand of cDNA was synthesized with six base random primers using the interrupted mRNA as a template. Double-strand cDNA was purified using a commercial kit (Promega Corp, Beijing, China); the purified double-strand cDNA was repaired, combined with an A-tail, and connected with a sequencing connector. The constructed library was qualified by an Agilent 2100 Bioanalyzer and sequenced by Illumina HiSeq 2500 (19).

Gene Expression Abundance

For transcriptomic sequencing analysis, we estimated gene expression levels by counting the sequencing sequences (reads) located in the genomic region or gene exons. In addition to the true expression levels of genes, read count is also positively related to gene length and sequencing depth.

Analysis of Differential Gene Expression

RNA-Seq data were used to compare and analyze whether there was differential expression of the same unigene in two samples. Two criteria were used to determine differentially expressed genes: one is fold change and the other is *P*-value or false discovery rate (FDR) (padjust). The calculation method of the FDR value should first calculate the *P*-value for each unigene and then use the FDR error control method to test and correct multiple assumptions for the *P*-value.

Cluster Analysis

Differentially expressed genes were clustered by unsupervised hierarchical clustering. Based on calculating the distance between two samples, a distance matrix is constructed. The samples can appear in the same cluster through clustering, and genes in the same cluster may have similar biological functions.

Gene Ontology and Enrichment Analysis of Kyoto Encyclopedia of Genes and Genomes

We analyzed differentially expressed genes using gene ontology (GO) and described their functions. The number of differential genes in each GO item was counted, and the significance of differential gene enrichment in each GO item was calculated by the hypergeometric distribution test. According to the results of GO analysis and biological significance, we selected genes for a follow-up study.

The Kyoto Encyclopedia of Genes and Genomes (KEGG) is mainly a public database on pathways. We used the KEGG database to analyze differentially expressed genes and used the hypergeometric distribution test to calculate the significance of differentially expressed gene enrichment in each pathway entry. Pathway analysis is helpful to the experimental results. According to the pathway analysis, we found the pathway items that enrich differential genes and determine which molecular pathway alteration might be related to the differentially expressed genes in different samples.

Fluorescent Quantitative PCR Detection

According to the results of transcriptomic sequencing, five important differentially expressed genes (*LEPR*, *STAT3*, *leptin*, *HSPA12A*, and *CAPN1*) were selected. Premier 6.0 was used to design primers for the selected genes (**Table 1**) (Tiangen, Shanghai, China). β-Actin was used as an internal reference gene. The reaction system was 20 μL: 1 μL of cDNA, 0.4

μL (10 μmol/L) of upstream and downstream primers, 0.4 μL of Rox reference dye II (50x), 10 μL of SYBR green real-time PCR Master Mix (2x), and 7.8 μL of ddH_2O (Tiangen, Shanghai, China). Real-time PCR running conditions are as follows: predenaturation at 95°C for 15, 5 s at 95°C, and 34 s at 60°C, 40 cycles. The dissolution curve was analyzed after amplification. Each sample was measured in triplicate, and the average value was taken. Relative quantitative results were calculated by the $2^{-\Delta\Delta CT}$ method (20).

Statistical Analysis

Statistical analysis was performed by two-way analysis of variance (ANOVA) using the Tukey–Kramer adjusted generalized linear model (GLM) procedures of Statistical Analysis Software (SAS) 9.4 (SAS Institute, Cary, NC, USA). $P < 0.05$ indicates significant differences, and $P < 0.01$ indicates highly significant differences (21).

RESULTS

Three Cattle Breeds' Growth Performance and Carcass Traits

Body weight of Simmental and Yunling cattle was significantly greater than that of Wenshan cattle ($P < 0.05$; **Table 2**). The growth rate between 1 and 15 months of age was faster than that observed after 17 months of age regardless of breed (**Figure 1**).

At the age of 12 and 18 months, body size indexes including body height, body slant length, chest width, chest depth, chest circumference, abdominal circumference, hip circumference, waist angle width, ischial end width, tube circumference, and rump length of Simmental and Yunling cattle were significantly higher than those of Wenshan cattle ($P < 0.05$); however, there were no significant differences between Simmental and Yunling cattle ($P > 0.05$, **Supplementary Table 1**). In addition, the growth rate of Wenshan cattle was significantly higher than that of Simmental and Yunling cattle at the age of 12 and 18 months ($P < 0.05$).

Differences were detected in hormones associated with growth and development among Wenshan, Simmental, and Yunling cattle. Yunling cattle had the highest concentrations of insulin (INS), TSH, T4, T3, GH, and IGF-1 in plasma at the age of 12 months ($P < 0.05$; **Supplementary Table 1**). Additionally, T4 and INS levels in Yunling cattle were significantly higher than those in Wenshan cattle and Simmental cattle at the age of 18 months ($P < 0.05$; **Table 3**). Yunling cattle and Simmental cattle had greater concentrations of TSH, T3, GH, and IGF-1 than Wenshan cattle ($P < 0.05$). The hormone levels in Wenshan cattle showed a decreasing tendency from 12 to 18 months of age. By contrast, they showed an opposite trend in Simmental cattle and Yunling cattle.

The L^* values of Yunling cattle, Simmental cattle, and Wenshan cattle showed an increasing trend from 0 to 5 days postmortem. After 5 days postmortem, the L^* value of Wenshan cattle showed a significant reduction, and it displayed a slight decline in Yunling cattle and Simmental cattle (**Figure 2A**). The a^* value of Yunling cattle, Simmental cattle, and Wenshan cattle showed an increasing trend from 0 to 1 day after slaughter. After 5 days postmortem, Yunling cattle showed a steady state, while Simmental cattle and Wenshan cattle were characterized by a fluctuant trend. Overall, the a^* value of Simmental cattle was higher than that of the other two breeds postmortem (**Figure 2B**). The b^* value showed an increasing trend from 0 to 1 day in all breeds after slaughter. Moreover, the b^* value in Simmental cattle maintained an increasing trend 1–2 days after slaughter; conversely, it began to decline in the other two breeds (**Figure 2C**). It can be seen from **Figure 2D** that the C value of these three cattle breeds is highly similar to the a^* value. Additionally, it can be observed from **Figure 2E** that the H value of these cattle breeds is very similar to the b^* value.

The pH value in the muscle of Yunling cattle, Simmental cattle, and Wenshan cattle decreased significantly from 0 to 1 day postmortem. After 3 days postmortem, the change in pH tended to be stable (**Figure 3A**).

The shear force of Yunling cattle, Simmental cattle, and Wenshan cattle was all decreased between 0 and 5 days postmortem. Shear force decreased slightly during the first 5 days postmortem. As a whole, the shear force value at the same time point during acid excretion was Simmental cattle > Wenshan cattle > Yunling cattle. The results showed that the tenderness of Yunling cattle was better than that of Simmental cattle (**Figure 3B**).

The water loss rate of Yunling cattle, Simmental cattle, and Wenshan cattle increased markedly from 0 to 1 day postmortem. Overall, the water loss rate of Yunling cattle was higher than that of Simmental and Wenshan cattle during acid discharge (**Figure 3C**).

The cooking loss of Yunling cattle and Wenshan cattle increased, then it decreased followed by an increase. By contrast, cooking loss in Simmental cattle exhibited a clear increasing trend from 0 to 2 days and then began to decline (**Figure 3D**).

Correlations between the time of acid excretion and meat quality indexes (pH, water loss %, cooking loss %, muscle tenderness, L^* value, a^* value, and b^* value) in the three cattle breeds (Yunling cattle, Simmental cattle, and Wenshan cattle) were analyzed. The pH value and tenderness of Yunling cattle muscle were negatively correlated with the time of acid excretion ($P < 0.01$), whereas water loss rate, cooking loss, and meat color (L^* value, a^* value, and b^* value) were positively correlated with the time of acid excretion (all $P < 0.05$; **Table 4**). pH value had a significantly negative correlation with water loss rate and meat color (L^* value, a^* value, and b^* value) in Yunling cattle ($P < 0.01$), indicating that when the pH value of Yunling cattle muscle decreased, the water loss rate increased, and the meat color changed (**Table 4**).

There was a significantly negative correlation between pH value and acid excretion time ($P < 0.01$), while there was no significantly negative correlation between pH value and muscle tenderness ($P < 0.05$; **Table 5**). The results showed that pH value decreased, but muscle tenderness did not change notably with prolonged acid excretion time. The pH value of Simmental cattle had a significantly negative correlation with water loss rate and meat color (L^* value, a^* value, and b^* value, **Table 5**).

Acid excretion time was significantly negatively correlated with pH value and muscle tenderness ($P < 0.01$). Meanwhile,

TABLE 1 | Information of primer sequences.

Gene	Accession number	Primer sequence	Length (bp)
Leptin	XM_010804453.2	F: 5′-CAATGACATCTCACACACGCAG-3′ R: 5′-TCGCCAATGTCTGGTCCATC-3′	116
LEPR	NM_001206441.1	F: 5′-CTGTGCCACCTTTCTCGTGG-3′ R: 5′-TTGGAAAGAAGGACCCTCTGC-3′	70
STAT3	NM_001012671.2	F: 5′-AAGGGATTCCCAAGGATGCC-3′ R: 5′-AATTTGAATGCAGTGGCCAGG-3′	75
HSPA1A	NM_203322.2	F: 5′-AGCCTGGAGAGAGCTGATAAAA-3′ R: 5′-CCCACAGGATCAACGACGTA-3′	118
CAPN1	NM_174259.2	F: 5′-GCTGACCATGTTTGCGTGAG-3′ R: 5′-AGAGCAAATGAAACACGGCG-3′	190
β-actin	XM_003124280.3	F: 5′-TGGCGCCCAGCACGATGAAG-3′ R: 5′-GATGGAGGGGCCGGACTCGT-3′	149

TABLE 2 | Body mass index at different growth periods in three cattle breeds.

Age (months)	Breed		
	Yunling cattle (kg)	Wenshan cattle (kg)	Simmental cattle (kg)
12	305.2 ± 19.1^{b}	160.3 ± 32.2^{a}	312.2 ± 38.3^{b}
13	328.1 ± 14.4^{b}	176.6 ± 32.5^{a}	345.7 ± 35.5^{b}
14	352.8 ± 14.8^{b}	200.6 ± 37.9^{a}	381.2 ± 28.8^{b}
15	425.0 ± 15.7^{b}	253.5 ± 48.7^{a}	453.4 ± 32.3^{b}
16	444.4 ± 98.0^{b}	273.2 ± 46.0^{a}	469.4 ± 34.5^{b}
17	478.6 ± 10.4^{b}	300.8 ± 48.9^{a}	505.4 ± 41.5^{b}
18	487.8 ± 14.0^{b}	312.7 ± 50.1^{a}	526.2 ± 38.6^{b}

Means with different letters (a, b) differ ($P < 0.05$). Data were presented as means ± SD of at least three independent experiments.

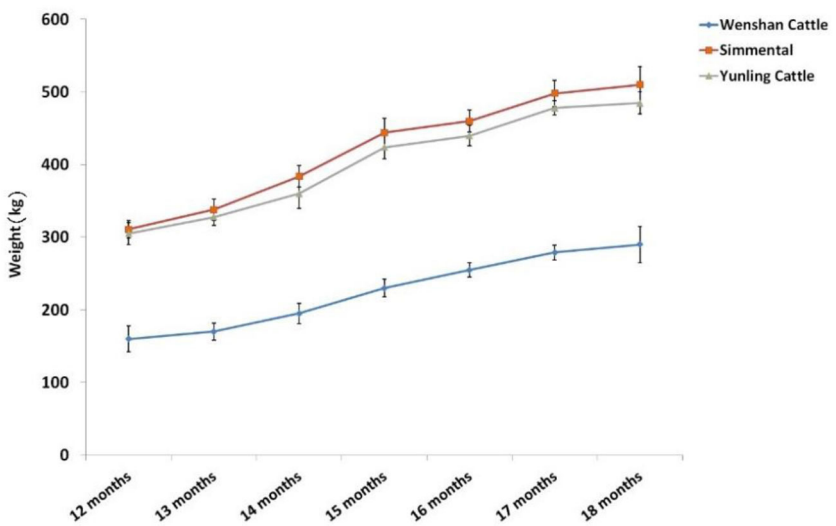

FIGURE 1 | Trend of body weight change at different growth periods in the three cattle breeds. Data were presented as means ± SD of at least three independent experiments.

there was a significantly positive correlation between water loss (%) and acid excretion time ($P < 0.01$) and a significantly positive correlation between meat color (L^* value, a^* value, and b^* value) and water loss (%) ($P < 0.05$; **Table 6**). Thus, data suggest that with different time points, both pH value and muscle tenderness decrease, and the water loss rate under

TABLE 3 | Growth hormone levels of three cattle breeds at the age of 18 months.

Hormones	Breed		
	Wenshan cattle	**Simmental cattle**	**Yunling cattle**
TSH, μIU/L	339.7 ± 11.2[a]	407.6 ± 15.1[b]	470.5 ± 12.4[b]
T4, μg/L	207.6 ± 11.3[a]	202.1 ± 5.2[a]	287.1 ± 42.9[b]
T3, pmol/L	49.3 ± 3.8[a]	59.8 ± 1.9[b]	54.6 ± 5.1[b]
GH, μg/L	23.3 ± 1.1[a]	27.1 ± 0.6[b]	28.7 ± 3.8[b]
INS, mIU/L	31.4 ± 1.5[a]	30.7 ± 1.4[a]	40.8 ± 3.3[b]
IGF-1, μg/L	18.5 ± 0.1[a]	22.4 ± 0.9[b]	23.1 ± 3.8[b]

Means with different letters (a, b) differ ($P < 0.05$). Data were presented as means ± SD of at least three independent experiments.

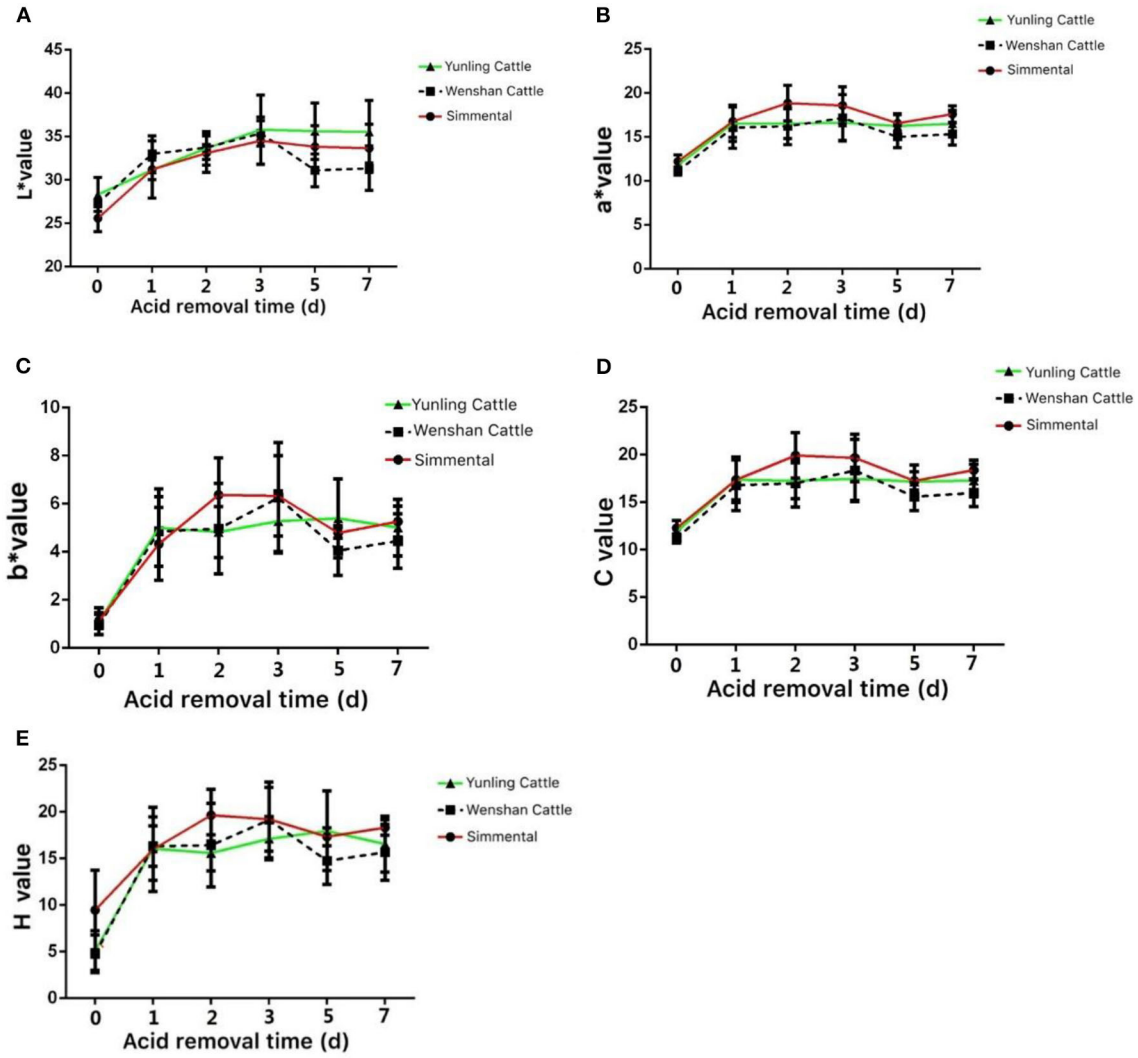

FIGURE 2 | Meat color at different growth periods in the three cattle breeds. **(A)** Change in the *L** value in the muscle aging process of the three cattle breeds; **(B)** change in the *a** value during muscle aging in the three cattle breeds; **(C)** change in the *b** value during muscle aging in the three cattle breeds; **(D)** change in the *c** value during muscle aging in the three cattle breeds; **(E)** change in the *H* value during muscle aging in the three cattle breeds. Data were presented as means ± SD of at least three independent experiments.

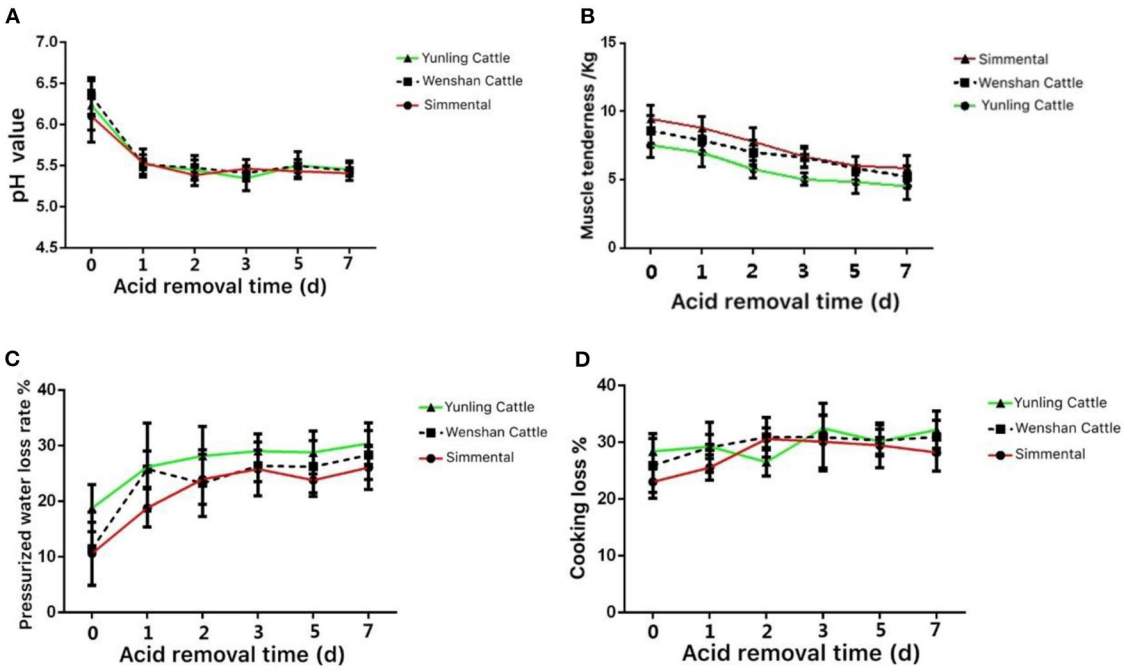

FIGURE 3 | Meat quality of the three cattle breeds. **(A)** Change in pH value in the muscle aging process of different breeds of cattle; **(B)** change in muscle tenderness in the muscle aging process of different breeds of cattle; **(C)** change in pressurized water loss rate in the muscle aging process of different breeds of cattle; **(D)** change in weight while cooking in the muscle aging process of different breeds of cattle. Data were presented as means ± SD of at least three independent experiments.

TABLE 4 | Correlation analysis of meat quality index in Yunling beef in the process of maturity.

Index	Acid excretion time	pH	Water loss rate %	Cooking loss %	Tenderness	*L** value	*a** value	*b** value
Acid excretion time	1	−0.494**	0.399*	0.419*	−0.510**	0.607**	0.411*	0.472**
pH		1	−0.600**	−0.354	0.163	−0.622**	−0.677**	−0.662**
Water loss %			1	0.211	−0.264	0.539**	0.587**	0.514**
Cooking loss %				1	−0.084	0.316	0.135	0.221
Tenderness					1	−0.502**	−0.057	−0.067
*L** value						1	0.591**	0.628**
*a** value							1	0.932**
*b** value								1

Asterisk indicates significant difference ($P < 0.05$). Data were presented as means ± SD of at least three independent experiments.

TABLE 5 | Correlation analysis of meat quality index in Simental beef in the process of maturity.

Index	Acid excretion time	pH	Water loss rate %	Cooking loss %	Tenderness	*L** value	*a** value	*b** value
Acid excretion time	1	−0.546**	0.615**	0.367*	−0.410	0.553**	0.391*	0.417*
pH		1	−0.621**	−0.527**	−0.051	−0.728**	−0.629**	−0.653**
Water loss %			1	0.656**	−0.178	0.741**	0.769**	0.791**
Cooking loss %				1	0.181	0.590**	0.473**	0.558**
Meat tenderness					1	−0.214	−0.134	−0.109
*L** value						1	0.712**	0.782**
*a** value							1	0.962**
*b** value								1

Asterisk indicates significant difference ($P < 0.05$). Data were presented as means ± SD of at least three independent experiments.

TABLE 6 | Correlation analysis of meat quality index in Wenshan beef in the process of maturity.

Index	Acid excretion time	pH	Water loss rate %	Cooking loss %	Tenderness	*L** value	*a** value	*b** value
Acid excretion time	1	−0.560**	0.534**	0.295	−0.598**	0.078	0.268	0.277
pH		1	−0.770**	−0.418*	0.336	−0.379*	−0.685**	−0.677**
Water loss %			1	0.353	−0.221	0.148	0.553**	0.536**
Cooking loss %				1	−0.121	0.104	0.187	0.169
Meat tenderness					1	−0.12	−0.256	−0.219
*L** value						1	0.351	0.427*
*a** value							1	0.966**
*b** value								1

Asterisk indicates significant difference ($P < 0.05$). Data were presented as means ± SD of at least three independent experiments.

pressure increases; however, meat color and cooking loss may not be affected by acid discharge. The pH value of Wenshan beef showed a significantly negative correlation with water loss, cooking loss, and meat color (L^* value, a^* value, and b^* value, **Table 6**).

Sample RNA Quality

Total RNA integrity and purity of six muscle samples (S1–S3 and y1–y3) satisfied sequencing standards (RIN ≥ 8.0; OD260 nm/OD280 nm ≥ 2.0; 28S/18S ≥ 1.6), which could be used for further RNA-Seq transcriptomic sequencing.

Read Data Quality Control (Reads QC)

The base effective ratio and Q30 are above 90% (**Supplementary Table 1**). It can be seen from the base mass distribution diagram (**Figure 4A**) that the quality of sequencing data was above the average value. In addition to the first several bases, the transcripts obtained from the sequencing results had considerable fluctuation. The results showed that the sequencing data were suitable for further analysis.

Sequencing Quality Control

In genome alignment, the reads across introns are divided into multiple tags. The number of tags was enriched in different gene elements to determine whether most of the tested samples fell into the coding sequence (CDS) region. It is shown in **Figure 4B** that most reads were matched to the CDS exon, which is in line with the results predicted by transcriptomic sequencing.

Differentially Expressed Genes and Cluster Analysis

After sequencing, 890 differentially expressed genes were screened between the two groups according to the principle of $P < 0.05$ and $\log_2$ fold change >2. There were 356 upregulated genes and 534 downregulated genes (**Figure 4C**, **Supplementary Material 1**).

GO Function and Enrichment Analysis of KEGG Pathways

Blast2GO software was used to annotate the GO function of differentially expressed genes and analyze the biological process, cellular component, and molecular function. The biological process module (**Figure 4D**) mainly includes the regulation of cell growth involved in cardiac muscle, the cell component module (**Figure 4E**) mainly includes the calcineurin complex, and the molecular function module (**Figure 4F**) mainly includes lipoprotein particle binding. The KEGG database was used to analyze differentially expressed genes involved in pathway enrichment (**Figure 4G**). The genes were enriched in 20 pathways, mainly in the adipocytokine signaling pathway and protein processing in the endoplasmic reticulum. Five significantly differentially expressed genes, namely *LEPR*, *STAT3*, *leptin*, *HSPA12A*, and *CAPN1*, were screened out.

Quantitative PCR Verification of Differentially Expressed Genes

To further verify the reliability of sequencing results, five key differentially expressed genes (*LEPR*, *STAT3*, *leptin*, *HSPA12A*, and *CAPN1*) were selected based on function and pathway enrichment in the present study. Results showed that the expression levels of the five genes in Simmental and Yunling cattle longissimus dorsi muscle were largely consistent with the transcriptomic RNA-Seq results, indicating that the sequencing results were reliable (**Figure 5**). The correlation analysis between expression levels and muscle shear force showed that *HSPA12A* and *CAPN1* were positively correlated with tenderness ($P < 0.05$), and the correlation coefficients were 0.8980 and 0.8364, respectively (**Figure 6**). Therefore, *HSPA12A* and *CAPN1* can be used as important candidate genes to further study how they regulate beef quality in Chinese native cattle in future research.

DISCUSSION

The food quality of beef is affected by many factors, including palatability, flavor, juiciness, and tenderness. Chinese native cattle resources are abundant (22, 23). These cattle have a unique origin system and excellent genetic resources and ecological adaptability (24, 25). These native cattle breeds have many specific genes and potentials for meat production (26, 27). The meat quality

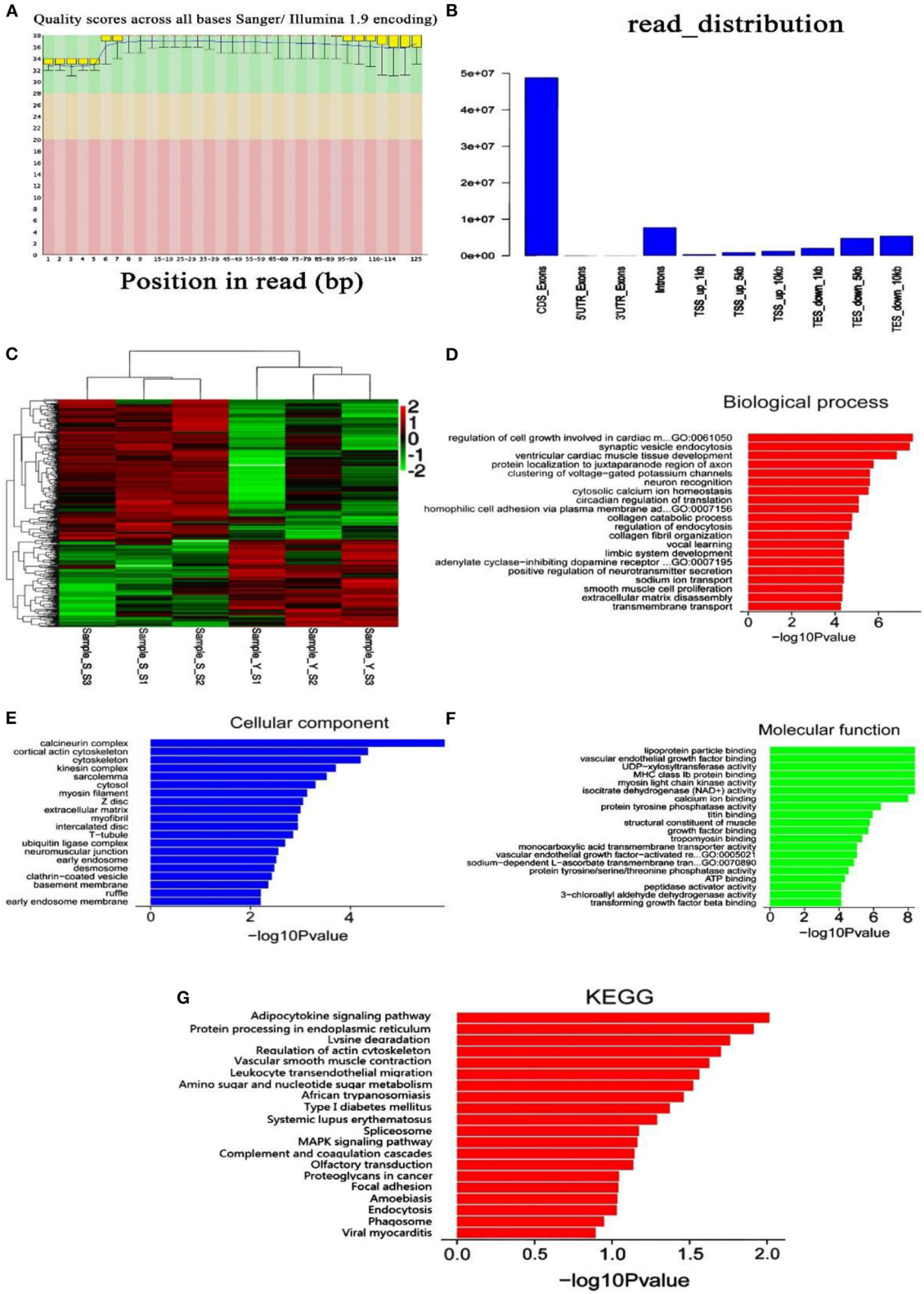

FIGURE 4 | Transcriptome sequencing analysis. **(A)** Quality distribution of the original database group; **(B)** distribution of the original samples in different patterns; **(C)** cluster analysis of differentially expressed genes; **(D)** annotation of biological process; **(E)** annotation of cellular components; **(F)** annotation of molecular function; **(G)** annotation of KEGG pathways.

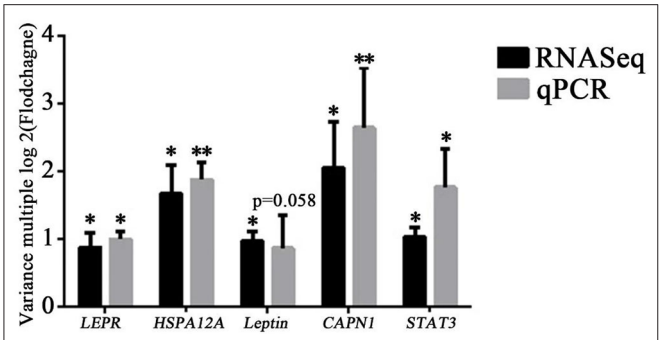

FIGURE 5 | Verification of differentially expressed genes by qPCR. Data were presented as means ± SD of at least three independent experiments.

analysis of Chinese native cattle (Yunling cattle, Wenshan cattle), a new hybrid beef cattle bred by Chinese researchers, is still a research gap meat quality. To explore Chinese native cattle initially, indicators were measured associated with meat quality of Yunling, Simmental, and Wenshan cattle. Simmental is an important reference. This study measured a set of indicators of Yunling beef, Wenshan cattle, and other breed as a control (Simmental cattle, an excellent beef cattle breed in the world), including pH, muscle tenderness, water loss rate, cooking loss, correlation analysis of acid excretion time and meat quality indexes, and mRNA expression. Physical and chemical indexes, such as meat color, pH value, water loss rate, tenderness, and cooking loss, were measured. This study reveals the changing pattern of meat quality in Chinese native cattle during acid discharge and lays a theoretical foundation for further development of high-quality native beef in China. Meat color mainly reflects changes in biochemical, physiological, and microbiological properties in muscle (28). The observation of meat color is widely used as an effective way to evaluate meat quality by consumers. The L^* value of Yunling cattle was significantly higher than that of Simmental and Wenshan cattle after 2 days postmortem. By contrast, the a^* value in the muscle of Simmental cattle was higher than that of Yunling cattle and Wenshan cattle. The b^* value of Yunling cattle, Simmental cattle, and Wenshan cattle showed an increasing trend between 0 and 1 day postmortem. The b^* value of Simmental cattle continued to rise between 1 and 2 days postmortem, while it began to decrease in the other two cattle breeds. The increase of L^* value and the decrease of a^* value are mainly due to the infiltration of water in muscle, thereby increasing the reflection ability of light.

Cooking loss is an effective index to evaluate the water-holding capacity in muscle (29). It has been well-established that water is the major lost substance during cooking (30, 31). Generally, acid excretion time is positively related to cooking loss, which is partly explained by the changes of material composition, pH value, and heating temperature in muscle. The cooking loss of Yunling cattle and Wenshan cattle fluctuated postmortem. For instance, Simmental cattle showed an obvious increasing trend during the first 2 days postmortem and then began to decline. Overall, the cooking loss of Yunling cattle was greater than that of Wenshan cattle and Simmental cattle. In addition, the shear force value of Simmental and Wenshan cattle showed a significant downward trend. After 5 days postmortem, the shear force values decreased slightly and tended to be stable. As a whole, the shear force value at the same time point is as follows: Simmental cattle > Wenshan cattle > Yunling cattle. Therefore, data suggest that the muscle tenderness of Yunling cattle was better than that of Simmental cattle. Various biochemical reactions still exist in muscle postmortem but mainly anaerobic metabolism, constantly generating plenty of lactic acids and other substances, resulting in decreased pH value in muscle (32, 33). It was found that the tenderness of muscle was the highest at the beginning of slaughter and then reduced along with the decrease of pH. In the current study, the pH value of Yunling cattle, Simmental cattle, and Wenshan cattle decreased in the course of 0–7 days postmortem. In addition, changes in meat color, water-holding capacity, microbial growth rate, and protein solubility are all affected by decreased pH.

With the development of high-throughput sequencing technology, transcriptomic RNA-Seq sequencing has been widely used to determine gene expression levels in different species or tissues (34, 35). Bioinformatics is applied to elucidate the molecular mechanism on the formation of specific traits, which provides theoretical guidance for cattle breeding in the future. With the improvement of living standards, consumers are increasingly pursuing high-quality beef. To meet consumers' needs, it is highly important to cultivate beef breeds with good meat quality. Different growth traits, meat quality, and flavor in the three cattle breeds suggest potentially different mechanisms on regulating meat quality among different breeds. Therefore, we further analyzed differentially expressed genes in the longissimus dorsi muscle between Chinese native cattle and Simmental cattle. It is noteworthy that we found two important regulatory pathways (e.g., adipocytokine signaling pathway and protein processing in the endoplasmic reticulum) and screened some important differentially expressed genes, such as *LEPR*, *STAT3*, *leptin*, *HSPA12A*, and *CAPN1*. LEPR is a key receptor for leptin (36). STAT3, a signal transducer and activator of transcription, plays an important role in modulating cell proliferation, differentiation, and migration (37). *CAPN1*, as a cysteine-sparse endopeptidase, regulates biological processes of muscle growth and development, cell differentiation and apoptosis, and signal transduction (38). It was reported that calpain was the main enzyme that degraded muscle fiber, and enzyme activity was closely related to the change of meat tenderness. Moreover, it was also found that calpain could effectively improve feed efficiency in cattle. Heat shock proteins are proteins produced by stimulation of the external environment (39). Generally, different proteins in this family are highly conserved. These proteins are important nonspecific cytoprotective proteins that have a crucial influence on the maintenance of cell metabolism, apoptosis, and the stability of the internal environment. Thus, based on the correlation analysis between gene expression levels and tenderness, *HSPA12A* and *CAPN1* genes may play a critical role

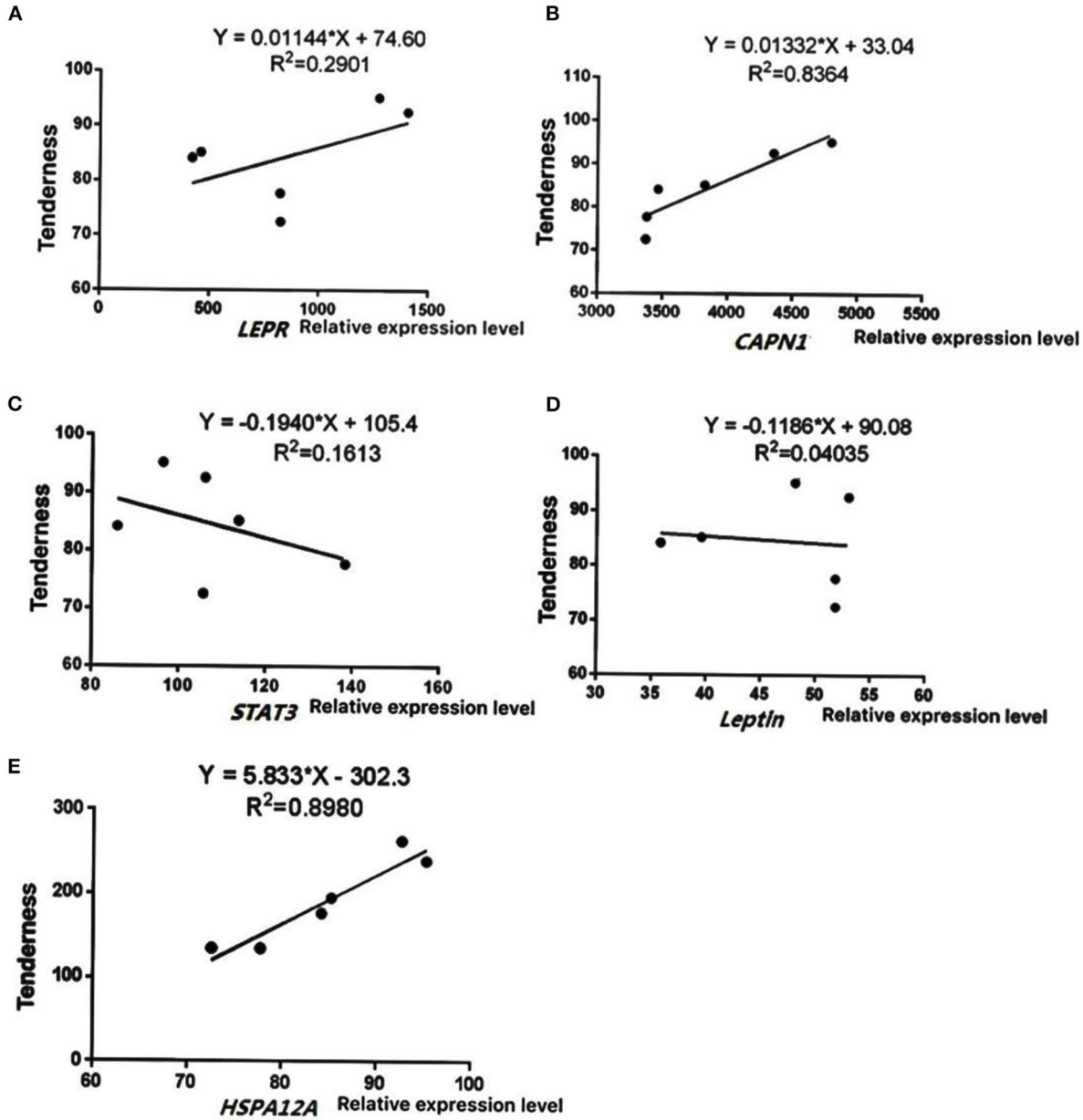

FIGURE 6 | Correlation analysis between gene expression levels and tenderness. Data were presented as means ± SD of at least three independent experiments. **(A)** The correlation analysis between *LEPR* levels and muscle shear. **(B)** The correlation analysis between *CAPN1* levels and muscle shear. **(C)** The correlation analysis between *STAT3* levels and muscle shear. **(D)** The correlation analysis between *Leptin* levels and muscle shear. **(E)** The correlation analysis between *HSPA12A* levels and muscle shear.

in changing native beef quality in China. Future research should be done using RNA interference and gene knockout technology at the cellular level to further clarify the specific functions of these target genes.

CONCLUSION

Overall, potential genetic markers related to meat quality in Chinese native cattle were selected, which can provide a theoretical reference for genetic improvement and breeding in cattle in the future.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are publicly available. This data can be found at https://www.ncbi.nlm.nih.gov/ using the following codes: SRR13156118, SRR13156117,

SRR13156116, SRR13156154, SRR13156153, SRR13156152, SRR13156151, SRR13156150, SRR13156149.

ETHICS STATEMENT

The animal study was reviewed and approved by Institutional Animal Care and Use Committee of the College of Animal Science and Technology, Yangzhou University, Yang Zhou, China.

AUTHOR CONTRIBUTIONS

XM, ZG, and ZY: conceptualization and writing—review and editing. YL and CZ: methodology. ZC, YM, and CZ: investigation. ZY: resources, project administration, and funding acquisition. XM: writing—original draft preparation. ZC: supervision. 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.2020.601064/full#supplementary-material

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Moderate Coconut Oil Supplement Ameliorates Growth Performance and Ruminal Fermentation in Hainan Black Goat Kids

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The study investigated amelioration effects of coconut oil (CO) on growth performance, nutrient digestibility, ruminal fermentation, and blood metabolites in Hainan Black goat kids. Twenty-four Hainan Black goat kids (10 days of age) were assigned randomly to four treatments for 90 days, including pre-weaning (10–70 d of age) and post-weaning (70-100 d of age) days. The treatment regimens were control (CON), low CO (LCO), medium CO (MCO), and high CO (HCO) with 0, 4, 6, 8 g CO per goat per day, respectively. During the pre-weaning period, the average daily gain (ADG) linearly and quadratically increased ($P < 0.05$), whereas the average daily feed intake (ADFI) linearly decreased, and the feed conversion ratio (FCR) also decreased linearly and quadratically by increasing CO supplementation ($P < 0.05$). During the post-weaning period, increasing CO supplementation linearly and quadratically increased the BW at 100 days and ADG ($P < 0.05$), but quadratically decreased the ADFI and FCR ($P < 0.05$). The digestibility of ether extract (EE) linearly and quadratically increased with increasing CO supplementation ($P < 0.05$). Supplementation of CO linearly increased ruminal pH ($P<0.05$), but linearly decreased ($P < 0.05$) ammonia-N, total VFAs, molar proportions of acetate, ruminal microbial enzyme activity of carboxymethyl-cellulase, cellobiase, xylanase, pectinase and α-amylase, and number of total protozoa, the abundance of *Ruminococcus albus*, *Ruminococcus flavefaciens*, *Fibrobacter succinogenes*, *Butyrivibrio fibrisolvens*, *Prevotella ruminicola,* and *Ruminobacter amylophilus*. The estimated methane emission decreased linearly and quadratically with increasing CO addition ($P < 0.05$). The serum concentration of triglycerides (TG), non-esterified fatty acids (NEFA) and growth hormone (GH) linearly ($P < 0.05$) increased by raising the CO supplementation. The present results indicate that CO supplementation at 6 g/day per goats is optimum due to improved growth performance and decreased estimated methane emission. Supplementation CO up to 8 g/day depressed growth and feed conversion due to its suppression of growth performance, rumen protozoa, cellulolytic bacteria and microbial enzyme activity, and reduced ADF and ADF digestibility.

Keywords: coconut oil, growth performance, nutrient digestibility, ruminal fermentation, blood metabolites

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INTRODUCTION

Goats are important meat-producing animals and goat meat is well-appreciated by consumers worldwide, especially in developing countries (1). Hainan Black goats are the main goat breed in South China, characterized by a good adaptability to the local hot and wet weather (2). Hainan Black goat meat is also very popular in South China because of its delicious flavors. However, Hainan Black goats exhibit slow growth rates and small body sizes, resulting in poor carcass characteristics (3).

Manipulation of the rumen microbial ecosystem to enhance fiber digestion, reduce the excretion of methane and urea, in order to improve the production performance of ruminants is one of the most important goals for animal nutritionists (4, 5). Dietary fats have been used to improve ruminant growth performance and modify meat characteristics with human health benefits (6). Moreover, fat supplementation in the diet of newborn lambs would be considered an effective mechanism to modify the rumen microbiome (7, 8). Therefore, further understanding of the effects of fat on rumen fermentation may help to offer a nutritional strategy to reduce rumen methane emissions and improve the quality of ruminant products.

Among all the lipid feedstocks, vegetable oils, oilseed, and calcium salts of fatty acids are the most appropriate for application in ruminant diets (9). Coconut oil is a cheaper, tastier, and readily available feed resource for ruminants (10). Coconut oil is a highly saturated oil (About 90% saturation), which is rich in medium chain fatty acids (MCFAs) (11). MCFA have been reported to reduce fat deposition due to their faster metabolism and reduced storage in adipocytes (12). Furthermore, coconut oil has been proven to exert positive environmental effects by enhancing rumen fermentation via limiting the production of methane and modifying microbial populations (13–15). There have been discrepancies in the results obtained by studies conducted to evaluate the effects of coconut oil supplementation on nutrient digestibility, growth performance and body composition of ruminants. The studies reported by Ding et al. (16) found that supplementing 12 g CO or 0.48 g/kg BW daily, showed a strong methane reduction as well as a decrease in the number of methanogen and *Fibrobacter succinogenes* in Tibetan sheep. Similar effects were also observed by Liu et al. (17) who reported that supplementation with 0.52 g/kg BW CO in sheep decreased methane emissions by reducing the methanogen and protozoa populations without negatively affecting the growth performance or reduction of rumen total VFA. Besides, the anti-methane effects of CO were also observed in swamp buffalo (18) and dairy cows (19), and neither study identified negative effects of CO on DMI, nutrient digestibility or ruminal fermentation. However, a study on beef heifers with different levels of CO demonstrated a linear decrease in CH_4 production without affecting the DMI or giving rise to negative effects on DMI and digestibility at lower doses, with only the highest dose of 375 g/d yielding undesirable effects on the DMI and digestibility (20). Another study in lambs revealed that CO supplementation at 50 g/kg in the concentrate improved the feed conversion ratio and carcass traits of lambs, but its higher inclusion in ruminant diets has negative effects on growth and feed conversion due to its depressing impact on rumen protozoa which results in lower fiber digestibility (10). We hypothesized that in ruminant species, the level of fat, and the nature of the basal diet may determine the variable effects of CO on ruminal microbes.

Considering the inconsistent results regarding the impact of CO supplementation on growth performance, nutrient digestibility, and ruminal fermentation, as well as the limited research performed in goat kids, this study was undertaken to investigate the effects of coconut oil on growth performance, nutrient digestion, ruminal fermentation, and blood metabolites in Hainan Black goat kids.

Abbreviations: ADF, acid detergent fiber; ADG, average daily gain; BW, body weight; CO, coconut oil; CP, crude protein; DM, dry matter; DMI, dry matter intake; average daily feed intake, ADFI; EE, ether extract; FCR, Feed conversion ratio; GH, growth hormone; NDF, neutral detergent fiber; NEFA, non-esterified fatty acid; OM, organic matter; RT-PCR, real time polymerase chain reaction; TG, triglyceride; VFA, volatile fatty acids.

MATERIALS AND METHODS

Animals and Experimental Design

The animal and experiment protocols were approved by the Animal Care and Use Committee of Chinese Academy of Tropical Agricultural Sciences (ACUCC), Hainan, PR China. Twenty-four Hainan Black goat kids averaging 10 days of age and 2.05 ± 0.16 kg of body weight (BW) were randomly assigned to four treatment regimens. The treatments consisted of control (CON), low CO (LCO), medium CO (MCO) and high CO (HCO) dosages containing 0, 4, 6, 8 g of CO per goat daily, respectively. The CO supplement was purchased commercially and sprayed into the back of the kids' mouth using a small syringe, twice a day at 0700 and 1700 h throughout the experimental period. From 10 to 70 days of age (weaning), the goat kids were fed with a milk replacer (2% of BW) twice a day at 0800 h and 1800 h for 30 days, after which the daily milk portion was decreased by half until weaning. The goats were weighed weekly to calculate the amount of milk replacer to be administered. The goats were also offered an *ad libitum* concentrate and dried king grass in a cafeteria system during the whole experimental period, and the dietary concentrate to forage ratio was maintained at 50:50 based on an air-dry matter. All goats were fed the same concentrate mixture. The post-weaning feeding management for all goats was kept identical that of the pre-weaning phase, except for the fact that administration of the milk replacer stopped at 70 days of age. The ingredients and chemical composition of the experimental diets were illustrated in **Table 1**. Fresh water was available to the goats for drinking throughout the experimental period. The animals were weighed at 10, 70, and 100 days of age before feeding, and the average daily gain (ADG) was recorded.

Data Collection and Sampling Procedures

The milk intake of individual goats was measured during the pre-weaning period. Feed offered and refusals for each goat were also recorded on a daily basis throughout the experimental

TABLE 1 | Ingredient and chemical composition of basal diets (Air-dry matter basis).

Item	Content
Ingredients of diet (%)	
Dried king grass	50.00
Corn	34.00
Soybean meal	9.00
Wheat bran	4.90
Shell powder	0.70
Sodium bicarbonate	0.30
Salt	0.70
Calcium carbonate	0.40
Chemical composition of diet	
Organic matter (%)	93.34
Crude protein (%)	16.55
Ether extract (%)	2.64
Neutral detergent fiber (%)	39.63
Acid detergent fiber (%)	26.45
Calcium (%)	0.32
Phosphorus (%)	0.22
Gross energy (MJ/kg)	18.12

period so as to calculate the daily DM intake (DMI). The goats were dosed via the esophagus with 1 g of chromic oxide in a paper capsule twice daily (07:00 and 19:00 h) from 78–87 days of age. The chromic oxide powder was used as a digestion marker to estimate the fecal excretion. From 83–87 days of age, Fecal pellets were collected from the rectum at 7:00, 15:00, and 24:00, then representative samples of the feces were pooled. The samples of feeds, refusals and feces were pooled for each goat, dried at 60°C for 48 h, ground to pass a 1 mm sieve, and preserved for chemical composition analysis. The apparent nutrient digestibility was calculated according to our prior studies (21).

Samples of rumen fluid were collected using an oral stomach tube at 07:00 by 70 days of age. The initial 100 mL ruminal fluid extracted was discarded, and the next 100 mL was retained. The fluid's pH values were immediately measured using a pH meter (PHS-3C, Shanghai Leijun experimental instrument Co., Ltd., Shanghai, China). After pH measurement, the rumen fluid was filtered through four layers of cheesecloth and subsampled for various determinations. A 5 ml filtrate was preserved by adding 1 mL of 250 g/L meta-phosphoric acid or 1 mL of 20 g/L H_2SO_4 to determine the VFA and NH_3 concentrations, respectively. These samples were then frozen at −20°C until further analysis. About 50 mL of filtrate was collected and frozen at −80°C for DNA extraction, and another 40 mL of filtrate was used to determine the activity of ruminal enzymes according to the method described by Agarwal (22).

At 70 and 100 days of age, about 5 ml of blood was collected from the jugular vein and harvested into tubes without anticoagulant before the morning feeding at 100 days of age. Serum samples were then centrifuged at 3,000 × g for 15 min at 4°C and stored at −20°C until the assay.

Chemical Analyses

Oven-dried samples were analyzed for DM method 934.01), OM (method 942.05), nitrogen (method 976.05), ether extract (method 973.18) and acid detergent fiber (ADF; method 973.18) according to AOAC methods (23). The neutral detergent fiber (aNDF) was analyzed using methods described by Van Soest et al. (24) with heat stable alpha amylase and sodium sulfite utilized in the NDF procedure, and results were expressed inclusive of residual ash. Ruminal VFA concentration was measured by gas chromatography (HP Agilent 6890N, Santa Clara, CA, USA) with a flame ionization detector equipped with an HP-INNOWAX (19091N-133) capillary column (30 m × 0.25 mm × 0.25 μm). Two microliter of fluid samples were injected with a syringe, and the injector and detector temperature were programmed at 200 and 220°C, respectively. Nitrogen was used as a carrier flowing at 5.5 mL/min. A program altered oven temperature from 80 to 170°C at 15°C/min and then held it at 170°C for 1.5 min. Ruminal VFA were expressed on the basis of absolute concentrations (mM) and molar proportions (mol/100 mol total VFA). Ruminal ammonia-N concentration was determined by a colorimetric spectrophotometer (UV2100, Shanghai Younike instrument Co., Ltd., Shanghai, China) according to AOAC methods (2000). Subsequently ruminal fluid samples were sonicated at 4°C in an ice bath with a 30 s pulsation rate for 10 min, then centrifuged at 3,000 × g at 4°C for 20 min. The resulting supernatant was used for estimation of the enzyme activity (carboxymethyle cellulase, cellobiase, xylanase, pectinase, α-amylase and protease) as described by Agarwal et al. (22). Serum parameters including glucose, cholesterol, and triglycerides were determined by using the BH13 MD 1600 (America) automatic biochemical analyzer. Serum level of non-esterified fatty acids (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) and growth hormone (Shanghai Fankel Industrial Co., Ltd, Shanghai, China) were determined by using enzyme-linked immunosorbent assay (ELISA) kits according to the manufacturer's instructions.

DNA Extraction and Quantitative Real-Time PCR

Microbial DNA was extracted from 0.5 g of rumen fluid by using a Fastpure Bacteria DNA Isolation Mini Kit (Vazyme, Version 8.1). Subsequently, agarose gel electrophoresis and the NanoDrop 2000 Spectrophotometer (NanoDrop Technologies, USA), were used to evaluate the quality and quantity of DNA, respectively. The extracted DNA was then kept frozen at −20°C for real time PCR analysis. Populations of *Ruminococcus albus*, *Ruminococcus flavefaciens*, *F. succinogenes*, *Butyrivibrio fibrisolvens*, *Prevotella ruminicola,* and *Ruminobacter amylophilus* were estimated using real time PCR as a proportion of the total number of bacteria. The sequences of all primers were synthesized by Tianyi Huiyuan Biotechnology Co., Ltd and displayed in **Table 2**. All real-time PCR reactions were carried out in triplicate and run on Applied Biosystems 7500 Fast real-time quantitative PCR systems. The reaction mixture (20 μL) contained 10 μL SYBR Color qPCR Master Mix (Vazyme Biotechnology Co., Ltd., Nanjing, China), 0.4 μL 10 μmol/L PCR Forward Primer, 0.4 μL 10 μmol/L

TABLE 2 | The primer of ruminal bacteria and 16s rRNA genes.

Target species	Primer sequence	GeneBank accession no.	Size (bp)
Total bacteria	F: CGGCAACGAGCGCAACCC	AY548787.1	147
	R: CCATTGTAGCACGTGTGTAGCC		
Total protozoa	F: GCTTTCGWTGGTAGT GTATT	HM212038.1	234
	R: CTTGCCCTCYAATCGTWCT		
R. albus	F: CCCTAAAAGCAGTCTTAGTTC G	CP002403.1	175
	R: CCTCCTTGCGGTTAGAACA		
R. flavefaciens	F: ATTGTCCCAGTTCAGATTGC	AB849343.1	132
	R: GGCGTCCTCATTGCTGTTAG		
F. succinogenes	F: GTTCGGAATTACTGGGCGTAA A	AB275512.1	121
	R: CGCCTGCCCCTGAACTATC		
B. fibrisolvens	F: ACCGCATAAGCGCACGGA	HQ404372.1	65
	R: CGGGTCCATCTTGTACCGATA AAT		
R. amylophilus	F: CTGGGGAGCTGCCTGAATG	MH708240.1	102
	R: GCATCTGAATGCGACTGGTTG		
P. ruminicola	F: GAAAGTCGGATTAATGCTCTATGTTG	LT975683.1	74
	R: CATCCTATAGCGGTAAACCTTTGG		

PCR Reverse Primer, 0.4 μL ROX Reference Dye (50×), 6.8 μL ddH_2O and 2 μL of the template DNA. The quantity of DNA was measured in triplicate for each sample using the ND-1000 UV spectrophotometer (NanoDrop Technologies, USA), and the mean values were estimated. PCR was implemented according to the following conditions: Degeneration at 95°C for 60 s; PCR reaction at 95°C for 15 s and 60°C for 30 s, with 40 cycles; dissociation stage.

Statistical Analyses

Data analysis was conducted using the SAS mixed model procedure (Proc Mixed; SAS, 2002). Analysis of variance (ANOVA) was performed to examine the effects of the respective treatment regimens on growth performance, nutrient digestibility, ruminal fermentation, and blood metabolites. Linear and quadratic effects were tested using the CONTRAST statement of SAS with coefficients estimated based on the CON application rates. Differences between the treatment regimens were detected by the Duncan's multiple range test. The *P*-value for statistical significance was set at $P \leq 0.05$, unless otherwise noted $P \leq 0.10$ was considered as a tendency approaching significance.

RESULTS

Dry Matter Intake, Average Daily Gain, and Feed Conversion Ratio

Dry matter intake, average daily gain and feed conversion ratio were delineated in **Table 3**. The dry matter intake (DMI) exhibited a linear decline ($P < 0.05$) with increasing CO supplementation for pre-weaned and post-weaned goats, and was lower for HCO than for control, LCO, and MCO ($P < 0.05$). Meanwhile the average daily gain (ADG) for pre-weaned and post-weaned goats increased linearly ($P < 0.05$) and quadratically ($P < 0.05$) with increasing CO supplementation and was higher for MCO than that for control, LCO, and HCO ($P < 0.05$). The feed conversion ratio (FCR) for pre-weaned and post-weaned goats decreased linearly ($P < 0.05$) and quadratically ($P < 0.05$) with increasing CO supplementation, and was lower for MCO group than control and HCO ($P < 0.05$).

Nutrient Digestibility and Ruminal Fermentation Parameters

As presented in **Table 4**, the digestibility of crude protein (CP) was not affected by CO addition. The digestibility of DM, OM, aNDF and ADF decreased linearly ($P < 0.05$) with increasing CO supplementation, and was lower for MCO than that of control, LCO and HCO groups ($P < 0.05$). However, the digestibility of EE increased linearly ($P < 0.05$) and quadratically ($P < 0.05$) with increasing CO supplementation, and was higher for MCO and HCO than control and LCO ($P < 0.05$).

Furthermore, ruminal pH increased linearly ($P < 0.05$) with increasing CO supplementation and was higher for HCO and LCO than control ($P < 0.05$). Total ruminal VFA concentration linearly decreased ($P < 0.05$) and was lower for HCO group than other three groups ($P < 0.05$). The molar proportions of propionate, valerate, and the ratio of acetate to propionate were not affected ($P > 0.05$), but the molar proportions of acetate, butyrate, isobutyrate and isovalerate linearly ($P < 0.05$) decreased with increasing CO supplementation, and was lower for the HCO than for control, LCO and MCO ($P < 0.05$). Ruminal ammonia N content linearly reduced by increasing CO supplementation ($P < 0.05$). The estimated methane emission decreased linearly ($P < 0.05$) and quadratically ($P < 0.05$) with increasing CO supplementation and was lower for the LCO, MCO, and HCO than control ($P < 0.05$).

Ruminal Microbial Enzyme Activity and Populations of Ruminal Cellulolytic Bacteria

The enzymatic activities of caboxymethyl-cellulase, cellobiase, xylanase, pectinase and α-amylase

TABLE 3 | Effects of coconut oil on dry matter intake, average daily gain and feed conversion ratio in goat kids.

	Treatment[e]					*P*-value[f]		
Item[f]	CON	LCO	MCO	HCO	SEM[g]	Treatment	Linear	Quadratic
Pre-weaning (10∼70 days of age)								
Body weight (kg)								
Birth weight	1.82	1.91	1.93	1.87	0.157	0.982	0.921	0.775
10 days	2.05	2.13	2.17	2.03	0.086	0.994	0.956	0.786
70 days	5.57^{b}	6.23^{ab}	7.57^{a}	6.10^{ab}	0.249	0.020	0.134	0.020
ADG (g/d)	60.15^{b}	69.47^{b}	91.12^{a}	68.30^{b}	2.808	0.001	0.004	0.001
DMI (g/d)	325.17^{a}	325.50^{a}	323.17^{a}	309.33^{b}	2.184	0.014	0.006	0.152
FCR (kg/kg)	5.47^{a}	4.70^{b}	3.56^{c}	4.62^{b}	0.176	0.001	0.001	0.001
Post-weaning (70∼100 days of age)								
Body weight (kg)								
100 days	7.90^{d}	8.83^{b}	10.22^{a}	8.18^{c}	0.190	0.001	0.001	0.001
ADG (g/d)	77.67^{b}	86.45^{a}	88.52^{a}	68.78^{c}	1.982	0.001	0.034	0.001
DMI (g/d)	436.17^{a}	434.50^{a}	435.00^{a}	423.17^{b}	1.350	0.001	0.001	0.033
FCR (kg/kg)	5.67^{a}	4.88^{b}	4.95^{b}	6.22^{a}	0.151	0.001	0.086	0.001

^{a,b,c,d}*Means with different superscripts in each row differ significantly (P < 0.05).*
[e]*Control (without CO), LCO, MCO and HCO with 4, 6, and 8 g CO per goat per day, respectively.*
[f]*ADG, average daily bodyweight gain; DMI, dry matter intake; FCR, feed conversion ratio.*
[g]*SEM, standard error of the mean (n = 6).*

TABLE 4 | Effects of coconut oil on nutrient digestibility and ruminal fermentation in goat kids.

	Treatment[e]					*P*-value		
Item	CON	LCO	MCO	HCO	SEM[f]	Treatment	Linear	Quadratic
Nutrient digestibility (%)								
Dry matter	0.63^{a}	0.62^{a}	0.62^{a}	0.60^{b}	0.003	0.001	0.001	0.121
Organic matter	0.62^{a}	0.61^{ab}	0.61^{a}	0.59^{b}	0.004	0.014	0.005	0.461
Crude protein	0.74	0.73	0.73	0.73	0.005	0.875	0.456	0.835
Ether extract	0.61^{d}	0.66^{c}	0.72^{b}	0.79^{a}	0.014	0.001	0.001	0.042
Neutral detergent fiber	0.57^{a}	0.52^{c}	0.54^{b}	0.51^{d}	0.005	0.001	0.001	0.301
Acid detergent fiber	0.43^{a}	0.41^{b}	0.41^{b}	0.39^{c}	0.005	0.001	0.001	0.775
Ruminal fermentation pH	6.35^{b}	6.46^{a}	6.44^{ab}	6.48^{a}	0.018	0.033	0.013	0.276
Total volatile fatty acid (mmol/L)	91.15^{a}	90.16^{a}	90.53^{a}	88.01^{b}	0.423	0.016	0.006	0.192
Mol/100 mol								
Acetate (A)	72.41^{a}	70.74^{a}	70.09^{ab}	68.79^{b}	0.491	0.011	0.002	0.464
Propionate (P)	20.94	20.31	20.00	20.34	0.188	0.385	0.234	0.221
Butyrate	13.84^{a}	13.18^{ab}	13.02^{b}	11.77^{c}	0.236	0.001	0.001	0.107
Valerate	1.74	1.72	1.72	1.70	0.008	0.547	0.221	0.789
Isobutyrate	1.12^{a}	1.05^{b}	1.07^{b}	0.96^{c}	0.021	0.020	0.005	0.527
Isovalerate	1.53^{a}	1.46^{ab}	1.50^{a}	1.41^{b}	0.017	0.032	0.016	0.833
Acetate/Propionate	3.47	3.46	3.50	3.38	0.024	0.457	0.349	0.320
Ammonia-N (mg/100 ml)	12.71^{a}	12.50^{ab}	12.39^{ab}	12.13^{b}	0.092	0.158	0.032	0.886
Methane (mol/mol TVFA)	32.04^{a}	31.52^{b}	31.29^{b}	30.07^{c}	0.224	0.001	0.001	0.012

^{a,b,c,d}*Means with different superscripts in each row differ significantly (P < 0.05).*
[e]*Control (without CO), LCO, MCO and HCO with 4, 6, and 8 g CO per goat per day, respectively.*
[f]*SEM, standard error of the mean (n = 6).*

linearly ($P < 0.05$) decreased with increasing CO supplementation, and were lower for HCO than the control ($P < 0.05$) (**Table 5**).

Total bacterial and *F. succinogenes* populations linearly ($P < 0.05$) and quadratically ($P < 0.05$) decreased, and the populations of *R. albus*, *R. flavefaciens*, *B. fibrisolvens*, *P. ruminicola,* and

TABLE 5 | Effects of coconut oil on rumen microbial enzyme activity and ruminal microflora in goat kids.

Item	Treatment[e]				SEM[h]	*P*-value		
	CON	LCO	MCO	HCO		Treatment	Linear	Quadratic
Microbial enzyme activity[f]								
Caboxymethyl-cellulase	0.32[a]	0.28[b]	0.28[b]	0.23[c]	0.010	0.001	0.001	0.658
Cellobiase	0.14[a]	0.13[ab]	0.13[ab]	0.11[b]	0.004	0.129	0.041	0.420
Xylanase	0.45[a]	0.39[b]	0.39[b]	0.35[b]	0.012	0.011	0.002	0.559
Pectinase	0.33[a]	0.29[b]	0.24[c]	0.21[d]	0.014	0.001	0.001	0.688
α-amylase	1.56[a]	1.46[b]	1.46[b]	1.40[c]	0.019	0.001	0.001	0.343
Protease	0.47[a]	0.46[a]	0.43[b]	0.42[b]	0.007	0.001	0.001	0.706
Microbiota (copies/ml)[g]								
Total bacteria $\times 10^{11}$	1.77[a]	1.46[b]	1.44[b]	1.40[b]	0.046	0.001	0.001	0.001
protozoa $\times 10^5$	5.42[a]	4.69[b]	4.12[c]	2.71[d]	0.308	0.001	0.001	0.076
R. albus $\times 10^8$	0.71[a]	0.36[b]	0.35[b]	0.04[c]	0.079	0.002	0.001	0.816
R. flavefaciens $\times 10^8$	0.74[a]	0.32[b]	0.35[b]	0.09[c]	0.077	0.001	0.001	0.291
F. succinogenes $\times 10^8$	0.51[a]	0.49[a]	0.48[a]	0.15[b]	0.046	0.001	0.001	0.001
B. fibrisolvens $\times 10^8$	1.75[a]	1.58[b]	1.54[b]	1.39[c]	0.039	0.001	0.001	0.524
P. ruminicola $\times 10^8$	0.82[a]	0.41[bc]	0.37[bc]	0.20[c]	0.072	0.001	0.001	0.052
R. amylophilus $\times 10^9$	0.74[a]	0.38[b]	0.17[c]	0.03[d]	0.087	0.001	0.001	0.195

[a,b,c,d] *Means with different superscripts in each row differ significantly ($P < 0.05$).*

[e] *Control (without CO), LCO, MCO and HCO with4, 6and 8 g CO per goat per day, respectively.*

[f] *Units of enzyme activity are: carboxymethyl cellulase (μmol glucose/min/ml), cellobiase (μmol glucose/min/ml), xylanase (μmol xylose/min/ml), pectinase (μmol D-galactouronic acid /min/ml), α-amylase (μmol glucose/min/ml) and protease (μg hydrolysed protein/min/ml).*

[g] *R. albus, Ruminococcus albus; R. flavefaciens, Ruminococcus flacefaciens; B. fibrisolvens, Butyrivibrio fibrisolvens; F. succinogenes, Fibrobacter succinogenes; R. amylophilus, Ruminobacter amylophilus; P. ruminicola, Prevotella ruminicola.*

[h] *SEM, standard error of the mean (n = 6).*

R. amylophilus decreased linearly ($P < 0.05$) with increasing CO supplementation (**Table 5**).

Blood Metabolites

The serum concentration of TGs, NEFAs, and GH linearly ($P < 0.05$) increased with increasing CO supplementation and was higher for HCO group than for the control ($P < 0.05$). Nevertheless, serum glucose and TC were not affected by CO supplementation ($P > 0.05$) for pre-weaning and post-weaning goats (**Table 6**).

DISCUSSION

Growth Performance of Goat Kids

An appropriate amount of energy supply is the key to ensure and promote the healthy and rapid growth and development of young ruminants. Dietary MCFA (medium chain fatty acid) can effectively reduce body fat deposition and improve lipid concentration. However, there have been discrepancies in the results of coconut oil supplementation on nutrient digestibility, growth performance, etc. The decrease in DM intake with increasing CO supplementation was consistent with the findings of other studies, in which DM intake was decreased by CO supplementation (25, 50, and 75 g/kg of concentrate) in the diet of lambs (10). This reduced DM intake was not surprising, since the negative effects of CO on DMI may be a consequence of higher energy density in the diet (25, 26). Moreover, higher CO inclusion have been shown to be related to decreased NDF digestion (27) and palatability (28). Hollmann and Beede (29) also reported that CO replacement of ground corn in the diets of lactating dairy cows with CO lead to a significant reduction in the DMI. Linear and quadratic increments in the ADG with higher CO supplementation were observed in our experiment. Meanwhile, FCR in this study quadratically decreased with CO supplementation during the pre-weaning and post-weaning periods. This could be attributed to the improvement in the energy intake level and EE digestibility by CO supplementation, essentially due to the higher EE levels of CO-supplemented diets. Similarly, Dutta et al. (30) reported a gradual increase in ADG up to 50 g/kg fat supplementation, but above this level it declined. Unlike our finding, Bhatt et al. (10) reported that increasing CO supplementation had no effect on ADG of lambs during the pre-weaning and post-weaning periods, which might be due to the heat stress in their study.

Nutrient Digestibility

Adding appropriate fat or fatty acid into ruminates can promote nutrient digestibility (8). The linear EE digestibility increments with higher CO supplementation observed in this study were also reported by Bhatt et al. (10). No change was observed in CP digestibility, but DM, OM, ADF, and NDF digestibility linearly decreased when increasing CO feeding portions. Similar results have been reported after CO addition (29, 31). This phenomenon could be the result of several factors. For instance, CO supplementation could markedly reduce the number of rumen protozoa (10). Rumen protozoa exhibited

TABLE 6 | Effects of coconut oil on serum biochemical indices and hormone secretion in goat kids.

	Treatment[c]					*P*-value		
Item[d]	CON	LCO	MCO	HCO	SEM[e]	Treatment	Linear	Quadratic
70 days of age								
TC (mmol/L)	3.69	3.49	3.86	4.20	0.174	0.571	0.261	0.468
TG (mmol/L)	0.58[b]	0.67[ab]	0.75[ab]	0.82[a]	0.045	0.136	0.024	0.830
Glucose (mmol/L)	4.50	3.85	3.99	4.08	0.113	0.155	0.226	0.077
NEFA (mmol/L)	0.19[b]	0.21[b]	0.27[ab]	0.32[a]	0.020	0.037	0.007	0.560
GH (ug/L)	5.60[b]	6.59[ab]	7.47[a]	6.82[ab]	0.267	0.067	0.040	0.086
100 days of age								
TC (mmol/L)	3.77	3.72	3.94	4.31	0.162	0.217	0.070	0.316
TG (mmol/L)	0.65[b]	0.72[b]	0.79[ab]	0.90[a]	0.034	0.027	0.004	0.679
Glucose (mmol/L)	4.62	4.43	4.28	4.23	0.087	0.285	0.089	0.485
NEFA (mmol/L)	0.25[b]	0.29[ab]	0.32[ab]	0.38[a]	0.019	0.081	0.015	0.751
GH (ug/L)	6.41[b]	6.94[b]	7.68[a]	7.04[ab]	0.161	0.014	0.019	0.028

[a,b] *Means with different superscripts in each row differ significantly ($P < 0.05$).*
[c] *Control (without CO), LCO, MCO and HCO with4, 6, and 8 g CO per goat per day, respectively.*
[d] *TC, Total-cholestero; NEFA, non-esterified fatty acid; TG, triacylglycerols; GH, Growth hormone.*
[e] *SEM, standard error of the mean ($n = 6$).*

cellulase, hemicellulase, and pectinase activities (32–34), which may explain their role in NDF digestion. Additionally, rumen protozoa may also alter the number of cellulolytic bacteria, and thus affect the extent of ruminal fiber fermentation (35). In the present study, the reduction in NDF digestibility was consistent with a reduction in protozoal numbers by increasing CO supplementation, and this maybe a reason for reduced NDF and ADF digestibility by increasing CO feeding portions.

Ruminal Fermentation, Microorganism Population and Enzyme Activities

Fat can be used as carrier of fat-soluble vitamins and promote the absorption and utilization of fat-soluble vitamins, negative effects on rumen microbes, fiber digestion, and fermentation (10, 14). The pH in rumen fluid linearly increased with increasing CO supplementation and was higher for LCO and HCO than MCO and control group, which was similar with the observation of Pilajun et al. (36), who reported that ruminal pH was directly proportional to the dosage of CO replacing sunflower oil from 250 to 750 g/kg in steers. Ruminal microbes can utilize ruminal ammonia-N derived from protein degradation for microbial protein synthesis (37). The lower ammonia-N levels produced by goats receiving CO supplementation were likely due to decreased protease activity, and reduced rumen bacterial and protozoal populations.

CO supplementation linearly decreased the total VFA concentration in the rumen. The results of this study were consistent with those obtained by Machmüller et al. (38), who found that CO supplementation tended to decrease the total VFA concentration. This finding could be due to the inhibitory effect of fatty acids on fiber digestion (39) and toxicity of fats to microorganisms (40). Moreover, no differences were observed in the molar proportion of propionate, but the molar proportions of acetate and butyrate were decreased with higher levels of CO supplementation. The possible explanation for this phenomenon was that CO inhibited bacteria and protozoa that are not related to *Selenomonas ruminantium*, which is essential to propionate production (41). Ruminal cellulolytic bacteria and protozoa produce cellulolytic enzymes and degrade dietary fiber to acetate (42). Thus, the lower acetate molar proportion resulted from the decrease in activity of carboxymethyl-cellulase, cellobiase and xylanase as well as the total population of bacteria, protozoa, and cellulolytic bacteria (*R. albus*, *R. flavefaciens*, *B. fibrisolvens,* and *F. succinogenes*) following CO addition. Being a by-product of carbohydrate fermentation, butyrate is produced by ruminal protozoa (43). Similarly, Hristov et al. (41) reported that CO supplementation inhibited both protozoa and important butyrate producers in the rumen, such as *B. fibrisolvens*. Hence, the decrease in the population of protozoa with CO supplementation observed in the present study also provides evidence for the reduction in the molar proportion of butyrate. In agreement with this study, similar findings in total VFA concentration (38), proportion of acetate (17), propionate (17), and A:P ratio (17) were reported in other previous studies. In contrast, Bozzolo et al. (44) found that dietary supplementation of 50 g/kg of CO had no significant effect on the concentration of VFA in the rumen of lambs for a period of 2 weeks directly after weaning. The inconsistency in these results could be due to that in their experiment, the lambs among the treatment fed the same level of fatty acids included in the diet, whereas in our experiment, goat kids in each treatment were fed a diet with different levels of total energy intake.

Calculation of ruminal methane production using VFAs based on this study's procedure demonstrated that CO supplementation elicited a significantly linear and quadratic decline in methane production. The protozoa populations were also linearly reduced by CO addition. These results were consistent with those of *in vitro* (45) and *in vivo* studies (15, 28), which have confirmed

the methane-suppressing effect of CO supplementation in ruminants.

The linear decrease in the total population of bacteria, protozoa, *R. amylophilus*, and predominant cellulolytic bacteria (*R. albus, R. flavefaciens, B. fibrisolvens, and F. succinogenes*) with increasing CO supplementation suggested that CO modulates the ruminal microorganisms in a dose-dependent manner. The toxicity of Medium-chain saturated FAs to the ruminal microbiota has been well-documented. Work by Hristov et al. (41) has confirmed that CO supplementation results in statistically significant suppression of microbial flow. Inhibition of total bacterial counts, cellulolytic and amylolytic species secondary to CO administration was reported by Dong et al. (45). *In vitro* study carried out by Patra and Yu (8) also reported that CO exerted inhibitory effects on protozoa and cellulolytic bacteria (*F. succinogenes* and *R. flavefaciens*). This decrease might be explained by the inhibitory effect of CO on protozoa or certain bacteria species that suppress the growth of cellulolytic bacteria in the rumen. The linear decrease in NDF digestibility with CO supplementation also provides evidence for the potential inhibitory effects of CO on rumen cellulolytic bacteria.

Rumen enzyme activity is closely related to the growth status of ruminal bacteria and then affects the degradation ability to nutrient (46). In the present study, the linear decrease in the enzymatic activities of caboxymethyl-cellulase, cellobiase, xylanase, pectinase, α-amylase, and protease with increasing CO supplementation confirmed the modulation of ruminal microbial activity by CO. Additionally, the decreased enzymatic activities of caboxymethyl-cellulase, cellobiase, xylanase, and pectinase were primarily attributed to the suppression of cellulolytic bacteria growth, hence resulting in a decreased NDF and ADF digestibility. *P. ruminicola* and *R. amylophilus* are able to secrete large amounts of α-amylase (47). The linear decrease in the enzymatic activities of α-amylase noticed in this study coincided with the decrease in the total number of *P. ruminicola* and *R. amylophilus* with increasing CO supplementation. In addition, the linear decrease in protease enzymatic activities was related to the inhibitory effect of CO on proteolytic bacteria. This finding was supported by the decreased ruminal ammonia-N concentration and CP digestibility.

Serum Biochemical Parameters

The serum concentration of glucose and TC were not affected by the treatments. In contrast, studies conducted in finishing heifers (48) and lambs (10) found an increase in serum cholesterol levels following CO supplementation. The discrepancy is attributed to the difference in animals in these studies. However, the serum concentration of TGs and NEFAs linearly increased with augmentation of CO supplementation. Circulating NEFAs derived from digestive tract absorption and adipose tissue release could be used to reflect the mobilization of body fat and metabolism of fatty acids (49, 50). In the present study, the higher blood concentrations of NEFA in HCO supplementation reflected the promoting of body fat mobilization as indicated by negative BW changes compared with the goats in MCO group. Furthermore, serum concentrations of GH are affected by the nutrient level and growth performance (51). In this present study, serum concentrations of GH exhibited a linear increase with increments in CO supplementation. This supports the hypothesis that optimum CO supplementation could result in positive responses of serum GH concentration and improvement of the goat kids' growth performance.

CONCLUSION

In summary, CO supplementation at 6 g/day per goats is optimum in goat kids due to improved ADG and feed conversion efficiency and decreased estimated methane emission. Supplementation CO up to 8 g/day depressed growth and feed conversion due to its suppression of growth performance, rumen protozoa, cellulolytic bacteria (*R. albus, R. flavefaciens, B. fibrisolvens,* and *F. succinogenes*) and microbial enzyme activity (caboxymethyl-cellulase, cellobiase, xylanase, pectinase, α-amylase, and protease), and reduced ADF and ADF digestibility.

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 Committee on laboratory animal ethics of Tropical Crops Genetic Resources Institute (TCGRI). Written informed consent was obtained from the owners for the participation of their animals in this study.

AUTHOR CONTRIBUTIONS

LS, WX, and QL designed the experiment. LS, YZ, and LW conducted the experiment. YZ, TC, and GH collected and analyzed data. LS and WX prepared the manuscript. All authors contributed to the article and approved the submitted version.

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The Effects of Feeding Antibiotic on the Intestinal Microbiota of Weanling Pigs

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This study investigated the use of carbadox in the diet of nursery pigs. Ten pens of weanling piglets were assigned to 2 treatments: one containing carbadox and another without it. From days 21 to 35 of age, the first group of piglets was fed carbadox at 55 mg/kg of diet; followed by 27.5 mg/kg from days 36 to 49; and 0 mg/kg from days 50 to 63. The second group of pigs was fed a control diet without carbadox from days 21 to 63 of age. On days 35, 49, and 63, fecal samples were collected directly from the rectum of 2 piglets in each pen, and the samples were subjected to microbial DNA sequencing and metagenomic functional analysis using the 16S rRNA gene. Feed conversion from days 21 to 63 was improved ($P = 0.04$) in the group of piglets fed carbadox. Faith's phylogenetic diversity was similar ($P = 0.89$) for both groups of piglets on day 35, but it was diminished ($P = 0.01$) in the carbadox-fed group on day 49; however, following the complete removal of carbadox from their diets, this microbial diversity index was once again found to be similar ($P = 0.27$) in both groups on day 63. Likewise, abundances of *Slackia*, *Peptococcus*, *Catenibacterium*, *Coprococcus,* and *Blautia* were all similar between the two groups ($P \geq 0.40$) on day 35, but were smaller in the carbadox group ($P \leq 0.05$) on day 49; however, on day 63, abundances of all these genera were once again similar ($P \geq 0.29$). Metabolic pathways involved in cellular growth, death, and genetic information processing (translation) were found to be similarly expressed in the microbiota of piglets from both groups on day 35 ($P \geq 0.52$), but decreased in the carbadox group on day 49 ($P \leq 0.05$), and were similar again in both groups on day 63 ($P \geq 0.51$). These results revealed that feeding carbadox to piglets during the first 4 weeks after weaning significantly affected their fecal microbiotas; however, 2 weeks after the removal of carbadox, those changes tended to disappear, indicating that the shifts were carbadox-dependent.

Keywords: antibiotic, bacteria, carbadox, feed efficiency, metabolic pathways, microbiome, microbial diversity, piglet

INTRODUCTION

Antibiotics have given significant contributions to the human food production chain during their almost 80 years of use. Such contributions include a reduced incidence of bacterial disease, improved animal health status, and an overall enhancement in production efficiency (1, 2). However, due to concerns of antimicrobial resistance and its consequences to human health, the entire food-producing industry is under pressure to remove antibiotics from animal production (3). Nevertheless, the complete removal of antibiotics from food-producing systems is not a simple task given that this removal normally results in some loss of productivity and negative economic impacts. In addition, only a limited number of reliable alternatives to antibiotics are currently available (4).

In swine-producing farms, antibiotics are typically used to control diarrhea and improve feed efficiency, which is a trait of utmost importance given that 60–70% of the total cost of production corresponds to feed (5). In US commercial farms, nursery pigs typically gain about 0.667 kg of body weight for each kg of feed consumed [or 1.5 kg of feed for each kg of gain; (6)]. One component that can impact feed efficiency in swine is the bacterial population of their gastrointestinal tracts, as recent studies have shown that pigs with distinct microbiotas have different feed efficiencies (7, 8).

Carbadox is an antibiotic that is widely used in the US swine production, and it is included in feeds to prevent dysentery and improve feed efficiency (9). Currently, the United States Food and Drug Administration (FDA) is evaluating the removal of carbadox from the market. Although this controversial topic is still being debated, carbadox was available in the US market when this study was performed (2019), and it currently is, so there is merit in gaining a better understanding of how this antibiotic works. Furthermore, the exact way by which carbadox improves animal performance is still unclear, and more research is needed to establish how long it takes for the gut microbiota to return to its original state after being exposed to carbadox. Therefore, the present study was performed to evaluate the effects of including carbadox in swine diets during the first two phases after weaning, followed by a removal of this product in a subsequent third phase. To that end, the effects of carbadox on growth performance, feed conversion, and the gastrointestinal microbiota of piglets were assessed during a three-phase nursery rearing system.

MATERIALS AND METHODS

Animals and Treatments

All procedures involving animals performed in this study were approved by the University of Georgia Institutional Animal Care and Use Committee (AUP A2018 01-033-Y1-A0).

Newly-weaned piglets ($n = 40$; 21 days of age; Yorkshire x Duroc crossbred) were obtained from the University of Georgia swine farm and housed in environmentally controlled facilities located at University of Georgia's Large Animal Research Facility in Athens, GA. The piglets were from Choice Genetics (Choice USA, West Des Moines, IA 50266) and were the progeny of the CG36 dam and P26 sire. Upon arrival, piglets were weighed and assigned to 1 of 10 pens, with 4 animals per pen. Each pen (∼1.5 × 3 m) hosted 2 males and 2 females and allowed *ad libitum* access to feed and water. Rations for the piglets were formulated to meet National Research Council's recommendations (10) for the duration of the study (i.e., piglets in the nursery stage; 21–63 days-old) and are presented in **Supplementary Tables 1–3**.

For the first 2 weeks of the nursery period (21–35 days-old), piglets were fed a phase 1 diet. A phase 2 diet was offered from days 36 to 49, and a phase 3 diet from days 50 to 63. Of the 10 pens participating in the study, 5 were randomly assigned to receive a control diet (no antibiotic) and the other 5 were assigned to a diet containing antibiotic. The antibiotic used in this study was carbadox (Mecadox®, Phibro Animal Health Corporation, Teaneck, NJ). It was included in the phase 1 diet at 55 mg/kg, and at 27.5 mg/kg in the phase 2 diet. No antibiotic was offered to piglets in any of the pens for the last 2 weeks of the study, therefore, all animals in the study were fed the same phase 3 diet—which did not contain carbadox—during the last 14 days of the study (between days 50 and 63). During the entire study, the amount of feed provided, the orts, and the weights of the piglets were assessed on a weekly basis to calculate animal performance and feed efficiency-related parameters.

Collection of Fecal Samples, DNA Extraction and Analysis

On days 35, 49, and 63 of age, fecal samples were collected from 2 piglets in each pen. The 2 piglets (one male and one female from each pen) were randomly selected on day 35, and subsequently used again on days 49 and 63. The fecal samples were collected by rectal swabs, immediately placed in sterile tubes, and frozen at −20°C. Afterwards, samples were thawed, combined to represent each individual pen, and their microbial DNA was extracted. DNA extractions were performed using the methodology described by Rothrock et al. (11), which uses a combination of mechanical and enzymatic methods to obtain the genomic DNA. Briefly, 0.33 g of fecal material was removed from the surface of the swab and placed into a Lysing Matrix E Tube (MP Biomedicals, Solon, OH), which were homogenized and further processed using an automated procedure. DNA purification was achieved using the DNA Stool–Pathogen detection protocol of the QIAcube Robotic Workstation (Qiagen Inc., Germantown, MD). After purification, DNA concentrations were determined spectrophotometrically using the Synergy H4 Hybrid Microplate Reader (BioTek Instruments, Inc., Winooski, VT). Samples with a minimum volume of 20 μL and 10 ng/μL of DNA were stored at 4°C until the following day. Samples that failed to meet these requirements were rejected and subjected to a new DNA extraction cycle.

One day after the DNA extractions, all samples were taken to the Georgia Genomics and Bioinformatics Core (https://dna.uga.edu) for library preparation and 16S rRNA gene sequencing. PCR libraries were generated using the S-D-Bact-0341-b-S-17 (5′-CCTACGGGNGGCWGCAG-3′) forward and S-D-Bact-0785-a-A-21 (5′-GACTACHVGGGTATCTAATCC-3′) reverse primer pair (12); followed by a PCR clean-up using AMPure XP beads (Beckman Coulter Life Sciences, Indianapolis, IN, USA).

Libraries were quantified using qPCR, and nucleotides were then sequenced using an Illumina MiSeq instrument and a MiSeq v3 reagent kit (Illumina Inc., San Diego, CA, USA). A bacteriophage PhiX genome (PhiX Control v3 Library; Illumina Inc., San Diego, CA, USA) was used as a control for the sequencing runs.

Sequencing data were demultiplexed and converted into FASTQ files. Paired-end sequencing reads were imported into the software Geneious v11.1.5 (Biomatters Ltd., Auckland, New Zealand) and then merged. Merged files were exported from Geneious as individual FASTQ files and were quality-filtered according to the default values provided in the "multiple_split_libraries_fastq.py" script in the QIIME pipeline v1.9.1 (13). The files were then converted into the FASTA format, and sequences were clustered into operational taxonomic units (OTU) at 97% similarity using the Uclust OTU picking method. Samples that did not align to PyNAST were excluded from the analysis. The sequencing depth was set at 33,200 sequences per sample for further analysis. Bacterial groups that had relative abundances smaller than 0.1% were shown as parts per million (PPM). Phylogenetic Investigation of Communities by Reconstruction of Unobserved States (PICRUSt) was carried out to make inferences about the metabolic functions of the microbial community (14, 15). Metagenome metabolic functions were assessed using the Kyoto Encyclopedia of Genes and Genomes (KEGG) second-level pathways.

Data Availability and Statistical Analysis

Nucleotide sequencing data was deposited in a public repository (www.mg-rast.org) under accession number mgm4906046.3. Statistical analyses were performed using the software Minitab® v18.1 and R (v3.3.3). Data were analyzed by ANOVA for each individual day (i.e., ages of 21, 35, 49, and 63 days-old) using the two groups—Antibiotic or No Antibiotic—as factors, and pens were considered the experimental unit. Average daily feed intake was evaluated using linear regression to assess the progression of feed intake over time. All results were treated as trends when $P \leq 0.10$, and were declared statistically significant when $P \leq 0.05$.

RESULTS

Animal Performance

Piglet body weight was similar at the beginning of the study (day 21; **Figure 1**); however, as the study progressed, piglets in pens treated with antibiotics had numerical greater body weights compared to the ones not receiving it, but this numerical increase was not statistically significant for any of the days evaluated ($P \geq 0.46$). Similarly, in **Figure 2**, it can be seen that there were no differences ($P \geq 0.28$) in average feed intake between piglets in the two treatment groups. But when comparing the three phases of the study, feed intake linearly increased ($P < 0.001$) in both groups as the piglets got older. Although no antibiotic was offered during the last 14 days of the study (phase 3), feed efficiency was calculated for the entire course of study and is shown in **Figure 3**. Significant differences ($P = 0.04$) were found between the two treatment groups, with piglets that received antibiotics being more efficient than the ones that never received antibiotics (gain:feed = 0.7075 and 0.6564, respectively).

Changes in the Microbiota

Figure 4 summarizes microbial richness (the number of OTUs) and microbial diversity (Faith's phylogenetic diversity) of the piglet gut microbiomes. The overall behavior of those two indexes was similar throughout the study: No differences were observed on day 35 ($P \geq 0.89$); but on day 49, Faith's phylogenetic diversity was significantly decreased ($P = 0.01$) in the group fed antibiotic. Likewise, the number of observed OTUs tended to be lower ($P = 0.06$) on day 49 in piglets fed antibiotic. However, on day 63, no differences were detected ($P \geq 0.20$) between piglets from the two groups.

Figures 5, **6** show the changes in abundance observed in specific bacterial genera during the course of the study. While all the bacteria shown there had the same abundance on day 35 ($P \geq 0.40$), significant decreases ($P < 0.05$) in the populations of *Slackia*, *Peptococcus*, *Catenibacterium*, *Coprococcus*, and *Blautia* were observed in the group fed antibiotics by day 49, as well as a tendency for smaller abundance of *Dorea* ($P = 0.08$). On day 63 however, the abundances of all those bacterial genera were found to be similar again ($P \geq 0.29$).

Changes in Expression of Metabolic Pathways

Figure 7 shows the expression of five metabolic pathways from the gut microbiomes of the antibiotic and antibiotic-free piglets throughout the study. The five level-2 KEGG pathways are related with cellular growth and death, genetic information and processing, nucleotide metabolism, and environmental information processing. For all of them, there was no difference in expression on day 35 ($P \geq 0.52$); but as was observed with the other microbiome data, significant ($P < 0.05$) or trending toward significant ($P < 0.07$) changes were observed for all pathways on day 49. However, once again, no differences were observed on day 63 ($P \geq 0.50$) between the two piglet treatment groups.

DISCUSSION

Effects of Carbadox on Performance of Piglets

Carbadox has been used as a feed additive in diets of young pigs for over 50 years, and its use is well-documented (9, 16–18). In the current study, carbadox was fed at 55 mg/kg in phase 1 (21–35 days old) and 27.5 mg/kg in phase 2 (36–49 days old) in the ration of the piglets that were treated. Thrasher et al. (16) conducted several growth trials using weanling pigs and tested inclusion levels of carbadox ranging from 18.3 to 110 mg/kg of feed. The authors found that the greatest improvements in weight gains were obtained by including it in the range of 27.5–55 mg/kg of feed. In addition, authors reported that the average daily gain in piglets suffering from diarrhea was 60% greater when carbadox was present at 27.5 mg/kg, compared to the control group (i.e., no antibiotics). Moreover, authors observed improved feed efficiency when carbadox was included in the diets of piglets. Differently than what was reported by Thrasher et al. (16), the current study did not detect differences in average daily gain for piglets receiving carbadox; however, we observed a significant

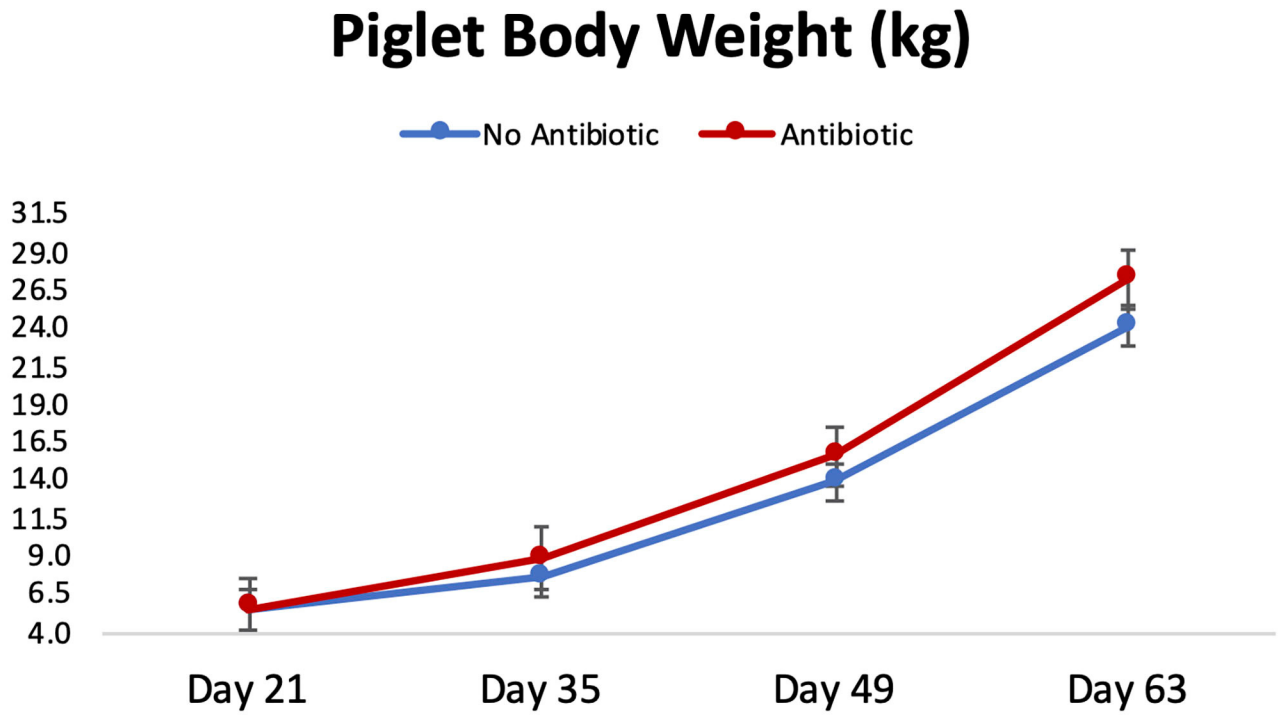

FIGURE 1 | Body weight progression observed for piglets in the No Antibiotic and Antibiotic groups during the study. No significant differences were observed between groups for all the days shown ($P \geq 0.46$).

improvement in feed efficiency for the group of pigs that received carbadox during phases 1 and 2.

Yen et al. (17) performed five feeding trials using carbadox, and all of them had this antibiotic added at 55 mg/kg of feed. Two of those trials were conducted with older pigs (initial age: 10–11 weeks old), but the other three trials were conducted using 4–5 week-old pigs, which are comparable to the present study. In two of those three trials, the authors did not find differences in animal performance, but in one of them, they reported significant improvements in average daily gain, feed efficiency, and also an increased daily feed intake due to inclusion of carbadox. Harper et al. (18) also tested the inclusion of 55 mg of carbadox per kg of diet in two trials using crossbred weanling pigs and found positive results. In both of their trials, authors observed increased average daily gains in the first 5 weeks post-weaning, and in their second trial, inclusion of carbadox improved feed efficiency in the first 3 and in the first 5 weeks post-weaning, which is in line with our findings.

Changes in Piglet's Microbiota During the Study

As can be seen in **Figures 4–6**, when the piglets were on average 35 days-old, the fecal microbiotas from both groups was very similar. In fact, except for some numerical differences, no significant dissimilarities were observed when comparing the antibiotic to the no antibiotic group. At that age, piglets had been fed the phase 1 diet for 2 weeks, and the level of carbadox in the diet of the antibiotic-fed group was 55 mg/kg; however, as shown in **Figure 2**, feed intake during phase 1 was very low compared to the following two phases, since the piglets consumed on average only 240 g feed/day during this initial phase. Thus, although the level of carbadox in the antibiotic group was the highest in this study during phase 1, the amount of feed piglets consumed in that period was likely not enough to generate important shifts in their microbiomes. However, feed intake linearly increased in both groups as the study progressed (**Figure 2**). Consequently, compared to phase 1, feed intake more than doubled during phase 2 as pigs consumed an average of 610 g of feed per day. Despite the lower level of carbadox used in the antibiotic group during phase 2 (i.e., 27.5 mg/kg), the greater intake of feed experienced by the piglets led them to consume greater amounts of antibiotics daily. This greater intake likely played a major role in generating the distinctions in the microbiotas of the piglets that were observed on day 49, particularly the reduction in microbial richness and diversity in the antibiotic-fed group. Another possible explanation for the observed differences is that it may take some time for the cumulative build-up effects of carbadox on the microbiome to take place. Nevertheless, despite the differences seen on day 49, at the end of phase 3 (i.e., day 63), when both groups of pigs were fed the same diet—which did not have carbadox included—no significant differences in their microbiotas were observed. This lack of differences indicates that feeding piglets the same diet for a period of 2 weeks is likely enough time to equalize their gastrointestinal microbiomes, despite the existence of previous differences.

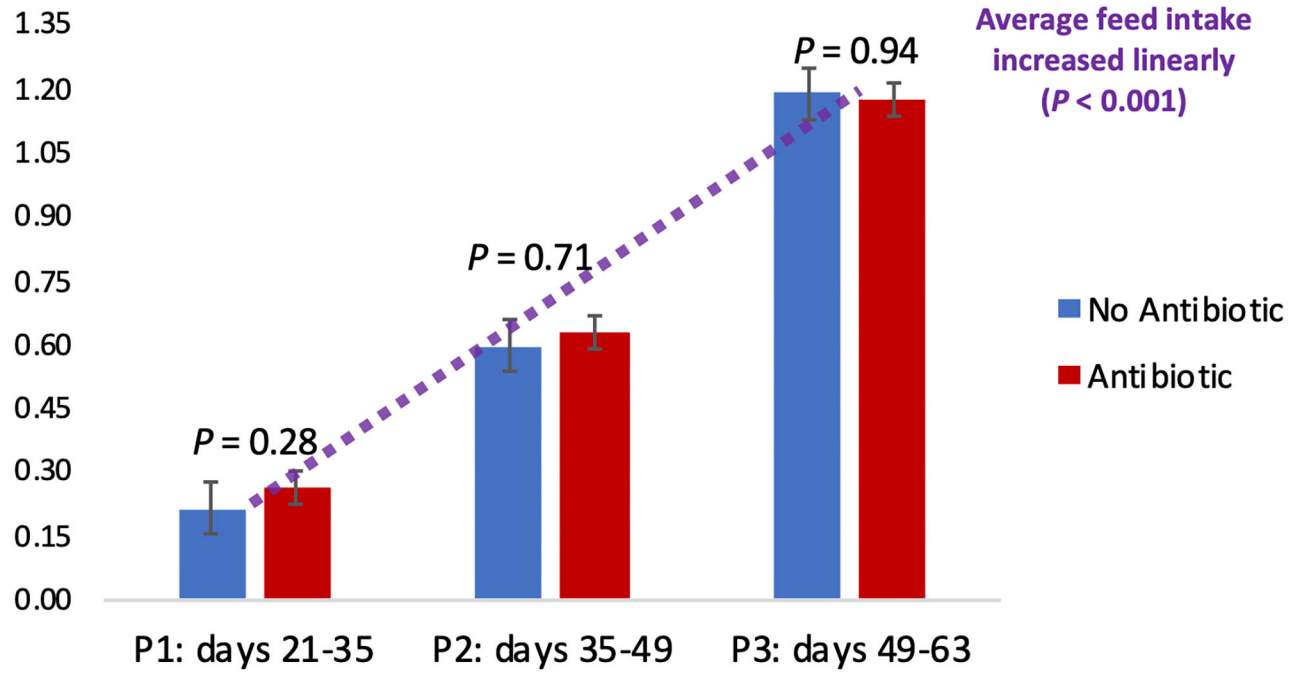

FIGURE 2 | Average feed intake observed in phases 1, 2, and 3 of the study (21–35; 35–49; and 49–63 days-old, respectively). No differences ($P \geq 0.28$) were observed between the No Antibiotic and Antibiotic groups, but feed intake linearly increased ($P < 0.001$) as piglets went from phase 1 to phase 3. Average intake for phases 1, 2, and 3 were 0.24, 0.61, and 1.18 kg/day, respectively.

Microbial richness and diversity followed a similar pattern during the 3 phases of the study: While no differences were detected on day 35, significant alterations were observed 2 weeks later, with the antibiotics group having a lower diversity and a tendency to have lower richness compared to the control group (**Figure 4**). In line with our findings (9), reported that feeding piglets a ration with 50 mg of carbadox per kg of feed significantly decreased their intestinal microbial richness (number of OTUs) compared to a control group; however, they observed this effect earlier than we did: While those authors saw a reduction in the number of OTUs in the 1st week after the inclusion of carbadox in the piglets' diet, in the present study, we observed this effect only on day 49, when carbadox had already been fed for 4 weeks. Despite this distinction between the two studies concerning the number of days it took to observe changes in piglet's microbiomes, both (9) and the present study found that the removal of carbadox from the diet resulted in an equalization in the number of OTUs in the feces of piglets, indicating a short-term nature of the effects of carbadox.

Microbial diversity, expressed as Faith's phylogenetic diversity, was also found to be lower in the antibiotic-fed group on day 49; but once again, this difference in diversity was gone by day 63, when carbadox had been removed from the feed. Once again, this shows that feeding the common phase 3 diet for the last 2 weeks of the study was enough to eliminate differences in the gastrointestinal microbiomes of the piglets. A previous study from our group (19) observed a similar effect on alpha-diversity in cattle. In that study, commingling and feeding weanling cattle the same diet for a period of 4 weeks completely eliminated the initial differences in alpha-diversity existing in their ruminal microbiotas (i.e., Shannon diversity index and Faith's phylogenetic diversity). Similarly (9), reported a reduction of 31.3% in the number of OTUs in piglets that received carbadox compared to a control group that did not receive this antibiotic, and this difference disappeared after the removal of carbadox from the piglets' diet. Therefore, our results are in line with the ones previously observed in the literature, given that feeding the piglets the same diet for 2 weeks resulted in the equalization of their gastrointestinal microbial diversity, regardless of the previous differences.

According to Riviere and Papich (20), carbadox is an antibacterial agent that is primarily active against gram-positive bacteria. Thus, not surprisingly, at the end of phase 2 (day 49), the presence of carbadox in the diet had significantly decreased the populations of *Slackia*, *Peptococcus*, *Catenibacterium*, *Dorea*, *Coprococcus*, and *Blautia*, which are all gram-positive bacteria. However, interestingly, the 14-day withdrawal period that took place when piglets were between 49 and 63 days-old was enough time to re-establish the abundance of all those bacterial genera to the same levels of the no antibiotic group. This

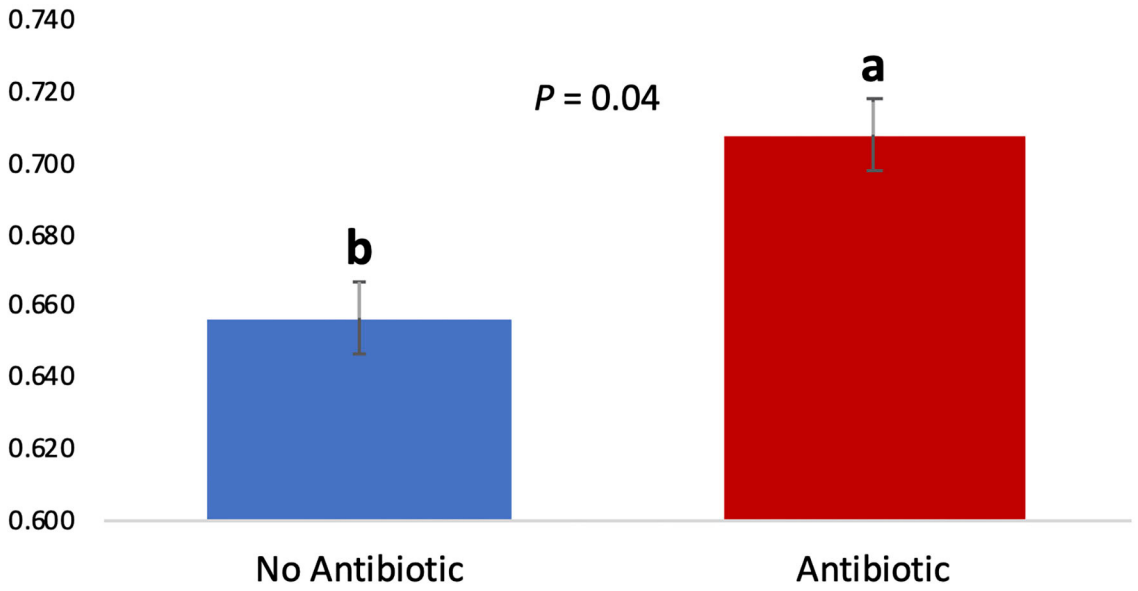

FIGURE 3 | Feed efficiency (expressed as gain: feed ratio) for piglets in the No Antibiotic and Antibiotic groups observed over the course of the entire study. Feed efficiency was significantly improved in the Antibiotic, compared to the No Antibiotic group (0.7075 vs. 0.6564; $P = 0.04$; which is equivalent to 1.41 and 1.52 kg feed:1 kg body weight gain, respectively). No antibiotic was given in the phase 3 of the study.

indicates that these bacterial groups have great plasticity, and that a 14-day removal is enough time for them to have their populations re-established.

In general, antibiotics cause a decrease in gut microbial diversity (21); however, they normally induce piglets to greater rates of body weight gain and feed efficiency (18, 22). Although this seems counterintuitive, it has been established that more efficient microbiomes are not necessarily more diverse (23). In fact (23), have found that more efficient microbiomes can be less diverse and produce a smaller range of output metabolites; however, in that smaller pool of metabolites there can be a larger amount of biologically relevant metabolites, which are more readily available to be utilized by the host animal. Similarly (24), found that nursing calves that received supplementation in their diets had greater amounts relevant metabolites (i.e., short chain fatty acids; which can readily be utilized by the animal for energy), which resulted in a numerically greater body weight gain, despite the fact that the gut microbiota of supplemented calves had lower diversity. Likewise, it has been demonstrated that pigs receiving antibiotics had increased expression of functional genes of their microbiota related to energy production and conversion, indicating that the consumption of antibiotics results in a more efficient capture of energy from the feed (25). Therefore, less diverse microbiomes can in fact be more efficient than the ones with greater diversity due to the quality of the metabolites that are generated, and this fact can explain why animals that received carbadox in this study had better feed conversion despite having a less diverse intestinal microbiota.

Metagenomic Predictions

As revealed by the analysis of the level-2-KEGG metabolic pathways, overall, the pathways involved in cellular processes such as cell growth and death, and processing of genetic and environmental information were all affected, or tended to be affected on day 49, despite the lack of differences observed earlier (i.e., day 35). Similar to what was observed in the overall microbial diversity, the constant presence of carbadox for 28 days resulted in a significant decrease in the expression of the studied pathways in the intestinal environment of antibiotic-fed pigs. However, 14 days after the removal of carbadox from the diets of the pigs (i.e., day 63), all the metabolic pathways returned to similar levels of expression in both treatment groups. These results indicate that the gut microbiota of young pigs have a great degree of malleability. In addition, the gene expressions and metabolism of such microbiota also have this attribute.

According to Constable et al. (26), the exact mechanism of action by which carbadox kills bacteria (primarily gram-positive) is not known. In spite of that, it is known that antibiotics act by disrupting essential processes in the bacterial cells, which impair some bacteria and kill others. Life within a cell requires a delicate balance between the promotion and inhibition of growth (27). Thus, although its specific mode of action is not completely elucidated, by day 49 carbadox was able to decrease the number of genes involved in cellular processes controlling growth and death. In addition, the processing of genetic information (translation) and the processing of environmental information were also affected by the presence of carbadox. These results not only

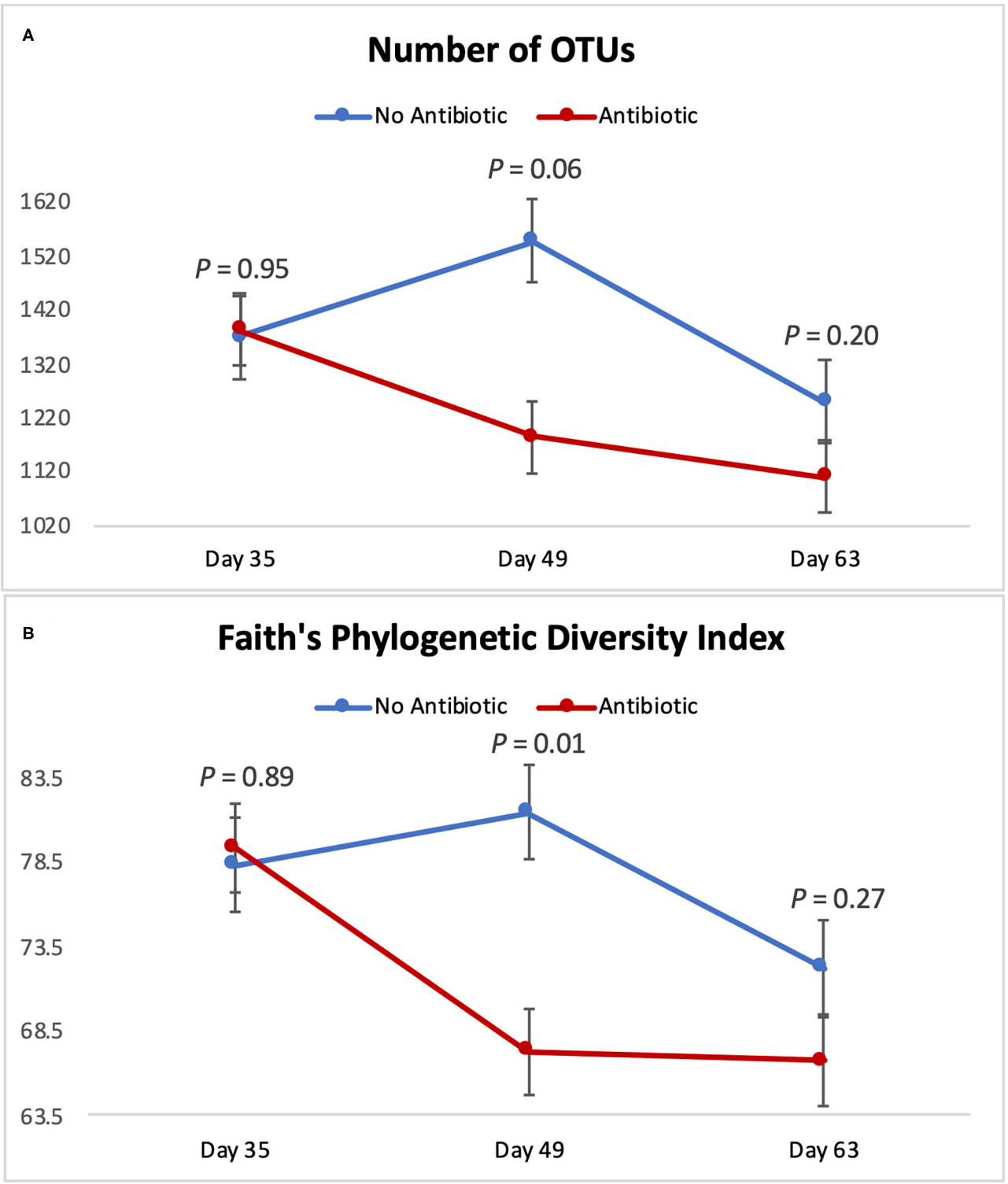

FIGURE 4 | Indicators of microbial richness and diversity for piglets in the No Antibiotic and Antibiotic groups: **(A)** Number of OTUs (richness indicator); **(B)** Faith's Phylogenetic Diversity Index (diversity indicator). *P*-values indicate the contrast between piglets in the two groups on each (day 35: end of phase 1; day 49: end of phase 2; day 63: end of phase 3).

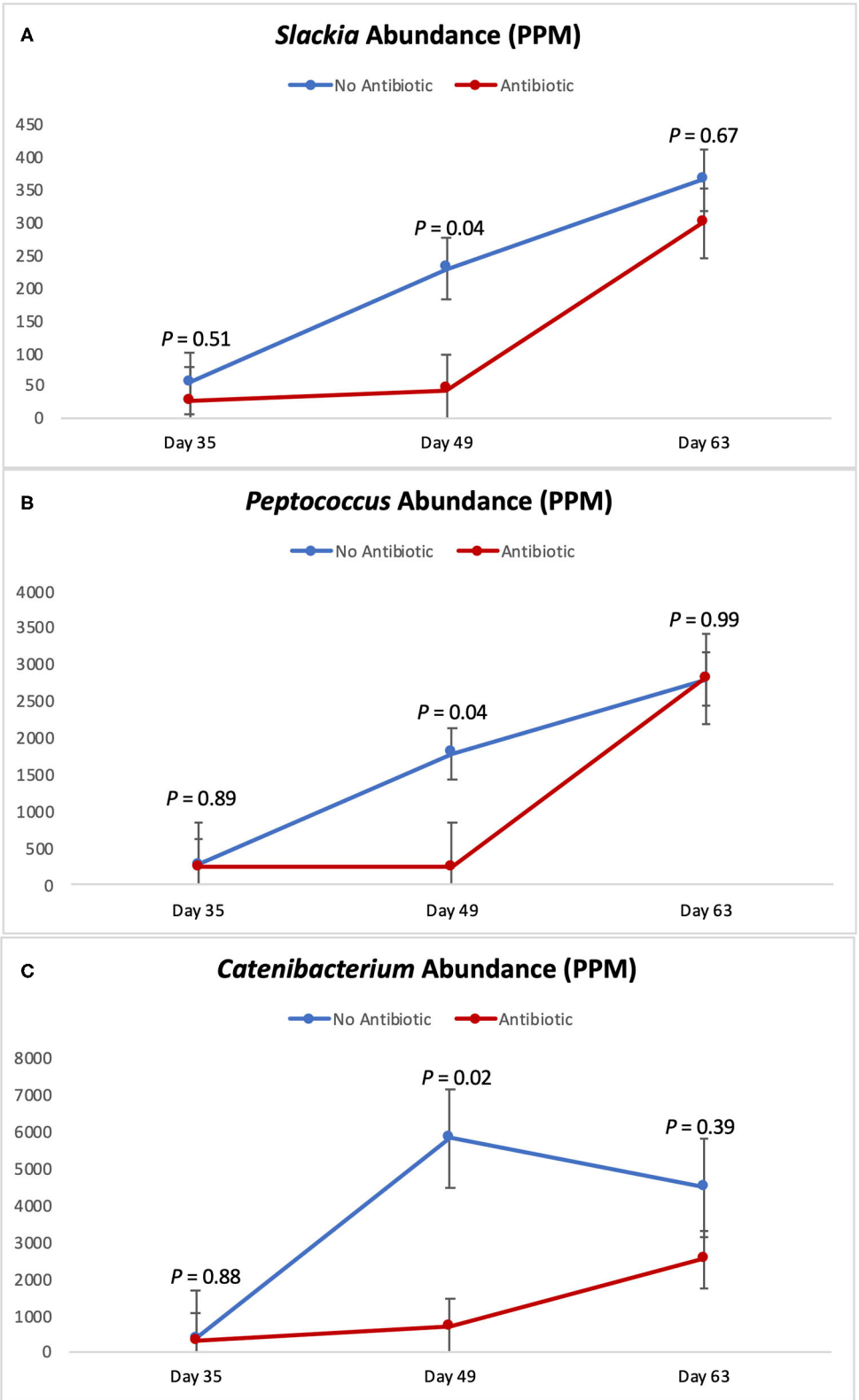

FIGURE 5 | Abundance of the genera *Slackia* **(A)** *Peptococcus* **(B)** and *Catenibacterium* **(C)** observed in the feces of piglets in the No Antibiotic and Antibiotic groups. *P*-values indicate the contrast between piglets in the two groups on each (day 35: end of phase 1; day 49: end of phase 2; day 63: end of phase 3).

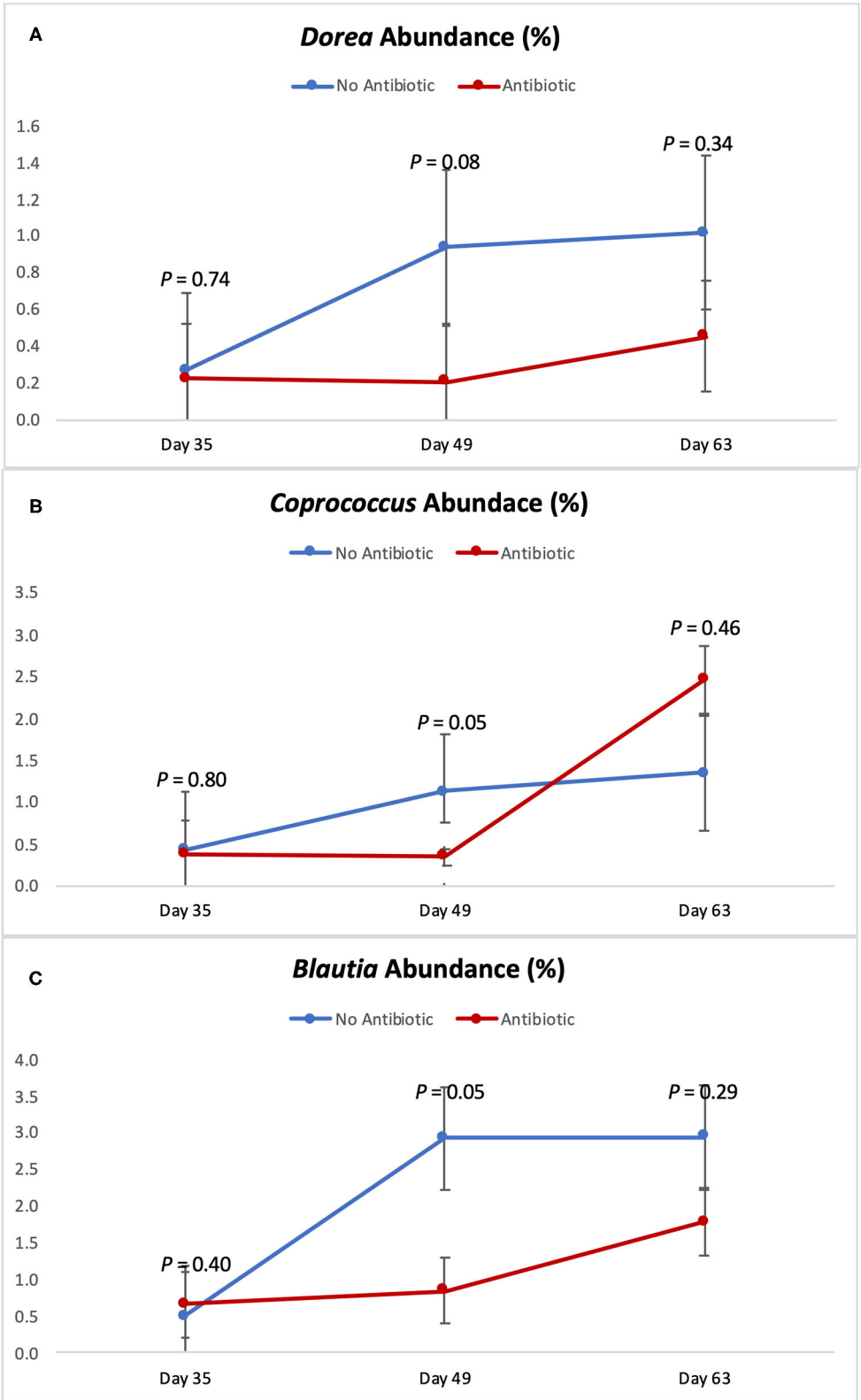

FIGURE 6 | Abundance of the genera *Dorea* **(A)** *Coprococcus* **(B)** and *Blautia* **(C)** observed in the feces of piglets in the No Antibiotic and Antibiotic groups. *P*-values indicate the contrast between piglets in the two groups on each (day 35: end of phase 1; day 49: end of phase 2; day 63: end of phase 3).

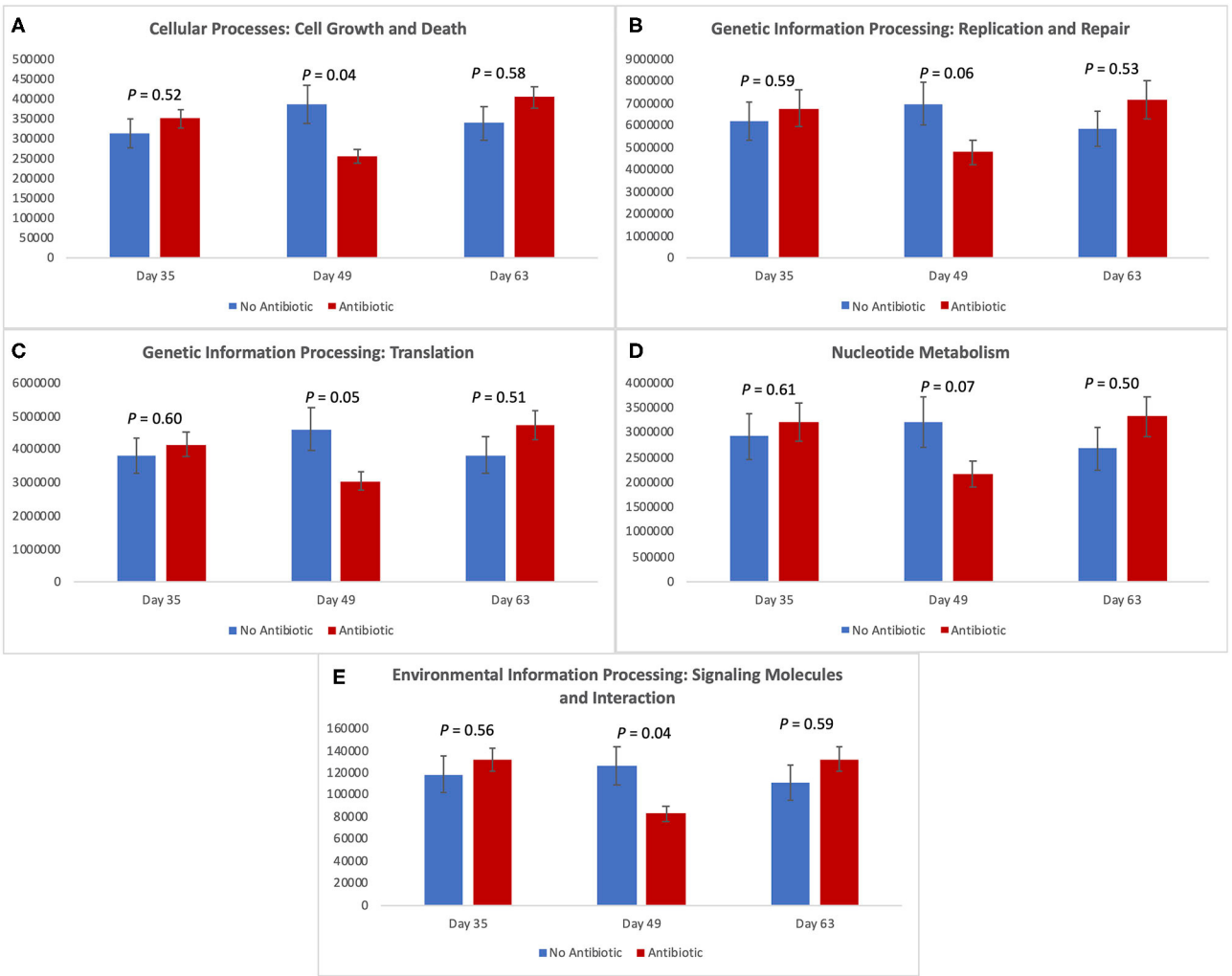

FIGURE 7 | Expression of level-2-KEGG pathways in the fecal microbiota of piglets in the No Antibiotic and Antibiotic groups: **(A)** Cell growth and death; **(B)** Genetic information replication and repair; **(C)** Translation; **(D)** Nucleotide metabolism; **(E)** Signaling molecules and interaction. *P*-values indicate the contrast between piglets in the two groups on each (day 35: end of phase 1; day 49: end of phase 2; day 63: end of phase 3).

confirm, but they also complement, the ones obtained by evaluating the piglet's microbiota; however, they are not totally unexpected given that the metagenomes were predicted using the same sequencing data used for the taxonomic analysis. In spite of that, our results indicate the potential mechanisms and pathways that carbadox tend to inhibit.

CONCLUSION

Overall, although performed with a relatively small sample size, our study has consistently shown that the gut microbiota of weanling pigs have a great degree of malleability. When the antibiotic carbadox was present in their diets for a total of 28 days after weaning, important changes were observed in their intestinal microbiotas, both at the genus level and in the overall microbial richness and diversity. In addition, the metabolic pathways expressed by their intestinal microbiotas were also affected by the presence of carbadox. However, after a complete removal of carbadox from their diets for 14 days (during phase 3), virtually all of the observed differences disappeared, indicating the ability that the intestinal microbiota of piglets has to return to its normal state in a relatively small amount of time. These results indicate the potential for short-term applications of carbadox, which may benefit piglets in terms of reduction of diarrhea and increase of feed efficiency, while not affecting piglet gut microbiomes. But if a longer term treatment ($\geq$ 4 weeks) is needed, up to 2 weeks is needed for the gut microbiome to return to normal after the end of the antibiotic treatment. No further information regarding the health and physiology of the pigs were evaluated, so further studies dealing with these topics should be performed given the close connection between microbiota and host health.

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 University of Georgia Institutional Animal Care and Use Committee.

AUTHOR CONTRIBUTIONS

MA, TC, and JL were responsible for the conception of the study. MA, RH, and JL were responsible for acquisition of data. JL was responsible for the statistical and data analysis. MR, JL, and RH were responsible for laboratory work. MA, RH, HJ, MR, TC, and JL were responsible for writing the manuscript. MA supervised the collection of data and manuscript editing. All authors have read and agreed to the published version of the manuscript.

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SUPPLEMENTARY MATERIAL

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Rumen Microbiota of Tibetan Sheep (*Ovis aries*) Adaptation to Extremely Cold Season on the Qinghai-Tibetan Plateau

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The Qinghai-Tibet Plateau is characterized by low temperatures and hypoxia, and this feature is more obvious in the winter. However, it is not clear how Tibetan sheep adapt to extreme cold climates. To address this, we used physiological methods combined with next-generation sequencing technology to explore the differences in growth performance, forage nutrient digestion, serum biochemical indexes, and rumen microbial communities of Tibetan sheep (*Ovis aries*) between the summer and winter. In the summer, owing to the high nutritional quality of the forage, the Tibetan sheep showed enhanced forage degradation and fermentation though increased counts of important bacteria in the rumen, such as Bacteroidetes, *Prevotella_1*, *Prevotellaceae_UCG-003*, *Ruminococcus_1*, *Saccharofermentans*, and *Ruminococcaceae_UCG-014*, to improve the growth performance and increase serum immunity and antioxidant status. In the winter, owing to the low nutritional quality of the forage, the Tibetan sheep presented low values of forage degradation and fermentation indicators. The relative abundance of Firmicutes, the Firmicutes/Bacteroidetes ratio, microbial diversity, interactive activity between microorganisms, and metabolism were significantly increased, implying that the rumen microbiota could promote the decomposition of forage biomass and the maintenance of energy when forage nutritional value was insufficient in the winter. Our study helps in elucidating the mechanism by which Tibetan sheep adapt to the high-altitude harsh environments, from the perspective of the rumen microbiota.

Keywords: Tibetan sheep, growth performance, digestibility, Firmicutes/Bacteroidetes, VFA profiles

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INTRODUCTION

Interactions between plants and animals are one of the key forces promoting ecosystem evolution and maintaining the structure and function of ecosystems (1). Grasslands are the largest of the terrestrial ecosystems, occupying over half of the earth's land surface. Ruminant livestock depend on grasslands to produce food, milk, wool, and leather worldwide (2). As one of the most critical conflicts in grassland management, seasonal disequilibrium between forage supply, and livestock nutrient demand of livestock inevitably occurs annually; the uneven seasonal distribution of annual

precipitation or heat results in excessive forage supply in the wet/warm season and insufficient forage production in the dry/cold season, whereas the nutritional demand of livestock remains relatively steady or only slightly fluctuates throughout the year (3). Unreasonable solutions have caused overgrazing and immediate grassland degradation, which have markedly elevated the risk of ecological disequilibrium and reduction of animal food production (4). Previous studies have mainly focused on the equilibrium of forage mass and quality between grassland supply and livestock demand generally in terms of grassland productivity at the farm scale; however, it is necessary to understand the mechanism of forage mass and quality disequilibrium between grassland supply and livestock demand, especially with respect to rumen microbes and nutrient physiology of livestock.

The rumen is an important digestive compartment that contains diverse microorganisms. Rumen microorganisms consist mainly of obligatory anaerobes (bacteria, protozoa, archaea, and fungi), and bacteria represents the highest proportion of the microbial population (5). Previous research has shown that ruminants are born without bacteria in the rumen, but microorganisms in the environment will soon enter and colonize it (6). Rumen microbial fermentation and degradation of plant fibers is a complex and coordinated process; the feed is converted into digestive compounds, particularly volatile fatty acids (VFAs) and microbial proteins. Rumen microbiota are the link between ruminants and their diets, playing a key role in ruminant nutrition (7, 8), food digestion (9), physical development, energy balance (10), immunity regulation (8), and pathogen resistance (11). Previous research suggests that changes in rumen microbiota can alter its function (12); however, there are relatively few studies on how the composition and function of rumen microbes respond to extremely cold seasons.

The Qinghai-Tibet Plateau (QTP) has the highest altitude and the largest area of continuous grazing grassland in the world, accounting for 44% of China's 40 million hectare grasslands, and maintains ∼20 million heads of yaks (5) and 50 million heads of Tibetan sheep (13). Forage mass and quality are strongly restricted by the great temperature difference between summer and winter in the QTP (3), resulting in approximately 37% nutrient-deficient livestock in northeastern QTP annually; this ultimately determines the productivity of livestock through rumen microbes and nutrient physiology, to a certain extent. In traditional ranches in the QTP, Tibetan sheep (*Ovis aries*) graze on natural pastures at >3,000 m above sea level without supplementary feeding, and are well adapted to the harsh high altitude environment, such as the lower temperature and risk of hypoxia (13). Tibetan sheep can adapt to extreme environments, including physiological adaptations, such as developed heart and molecular regulation mechanisms; for example, mutations in EPAS1 can enhance the average red blood cell hemoglobin concentration and average red blood cell volume (14). However, there is still a lack of in-depth research on the adaptability of Tibetan sheep based on rumen microorganisms. To explore the adaptation of Tibetan sheep to extremely cold seasons, we aimed to assess the differences in growth performance, forage digestion, serum biochemical indices, and rumen microbial communities of Tibetan sheep between summer and winter. We hypothesized that the decline in the nutritional quality of forage in the winter would cause a decrease in the digestibility of forage, serum immunity, and antioxidant status, which would ultimately lead to a reduction in growth performance. Our results show that in Tibetan sheep, the composition and function of rumen microorganisms are adjusted in different seasons, which might contribute to the animals' adaptation to extremely cold climates. The research results have great significance in explaining the survival adaptability of Tibetan sheep in different seasons on the QTP.

MATERIALS AND METHODS

Study Site, Animals, and Experimental Diets

The study site was located in the QTP base of Lanzhou University in Maqu County, Gansu Province (33°404′N, 101°5212′E) in the northeastern part of the QTP, with an elevation of 3,700 m asl (15). The average temperature and relative humidity during the experimental period in the summer (July 1 to August 23, 2019) was 12.6°C and 72%, and the corresponding values were −2.5°C and 46%, respectively, during the experimental period in the winter (November 1 to December 24, 2019). All trial procedures were approved by the Animal Ethics Committee of the Lanzhou University (file No: 2010-1 and 2010-2). Twelve 24-month-old Tibetan sheep (38 ± 1.46 kg summer liveweight and 38 ± 1.32 kg winter liveweight; mean ± SD) were selected from a local herdsman in each season. Before the start of the experiment, the animals were ear-tagged, and drenched. The Tibetan sheep were fed in individual metabolic cages (1.0 ×1.5 m) with a water tank and a feed trough. They were fed for 54 days, which included 14 days of adaptation to the forage and conditions and a 40-day trial. They were fed 800 g dry matter (DM)/day (∼2% of BW/days), three times per day, at 08:00, 12:00, and 18:00. Forage was collected daily in the morning; it consisted of natural pasture from 30 ha of a fenced alpine meadow from a native pasture, including grasses, sedges, and forbs. The species and proportions of the forage are listed in **Supplementary Table 1**. During the experiment, Tibetan sheep had no supplementary feed and had free access to fresh water.

Forage Intake and Digestibility

During the formal experimental period, were performed accurate recording of the amount of forage before feeding and the amount of leftover feed after feeding to calculate the DM intake by experimental animals; then, 100 g of forage was sampled. The digestibility trial consisted of 7 days for sampling to determine the apparent digestibility of forage nutrients from days 33–39 of the experimental period, and daily feed intake and feces excretion were recorded. Before the morning feeding, the feces samples were collected, weighed, and mixed, and samples (100 g each) were mixed with 10 mL of 10% hydrochloric acid and stored in a plastic bag. The forage and feces samples were oven dried (60°C, 48 h), ground (<1 mm), and sieved before chemical analysis. DM, organic matter (OM), and crude fat ether extract (EE) were measured according to the methods described by

the AOAC (16). The Kjeldahl method was used to determine the nitrogen content in the forage, and the crude protein (CP) content of the forage was calculated as 6.25 × N. The content of acid detergent fiber (ADF) and neutral detergent fiber (NDF) were measured and determined using the Van Soest method (17). Sodium sulfite (10 g/L of NDF solution) was added to the solution but without heat-stable α-amylase. Nutrient apparent digestibility was calculated from contents in the forage intake and the contents in fecal output.

Growth Performance

To evaluate growth performance, the animals were weighed at the beginning (after 6 h starvation) and at the end (2 h before feeding in the morning) of the experimental period of each season. The average daily gain (ADG) changes were calculated based on the difference between the final and initial weights.

Blood Samples

Jugular blood samples were collected into 10 mL evacuated collection tubes without anticoagulant. The collection was performed before morning feeding on day 40 of the experimental period. The samples were centrifuged (5,000 × *g*, 20 min, 4°C); the serum was then collected and frozen at −20°C for subsequent analysis. The concentrations of serum glucose (GLU), albumin (ALB), total protein (TP), globulin (GLO), superoxide dismutase (SOD), glutathione peroxidase (GSH-PX), malondialdehyde (MDA), and total antioxidant capacity (T-AOC) were analyzed using commercial kits (Model 100T/96S, Suzhou Keming Biotechnology Co. Ltd. China). Serum immunoglobulin (Ig) A, M, and G concentrations were measured using a microplate reader (Keda Biotechnology, Shanghai, China). Serum growth hormone (GH) levels were tested using commercial kits (Model 100T/96S, Jiangsu, China).

VFA Profile Measurement and DNA Analysis

Rumen fluid samples (∼50 mL) were collected 2 h after morning feeding using an oral stomach tube (5) on day 40 of the experimental period. Between the collection of samples, the oral tube was thoroughly cleaned with clean water, and the first 50 mL samples of each yak will be discarded to ensure that it is not contaminated by previous animals and its own saliva. The sample pH was immediately measured using a pH meter (Model 206-pH2, Testo, Germany), as previously described (5, 9, 12). The rumen-fluid samples were used for VFA testing, ammonia nitrogen (NH_3-N) concentration analysis, and DNA extraction. For the analysis of ruminal VFA concentrations, the filtrate was thawed and centrifuged at 1,000 × *g* for 15 min and then analyzed by gas chromatography (GC-MS522; Wufeng Instruments, Shanghai, China) as described by Fan et al. (5). The NH_3-N concentration was determined by colorimetry (UV-VIS8500, Tianmei, Shanghai, China) as described by Chaney and Marbach (18).

Total DNA was extracted from 2 mL of rumen liquid using the E.Z.N.A. DNA kit (Qiagen, Hilden, Germany). The V3-V4 hypervariable regions of the bacterial 16S rRNA gene were amplified using a thermocycler PCR system (GeneAmp 9700, Applied Biosystems, Foster City, CA, USA) and the following universal primers: forward (338F) 5′-ACTCCTAG GGAGGCAGCAG-3′; reverse (806R) 5′-GGACTACHVGGG TWTCTAAT-3′ (19). PCR amplification for next-generation sequencing (high-throughput sequencing) has been previously described (12). The 18 samples (12 samples from summer and 6 samples from winter) and the 6 samples (winter) were sequenced, respectively. Sequences were sorted based on their unique barcodes, followed by the removal of barcodes and primer sequences using QIIME (version 1.9.0; http://qiime.org). Raw tags were merged using FLASH (version 1.2.11) with default parameters (20). Low-quality reads were eliminated using QIIME (version 1.7.0) (21). Clean tags were compared to the Gold database using the UCHIME algorithm to eliminate chimera sequences. The sequences that possibly came from the mitochondrion and Chloroplast of the forage were moved. Effective tags were obtained for further analysis. These effective tags were clustered into operational taxonomic units (OTUs) with ≥97% similarity using UPARSE (version 7.0; http://drive5.com/uparse/) (22). Based on the SILVA (SSU123) database, the RDP classifier (version 2.2; https://sourceforge.net/projects/rdp-classifier/files/rdp-classifier/) was used to classify the representative sequences. Alpha diversity analysis including Chao1, Shannon, PD_whole_tree, and observed_species were calculated with QIIME (version 1.9.0). Principal coordinates analysis (PCoA) was used to compare treatments based on the weighted Uni-Frac distance metric (23).

Statistical Analysis

The difference of growth performance, forage nutrient digestion, serum biochemical indexes, VFAs and alpha diversity of Tibetan sheep between seasons was compared using an independent sample t test based on SAS (SAS, version 9.2; SAS Institute Inc., Cary, NY, USA). Differences were considered statistically significant at $P < 0.05$. Microbial networks were generated to calculate the correlations between predominant taxa using Gephi software (version 0.9.2 https://gephi.org), and keystone taxa in the microbial communities were identified using the combined score of high mean degree, high closeness centrality, and low betweenness centrality (24). Pearson correlation coefficients between the relative abundances of the rumen bacteria (genus) and short chain fatty acids were calculated using the heatmap package in R software (version 4.0.2 https://CRAN.R-project.org). Structural equation model performed in the 'SEM' package of R was used to estimate the effect of seasons on forage nutrient compositions, rumen fermentation parameters, serum biochemical indexes, and microbial community diversity. The rumen microbiota functional pathways were predicted using PICRUSt2 (PICRUSt2 v2.3.0_b; https://github.com/picrust/picrust2) software based on 16S sequencing data (https://github.com/picrust/picrust2/wiki). PICRUSt2 was used to study the prediction of the bacterial community function in the rumen of Tibetan sheep in two seasons, and the difference in the abundance of KOs (KEGG orthology groups) in KEGG (Kyoto Encyclopedia of Genes and Genomes) level 2 between summer and winter were determined.

RESULTS

Nutrient Composition of Forage

The chemical composition of the forage in the summer and winter is presented in **Table 1**. The contents of CP ($P < 0.01$) and EE ($P < 0.01$) were higher in the summer than in the winter. The opposite was found for the levels of NDF ($P = 0.013$) and ADF ($P < 0.01$). There were no differences in DM ($P = 0.921$) and OM ($P = 0.767$) between the two seasons.

Serum Profiles of Tibetan Sheep

The serum profile variables of Tibetan sheep in the summer and winter are presented in **Table 2**. The concentrations of GH ($P = 0.002$), TP ($P = 0.037$), ALB ($P = 0.046$), SOD ($P < 0.01$), GSH-PX ($P = 0.014$), IgG ($P < 0.01$), and IgM ($P = 0.043$) were higher in the summer than in the winter. However, there was no difference in serum GLO ($P = 0.066$), GLU ($P = 0.069$), MDA ($P = 0.727$), T-AOC ($P = 0.746$), and IgA ($P = 0.134$) between the two seasons.

Nutrient Intake and Apparent Digestibility in Tibetan Sheep

ADG was significantly higher in the summer than in the winter ($P < 0.01$; **Table 3**). The intake of DM ($P < 0.01$), CP ($P < 0.01$), and EE ($P < 0.01$) was also higher in the summer than in the winter. However, the intake of NDF ($P < 0.01$) and ADF ($P < 0.01$) was higher in the winter than in the summer. There was no difference in OM ($P = 0.056$) between the two seasons. Apparent digestibility of DM ($P < 0.01$), OM ($P = 0.010$), CP ($P < 0.01$), NDF ($P < 0.01$), ADF ($P < 0.01$), and EE ($P < 0.01$) was higher in the summer than in the winter.

Rumen Fermentation Parameters

The rumen fermentation parameters for Tibetan sheep in the summer and winter are presented in **Table 4**. The concentrations of rumen NH_3-N ($P < 0.01$) and TVFA ($P < 0.01$) and the proportion of propionate ($P = 0.031$), butyrate ($P = 0.024$), and valerate ($P < 0.01$) were higher in the rumen of Tibetan sheep in the summer than in the winter. The proportion of acetate ($P < 0.01$) and the ratio of acetate to propionate were higher in the winter than in the summer.

TABLE 1 | Common forage nutrient composition in the summer and winter (% of dry matter).

Chemical composition	Season		SEM	*p*
	Summer	Winter		
DM	38.36	38.61	0.8562	0.9214
OM	89.29	89.09	0.3346	0.7672
CP	12.27	6.38	0.6672	<0.01
EE	1.85	1.20	0.0718	<0.01
NDF	48.97	53.62	0.9742	0.0131
ADF	27.63	32.04	0.7512	<0.01

DM, dry matter; OM, organic matter; CP, crude protein; EE, ether extract; NDF, neutral detergent fiber; ADF, acid detergent fiber; SEM, standard error of the mean.

Microbial Community Composition

According to the Venn diagram, 2,146 OTUs in the rumen of Tibetan sheep were common to both seasons, and there were 396 and 658 unique OTUs in the summer and winter, respectively (**Figure 1A**). According to the PCoA (**Figure 1B**), obvious differences in the microbial communities in the

TABLE 2 | Comparison of serum biochemical indexes influenced by the summer and winter.

Chemical composition	Season		SEM	*p*
	Summer	Winter		
GH (ng/mL)	31.42	26.18	0.9926	0.0019
TP (g/L)	77.61	64.86	3.1762	0.0370
ALB (g/L)	31.48	26.35	1.2842	0.0463
GLO (g/L)	46.83	38.72	2.1421	0.0656
GLU (g/L)	3.74	2.58	0.1686	0.0691
MDA (nmol/mL)	0.86	0.79	0.1133	0.7272
SOD (U/ml)	225.38	113.26	15.6452	<0.01
T-AOC (U/ml)	6.58	6.47	0.2511	0.7464
GSH-PX (μmol/L)	960.53	840.28	12.4374	0.0143
IgA (μg/mL)	0.76	0.58	0.0614	0.1336
IgG (μg/mL)	31.57	18.65	1.8392	<0.01
IgM (μg/mL)	1.84	1.26	0.1411	0.0433

GH, growth hormone; TP, total protein; ALB, albumin; GLO, globulin; GLU, glucose; MDA, malondialdehyde; SOD, superoxide dismutase; T-AOC, total antioxidant capacity; GSH-PX, Glutathione peroxidase; IgA, immunoglobulin A; IgG, immunoglobulin G; IgM, immunoglobulin M; SEM, standard error of the mean.

TABLE 3 | Effect of season on average daily gain, dry matter intake, and apparent digestibility of nutrients.

Chemical composition	Season		SEM	*p*
	Summer	Winter		
BW (kg)	38.15	38.13	0.9982	0.2213
ADG (g/d)	96.28	−50.39	22.1251	<0.01
DM Intake (g/d, DM basis)				
DM	797.84	741.42	11.6083	<0.01
OM	716.85	672.94	10.3785	0.0561
CP	97.72	47.39	7.1761	<0.01
NDF	387.83	397.55	1.7514	<0.01
ADF	217.82	236.09	3.7826	<0.01
EE	14.82	10.07	0.6132	<0.01
Apparent digestibility (% DM)				
DM	65.69	55.89	1.8472	<0.01
OM	69.82	57.48	2.6285	0.0102
CP	66.41	40.13	4.1996	<0.01
NDF	64.45	54.26	2.0927	<0.01
ADF	61.10	51.48	1.9552	<0.01
EE	49.71	41.70	1.6531	<0.01

ADG, average daily gain; DM, dry matter; OM, organic matter; CP, crude protein; NDF, neutral detergent fiber; ADF, acid detergent fiber; EE, ether extract; SEM, standard error of the mean.

TABLE 4 | Rumen fermentation parameters in Tibetan sheep.

Chemical composition	Season		SEM	*p*
	Summer	Winter		
pH	6.43	6.81	0.041	0.037
NH_3-N (mg/dL)	10.03	4.26	0.751	<0.01
TVFA (mmol/L)	54.70	46.56	1.455	<0.01
Acetate (%)	71.4	77.98	1.195	<0.01
Propionate (%)	15.71	12.32	0.824	0.031
Butyrate (%)	9.98	7.56	0.523	0.024
Isobutyrate (%)	1.26	1.09	0.069	0.254
Valerate (%)	0.88	0.61	0.049	<0.01
Isovalerate (%)	0.75	0.92	0.041	0.052
Acetate/propionate	4.58	7.17	0.511	0.004

NH_3-N, ammonia nitrogen; TVFA, total volatile fatty acids; SEM, standard error of the mean.

different seasons were observed. Taxonomic analysis of the reads revealed 24 bacterial phyla. Bacteroidetes and Firmicutes were the predominant phyla, accounting for 43.73 and 41.36% of the relative abundances, respectively (**Figure 2A**); these were followed by Proteobacteria, Spirochaetes, and Tenericutes, representing 2.07, 1.49, and 1.18% of the relative abundances, respectively. At the genus level, 230 taxa were identified. *Rikenellaceae_RC9_gut_group* (11.75%) was the most dominant genus, followed by *Prevotella_1* (10.62%), *Christensenellaceae_R-7_group* (6.75%), *Ruminococcaceae_NK4A214_group* (4.31%), *Ruminococcaceae_UCG-014* (2.24%), and *Ruminococcaceae_UCG-005* (2.03%) (**Figure 2B**). The community richness estimates (Chao 1 estimator; $P = 0.049$), diversity indices (Shannon index; $P = 0.003$), Observed_species ($P = 0.005$), and PD_whole_tree ($P = 0.001$) were significantly higher in the winter than in the summer (**Figure 3**).

The effects of season on the prevalence of certain bacterial phyla and genera (average relative abundance >0.5% in one group) in the rumen of Tibetan sheep are presented in **Supplementary Tables 2**, **3**, respectively. At the phylum level, the relative abundance of Bacteroidetes (49.28% in the summer vs. 38.17% in the winter, $P = 0.011$) was higher in the summer than in the winter, whereas the relative abundance of Firmicutes (32.31% in the summer vs. 50.41% in the winter, $P = 0.014$) and the ratio of Firmicutes to Bacteroidetes (0.66 in the summer vs. 1.51 in the winter, $P = 0.004$) were higher in the winter than in the summer. At the genus level, the relative abundance of *Prevotella_1* (14.29% in the summer vs. 6.95% in the winter, $P = 0.017$), *Prevotellaceae_UCG-003* (2.24% in the summer vs. 1.12% in the winter, $P = 0.001$), *Ruminococcus_1* (0.87% in the summer vs. 0.47% in the winter, $P = 0.044$), *Saccharofermentans* (1.77% in the summer vs. 0.95% in the winter, $P = 0.025$), and *Ruminococcaceae_UCG-014* (3.17% in the summer vs. 1.30% in the winter, $P = 0.043$) were relatively higher in the summer than in the winter, whereas that of *Christensenellaceae_R-7_group* (4.01% in the summer vs. 9.48% in the winter, $P = 0.020$) presented the opposite pattern.

Network Analysis of Bacterial Communities

The microbial network was used to analyze the microbial interactions among rumen bacterial communities of Tibetan sheep and to statistically identify bacterial genera that are keystone taxa that regulate the fermentation process. The results showed that the season changed the correlation within the microbiota (**Figure 4**). We verified that the negative correlations in the winter were stronger than those in the summer. The putative drivers of keystone taxa in the rumen microbial communities of Tibetan sheep from the two seasons were estimated using the combined score of the high mean degree, high closeness centrality, and low betweenness centrality (**Supplementary Table 4**). The results indicated that *Prevotellaceae_YAB2003_group*, *Lachnospiraceae_XPB1014_group*, and *Pseudobutyrivibrio*—in the summer—and *Ruminococcus_1*, *Lachnospiraceae_AC2044_group*, and *Anaerovorax*—in the winter— could be considered keystone taxa in the rumen of Tibetan sheep.

Correlations Between Forage Nutrient Compositions, Rumen Fermentation Parameters, Serum Biochemical Indexes, and Bacterial Community Diversity

The correlation between the dominant rumen bacterial genera and pH, NH_3-N, or TVFA is shown in **Figure 5**. The pH was positively correlated with the genera *Ruminococcaceae_NK4A214_group* ($r = 0.705$), *Ruminococcus_1* ($r = 0.729$), and *Erysipelotrichaceae_UCG-004* ($r = 0.853$) and negatively correlated with *Papillibacter* ($r = -0.903$), *Quinella* ($r = -0.783$), *Prevotellaceae_UCG-003* ($r = -0.672$), and *Prevotella_1* ($r = -0.637$). NH_3-N was positively correlated with the genera *Prevotella_1* ($r = 0.827$), *Ruminococcaceae_UCG-014* ($r = 0.527$), *Ruminococcus_1* ($r = 0.801$), and *Treponema_2* ($r = 0.538$) and negatively correlated with *Erysipelotrichaceae_UCG-004* ($r = -0.495$). TVFA was directly correlated with *Prevotellaceae_UCG-003* ($r = 0.684$), *Prevotella_1* ($r = 0.856$), *Ruminococcaceae_UCG-014* ($r = 0.581$), and *Butyrivibrio_2* ($r = 0.537$) and negatively correlated with *Ruminococcaceae_UCG-005* ($r = -0.706$) and *Ruminococcus_2* ($r = -0.638$). Acetate was positively correlated with the genera *Ruminococcaceae_NK4A214_group* ($r = 0.795$), *Quinella* ($r = 0.831$), *Prevotella_1* ($r = 0.793$), *Ruminococcaceae_UCG-014* ($r = 0.752$), *Treponema_2* ($r = 0.574$), and *Butyrivibrio_2* ($r = 0.504$) and negatively correlated with *Prevotellaceae_UCG-001* ($r = -0.893$) and *Ruminococcus_1* ($r = -0.745$). Propionate was positively correlated with *Prevotella_1* ($r = 0.753$), *Ruminococcaceae_UCG-014* ($r = 0.837$), and *Treponema_2* ($r = 0.746$) and negatively correlated with *Ruminococcus_1* ($r = -0.648$) and *Ruminococcus_2* ($r = -0.624$). Butyrate was positively correlated with *Erysipelotrichaceae_UCG-004* ($r = 0.503$) and negatively correlated with *Lachnospiraceae_AC2044_group* ($r = -0.943$). Isobutyrate was positively correlated with *Rikenellaceae_RC9_gut_group* ($r = 0.735$) and

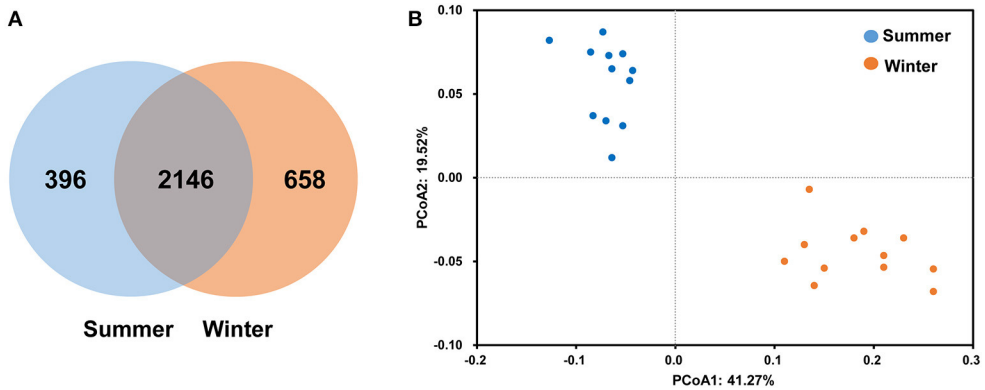

FIGURE 1 | Differences in community dissimilarities and operational taxonomic units (OTUs) between summer and winter. Venn diagram **(A)** indicates specific and shared OTUs in both seasons. The weighted UniFrac distance **(B)** was used to calculate the differences in Tibetan sheep rumen microbiota in the different seasons, and principal coordinate analysis (PCoA) was used to calculate the coordinates.

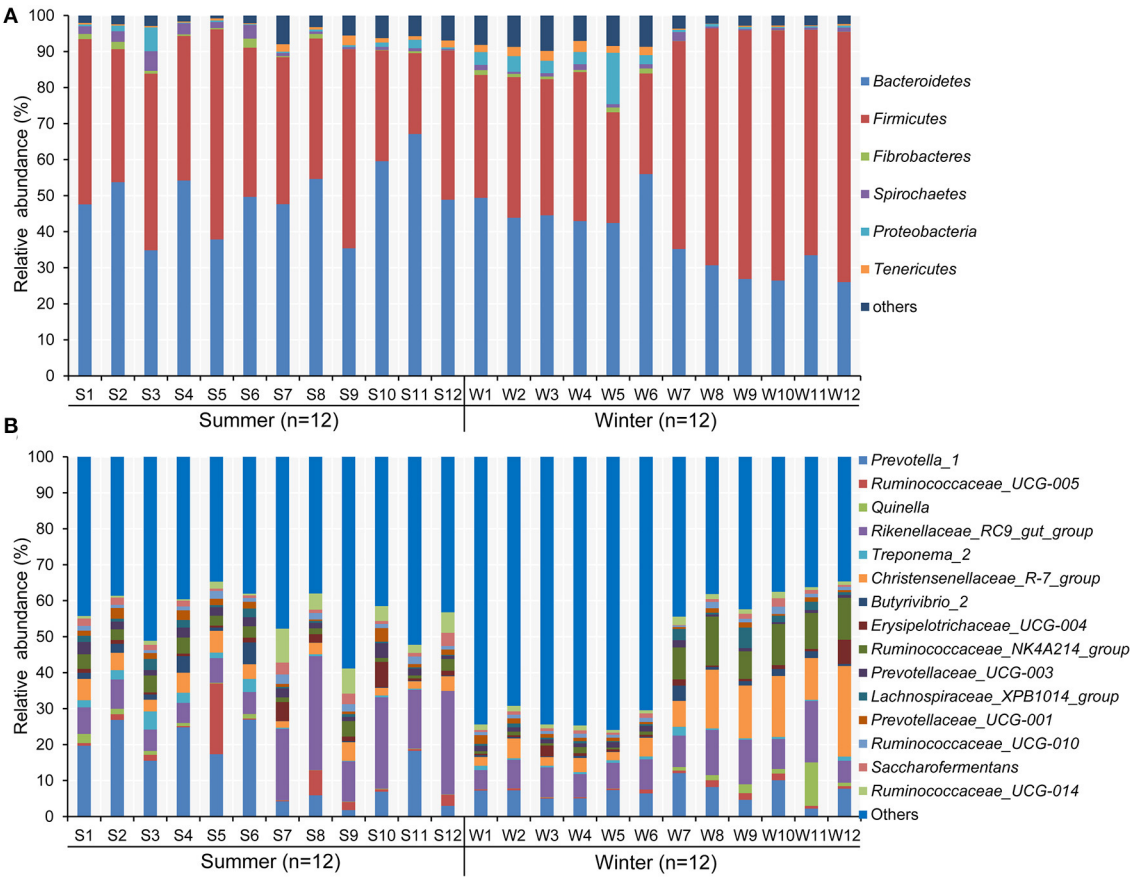

FIGURE 2 | Composition of bacterial communities in Tibetan sheep at **(A)** phylum and **(B)** genus levels in the summer and winter. Only taxa with an average relative abundance >0.5% are displayed.

Ruminococcaceae_UCG-014 ($r = 0.712$) and was inversely correlated with *Erysipelotrichaceae_UCG-004* ($r = -0.427$). Valerate was positively correlated with *Prevotella_1* ($r = 0.742$) and negatively correlated with *Papillibacter* ($r = -0.906$). Isovalerate was positively correlated with the genera *Lachnospiraceae_XPB1014_group* ($r = 0.493$), *Ruminococcus_2*

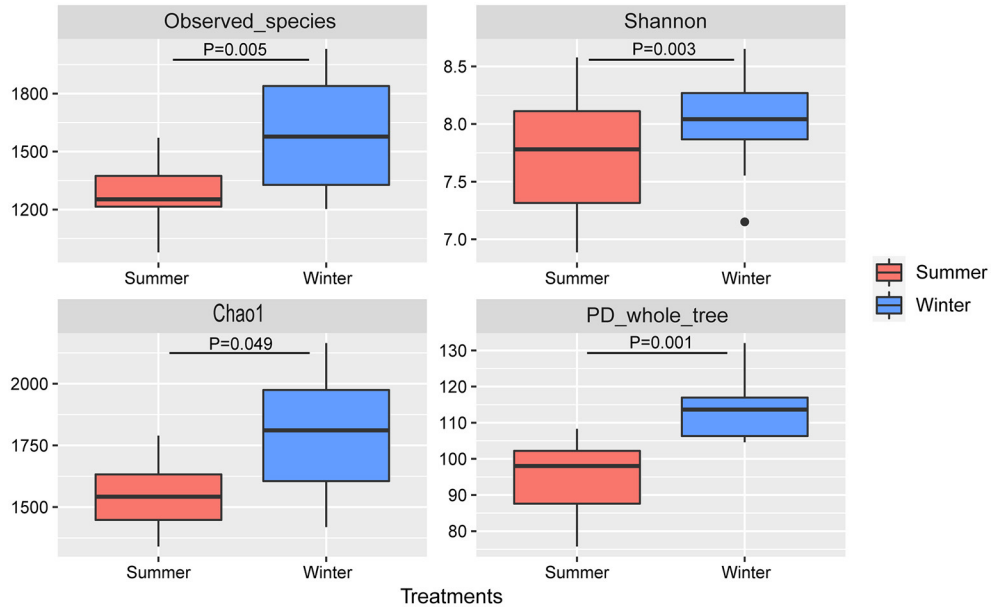

FIGURE 3 | Microbial community diversities in the summer and winter. A significant difference is indicated by $P < 0.05$.

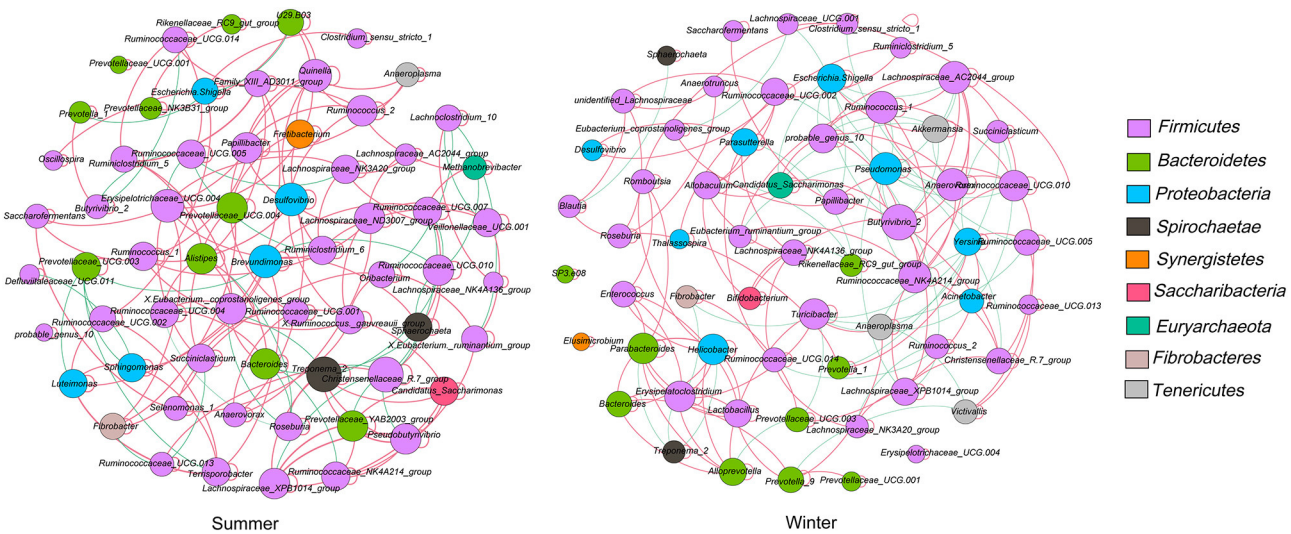

FIGURE 4 | Interaction networks of the rumen microbiota. 16S rRNA gene-based correlation network of the rumen microbiota, displaying statistically significant interactions with absolute value of correlation coefficients > 0.6. The node size was scaled based on the overall abundance of each taxa in the microbiota. A red edge indicates a positive correlation and green edge indicates a negative correlation.

($r = 0.472$), and *Fibrobacter* ($r = 0.407$) and negatively correlated with *Prevotellaceae_UCG-003* ($r = -0.915$) and *Saccharofermentans* ($r = -0.673$).

The correlation analysis among dominant rumen bacterial genus, forage nutrient compositions, rumen fermentation parameters, and serum biochemical indexes were investigated in summer and winter (**Supplementary Figure 1**). Overall, the interaction structure of the summer was more complex than that in winter. In addition, we found that *Prevotella_1* was negatively corrected with DM in summer, while positively corrected with EE in winter. *Prevotellaceae_UCG-003* was positively corrected with TVFA, GSH-PX, IGG and GH, and negatively corrected with ADF in summer, while *Prevotellaceae_UCG-001* was the dominant bacteria in winter and just negatively corrected with GLU and IGM. In addition, we estimated the relationships among the season, forage nutrient compositions, rumen fermentation parameters, serum biochemical indexes, and bacterial community diversity based on structural equation

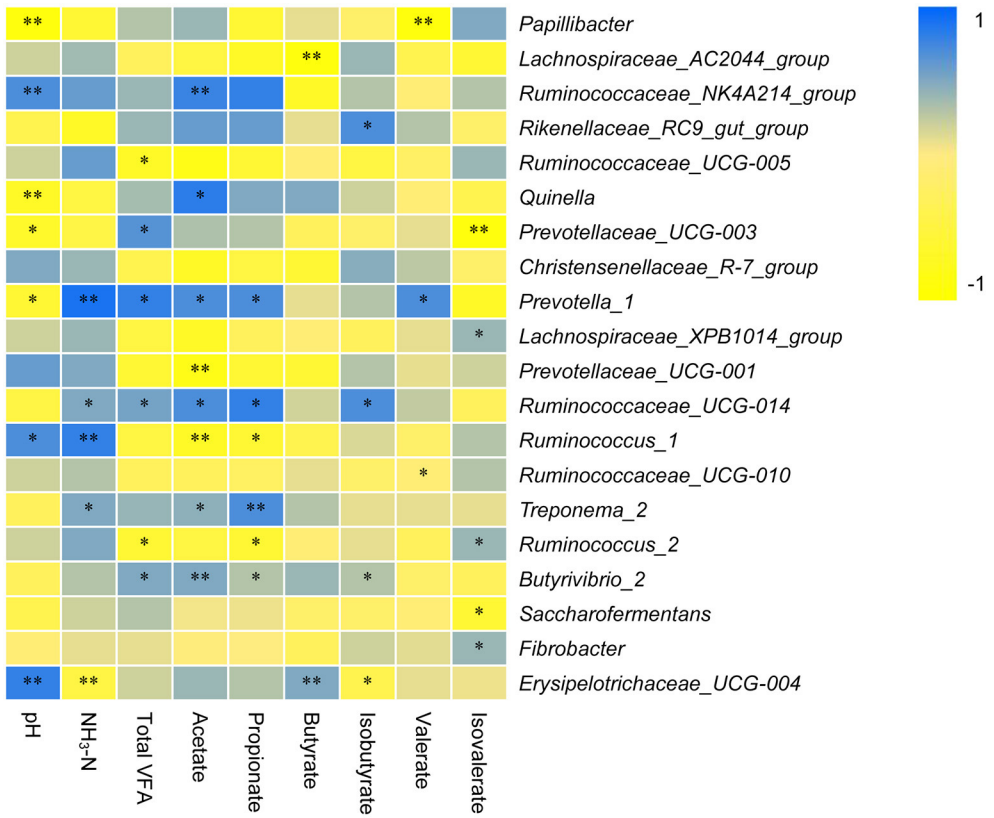

FIGURE 5 | Relationship among NH_3-N, bacterial Communities, and short-chain fatty acids (SCFAs). ** and * indicate significance levels at 0.01 and 0.05, respectively.

model (SEM) analysis. Our results showed that season may directly influence forage nutrient composition, rumen fermentation parameters, and serum biochemical indexes and indirectly shape rumen bacterial alpha diversity (Shannon index) by regulating forage nutrient compositions and serum biochemical indexes (**Supplementary Figure 2**).

PICRUSt2 Gene Function Estimation

KOs in level 2 (**Figure 6**) suggested that the pathways related to cell growth and death, transcription, amino acid metabolism, membrane transport, biosynthesis of other secondary metabolites, lipid metabolism, and cell motility were enriched differently between the summer and winter groups. The relative abundances of cell growth and death, transcription, amino acid metabolism, biosynthesis of other secondary metabolites, and cell motility terms were significantly higher in the summer than in the winter ($P < 0.05$). Conversely, the relative abundances of membrane transport and lipid metabolism terms were significantly higher in the winter than in the summer ($P < 0.05$).

DISCUSSION

The seasonal availability and quality of forage might have affected the performance of Tibetan sheep, corroborating the results of a previous study (2). The species and season of plants are important factors that affect forage quality (3). The results of this study showed that the CP content of forage grown in the summer was significantly higher than that in the winter, whereas the opposite was found for the fibrous fraction contents. This could be due to the low ratio of forage leaves to stems in plants in the winter, which would affect the intake of forage and hence reduce the forage DMI by the Tibetan sheep compared with the results of other studies (25, 26). Furthermore, the combined effects of low CP and high NDF and ADF contents of winter forage resulted in decreased ADG in Tibetan sheep. The forage nutritional CP fraction is the most important nutrient required by livestock. If dietary CP falls below 7.0%, rumen microbial activity will be suppressed and forage intake will be reduced (27). Moreover, the CP content of forage in this study was >7% in the summer, which consequently improved the ADG in Tibetan sheep. Ephrem et al. (27) reported that the nutritional values, including CP, NDF, and ADF contents, were higher in the summer than in the winter, affecting feed intake and the production efficiency of sheep grazing on the QTP. In this context, our results were in agreement with the work reported by Xue et al. (10).

GH is a peptide that is essential for stimulating the production and growth of IGF-1 (28). In this study, GH concentrations were higher in the serum of Tibetan sheep in the summer than in the winter. ALB is an important protein source for

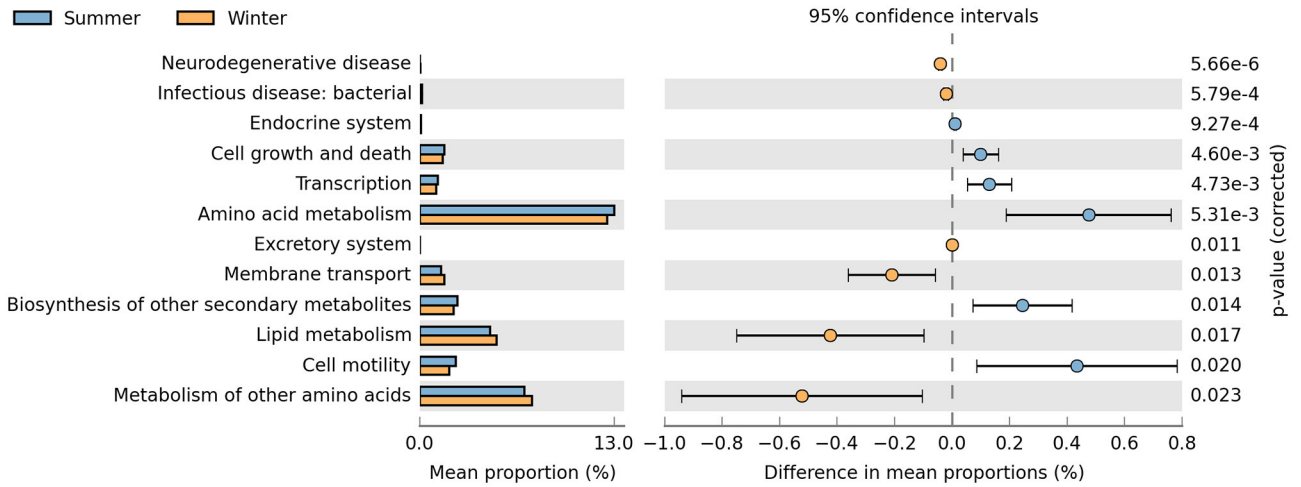

FIGURE 6 | Relative abundance of predicted functions and significance of KOs (KEGG orthology groups) at KEGG (Kyoto Encyclopedia of Genes and Genomes) level 2 between the summer and winter. Student's *t*-tests were used to compare the abundance changes two groups; only differences with a $P < 0.05$ are reported.

liver synthesis, and its main functions include providing energy, repairing tissues, and acting as a transport carrier of nutrients to maintain the dynamic balance of tissue proteins (29). In our study, the concentrations of ALB were higher in the serum of Tibetan sheep in the summer than in the winter, indicating that in the winter, the forage was deficient in essential nutrients. SOD is not only an enzyme that removes superoxide anions but also produces high amounts of H_2O_2, and it plays an important role in biological antioxidant systems (28); GSH-Px is an important indicator of antioxidant activity in livestock (30). In the present study, the concentrations of SOD and GSH-PX were higher in the serum of Tibetan sheep in the summer than in the winter, indicating that Tibetan sheep might have improved antioxidant capacity in the summer. Similarly, the concentrations of IgG and IgM, indicators of nonspecific humoral immunity in ruminants, were also higher in the summer than in the winter (28).

A previous study showed that forage presented higher levels of nutrients and were more digestible in the summer, when they were succulent and growing rapidly, than in the winter (31). This result was consistent with our findings in which the digestibility of forage decreased as the forage matured. This indicates that the nutrient quality of forage influences nutrient digestibility. An increase in forage NDF and ADF content resulted in linearly decreased CP digestibility because of the reduction in non-structural carbohydrates, and this consequently limited the supply of fermentable energy for rumen microorganisms to degrade dietary protein for their growth (3). This result corroborates our findings in which CP digestibility was lower in the winter than in the summer. Yang et al. (3) suggested that forage fiber content (NDF and ADF) would correlate negatively with the digestibility of all plant material. In this investigation, NDF and ADF contents were negatively correlated with their digestibility.

Ordinarily, ecosystems with high species diversity will be stable and show high levels of performance and function (32, 33). Although there are different interactions between species in different ecosystems, there is evidence that high species diversity provides more functional redundancy and buffers ecosystem functions to prevent the extinction of species (34). In the host-microbial system, different bacterial communities can contribute to a unique set of digestive enzymes to improve forage processing and digestion (35); thus, high microbial diversity is usually related to strong metabolic capacity and stability. A previous study showed that the rumen microbiota diversity of Tibetan sheep improves the fermentation efficiency of forage fiber and expedites the stability of the rumen microbial ecosystem (36). In the present study, higher rumen microbial diversity was found in the winter than in the summer, indicating that the rumen microbial community of Tibetan sheep might have enhanced ability to use high-fiber forage to help them meet their energy requirements in cold and harsh habitats during the winter (12). In addition, the predicted gene functional profiles of the rumen microbiota of Tibetan sheep showed that most of these genes were related to membrane transport and cell metabolism (e.g., lipid and protein metabolism) and were overrepresented in the winter. This result implies that Tibetan sheep improve metabolic functions of rumen microbiota to cope with the low nutritional quality of the forage in the winter (37). However, our results only predict genomic information and might not represent the true function of Tibetan sheep rumen bacteria. Therefore, it is necessary to further use metagenomics to study the role of these genes in the adaptation of animals to extreme environments.

In this study, Bacteroidetes and Firmicutes were the most predominant bacterial phyla in the rumen of Tibetan sheep, corroborating the results of previous studies on Tibetan sheep (38, 39), cattle (40), sheep (41), yaks (5, 9, 12), goats (42), and pikas (43), and indicating that these bacteria play an important role in the ecology and function of the mammalian gastrointestinal tract. Kim et al. (44) conducted a meta-analysis of all selected 16S rRNA sequences stored in the NCBI database to

summarize the distribution of rumen bacteria in major domestic animals and found that the proportions of Bacteroidetes and Firmicutes were approximately 31% and 56%, respectively. Interestingly, in the present study, the content of Bacteroidetes exceeded the mean proportion; however, the content of Firmicutes was lower than the mean proportion. A previous study showed that Firmicutes members are mainly responsible for energy conversion and harvesting (45), whereas Bacteroidetes members play an important role in carbohydrate degradation and protein hydrolysis (46). In this study, Bacteroidetes were more abundant in the summer, whereas Firmicutes were more abundant in the winter. The increase in the relative abundance of Firmicutes and the Firmicutes/Bacteroidetes ratio demonstrates that Tibetan sheep might exhibit improved energy utilization rates of forage and increased resistance to winter cold stress (12). Studies have confirmed that the ratio of Firmicutes/Bacteroidetes in goats and bovine is strong related to body fat storage and animal obesity (47–51).

At the genus level, *Ruminococcaceae_UCG-014* and *Ruminococcus_1*, which are related to plant cellulose fermentation, were more abundant in the summer than in the winter, demonstrating that these microorganisms might be involved in the degradation of forage (52). Importantly, the bacterial taxa *Prevotella_1* and *Christensenellaceae_R-7_group* in the rumen of Tibetan sheep were enriched in the winter, indicating that these microorganisms could help to adapt to harsh winter conditions such as low temperature and low oxygen (7, 53). The enrichment of these microorganisms in the rumen of winter-grazing sheep might be involved in the important functions of the host. For example, *Prevotella_1* degrades simple sugars, starches, and other polysaccharides as energy substrates to produce the glucogenic substrate succinate (53). The genus *Christensenellaceae_R-7_group* includes genes for critical hemicellulase and cellulase secretase, which could improve the ability of Tibetan sheep to degrade cellulose and obtain energy from indigestible polysaccharides (7). These genera in the rumen of Tibetan sheep can help improve host metabolic capacity and resistance to the low temperature and low oxygen environment in the winter. However, the ecological function of these bacteria in the rumen of Tibetan sheep requires further study. In the summer, *Saccharofermentans* were enriched in the rumen of Tibetan sheep, and these bacteria are considered VFA-producing (13), but as there is no known pure culture strain, the metabolic function of this genus is still unclear.

Rumen propionic acid production is enhanced and can lead to an increased concentration of glucose, as glucose can be produced by gluconeogenesis from propionic acid (29). Acetic acid production in the rumen is closely related to fiber degradation (54), and the values were higher in the winter than in the summer, which could be due to higher fiber degradation. Studies have shown that acetic acid in the rumen can significantly reduce the efficiency of energy utilization (28). In this study, the acetate:propionate ratio in the winter was enhanced, implying that the rumen profile shifted and the efficiency was reduced; however, in the summer, a lower acetate:propionate ratio and a higher TVFA concentration was verified, implying that the energy efficiency in the summer is higher than that in the winter. Some bacteria, including *Butyrivibrio_2*, *Prevotella_1*, *Treponema_2*, and *Ruminococcaceae_UCG-014*, were positively associated with the concentrations of acetate, propionate, and total VFA, indicating that these bacteria could be beneficial for VFA production. For example, *Butyrivibrio_2* might efficiently utilize fibrous and starchy substrates to produce butyrate (52); moreover *Prevotella_1* might utilize simple sugars and polysaccharides to produce propionate (55). However, because of the very complex interactions among bacteria, such as resource competition and cross-substitution, it is difficult to know which bacteria directly cause the production of a specific VFA (56). Through qPCR analysis, Liu et al. (36) found that the number of main cellulolytic and proteolytic bacteria in the rumen of grazing Tibetan sheep in the summer was significantly higher than that in the winter. This result was consistent with the higher nutrient digestibility and ADG of Tibetan sheep in the summer. Therefore, we hypothesize that in the warm season, a high abundance of rumen functional bacteria can improve the digestibility of forage and at the same time result in the production high concentrations of NH_3-N and VFAs, which can quickly improve the growth performance of Tibetan sheep.

The vital role of the microbial consortium in ecosystem functions has been defined, and the relationships between microorganisms in the rumen fermentation ecosystem are very complex (57). There might be some species for which their location in the rumen fermentation ecosystem is not proportionate with their abundance (58). The current study is the first to identify the keystone taxa with network topological properties in the rumen of Tibetan sheep. The results demonstrate that the season alters the correlations among microflora and showed that the keystone genus is different between the two seasons. Network analysis could reveal species interactions from both positive and negative aspects (36). Negative interactions might weaken competitive relations, whereas positive interactions could strengthen competitive relations (59). In our study, the number of negative links in the winter was higher than that in the summer. We speculate that owing to the low nutritional quality of forage in the winter and lack of forage, microorganisms can make full use of the limited low-quality forage resources by strengthening cooperation. This further shows that season can regulate the microbial dynamics of the Tibetan sheep rumen.

CONCLUSION

Our results indicate that rumen microorganisms of grazing Tibetan sheep have strong plasticity and might modulate function in response to environmental changes. In the summer season, the high relative abundance of Bacteroidetes, *Prevotella_1*, *Prevotellaceae_UCG-003*, *Ruminococcus_1*, *Saccharofermentans*, and *Ruminococcaceae_UCG-014* promotes forage degradation and fermentation to rapidly improve the growth performance and increase the serum immunity and antioxidant capacity of Tibetan sheep. In the winter season, the increase in Firmicutes, the Firmicutes/Bacteroidetes ratio, rumen microbial diversity, synergy between microorganisms,

and metabolic pathways could enable Tibetan sheep to maximize the usage of low-quality forage to cope with the cold conditions.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding authors at Fujiang Hou;cyhoufj@lzu.edu.cn.

ETHICS STATEMENT

All trial procedures strictly followed the rules and regulations of the Experimental Field Management protocols (File No: 2010-1 and 2010-2) of Lanzhou University and were approved by the Animal Ethics Committee of the University. Written informed consent was obtained from the owners for the participation of their animals in this study.

AUTHOR CONTRIBUTIONS

FH conceived and designed the experiments. XC and QF conducted animal experiments and sample collection. QF was responsible for data analysis and wrote the original manuscript. All authors have revised 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.2021.673822/full#supplementary-material

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Evaluation of the Fecal Bacterial Communities of Angus Steers With Divergent Feed Efficiencies Across the Lifespan From Weaning to Slaughter

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Numerous studies have examined the link between the presence of specific gastrointestinal bacteria and the feed efficiency of cattle. However, cattle undergo dietary changes during their productive life which can cause fluctuations in their microbial consortium. The objective of the present study was to assess changes in the fecal microbiome of beef steers genetically selected to be divergent in feedlot feed efficiency, to determine whether differences in their fecal microbiomes could be detected as early as weaning, and continued throughout the rearing process regardless of dietary changes. Fecal samples were collected at weaning, yearling age, and slaughter for a group of 63 steers. Based on their feedlot-finishing performance, the steers were selected and divided into two groups according to their residual feed intake (**RFI**): efficient steers (low-RFI; $n = 7$) and inefficient steers (high-RFI; $n = 8$). To ascertain the fecal microbial consortium and volatile fatty acid (VFA) content, 16S rRNA gene sequencing and VFA analysis were performed. Overall, bacterial evenness and diversity were greater at weaning compared to yearling and slaughter for both efficiency groups ($P < 0.001$). Feedlot RFI linearly decreased as both Shannon diversity and *Ruminococcaceae* abundance increased ($R^2 = 65.6$ and 60.7%, respectively). Abundances of *Ruminococcaceae*, *Rikenellaceae*, and *Christensenellaceae* were higher at weaning vs. yearling age and slaughter ($P < 0.001$); moreover, these families were consistently more abundant in the feces of the low-RFI steers (for most of the timepoints evaluated; $P \leq 0.05$), compared to the high-RFI steers. Conversely, abundances of *Bifidobacteriaceae* were numerically higher in the feces of the high-RFI steers throughout their lifespan. Total VFA concentrations increased at slaughter compared to weaning and yearling for both efficiency groups ($P < 0.001$). The acetate:propionate ratio decreased linearly ($P < 0.001$) throughout the life of the steers regardless of their efficiency, reflective of dietary changes. Our results indicate that despite fluctuations due to animal age and dietary changes, specific bacterial families may be correlated with feed efficiency of steers. Furthermore, such differences may be identifiable at earlier stages of the production cycle, potentially as early as weaning.

Keywords: beef cattle, *Bifidobacteriaceae*, *Christensenellaceae*, fecal microbiome, residual feed intake, *Rikenellaceae*, *Ruminococcaceae*, productive life

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INTRODUCTION

In beef production systems, feed represents the largest single cost and accounts for an estimated 60–75% of the total cost of production (1). In order to increase the profitability of beef operations, producers seek to improve the efficiency by which cattle convert ingested feed into body weight gain (2, 3). One method to determine the feed efficiency of cattle is to calculate their residual feed intake (RFI). Concisely, RFI is the difference between the observed and the expected feed intake, based on metabolic body weight and a certain level of gain. If an animal eats less than expected for that level of gain (low-RFI), it is considered more efficient (3, 4). Therefore, low-RFI animals are more efficient than animals that have high-RFI values.

An estimated 19% of the variation in RFI can be attributed to diet composition and digestibility of feed (5). Variation in RFI can also be linked to the microbial population within the gastrointestinal tract (**GIT**) of cattle because microbes produce 70% of the energy and 50% of the protein the ruminant animal uses (6, 7). Many studies have found a direct link between feed efficiency and the microbial population in cattle's GIT (8–10); however, most of these studies targeted on the ruminal microbial population. In contrast, a recent study has shown that certain bacterial families present in the hindgut of cattle can be correlated with feedlot RFI and may be important in driving the host's feed efficiency (11); however, it is still unknown at what point these bacterial families diverge within the hindgut of steers.

The present study was designed to evaluate the composition of the fecal microbial population of beef cattle that differed greatly in feed efficiency (as assessed by feedlot RFI). In practical beef production, obtaining fecal samples is substantially easier than taking ruminal samples. Therefore, fecal samples were collected from steers at three different times during their production cycle: at weaning, yearling age, and immediately post-slaughter. The fecal microbiome of the most efficient (low-RFI) and least efficient (high-RFI) steers were evaluated and compared at each stage. We hypothesized that fecal microbiomes would consistently differ at different points in the lifecycle of steers based on their feed efficiency evaluated during the feedlot-finishing phase, and that the fecal microbiomes would differ within those timepoints based on efficiency group.

MATERIALS AND METHODS

Animals, Diets, and Steer Selection

The steers utilized in this study were cared for using the guidelines approved by the University of Georgia's Animal Care and Use Committee (AUP #A2012 11-006-R1). The steers used in the present study are from the fifth generation of a genetic selection program involving Angus cattle being selected for residual average daily gain and intramuscular fat (marbling). All steers were born (12/7/2016–1/22/2017) and raised at the Northwest Georgia Research and Education Center, located in Calhoun, GA (34° 30 N, 84° 57 W) where they were reared in a pasture-based system until ∼10 months of age. The steers ($n = 63$) were then transported to a commercial feedlot located in Brasstown, NC (35° 10 N, 83° 23 W) where they were backgrounded prior to starting the feedlot trial. During the feedlot trial, the steers were maintained under a GAP 4 certification utilizing a non-hormone treated cattle program. Prior to the start of the finishing phase, steers were accustomed to high-grain diets over the course of 3 weeks. The finishing period lasted 110 days. The finishing diet contained 14.51% crude protein, 2.10 Mcal/kg NE_m, 1.43 Mcal/kg NE_g, 0.70% Ca, and 0.45% P on a DM basis. Further composition information on both the transition and finishing diets can be found in **Supplementary Table 1**. Additionally, body weight was recorded at birth, weaning, yearling age (start of the feedlot period), and the conclusion of the feedlot trial.

During the feedlot period, the feed intake of steers was individually measured using a GrowSafe System (GrowSafe Systems Ltd., Calgary, Canada). Intake data was then used to calculate their individual feed conversion rates, which were expressed as RFI. Feed intake data was also used to calculate the daily cost of feeding the steers. Upon conclusion of the feedlot period, steers were rank-ordered based on their feed efficiencies (i.e., RFI) and the 12 most efficient (lowest RFI values), along with the 12 least efficient (highest RFI values) were transported to the University of Georgia Meat Science Technology Center, a federally inspected meat plant located in Athens, GA (33° 57 N, 83° 22 W). The steers were housed on site overnight where they were fasted but given *ad libitum* access to water prior to slaughter the next morning. In order to create an even greater biological distinction between the two groups of steers and have a greater difference in the magnitude of their RFI values, further selection of the samples was performed, resulting in a total of 15 steers being used in this study: 7 classified as low-RFI (efficient steers), and 8 classified as high-RFI (inefficient steers).

Fecal Collection and Storage

The first set of fecal samples was collected at weaning (∼9 months of age) on all steers ($n = 63$). Fecal contents were aseptically collected via fecal grab (∼50 g) using a separate palpation sleeve for each sample. The feces were then placed in 50 mL conical tubes and stored on ice until the samples were transferred to the laboratory and stored at −20°C. After the steers were transported to the feedlot, backgrounded, and started on the finishing diet, the second set of fecal samples were collected on all steers ($n = 63$), which corresponded to the yearling phase (∼13 months of age). These samples were collected and prepared as described above. The final set of samples was collected from their rectum upon evisceration of the carcasses on slaughter day (∼18 months of age) as mentioned above, and immediately placed in a −20°C freezer for storage.

DNA Extraction and Sequencing

Microbial DNA was extracted from the feces of the 7 less-efficient and the 8 most-efficient steers using a hybrid DNA extraction protocol utilizing both mechanical and enzymatic methods as previously described by Rothrock et al. (12). This procedure uses 0.33 g of fecal material placed into 2-mL Lysing Matrix E tubes (MP Biomedicals LLC, Irvine, CA, USA) which are homogenized using a FastPrep 24 Instrument (MP Biomedical LLC, Irvine, CA, USA) to mechanically break

open the cells. InhibitEX Tablets (QIAGEN, Venlo, Netherlands) were used as the enzymatic means of increasing DNA yields. An automated robotic workstation (QIAcube; QIAGEN, Venlo, Netherlands) was used for elution and purification of DNA from the samples. DNA concentration and purity were determined spectrophotometrically using the Synergy H4 Hybrid Multi-Mode Microplate Reader along with the Take3 Micro-Volume Plate (BioTek Instruments Inc.; Winooski, VT, USA). The samples required at least 20 μL of volume and a concentration of 10 ng/μL of DNA in order to proceed to sequencing. Samples that failed to meet these minimum requirements were processed through a new cycle of DNA extraction. Once all samples were adequate in both volume and DNA concentration, they were stored at 4°C overnight.

After overnight storage, the samples were transported to the Georgia Genomics and Bioinformatics Core (https://dna.uga.edu) for library preparation and 16S rRNA gene sequencing. The library preparation included PCR replications using the forward primer: S-D-Bact-0341-b-S-17 (5′-CCTACGGGNGGCWGCAG-3′) and reverse primer: S-D-Bact-0785-a-A-21 (5′-GACTACHVGGGTATCTAATCC-3′) (13, 14). PCR conditions were: initial denaturation at 95°C for 3 min, followed by 25 cycles of 95°C for 30 s, annealing at 55°C for 30 s, extension at 72°C for 30 s, and then a final elongation step at 72°C for 5 min. PCR clean-up was performed using AMPure XP beads (Beckman Coulter Life Sciences, Indianapolis, IN, USA). The library was quantified using qPCR, and the V3-V4 variable regions of the 16S rRNA gene were sequenced using an Illumina MiSeq instrument with a MiSeq v3 reagent kit for lengths of 2 × 300 bp (Illumina Inc., San Diego, CA, USA). A well-characterized bacteriophage PhiX genome (PhiX Control v3 Library; Illumina Inc., San Diego, CA, USA) was used as a control for the sequencing runs.

Sequencing Data

After sequencing was performed, the data was demultiplexed and converted into FASTQ files. Pair-end reads were set and merged using BBMerge Paired Read Merger v37.64 with an expected insert size of 500 bp, and files were analyzed using QIIME pipeline v1.9.1 (15). The files were then filtered based on quality (minimum Phred quality score of 20) and merged into one single file that was converted into the FASTA format. Sequences were grouped together at 97% similarity into operational taxonomic units (OTU) using the Uclust method and the Greengenes database (gg_13_8_otus). Sequence depth was set at 17,542 sequences per sample for further analysis. This value was selected because it allowed the retention of all the samples while providing a minimum Good's coverage index of 0.95. The data was made publicly available, and readers can find it at: https://www.mg-rast.org using the accession number: mgm4909317.3. Phylogenetic Investigation of Communities by Reconstruction of Unobserved States (PICRUSt) was performed to make inferences about the metabolic pathways expressed within the microbiota (16, 17); and the metabolic functions were assessed using the Kyoto Encyclopedia of Genes and Genomes (KEGG) third-level pathways.

Volatile Fatty Acid Analysis

Analysis of volatile fatty acids (**VFA**) was performed according to the procedure described in Lourenco et al. (18). One gram of feces was diluted with 3 mL of distilled water and placed into 15-mL conical tubes. The tubes were vortexed for 30 s to produce a homogeneous sample and 1.5 mL of the mixture was transferred to microcentrifuge tubes. The tubes were centrifuged at room temperature at 10,000 × *g* for 10 min. One milliliter of the supernatant was transferred into a new microcentrifuge tube and mixed with 0.2 mL of metaphosphoric acid solution (25% v/v). The samples were vortexed for 30 s and stored at −20°C overnight. The next morning, samples were thawed and centrifuged at room temperature at 10,000 × *g* for 10 min. The supernatant was removed and transferred into polypropylene tubes combined with ethyl acetate in a 2:1 ratio of ethyl acetate to supernatant. Tubes were vortexed for 10 s to thoroughly mix them and allowed to settle for 5 min for optimum separation. Then 600 μL of the top layer was transferred into screw-thread vials. VFA analysis was performed using a Shimadzu GC-2010 Plus gas chromatograph (Shimadzu Corporation, Kyoto, Japan) with a flame ionization detector and a capillary column (Zebron ZB-FFAP; 30 m × 0.32 mm × 0.25 μm; Phenomenex Inx., Torrance, CA, USA). Sample injection volume was set to 1.0 μL, and helium was used as a carrier gas. Column temperature started at 110°C and increased to 200°C over the course of 6 min. The injector temperature was set to 250°C, and the detector temperature was set to 350°C.

Statistical Analysis

Statistical analyses were performed using Minitab v19.1. Animal performance data [birth weight, weaning weight, yearling age weight, weight at the end of the feedlot period, feedlot dry matter intake, feedlot feed costs, feedlot feed:gain, and feedlot residual feed intake], alpha-diversity indices, and bacterial abundances were analyzed using a one-way ANOVA with feedlot RFI classification (i.e., high- or low-RFI) as a factor. In addition, repeated-measures ANOVA were carried out for each group of steers to investigate potential differences across the 3 samples collected throughout their lifecycle (weaning, yearling, and slaughter), and Tukey's pairwise comparisons were performed to assess further differences. Multiple correlations between RFI values and the microbial traits were evaluated, and both Shannon diversity and the abundance of *Ruminococcaceae* at slaughter were found to be highly significant. Thus, linear regression analysis was performed to investigate the relationship between Shannon diversity index at slaughter and RFI; as well as between the abundance of *Ruminococcaceae* at slaughter and RFI. Multiple correlations were performed between bacterial abundance and the expression of metabolic pathways (**Supplementary Table 2**). Given that the abundance of *Rikenellaceae* was positively associated with glycosaminoglycan degradation, a linear regression was performed between these two traits in the feces of steers across all stages of production. Anderson-Darling Normality Tests were performed on the alpha diversity metrices and the bacterial abundances at each time point for both efficiency groups, and the majority were normally distributed (**Supplementary Figures 1–6**). Beta diversity

between all pairs of samples was calculated using QIIME's "beta_diversity_through_plots.py" script and results were visualized using 3-dimensional plots (**Figure 1**). Unweighted UniFrac distances were used for the beta diversity plots. This metric was chosen because it accounts for phylogenetic relationships when measuring beta diversity (19). For all statistical tests, results were considered significant at $P \leq 0.05$, and treated as trends when $0.05 < P \leq 0.10$.

RESULTS

Animal Performance

Body weight was consistent throughout the life cycle of the efficient and the inefficient steers (**Table 1**; $P \geq 0.51$). Dry matter intake during the feedlot-finishing period was lower for the low-RFI (efficient) steers ($P < 0.001$) compared to the high-RFI (inefficient) steers, resulting in a decrease in the daily feeding cost for the efficient steers ($P < 0.001$) compared to the inefficient steers. Feed conversion, expressed as a feed:gain ratio, was lower ($P = 0.001$) for the more efficient steers. Likewise, the efficient and inefficient steers were divergent ($P < 0.001$) in terms of feedlot RFI, with the efficient steers consuming 4.04 kg less dry matter per day when comparing to the inefficient steers (RFI = 2.02 and −2.02 for the inefficient and efficient steers, respectively).

Diversity Indices

The Principal Coordinate Analysis (PCoA) plot of Beta-diversity for the steers at weaning, yearling, and slaughter showed that fecal samples collected at weaning were different ($P < 0.001$); whereas, fecal samples collected at yearling and slaughter clustered together (**Figure 1**). There was no difference ($P = 0.080$) in beta diversity between efficiency group (**Supplementary Figure 7**). Alpha-diversity indices for the efficient and inefficient steers at weaning, yearling, and slaughter were examined (**Table 2**). In the inefficient steers, Chao 1, an indicator of microbial richness, was higher ($P = 0.02$) in the feces at weaning compared to the feces collected at slaughter. In the efficient steers, microbial richness at slaughter and yearling numerically decreased compared to weaning, but this was not significant ($P = 0.19$). In both the efficient and inefficient steers, the species within the feces at weaning were more evenly distributed ($P < 0.001$) than at yearling and slaughter. Similarly, the Shannon diversity index of fecal samples was higher ($P < 0.001$) at weaning than at the later timepoints, regardless of feedlot efficiency status of the steers. Regression analysis revealed that as the Shannon diversity index increased, RFI decreased (**Figure 2**; $R^2 = 65.6\%$). The Shannon diversity index did not differ between efficient and inefficient steers at weaning and yearling (**Table 2**; $P \geq 0.18$); however, at slaughter, fecal microbial diversity was greater ($P = 0.004$) in the efficient steers compared to inefficient steers. Chao 1 index did not differ between inefficient and efficient steers at any timepoint ($P \geq 0.13$). Microbial evenness did not differ between inefficient and efficient steers at weaning or yearling ($P \geq 0.15$) but was greater ($P = 0.001$) in the efficient steers compared to the inefficient steers at slaughter.

Bacterial Relative Abundance

The relative abundances of the bacterial families S24-7, *Bifidobacteriaceae*, and *Lactobacillaceae* were greater ($P \leq 0.05$) in the inefficient steers at weaning compared to the efficient steers (**Figure 3**). At both yearling and slaughter, abundance of the families *Ruminococcaceae*, *Rikenellaceae*, and *Christensenellaceae* were higher ($P \leq 0.05$) in feces of efficient steers than in feces of the inefficient steers.

As the population of *Ruminococcaceae* increased in the feces collected at slaughter, the RFI of the host was lower (**Figure 4**; $R^2 = 60.7\%$). *Ruminococcaceae* abundance was higher ($P < 0.001$) at weaning than at both yearling and slaughter in both feed efficiency groups (**Figure 5A**). At weaning, there were no differences ($P = 0.46$) in *Ruminococcaceae* abundance between efficient and inefficient steers. At yearling and slaughter, *Ruminococcaceae* abundance was higher ($P = 0.01$) in efficient steers compared to inefficient steers.

The fecal abundance of *Rikenellaceae* was higher at weaning than at yearling and slaughter for both groups of steers (**Figure 5B**; $P < 0.001$). Fecal abundance of *Rikenellaceae* tended to be higher ($P = 0.10$) at weaning in the efficient steers than in the inefficient steers. At yearling and slaughter, the most efficient steers had greater fecal abundances of *Rikenellaceae* compared to the least efficient steers ($P \leq 0.05$). As the relative abundance of *Rikenellaceae* increased in the feces during all stages of production, the expression of the gene responsible for glycosaminoglycan degradation increased (**Figure 6**; $r = 0.618$; $P < 0.001$; $R^2 = 38.2\%$). For both groups of steers, the abundance of *Christensenellaceae* was greater (**Figure 5C**; $P < 0.001$) in feces collected at weaning than in the feces collected at yearling and slaughter. Moreover, abundance of *Christensenellaceae* tended to be higher at weaning ($P = 0.08$) in efficient steers; and was higher ($P \leq 0.05$) at both yearling and slaughter in the efficient steers.

Fecal *Bifidobacteriaceae* abundance was higher ($P = 0.011$) at slaughter compared to weaning in the inefficient steers (**Figure 5D**). Conversely, abundances of *Bifidobacteriaceae* remained relatively consistent throughout the life of efficient steers ($P = 0.142$), although a numerical increase was observed. At weaning, there was a greater population of *Bifidobacteriaceae* present in the feces of inefficient steers compared to the feces of efficient steers ($P = 0.02$). *Bifidobacteriaceae* abundance was on average the same in the feces of the steers at the yearling stage regardless of their feed efficiency status ($P = 0.14$). Inefficient steers tended to have greater abundances of *Bifidobacteriaceae* in the feces at slaughter compared to efficient steers ($P = 0.06$).

Volatile Fatty Acid Concentrations

Fecal acetate concentrations were greater (**Table 3**; $P = 0.002$) at slaughter compared to weaning and yearling age in the inefficient steers, whereas, it was only greater ($P = 0.019$) at slaughter compared to the yearling stage in the efficient steers. Fecal acetate concentrations were greater ($P = 0.028$) in the inefficient steers compared to efficient steers at slaughter. More propionate and butyrate were present in the feces at slaughter compared to the feces at weaning and yearling ($P \leq 0.003$) in both efficient and inefficient steers. Valerate concentrations in the feces at slaughter were higher ($P < 0.001$) in the efficient steers compared to at the

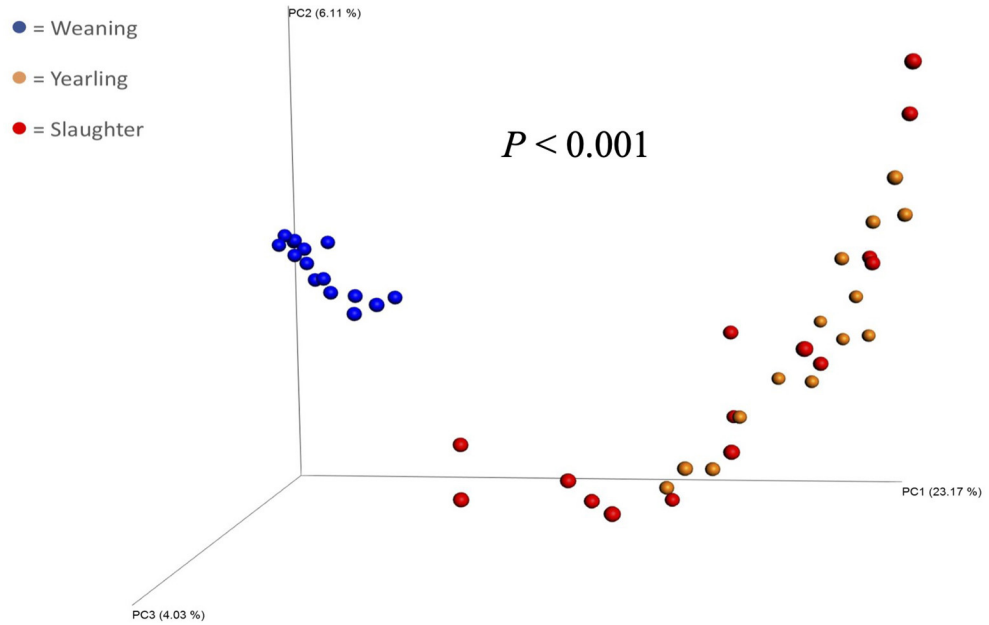

FIGURE 1 | Principal coordinate analysis plot of beta diversity (unweighted UniFrac) of fecal bacterial populations of steers ($n = 15$) collected at weaning, yearling, and slaughter. *P*-value indicates a difference in beta diversity between samples collected at each timepoint.

TABLE 1 | Performance of the efficient and inefficient steers ($n = 7$ efficient and $n = 8$ inefficient) at different points in the beef production continuum.

Performance trait	Inefficient	Efficient	SEM	*P*-value
Birthweight, kg	38.4	37.2	1.3	0.52
Weaning (9 months-old) weight, kg	300	298	10.5	0.90
Yearling weight (13 months-old), kg	501	487	14.4	0.51
Feedlot final body weight (18 months-old), kg	599	584	15.6	0.51
Feedlot dry matter intake, kg/d	13.6	9.7	0.87	<0.001
Feedlot daily feeding cost, US$/steer*	3.13	2.22	0.14	<0.001
Feedlot feed:gain ratio, kg	15.84	10.54	1.32	0.001
Feedlot residual feed intake (RFI), kg/d	2.02	−2.02	0.82	<0.001

**The feedlot-finishing diet had a cost of US$ 0.23/kg DM.*

weaning and yearling age; however, fecal valerate was only higher ($P = 0.049$) at slaughter compared to the yearling stage in the inefficient steers. Total VFA concentrations were increased ($P < 0.001$) in all steers, regardless of their feed efficiency, in the fecal samples collected at slaughter compared to either the weaning or yearling stages. Regardless of feedlot feed efficiency status, the ratio of acetate to propionate decreased throughout the life of the steers, being highest in the feces at weaning, intermediate in the feces at the yearling stage, and lowest in the feces at slaughter ($P < 0.001$). Correlations between VFA concentration and RFI at weaning, yearling, and slaughter can be found in **Supplementary Table 3**.

Bacterial Family and Volatile Fatty Acid Correlations

At weaning, the bacterial family *Christensenellaceae* was positively correlated with propionate concentration (**Table 4**; $r = 0.523$; $P = 0.046$). At yearling age, the acetate to propionate ratio was positively correlated with both *Ruminococcaceae* ($r = 0.579$; $P = 0.030$) and *Rikenellaceae* ($r = 0.675$; $P = 0.008$) abundances. *Ruminococcaceae* abundance was negatively correlated to acetate ($r = -0.578$; $P = 0.024$) and total VFA ($r = -0.536$; $P = 0.039$) concentration of the feces at slaughter. This bacterial family was positively correlated to valerate concentration at slaughter ($r = 0.515$; $P = 0.049$).

TABLE 2 | Alpha-diversity indices calculated for the fecal samples of efficient and inefficient steers at different stages of their lives: weaning, yearling, and kill floor (slaughter).

Index		Weaning	Yearling	Slaughter	SEM	*P*-value
Chao1	Inefficient	4360.6[a]	3956.9[ab]	3384.7[b]	206	0.02
	Efficient	4424.8	3621.4	3834.8	303	0.19
	SEM	178	109	205		
	P-value	0.86	0.13	0.29		
Species evenness	Inefficient	0.788[a]	0.687[b]	0.658[b]	0.009	<0.001
	Efficient	0.804[a]	0.710[b]	0.724[b]	0.006	<0.001
	SEM	0.006	0.009	0.012		
	P-value	0.15	0.20	0.001		
Shannon diversity	Inefficient	8.73[a]	7.42[b]	6.99[b]	0.14	<0.001
	Efficient	8.95[a]	7.61[b]	7.81[b]	0.11	<0.001
	SEM	0.080	0.100	0.159		
	P-value	0.18	0.36	0.004		

[ab] *Values not sharing a common superscript within each row significantly differ according to Tukey's pairwise comparison ($P \leq 0.05$).*

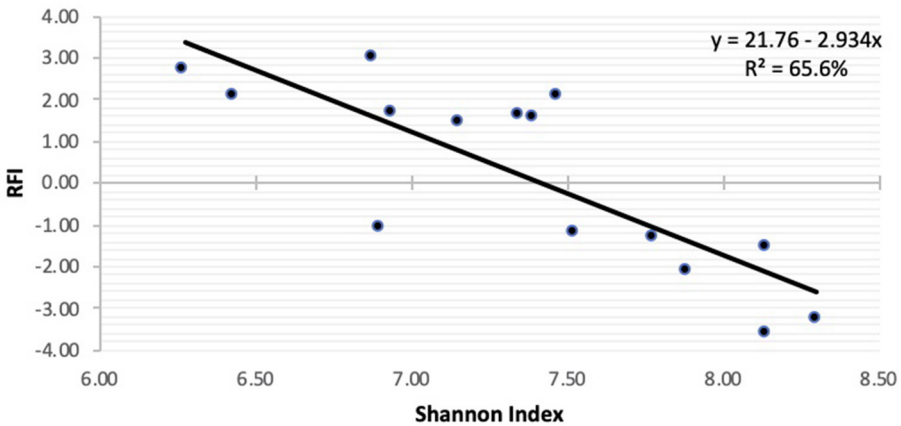

FIGURE 2 | Linear regression expressing the relationship between residual feed intake (RFI) and Shannon diversity in the feces of Angus steers observed at slaughter.

DISCUSSION

Animal Performance

As expected, based on previous generations' performance within this commercial Angus herd (20), the steers maintained similar body weights throughout production regardless of feed efficiency classification. However, steers differed in the amount of feed consumed and in their conversion of feed into body weight. During the feedlot-finishing phase, the efficient steers consumed on average 3.9 kg less feed per day (dry matter basis) while gaining approximately the same amount of weight as their counterparts. This translated into the efficient steers needing 5.3 kg less feed than their inefficient counterparts to gain 1 kg of body weight during the feedlot trial, given that their feed:gain ratios were 10.54 and 15.84, respectively. Similarly, the calculated RFI values were distinct between the 2 groups of steers, with the lowest RFI values observed in the efficient steers. Feed conversion differences are important for producers because feed is the most expensive input cost of animal production systems, therefore having cattle that can gain the same amount of weight while consuming less feed can have a significant impact on feeder profit margins (1, 21). For instance, in the present study, it cost US$0.91 less per day to feed the more efficient steers compared to the inefficient ones, resulting in a difference of US$100.10 per steer during the 110-day feedlot trial.

Diversity Indices

With 19% of the variation in RFI being attributed to diet composition and the digestibility of feed (5), our beta diversity results are to be expected. Since the steers in our study were transitioning from a forge-based diet at weaning to a grain-based diet at both yearling age and slaughter, it is reasonable that the differences in diets would drive the differences seen in beta diversity. The selection pressure placed on the fecal microbiota by the nutrient availability of diet selected for or against certain bacterial species causing the clustering of samples

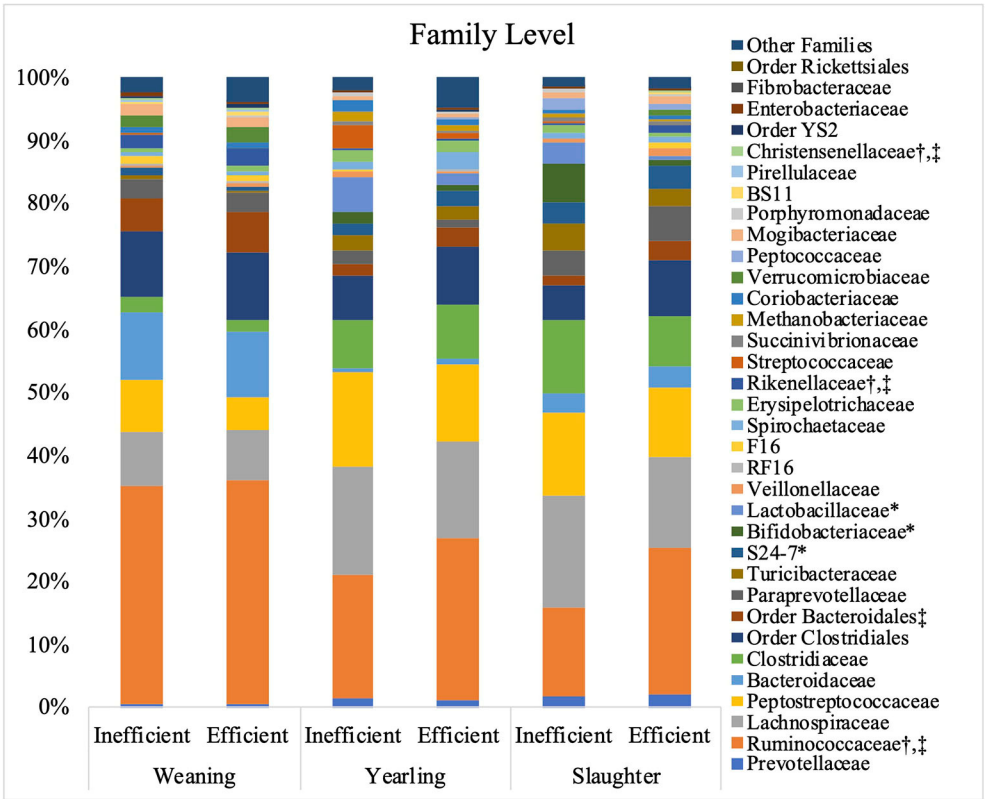

FIGURE 3 | Relative bacterial abundance at the family level found in the feces of efficient and inefficient steers at weaning, yearling, and slaughter. Differences between the two groups of steers ($P \leq 0.05$) are denoted by * at weaning, † at yearling, and ‡at slaughter.

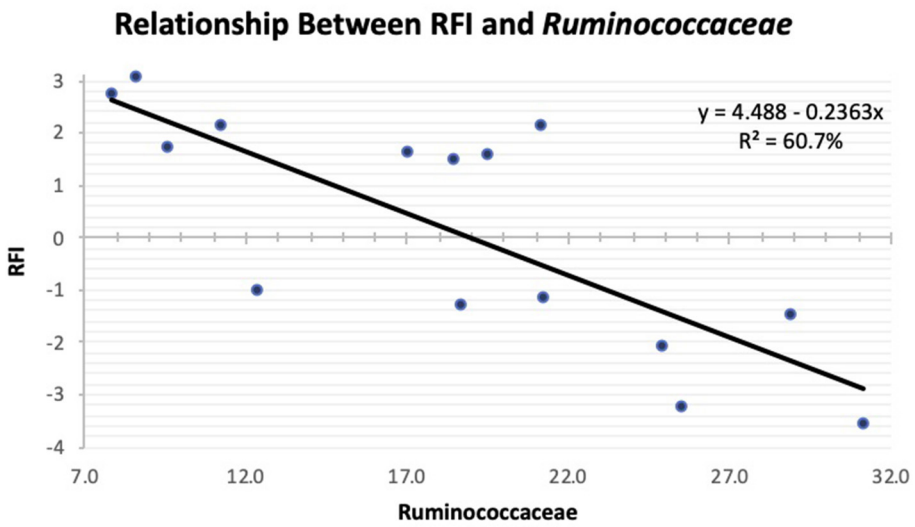

FIGURE 4 | Linear regression expressing the relationship between RFI and abundance of *Ruminococcaceae* in the feces of Angus steers at slaughter.

on a forage-based vs. a grain-based rations. This finding was corroborated by previous studies that found that both diet composition and age played important roles in the composition of the microbial population of the gastrointestinal tract (22, 23). So, with the age and diet of the steers changing, the microbial population within the feces is also expected to change.

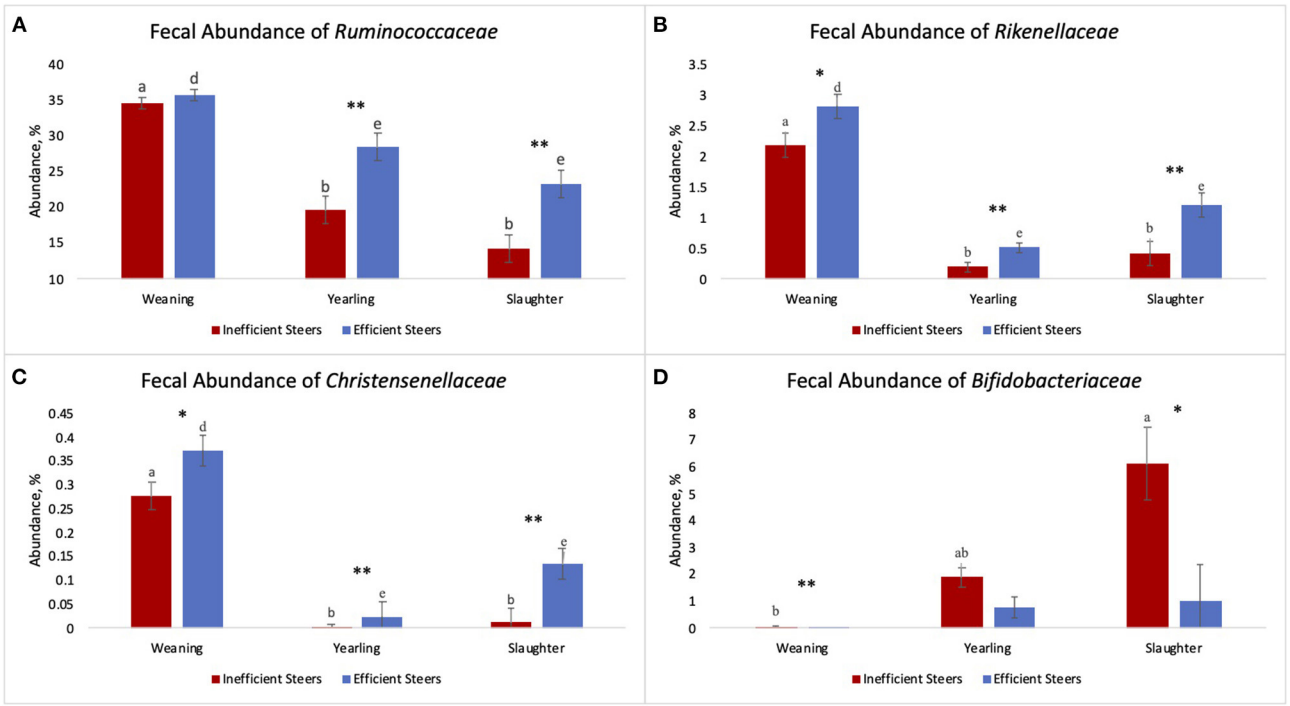

FIGURE 5 | Fecal abundance of *Ruminococcaceae* **(A)**, *Rikenellaceae* **(B)**, *Christensenellaceae* **(C)**, and *Bifidobacteriaceae* **(D)** at weaning, yearling, and slaughter of inefficient ($n = 8$) and efficient ($n = 7$) steers. ^{a,b,c} indicate a significant difference across timepoints ($P \leq 0.05$) of inefficient steers. ^{d,e,f} indicate a significant difference across timepoints ($P \leq 0.05$) of efficient steers. Asterisks indicate a difference between inefficient and efficient steers at individual timepoint with * indicating a trend ($0.10 \leq P \geq 0.05$) and ** indicating a significant difference ($P \leq 0.05$). Error bars indicate standard error.

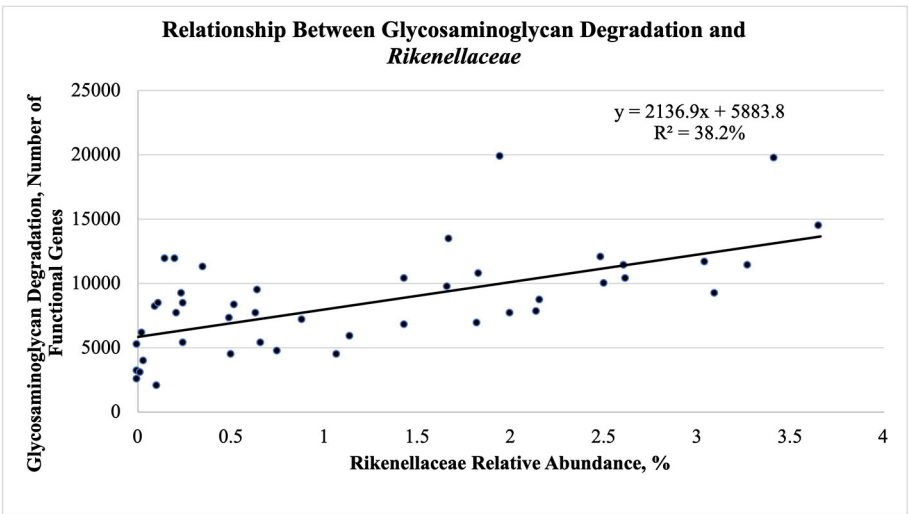

FIGURE 6 | Linear regression expressing the relationship between glycosaminoglycan degradation and *Rikenellaceae* abundance in the feces of Angus steers ($n = 15$) across all stages of production.

Research has broadly demonstrated that more efficient ruminants have comparatively low bacterial richness and diversity (9, 24). However, studies investigating the composition of the microbiome relative to efficiency of the host mainly focus on the ruminal microbial population, so the intestinal microbiome has not been extensively evaluated in this regard (25). Shabat et al. (9) hypothesized that ruminal microbial populations with less richness and diversity carried out fewer, but more relevant metabolic pathways, leading to a more limited metabolite pool but with greater biological relevance in the rumen. However, in the intestinal environment, Welch et al. (11) found that bacterial richness and diversity were greater in

TABLE 3 | Volatile fatty acid (VFA) concentration (m*M*) in the feces of efficient and inefficient steers at different stages of production: weaning, yearling, and kill floor (slaughter).

Volatile fatty acid		Weaning	Yearling	Slaughter	SEM	*P*-value*
Acetate	Inefficient	39.9^{b}	29.6^{b}	55.9^{a}	4.08	0.002
	Efficient	33.0^{ab}	30.6^{b}	43.8^{a}	2.95	0.019
	SEM	3.45	2.65	2.85		
	P-value	0.34	0.85	0.03		
Propionate	Inefficient	6.2^{b}	6.4^{b}	16.0^{a}	1.05	<0.001
	Efficient	6.0^{b}	5.9^{b}	14.1^{a}	0.82	<0.001
	SEM	0.692	0.71	1.09		
	P-value	0.91	0.73	0.41		
Butyrate	Inefficient	2.5^{b}	4.0^{b}	7.6^{a}	0.87	0.003
	Efficient	1.8^{b}	3.4^{b}	6.5^{a}	0.61	<0.001
	SEM	0.302	0.507	0.891		
	P-value	0.21	0.55	0.56		
Valerate	Inefficient	0.70^{ab}	0.40^{b}	1.09^{a}	0.18	0.049
	Efficient	0.5^{b}	0.3^{b}	1.4^{a}	0.10	<0.001
	SEM	0.098	0.137	0.182		
	P-value	0.31	0.83	0.48		
Total VFA	Inefficient	51.0^{b}	40.4^{b}	82.6^{a}	5.37	<0.001
	Efficient	42.7^{b}	40.2^{b}	68.7^{a}	3.54	<0.001
	SEM	4.52	3.78	4.08		
	P-value	0.38	0.98	0.09		
Acetate:Propionate	Inefficient	6.5^{a}	4.6^{b}	3.6^{c}	0.23	<0.001
	Efficient	6.3^{a}	5.2^{b}	3.2^{c}	0.28	<0.001
	SEM	0.202	0.189	0.200		
	P-value	0.54	0.05	0.37		

**P-value for the repeated measures ANOVA using collection time as a factor.*

^{abc}Values not sharing a common superscript within each row are significantly different ($P \leq 0.05$).

TABLE 4 | Correlation between volatile fatty acids concentration and bacterial families* in the feces of steers ($n = 15$) at weaning, yearling, and slaughter.

	Correlation coefficient	*P*-value
Weaning		
Christensenellaceae		
Propionate	0.523	0.046
Yearling		
Ruminococcaceae		
Acetate:Propionate	0.579	0.030
Rikenellaceae		
Acetate:Propionate	0.675	0.008
Slaughter		
Ruminococcaceae		
Acetate	−0.578	0.024
Valerate	0.515	0.049
Total VFA	−0.536	0.039

**Only bacterial families with significance to host efficiency and significant Pearson correlations ($P \leq 0.05$) with volatile fatty acids are shown.*

both the cecal contents and feces of steers with higher feed efficiency. Other researchers have also shown that microbial diversity measures differed between the rumen and the feces of cattle (10, 18, 26). The present study found bacterial evenness and diversity to be greater in the feces of the most efficient steers which somewhat contradicts the hypothesis proposed by Shabat et al. (9); however, our findings conform the biological theory regarding the intestinal environment outlined by Welch et al. (11). Nutrient availability differs widely throughout the GIT of cattle, and the digesta that reaches the large intestine contains less-digestible nutrients, which are essentially non-digested nutrients that escaped ruminal microbial degradation and small intestinal digestion. Therefore, increased bacterial evenness and diversity in the hindgut will, in turn, result in a greater array of microbial enzymes to degrade the intestinal digesta, allowing the more efficient steers to capture more non-digested nutrients that would have been unutilized in the GIT, and to convert them into metabolic end products that can be utilized by the host.

Beyond gastrointestinal anatomy and physiology there are many other contributing factors to microbial consortium composition variation, including animal age and diet composition (10, 22, 23). Thus, the changes in alpha diversity in the present study were also related to both changes in age of the steers and dietary changes during each growth phase. Overall, we found that steers had the greatest microbial richness, evenness,

and diversity in their feces at weaning, when they were younger and were reared on their dams in a pasture-based production system. During backgrounding, leading to the yearling stage, starch concentrations in their ration increased resulting in a selective pressure on the complex microbial ecosystem of their GIT. For instance, the availability of digestive enzymes (e.g., pancreatic amylase and maltase) can limit the breakdown of starch in the small intestine; therefore, starch that escapes microbial degradation in the rumen and is not digested in the small intestine, can reach the cecum and colon of cattle and impact the composition of the bacterial population colonizing them (27–29). Since the finishing feedlot ration contains an even higher concentration of starch, the selective pressure of this ration decreased the microbial evenness and diversity of all steers regardless of feed efficiency status and decreased bacterial richness (Chao1) in the inefficient steers. It has been found that as grain levels in diets increased to the point of incurring ruminal acidosis, the environmental pressures would select for reduced bacterial diversity and for a microbial population largely made up of lactic acid bacteria in the rumen (30–32). The present results tend to support this hypothesis as to a cause and type of change in the microbial consortium diversity in the feces of cattle.

Bacterial Relative Abundances

Diet composition alters not only the diversity of the gastrointestinal microbiome as a whole, but also causes fluctuations in many individual bacterial populations within the gastrointestinal tract (33, 34). Since the steers utilized in this study changed from a pasture to a feedlot-based system, the variation in bacterial populations at each timepoint can be explained by diet. However, the more novel results were that despite fluctuations in abundance throughout the steers' lives, certain bacterial families were consistently more abundant in one group of steers based on their efficiency status, regardless of the diet.

Ruminococcaceae is a family comprised of primarily cellulolytic and hemicellulolytic bacterial species that produce acetate, formate, and hydrogen as fermentation end products (35, 36). This acetate production could be driving the correlation of this bacteria with the acetate-to-propionate ratio observed during the study. *Ruminococcaceae* can degrade many substrates that other bacterial families cannot because it possesses many genes which allow them to bind to cellulose, hemicellulose, and xylan, allowing them to degrade plant materials more effectively (36–39). In the present study, regardless of feed efficiency status, abundances of *Ruminococcaceae* were greatest at weaning, while animals were consuming a forage-based diet, which contains substantially more fiber than feedlot finishing diets. Genetic diversity of CAZymes (carbohydrate active enzymes) provides *Ruminococcaceae* an advantage when it comes to nutrient uptake and utilization of diverse polysaccharides (40). The negative relationship between RFI and *Ruminococcaceae* found in the present study at slaughter corroborate with our previous findings (23) that have shown that as this bacterial family increased, RFI decreased resulting in the steer becoming more efficient by utilizing a broader nutrient spectrum, allowing the animal to absorb more energy from the diet. Therefore, we suggest in the present study that the greater abundance of *Ruminococcaceae* in the most efficient steers allowed them to extract greater amounts of energy from the digesta reaching their hindguts, resulting in greater metabolizable energy levels compared to the inefficient steers.

Rikenellaceae is a family consisting of bacteria found within the gastrointestinal tract and fecal material from animals and humans (41, 42). Bacteria within *Rikenellaceae* can utilize mucin as a source of carbohydrates and energy which provides them a competitive advantage over other bacteria (43). This is supported by the positive relationship seen between the relative abundance of *Rikenellaceae* and the expression of the gene responsible for glycosaminoglycan degradation shown in the present study. Glycosaminoglycans are essential to the development of gastrointestinal mucosa (44). *Rikenellaceae* abundance and the gene responsible for glycosaminoglycan degradation have previously been found to be important in feces of mice in terms of gut mucosa; however, there were no correlations provided to show if a relation existed between them (45). Although the amount of glycosaminoglycans was not quantified in the present study, the positive association between *Rikenellaceae* and this gene suggests that the more efficient steers had more glycosaminoglycans present in their hindgut that can be utilized by this bacterium. *Rikenellaceae* produce acetate, succinate, and propionate as fermentative end products (46), all of which can be utilized by the host animal. This bacterial family was found to be correlated with the acetate-to-propionate ratio at yearling which can be a result of an increase in its ability to produce acetate for the host. Moreover, *Rikenellaceae* was found to be more prevalent as a member of the core microbiome of heifers fed a forage-based diet, compared to a forage-grain mixed or all grain diet (47), which agrees with present results, given that the greatest abundances of *Rikenellaceae* were observed at weaning when the steers were consuming primarily forages. The abundance of *Rikenellaceae* was consistently higher in the feces of the most efficient steers at all timepoints evaluated, indicating that the efficient steers might have produced more mucin in their hindguts resulting in a greater population of *Rikenellaceae*. Furthermore, because the abundance of this bacterial family was consistently higher in the feces of the most efficient steers, this family is a candidate marker of cattle feed efficiency, even at early stages of growth.

Christensenellaceae has been associated with a healthy digestive system in humans, a reduction in adipose tissue, and a lower body mass index (48). The family *Christensenellaceae* consists of species which produce α-arabinosidase, β-galactosidase, and β-glucosidase (49), which break down components of plant fibers, thus it is logical that the highest fecal abundances were found at weaning when the steers were grazing forages. Additionally, a positive correlation was observed between *Christensenellaceae* abundance and propionate concentration at weaning when this bacterial family was able to efficiently extract energy from the feedstuffs. The decrease in abundance of *Christensenellaceae* seen in the feces at yearling and slaughter can be attributed to a decrease in the overall health of the digestive system during the feedlot-finishing period (50–52),

where the diet is much more concentrate-based and has been linked to a reduction in ruminal and intestinal health (53–56). Additionally, *Christensenellaceae* produce butyrate (57) which is used by the epithelial tissue for energy (58, 59). Increased butyrate production by *Christensenellaceae* may reduce the incidence of leaky gut which may be responsible for leakage of lipopolysaccharide (LPS) and resultant inflammation in the less efficient steers (55, 60–62).

Bifidobacteriaceae is comprised of a fructo- and galacto-oligosaccharide-fermenting bacteria to produce acetate (49). It is often associated with a healthy gastrointestinal tract and is often used as a probiotic (63–65), and has been found in the rumen, small intestine, and hindgut of ruminants (49, 66, 67). *Bifidobacteriaceae* were negatively correlated with *Christensenellaceae* populations in human fecal samples (48) which is in line with what we found in the present study. *Christensenellaceae* abundance was higher in the most efficient steers, whereas *Bifidobacteriaceae* abundance was higher in the inefficient steers. *Bifidobacteriaceae* was most abundant in the cecum of steers that had a high rate of gain and high feed intake but was not found in steers with high gain and low feed intake [i.e., more efficient; (68)]. Our present results support this finding since *Bifidobacteriaceae* populations were consistently lower in the more efficient steers.

Volatile Fatty Acid Production

Previous studies have found low-RFI (more efficient) steers to have an increase in energy converted in the rumen (2, 59), suggesting that host efficiency may be directly related to additional VFA produced by microbial fermentation of the rumen. Welch et al. (11) reported a numerical increase in VFA production in the rumen of efficient steers compared to inefficient steers; however, fecal samples collected post slaughter were reversed, with VFA concentrations being highest in the less efficient steers. The present study observed similar patterns, with the inefficient steers having more total fecal VFA than the efficient steers. Moreover, the lower concentration of acetate and lower numerical concentrations of other major VFAs such as propionate, and butyrate observed in the feces of the efficient steers suggests that the VFA values quantified in the fecal material are strong indicators of increased lower gut VFA absorption rather than production. This result suggests a need to investigate the linkage between the microbiome of the lower gut and gut epithelial integrity and health, potentially explaining differences in feed efficiency.

Diet composition greatly impacts VFA concentration (69, 70), and it is unsurprising that fecal VFA concentrations varied greatly throughout the steers lifetimes due to the different diets consumed at each stage of growth. Furthermore, most individual VFA (and total VFA) fecal concentrations were highest at slaughter, suggesting that the hindgut microbial activity increased with age, resulting in more VFA production. The acetate-to-propionate ratio in the rumen is directly related to energy availability to the host animal because propionate is glucogenic (71–73). In the present study, the ratio of acetate-to-propionate decreased throughout the life of the steers, regardless of their efficiency status, reflecting the dietary composition as a key factor in these changes. The acetate-to-propionate ratio was decreased in ruminants fed a high concentrate diet (reflective of an increase in propionate production) compared to cattle fed a high forage diet (27, 74, 75). Although our measurements were made in the fecal material, still, the acetate-to-propionate ratio was highest when the steers consumed predominantly pasture and was lower when the steers were fed a feedlot ration.

The results presented here provide a meaningful insight into the relationship between beef steers and their fecal microbiotas throughout their productive lives. While these results are meaningful, they are reflective of a limited sample size (i.e., fifteen steers). Additionally, the samples at slaughter were collected post-mortem and following a 24-h fasting period. Thus, it is possible that these conditions impacted the fecal microbiome and VFA concentrations assessed at slaughter. Lastly, the fact that the present study identified bacteria at the family taxonomic level may pose another limitation, as more specific taxonomic levels may be more informative. Therefore, despite these notable results, more research is necessary to draw irrefutable conclusions about the relationship between beef cattle and their fecal microbiota, and how it affects their feed efficiency.

Conclusions

Our results demonstrated that the fecal microbiota fluctuated throughout the life of beef steers, and that some specific bacterial families were consistently found at differential abundances in steers depending on their feed efficiency status. Surprisingly, for some bacterial families this holds true throughout the entire production continuum of beef steers, even when major diet changes occur. Abundances of *Ruminococcaceae*, *Rikenellaceae*, and *Christensenellaceae* were numerically greater in the feces of the steers with greater feed efficiency from weaning until slaughter. Conversely, *Bifidobacteriaceae* was more abundant in the feces of the less efficient steers at multiple stages of their lives, suggesting a potential negative impact on feed efficiency. Moreover, microbial diversity in the hindgut was strongly correlated with feedlot RFI, and it was consistently higher in the most efficient steers during their productive lives. Collectively, our results illustrate that the ruminants' intestinal microbiota can significantly impact feed efficiency, and some aspects of this microbiome divergence can be detected as early as weaning, leading to the opportunity for producers to utilize fecal samples as a selection tool for feed efficiency within their herd.

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: MG-RAST (accession number: mgm4909317.3).

ETHICS STATEMENT

The animal study was reviewed and approved by approved by the University of Georgia's Animal Care and Use Committee (AUP #A2012 11-006-R1).

AUTHOR CONTRIBUTIONS

CW wrote the manuscript with the help of all the authors. CW, JL, TK, and DS performed data analysis. JL, TK, DS, FF, TP, and TC revised the manuscript. All authors read and approved the final version for publication.

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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.2021.597405/full#supplementary-material

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