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Unlocking the power of gut microbiota to improving health and welfare in non-ruminant livestock

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Unlocking the power of gut microbiota to improving health and welfare in non-ruminant livestock

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Editorial: Unlocking the power of gut microbiota to improving health and welfare in non-ruminant livestock

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Editorial on the Research Topic
Unlocking the power of gut microbiota to improving health and welfare in non-ruminant livestock

Introduction

In the era of sustainable agriculture, improving the health and welfare of non-ruminant livestock—including poultry, rabbits, and swine—has become a central focus for researchers and producers alike. Among the many factors that influence these outcomes, the gut microbiota emerges as a key player, orchestrating processes ranging from nutrient utilization to immune function. Recent studies, including those featured in this Research Topic, highlight how manipulating the gut microbiome through dietary strategies can unlock significant improvements in animal health, productivity, and welfare. This collection includes nine new studies that thoroughly explore these topics and other related subjects. The aim is to deepen our understanding of the importance of non-ruminant animals in global livestock production and to underscore the crucial role of gut microbiota in maintaining their health and welfare. The nine articles focus on aspects such as gut microbial communities, nutritional metabolism, health status, and production performance in non-ruminant species—including piglets, broiler chickens, rabbits, and horses. Emphasis is placed on the significance of dietary management strategies, such as functional additives and rearing systems, in promoting gut health and overall animal wellbeing.

Gut microbiota: a driver of nutrient utilization

The microorganisms that reside in the gastrointestinal tract contribute significantly to the breakdown and absorption of nutrients. Non-ruminant livestock, including pigs and

poultry, have different digestive systems compared to their ruminant counterparts. Their gut microbiota plays a crucial role in nutrient utilization. Non-ruminant livestock rely on their gut microbiota to break down complex nutrients that their own digestive enzymes cannot process, directly impacting feed efficiency, growth performance, and health condition (1). Research consistently demonstrates that microbial communities maintained within a stable environment can not only enhance the utilization efficiency of energy and amino acids in feed but also improve animal health and welfare (1–3). Jia et al. found in a 28-day clinical trial on weaned piglets that dietary supplementation with 5% and 10% fermented wheat bran significantly reduced the incidence of diarrhea and markedly improved the efficiency of nutrient absorption. These effects were associated with alterations in the gut microbiota, notably a substantial proliferation of beneficial bacteria such as *Prevotellaceae* and *Succinivibrionaceae*, which effectively promoted the digestion and absorption of dry matter, crude protein, and energy. Similarly, in broiler chicken production, the addition of fermented Astragalus polysaccharides (FAP) and glycyrrhiza extract containing Lactobacillus acidophilus (GUE) significantly enhanced nutrient utilization (Liu Z. et al.; Li X. et al.). Broilers supplemented with FAP not only exhibited higher daily weight gains but also produced carcasses with superior weights at slaughter (Liu Z. et al.). This improvement is attributed to an optimized microbial community structure that decomposes nutrients more efficiently. These findings suggest that precise modulation of microbial communities can optimize nutrient utilization, reduce waste, and enhance production efficiency.

Safeguarding intestinal health through microbial balance

Intestinal health is a cornerstone of animal welfare, as a compromised gut barrier leads to inflammation, disease, and reduced productivity (4). The gut microbiota plays a critical role in maintaining this barrier by regulating tight junction proteins, inhibiting pathogens, and modulating immune responses (5). According to Liu Y. et al. weaned piglets fed a high-protein diet are at an increased risk of diarrhea; however, supplementation with Bacillus subtilis PB6 alleviated this Research Topic. This specific diet decreased diarrhea scores, enhanced growth performance, and modulated the gut microbiota, while also reducing the expression of inflammation-related genes. For broilers, Li X. et al. reported that in broiler chickens, a combination of *Glycyrrhiza uralensis extract* (GUE) and lactobacilli significantly increased the antioxidant enzyme levels in the intestinal mucosa, thereby reducing oxidative stress. Liu Z. et al. found that fermented *Astragalus* polysaccharides (FAP) also strengthened the intestinal barrier by upregulating tight junction proteins, which prevent pathogen entry. Wu et al. discovered that rearing systems influenced the ileal microbiota of rabbits. Forest-raised rabbits exhibit microbiota associated with reduced inflammation, whereas caged rabbits show alterations in metabolic pathways. These outcomes highlight the role of the microbiota as a "first line of defense" against intestinal dysfunction.

Enhancing animal welfare: beyond productivity

Animal welfare encompasses more than just growth—it also includes freedom from disease, stress, and discomfort. The gut microbiota influences welfare through its impact on immune function, stress response, and even behavior (6). Ma et al. reported that in neonatal foals, angular limb deformities (ALDs) are linked to gut microbiota imbalances. Diseased foals and their mothers show altered microbial communities, which affect metabolism and immunity, highlighting the role of the microbiota in overall health. In broiler chickens, studies by Yan et al. and Li J. et al. demonstrated that dietary supplementation with adjuncts such as fructooligosaccharides (FOS) and vitamin D3 enhances meat quality and mitigates physiological stress. FOS-supplemented broilers were found to have better muscle pH and tenderness, while vitamin D3 modulated the cecal microbiota to enhance immune competence. Furthermore, Al-abdullatif et al. demonstrated that the administration of probiotics, including multi-strain formulations such as multi-strain probiotics (RISCO-NUTRIFOUR®, RNF), exerts beneficial effects on broiler welfare. Specifically, these probiotics enhance meat quality by decreasing cooking losses and increasing tenderness. Different concentrations of RNF have varying effects on the quality characteristics of the meat, such as texture, juiciness, and sensory acceptability, all of which contribute to the overall welfare perception in broilers, both during rearing and in the final product. These studies confirm that a healthy microbiome is integral to reducing suffering and improving quality of life.

Harnessing microbial power: practical strategies

This Research Topic underscores actionable strategies to manipulate the gut microbiota to achieve better outcomes. Dietary additives (7)—including prebiotics (FOS), probiotics (Lactobacillus), plant extracts (GUE), and fermented polysaccharides (FAP)—consistently emerge as effective tools. For piglets, fermented wheat bran (FWB) and Bacillus subtilis PB6 are viable options. For poultry, a range of additives works: FAP for growth and antioxidant capacity, GUE-Lactobacillus combinations for immunity, and FOS for meat quality. Even rearing systems, such as forest-rearing for rabbits, can be leveraged. These strategies align with the push for antibiotic-free farming, as a balanced microbiota naturally suppresses pathogens.

Conclusion

The gut microbiota plays a crucial role in the health and welfare of non-ruminant livestock, with far-reaching impacts on nutrient utilization, intestinal integrity, and overall wellbeing. This Research Topic demonstrates that targeted microbial modulation—through dietary additives and improved rearing systems—can unlock significant benefits, from enhanced

productivity to reduced suffering. As the industry evolves, these insights will be key to raising healthier, more productive animals while meeting consumer demands for ethical and high-quality livestock products. Future research should focus on unraveling species-specific microbial interactions and refining interventions to maximize these gains for producers, animals, and consumers alike.

Author contributions

SL: Conceptualization, Formal analysis, Investigation, Methodology, Project administration, Funding acquisition, Supervision, Writing – original draft, Writing – review & editing. YTC: Formal analysis, Investigation, Methodology, Resources, Supervision, Writing – review & editing. JHL: Investigation, Methodology, Software, Supervision, Validation, Writing – review & editing. MYY: Formal analysis, Investigation, Methodology, Software, Supervision, Writing – review & editing.

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Effect of supplementation with *Glycyrrhiza uralensis* extract and *Lactobacillus acidophilus* on growth performance and intestinal health in broiler chickens

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Intestinal microbiota community is an important factor affecting the nutritional and health status of poultry, and its balance is crucial for improving the overall health of poultry. The study aimed to investigate the effect of dietary supplementation with *Glycyrrhiza uralensis* extract (GUE), *Lactobacillus acidophilus* (Lac) and their combination (GL) on growth performance and intestinal health in broilers in an 84-day feeding experiment. Supplementary 0.1% GUE and 4.5×10^7 CFU/g Lac significantly increased average daily gain (ADG), and GL (0.1% GUE and 4.5×10^7 CFU/g Lac) increased ADG and average daily feed intake (ADFI), and decreased feed conversion rate (FCR) in broilers aged 29 to 84 d and 1 to 84 d. Dietary GUE, Lac and GL increased the superoxide dismutase (SOD) and glutathione peroxidase (GSH-PX) activity and decreased Malondialdehyde (MDA) content in the jejunum mucosa of broilers, and increased secretory IgA (sIgA) content in broilers at 84 d. Moreover, GUE, Lac and GL increased cecal microbial richness and diversity, and modulated microbial community composition. Both GUE and Lac reduced the harmful bacteria *Epsilonbacteraeota*, *Helicobacter*, and *H. pullorum* at 28 d and *Proteobacteria*, *Escherichia*, and *E. coli* at 84 d, while Lac and GL increased beneficial bacteria *Lactobacillus* and *L. gallinarum* at 28 d. Compared with individual supplementation, GL markedly increased the SOD activity and the sIgA content, and reduced *Helicobacter* and *Helicobacter pullorum*. In conclusion, GUE and *Lactobacillus acidophilus* as feed additives benefit growth performance and intestinal health, and their combined use shows an even more positive effect in broilers.

KEYWORDS

***Glycyrrhiza uralensis* extract, *Lactobacillus acidophilus*, growth performance, intestine health, broiler chickens**

1 Introduction

With the continuous improvement of the scale and intensity of poultry farming, the production performance of poultry has been gradually improved. However, broilers are susceptible to external factors such as disease, nutrition, and environment due to the imperfect development of intestinal function and low immunity, which frequently result in poor health, enhanced stress response, and intestinal imbalance (1). In the era of a complete ban on antibiotics as feed additives and a focus on healthy breeding, the development and utilization of feed additives like medicinal plant extracts and probiotics have garnered much attention (2, 3).

Glycyrrhiza uralensis Fisch is a traditional medicinal and edible plant with a long history of dietary and pharmacological applications, and its edible and medicinal parts are the root and rhizome (4, 5). The primary active ingredients in *Glycyrrhiza uralensis* extract (GUE) include glycyrrhiza polysaccharides, triterpene saponins (glycyrrhizic acid, glycyrrhizinic acid, etc.), and flavonoids (chalcone, isoflavone, etc.) (6). GUE exhibits various pharmacological effects, such as anti-inflammatory (7), antioxidant (8), antiviral (9), immune regulation (10) and improvement intestinal microbiota (11). *Lactobacillus acidophilus* is a dominant microbiota in the gastrointestinal tract (GIT) of humans and animals, often used as a probiotics due to its health-promoting properties (12). Probiotics *Lactobacillus* is typically employed as Direct-Fed microbiota to poultry and other livestock to enhance intestinal health (13), enhancing immunity (14, 15) and reducing colonization of pathogens in the GIT (16).

The intestinal tract is a crucial organ for digestion and absorption of feed nutrients in animals, closely linked to a range of physiological and biochemical processes, contributing to the animal's nutrition and overall health (17). The intestinal microbiota is critical for maintaining host intestinal health, acting as the organism's "second brain," preventing pathogen colonization, modulating the intestinal epithelial barrier and inflammatory response (18, 19). The colonization of gut microbiota is relatively stable, and their composition and structure are influenced by factors such as diet, age, and feeding methods in animals (20). Changes in the GIT microbial community impact feed efficiency, productivity, and the health of chickens (21). The cecum, with the highest microbial colonization and the most abundant and diverse microbial community in the broiler gut, is crucial for the overall performance in poultry (22, 23). Generally, the chyme stays in the cecum for an extended period, allowing thorough decomposition of many feed ingredients by microbiota to improve nutrient utilization (24). Therefore, the balance of cecum microbiota is essential for enhancing overall performance of poultry.

Several recent studies have confirmed that GUE (25, 26) and probiotics (27, 28) promote growth and improve intestinal health in broilers. However, the potential effects of the combination of GUE and *Lactobacillus* on growth performance and intestinal health are not yet fully understood. Thus, the purpose of the present study was to investigate the impact of GUE, *Lactobacillus acidophilus* and their combination on growth performance and intestinal health in *Liangfenghua* broiler chickens, a medium-growing broiler strain known for its popularity due to the excellent meat quality. The findings of this study would be beneficial in comprehending the intestinal microecology of chickens and offering novel perspectives on the upkeep of intestinal wellness in broiler chickens that are fed antibiotic-free diets.

2 Materials and methods

2.1 Ethics statement

All procedures involving in animals were performed following the Regulations for the Administration of Affairs Concerning Experimental Animals (Ministry of Science and Technology, China, 2004) and were approved and supervised by the Northwest Minzu University Animal Care and Use Committee (Permit No. xbmu-sm-20210130).

2.2 Experimental design and animal management

The *Glycyrrhiza uralensis* extract (GUE; prepared from the root of GU using decoction extraction and ethanol precipitation methods; Yalan Pharmaceutical Co, Gansu, China) and the *Lactobacillus acidophilus* (Lac; Zhongxin Bio-Technology Co., Hebei, China; 3×10^9 CFU/g) used in the present study were commercial products.

A total of 420 healthy one-day-old male *Liangfenghua* broiler chickens were randomly allocated into 4 dietary treatments, each with 7 replicates and 15 chickens per replicate. The treatment groups included: (1) basal diet (Con group); (2) basal diet supplemented with 0.1% GUE (GUE group); (3) basal diet supplemented with Lac at 4.5×10^7 CFU/g (Lac group); and (4) basal diet supplemented with 0.1% GUE and 4.5×10^7 CFU/g Lac (GL group). Table 1 presented the composition and nutritional levels of the basal diet, formulated in accordance with the "Broilers Feeding Standard in China" (NY/T 33-2004). The trial was conducted at Gansu Agricultural Vocational Farm Co. in Gansu, China.

Before the experiment commenced, the chicken coops and internal equipment were cleaned and fumigated for disinfection. Chicks aged 1–28 days were reared in three-layer ladder cages ($1.2 \times 0.9 \times 1.0$ m, length × width × height), with the coop preheated before the chicks entering. During the first week, the coop temperature was maintained at 34°C with a relative humidity (RH) of 50%. Subsequently, the temperature was reduced by 2°C per week until reaching 26°C, with RH at 45%. From 29 to 84 days old, the broilers were raised on the ground, ensuring the coop remained dry, hygienic, and well-ventilated. All the chickens were kept in a single room comprising six floor pens, each measuring 300×350 cm. Each pen had solid white plastic walls and was divided by wire mesh into 10 compartments. These compartments were equipped with a round feeder pan (diameter = 30 cm) and one nipple drinker. Cork shavings were used as litter, and the litter was replaced every 3 days. The chickens were exposed to 12-h light/dark cycles daily throughout the test period and had *ad libitum* access to feed and water. The broilers were vaccinated with Newcastle disease vaccine and the infectious bursal polyvalent vaccine on d 7 and 14 of the experiment, respectively.

2.3 Growth performance determination

All broilers were weighed at 1, 28, and 84 days of age after a 12-h fast. The feed intake per replicate was recorded daily to calculate the

TABLE 1 Composition and nutrient levels of basal diets (air-dry basis).

Items	Content	
	1 to 28 d of age	29 to 84 d of age
Ingredients (%)		
Corn	55.00	57.50
Soybean oil	2.90	4.20
Soybean meal	29.00	24.00
Cottonseed meal	1.40	1.70
Rapeseed meal	1.90	3.00
Corn gluten meal	6.80	6.60
$CaHPO_4$	1.80	2.50
NaCl	0.78	0.08
L-Lys•HCL	0.15	0.11
DL-Met	0.00	0.08
Cys-Cys	0.07	0.03
Premix[a]	0.20	0.20
Total	100.00	100.00
Nutrient levels[b]		
ME/(MJ/kg)	12.49	12.86
CP (%)	21.46	19.91
Ca (%)	0.90	0.86
TP (%)	0.68	0.65
Lys (%)	1.15	1.00
Met (%)	0.70	0.40

[a]The premix provided the following per kg of diets: 1 to 28 d of age, VA 12,000 IU, VD_3 3,500 IU, VE 60 IU, VK_3 4 mg, VB_1 2.5 mg, VB_2 10 mg, VB_6 6 mg, VB_{12} 8 μg, D-pantothenic acid 40 mg, nicotinic acid 75 mg, folic acid 10 mg, biotin 0.8 mg, choline 700 mg, Zn 90 mg, Fe 110 mg, Cu 20 mg, Mn 100 mg, I 0.5 mg, Se 0.3 mg; 29 to 84 d of age, VA 10,000 IU, VD_3 3,000 IU, VE 50 IU, VK_3 3.5 mg, VB_1 2 mg, VB_2 10 mg, VB_6 5 mg, VB_{12} 6 μg, D-pantothenic acid 20 mg, nicotinic acid 60 mg, folic acid 8 mg, biotin 0.6 mg, choline 600 mg, Zn 80 mg, Fe 100 mg, Cu 15 mg, Mn 80 mg, I 0.5 mg, Se 0.3 mg.
[b]Nutrient levels were all calculated values.

average daily gain (ADG), average daily feed intake (ADFI) and feed conversion rate (FCR, feed/gain). Chicken mortality was recorded after which performance parameters were corrected for mortality.

2.4 Sample collection

All birds were fasted for 12 h (overnight) prior to collecting test samples. On d 28 and 84, two broilers with similar body weight per replicate from each group were selected and euthanized by severing the jugular vein. The abdomen was disinfected with 75% ethanol, then immediately dissected. The entire intestine was carefully removed from the abdominal cavity, and the jejunum and cecum were separated with a sterile scalpel. The middle part of jejunum was cut longitudinally, washed with 4% phosphate buffer solution (PBS), and jejunal mucosa was scraped with sterilized slides, put into RNAase-free tubes, snap-frozen in liquid nitrogen, and stored at −80°C for the determination of antioxidant indexes and secretory IgA (sIgA) content. The cecum contents were carefully collected, homogenized with a sterile spatula, transferred to CryoPure Tubes (Sarstedt AG + Co., Nümbrecht, Germany), snap-frozen in liquid nitrogen and stored at −80°C until they were processed for microbial DNA analysis.

2.5 Intestinal antioxidant and sIgA analysis

The activity of superoxide dismutase (SOD), glutathione peroxidase (GSH-PX), and the content of Malondialdehyde (MDA) in the jejunum mucosa were measured by assay kit (Shanghai Gantu Biotechnology Co., China). The level of intestinal sIgA was determined by double antibody one-step sandwich enzyme-linked immunosorbent assay (ELISA; Shanghai Liquid Quality Testing Technology Co., China). All detection methods were performed according to the manufacturer's instructions.

2.6 Intestinal microbial diversity analysis

Bacterial genomic DNA was isolated from the cecal contents using the TGuide S96 kit (DP812; Tiangen Biotech Co., China) following the manufacturer's instructions. The purity and quality of the DNA were verified in 0.8% agarose gels. Subsequently, the full-length 16S rRNA gene was amplified using the primers (27F, AGRGTTTGA TYNTGGCTCAG and 1492 R, TASGGHTACCTTGTTASGACTT). The purified PCR products were used to construct the single-read sequencing library on the PacBio platform (Biomarker-Technologies Co., China), following the manufacturer's specifications. SMRT-Link v8.0 was used to correct the original subreads to obtain Circular Consensus Sequencing (CCS) sequence. The lima v1.7.0 software was used to identify the CCS sequence of different samples through the barcode sequence and remove the chimera (UCHIME v4.2), and obtain effective-CCS sequences. The generated datasets were analyzed using USEARCH v10.0. High-quality sequences were clustered as operational taxonomic units (OTUs) based on 97% similarity. BMK Cloud[1] was used for Alpha diversity, Beta diversity, and microbial composition analysis to investigate differences in samples among groups.

2.7 Statistics analysis

All statistical analyses were conducted using SPSS software (version 26.0; IBM Corp., Armonk, NY, United States), with the results reported as mean ± Standard Error of Means (SEM). A one-way ANOVA test for multiple comparisons, followed by Dunnett's *post-hoc* test, was employed to assess statistical significance between groups. Histograms were plotted using GraphPad Prism 8.0 (GraphPad, Inc. La Jolla, CA, United States). A probability value of $p < 0.05$ or $p < 0.01$ was considered statistically significant. A trend in significance was acknowledged for $0.05 < p < 0.10$.

Alpha diversity indices (ACE and Shannon indices) of the samples were evaluated using QIIME2 2020 software, and the significance of differences was confirmed using the Wilcoxon rank sum test. After the alpha diversity analysis, Partialleast squares discriminant analysis (PLS-DA)was conducted with QIIME software based on OTU level.

1 http://www.biocloud.net/

TABLE 2 Effect of dietary GUE, Lac and their combination on the growth performance in broilers.

Items	Con group	GUE group	Lac group	GL group	SEM	*p*-value
Initial body weight, g	40.19	40.48	41.21	39.19	0.85	0.416
d 1–28						
BW, g	550.38	564.46	567.46	579.92	10.58	0.218
ADG, g/d	18.23	18.72	18.80	19.32	0.38	0.262
ADFI, g/d	48.30	48.10	48.06	48.23	1.51	0.999
F/G	2.65	2.58	2.57	2.51	0.10	0.829
d 29–84						
BW, g	3398.34^{c}	3553.77^{b}	3539.37^{b}	3780.79^{a}	27.21	0.019
ADG, g/d	51.10^{c}	53.38^{b}	53.12^{b}	57.13^{a}	0.188	0.042
ADFI, g/d	171.74^{b}	177.07^{ab}	172.97^{b}	181.80^{a}	2.48	0.035
F/G	3.36^{a}	3.32^{ab}	3.26^{ab}	3.18^{b}	0.05	0.041
d 1–84						
ADG, g/d	33.98^{c}	41.81^{b}	41.64^{b}	44.54^{a}	0.68	0.047
ADFI, g/d	130.60^{b}	134.08^{ab}	131.33^{b}	137.27^{a}	2.21	0.017
F/G	3.27^{a}	3.21^{ab}	3.15^{bc}	3.08^{c}	0.09	0.032

GUE, *Glycyrrhiza uralensis* extract; Lac, *Lactobacillus acidophilus*; GL, GUE and Lac; SEM, standard error of means. BW, body weight; ADG, average daily gain; ADFI, average daily feed intake; F/G, feed/gain. Values with the same or no letter superscripts in the same row mean no significant difference ($p > 0.05$), while with different letter superscripts mean significant difference ($p < 0.05$).

TABLE 3 Effect of dietary GUE, Lac and their combination on intestinal antioxidant indexes and sIgA content in broilers.

Items	Con group	GUE group	Lac group	GL group	SEM	*p*-value
28 d of age						
SOD, U/g	130.94^{c}	150.32^{b}	149.03^{b}	178.43^{a}	4.80	0.001
GSH-Px, nmol/min/g	157.19^{c}	188.07^{a}	178.49^{b}	195.59^{a}	5.89	0.009
MDA, nmol/g	13.13^{a}	8.54^{b}	9.60^{b}	8.68^{b}	1.01	0.040
sIgA, μg/g	85.56	87.43	85.47	85.27	3.34	0.963
84 d of age						
SOD, U/g	237.62^{c}	256.92^{b}	285.59^{a}	298.76^{a}	7.34	0.007
GSH-Px, nmol/min/g	344.04^{b}	381.63^{a}	357.76^{b}	393.75^{a}	6.03	0.002
MDA, nmol/g	15.74^{a}	12.31^{b}	13.60^{b}	11.71^{b}	0.88	0.048
sIgA, μg/g	78.65^{c}	96.44^{b}	94.64^{b}	107.37^{a}	1.84	0.001

SOD, superoxide dismutase; GSH-Px, glutathione peroxidase enzyme; MDA, malondialdehyde. Values with the same or no letter superscripts in the same row mean no significant difference ($p > 0.05$), while with different letter superscripts mean significant difference ($p < 0.05$).

3 Results

3.1 Growth performance

Throughout the entire experiment, the broiler chickens maintained good health. The effects of supplements on the growth performance of broilers were presented in Table 2. There were no significant differences in body weight (BW) of broilers at 1 and 28 d of age among groups ($p > 0.05$). Likewise, no significant differences were observed in ADG, ADFI, and F/G among groups from 1 to 28 d of age ($p > 0.05$). At 84 d of age, the BW in the GUE, Lac and GL groups significantly increased ($p < 0.05$) compared to the Con group, with the GL group showing the greatest improvement ($p < 0.05$). From day 29 to 84 and from day 1 to 84, the ADG of broilers in the GUE and Lac groups significantly increased ($p < 0.05$) compared to that in the Con group, while there was no significant difference in ADFI ($p > 0.05$). However, the GL group had a significant increase ($p < 0.05$) in both ADG and ADFI, along with a marked decrease ($p < 0.05$) in F/G compared to the Con group. Additionally, the F/G from day 1 to 84 in the Lac group was significantly lower ($p < 0.05$) than in the Con group.

3.2 Intestinal antioxidant and sIgA

The effects of GUE, Lac and their combination on intestinal antioxidant capacity and sIgA content of broilers were presented in Table 3. In comparison to the Con group, the GUE, Lac and GL groups exhibited a significant increased SOD activity and a significant decreased MDA content ($p < 0.05$) in the jejunum mucosa of broilers at 28 and 84

d of age; the GSH-Px activity in broilers at 28 and 84 d of age in GUE and GL groups, and in broilers at 28 d of age in Lac group, significantly increased ($p < 0.01$). Additionally, a significantly higher SOD activity in broilers at 28 d of age was observed in the GL group compared to the GUE and Lac groups ($p < 0.01$). There was no significant difference in the sIgA content in the jejunum mucosa of broilers at 28 d of age among the groups ($p > 0.05$). However, the GUE, Lac and GL groups exhibited a significant increase of sIgA content at 84 d of age compared to the Con group ($p < 0.01$). Additionally, the sIgA content in the GL group was significantly higher than in the GUE group or Lac group ($p < 0.05$).

3.3 Variation in cecal microbiota diversity

3.3.1 Variation in alpha diversity

In the microbiome analysis, a total of 256,857 original CCS sequences were obtained through full-length 16S rRNA gene amplification on 40 cecal content samples using the PacBio platform. On average, 6,517 CCS sequences were generated in broilers at 28 d of age, with at least 4,400 CCS sequences for each sample. Similarly, an average of 6,326 CCS sequences were obtained in broilers at 84 d of age, with at least 4,074 CCS sequences for each sample. Following size filtering, quality control and chimera removal, a total of 199,146 high-quality sequences were retained, including 104,911 sequences at 28 d of age and 94,235 at 84 d of age.

At a 97% sequence similarity threshold, 322 OTUs were identified in samples from 28-d-old broilers in Con, GUE, Lac, and GL groups, with 5, 7, 13, and 17 unique OTUs, respectively (Figure 1A). At 84 d of age, 349 OTUs were identified in the four groups, with 13, 5, 5 and 10 unique OTUs, respectively (Figure 1B). The Shannon curves and the Rank abundance curve of cecal samples indicated that the sample size was reasonable, and the sequencing depth was sufficient for all samples based on a saturated trend (Figures 1C–F).

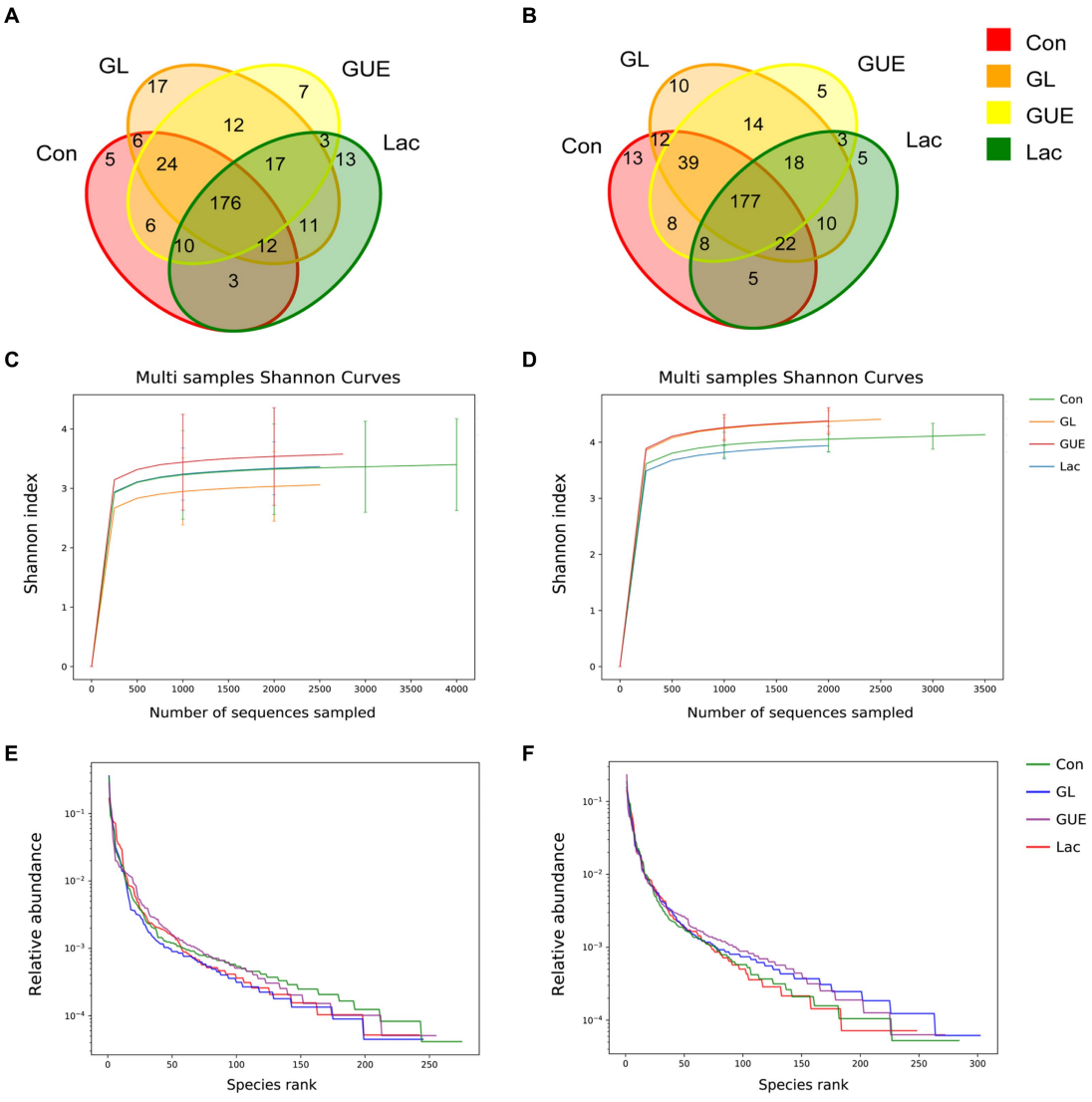

FIGURE 1
The Venn diagram, Shannon curves and Rank abundance curve of OTUs. **(A,B)** The Venn diagrams at 28 and 84 d of age. **(C,D)** The Shannon curves at 28 and 84 d of age. **(E,F)** The Rank abundance curve at 28 and 84 d of age.

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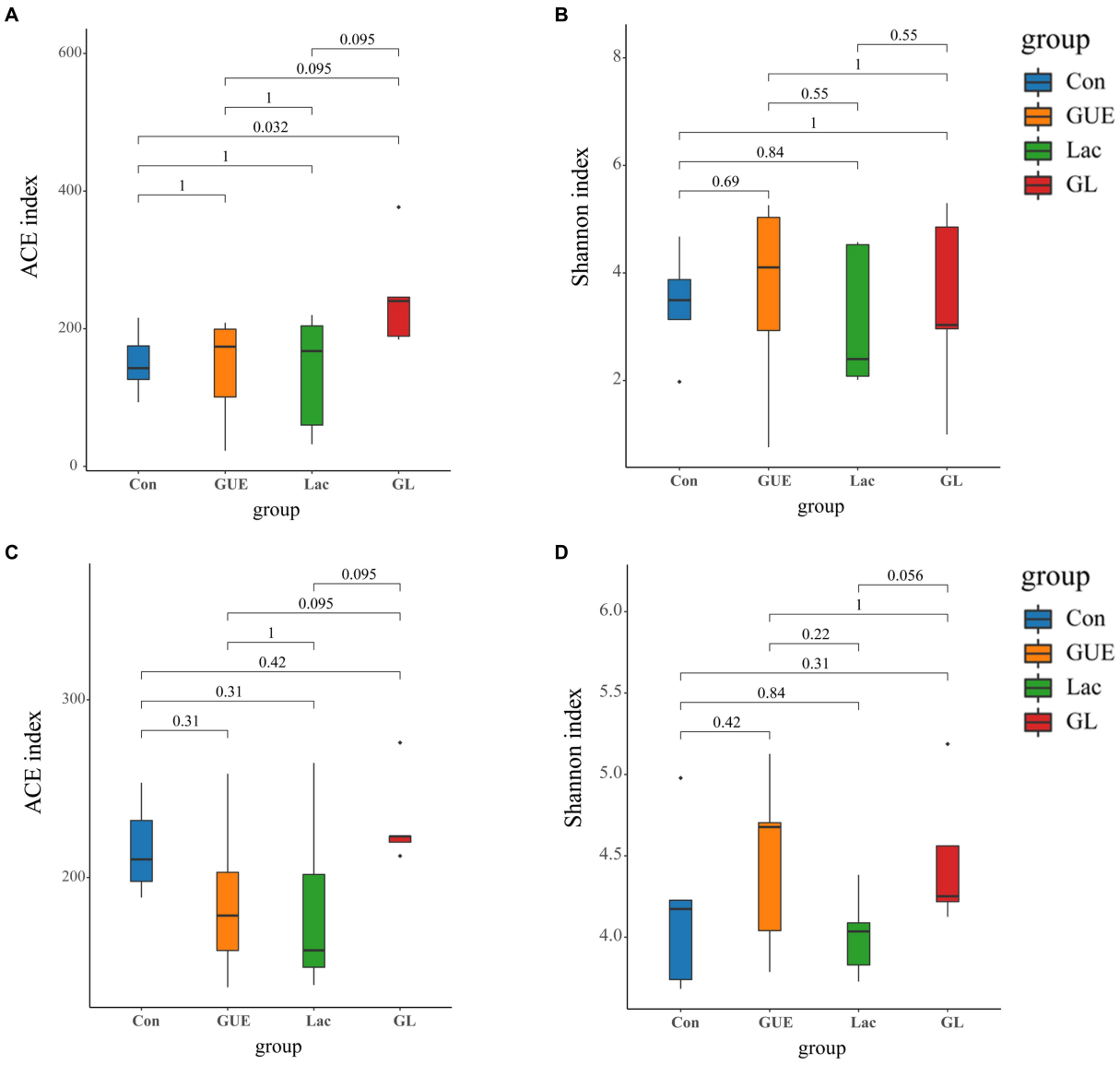

FIGURE 2
Alpha diversity of the microbiome residing in the cecal contents of broilers. **(A,B)** ACE and Shannon index in 28-d-old broilers. **(C,D)** ACE and Shannon index in 84-d-old broilers.

The alpha diversity index serves as an indicator of the species richness and diversity within individual samples (29). To assess the alpha diversity of the samples, the ACE and Shannon indices were determined. At 28 d of age, there was no significant difference in the Shannon index among the groups ($p > 0.05$; Figure 2B). However, the ACE index in the GL group was significantly higher than in the Con group ($p < 0.05$; Figure 2A). In comparison with the GUE or Lac groups, there was a tendency to increase in the ACE index in the GL group ($p = 0.095$; Figure 2A). At 84 d of age, the ACE index ($p = 0.095$) and Shannon index ($p = 0.056$) showed a tendency to increase in the GL group compared to the GUE group or Lac group (Figures 2C,D).

3.3.2 Variation in beta diversity

Beta diversity, a measure of variance in taxa composition between sampling sites (30), was visualized by plotting the distances between samples on Partial Least Squares Discriminant Analysis (PLS-DA) biplot. At 28 d of age, there was no significant separation observed between different treatment groups, and the distribution of samples within each group was discrete (Figure 3A). At 84 d of age, the intestinal microbial community exhibited a distinct separation among the Con, GUE, Lac, and GL groups, with samples clustering within each group (Figure 3B).

3.4 Variation in cecal microbiota composition

3.4.1 Phylum level

The relative abundance of microbial composition at the phylum level was depicted in Figure 4. At 28 d of age, cecal samples in the Con, GUE, Lac, and GL groups were primarily dominated by five bacterial phyla: *Firmicutes* (59.24, 63.21, 78.93, and 86.13%,

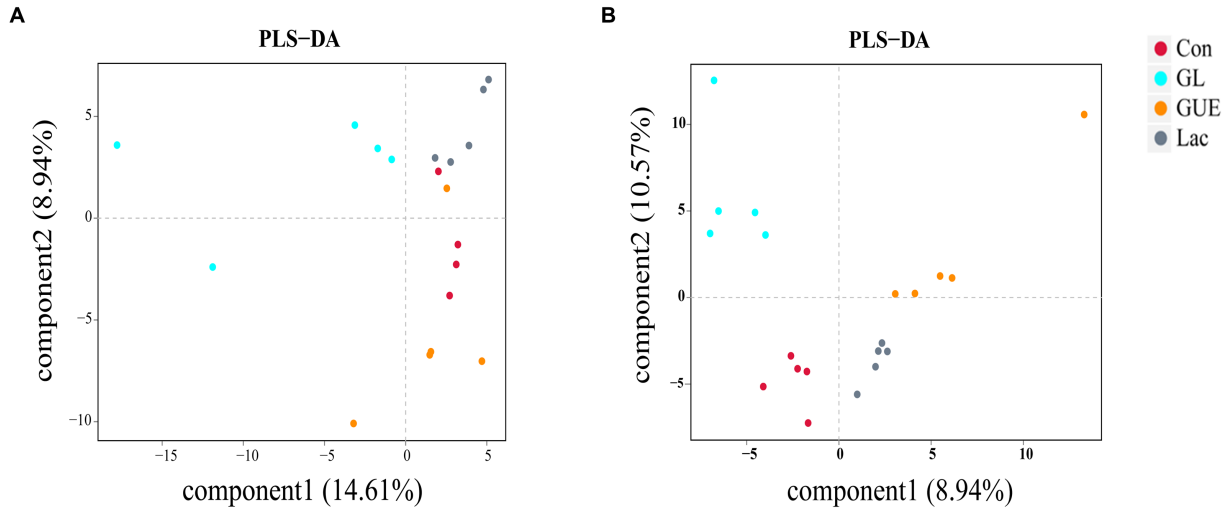

FIGURE 3
Beta diversity of the microbiome residing in the cecal contents of broilers. **(A,B)** Partial Least Squares Discriminant Analysis (PLS-DA) biplot at 28- and 84-d-old broilers.

respectively), *Bacteroidetes* (28.16, 31.37, 16.27, and 8.52%, respectively), *Epsilonbacteraeota* (10.97, 1.30, 0.98, and 0.46%, respectively), *Tenericutes* (0.95, 1.98, 3.29, and 1.22%, respectively) and *Proteobacteria* (0.61, 2.00, 0.13, and 2.93%, respectively) (Figure 4A). Compared to the Con group, the relative abundance of *Tenericutes* and *Proteobacteria* significantly increased, while that of *Epsilonbacteraeota* significantly decreased in the GUE group. Similarly, the relative abundance of *Firmicutes* and *Tenericutes* significantly increased, while that of *Bacteroidetes* and *Epsilonbacteraeota* significantly decreased in the Lac group. In the GL group, the relative abundance of *Firmicutes* and *Proteobacteria* significantly increased, and that of *Bacteroidetes* and *Epsilonbacteraeota* significantly decreased ($p < 0.05$) (Figure 4C). Moreover, the relative abundance of *Firmicutes* and *Proteobacteria* significantly increased, while that of *Bacteroidetes* and *Tenericutes* significantly decreased in the GL group compared to the GUE or Lac group ($p < 0.05$) (Figure 4C).

At 84 d of age, cecal samples in the Con, GUE, Lac, and GL groups were dominated by four bacterial phyla: *Bacteroidetes* (50.06, 56.07, 52.55, and 44.55%, respectively), *Firmicutes* (37.68, 33.59, 35.73, and 35.65%, respectively), *Proteobacteria* (10.63, 2.36, 3.48, and 12.90%, respectively) and *Tenericutes* (0.66, 2.65, 7.17, and 5.87%, respectively) (Figure 4B). Compared to the Con group, the relative abundance of *Bacteroidetes* and *Tenericutes* significantly increased, while that of *Firmicutes* and *Proteobacteria* significantly decreased in the GUE group. Similarly, the relative abundance of *Tenericutes* significantly increased, while that of *Proteobacteria* significantly decreased in the Lac group. In the GL group, the relative abundance of *Proteobacteria* and *Tenericutes* significantly increased, while that of *Bacteroidetes* significantly decreased ($p < 0.05$) (Figure 4D). Additionally, the relative abundance of *Proteobacteria* and *Tenericutes* significantly increased, and that of *Bacteroidetes* significantly decreased in the GL group compared with the GUE or Lac group ($p < 0.05$) (Figure 4D). Thus, at the bacterial phylum level, the dietary supplements had a significant impact on the cecal microbial composition of broilers.

3.4.2 Genus level

The relative abundance of the microbial composition at the genus level was showed in Figure 5. At 28 d of age, cecal samples in the Con, GUE, Lac and GL groups were primarily dominated by five bacterial genera: *Lactobacillus* (33.23, 32.67, 54.19, and 50.98%, respectively), *Barnesiella* (13.01, 16.47, 7.74, and 0.12%, respectively), *Alistipes* (5.56, 11.58, 7.03, and 1.88%, respectively), *Bacteroides* (9.49, 3.22, 0.88, and 6.51%, respectively) and *Helicobacter* (10.95, 1.30, 0.98, and 0.23%, respectively) (Figure 5A). Among them, the relative abundance of *Alistipes* in the GUE group and *Lactobacillus* in Lac and GL groups was higher than in the Con group ($p < 0.05$), while the relative abundance of *Helicobacter*, *Bacteroides* and *Barnesiella* was lower in the GUE, Lac and GL groups than in the Con group ($p < 0.05$) (Figure 5C). Additionally, the relative abundance of *Lactobacillus* and *Bacteroides* was higher, and that of *Barnesiella* and *Alistipes* was lower in the GL group than in the GUE or Lac group ($p < 0.05$) (Figure 5C). The relative abundance of other genera in the top 20 taxa varied among groups.

At 84 d of age, the most abundant taxa in the Con, GUE, Lac, and GL groups were *Bacteroides* (31.75, 12.93, 25.71, and 24.04%, respectively), *Barnesiella* (8.54, 23.15, 12.67, and 8.10%, respectively), *Alistipes* (6.15, 9.07, 11.40, and 9.24%, respectively), *Escherichia* (10.32, 1.82, 2.57, and 12.52%, respectively) and *Megamonas* (9.33, 1.12, 5.91, and 9.17%, respectively) (Figure 5B). These main bacterial genera showed significant differences among different treatment groups ($p < 0.05$) (Figure 5D). Additionally, the remaining bacterial genera also exhibited differences in relative abundances, such as *[Ruminococcus]_torques_group*, *Phascolarctobacterium* and *Lactobacillus*. In summary, the difference in supplements also affected the relative abundance of broiler cecal microbiota at the genus level.

3.4.3 Species level

The relative abundance of the microbial composition at the species level was presented in Figure 6. At 28 d of age, cecal samples in the Con, GUE, Lac, and GL groups was dominated by *Lactobacillus_gallinarum*, *Alistipes_sp.*, *Barnesiella_viscericola*, *Lactobacillus_salivarius*,

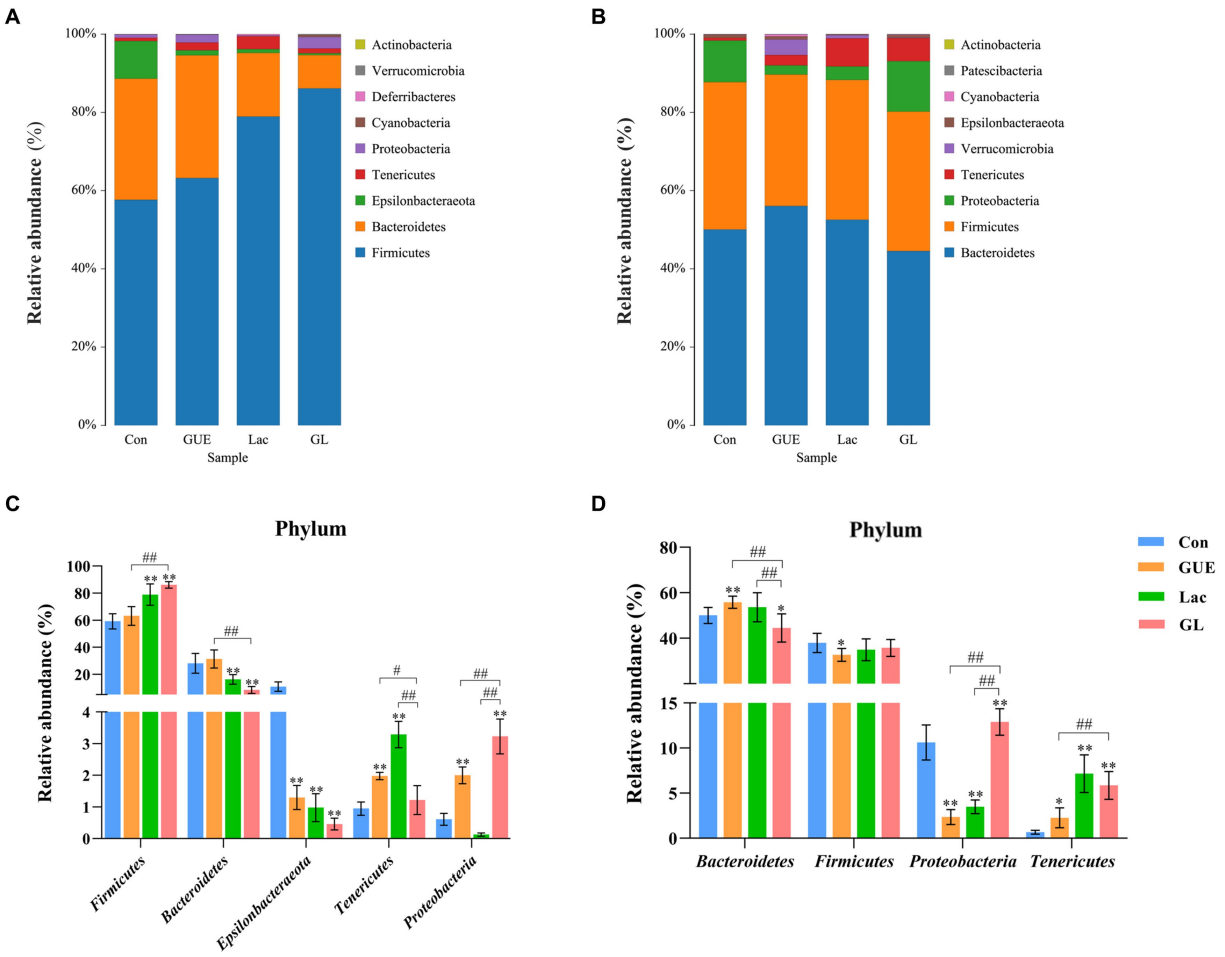

FIGURE 4
The relative abundance taxa of cecal microbiota in broiler chickens at the phylum level. **(A,B)** Relative abundance taxa at 28 and 84 d of age. **(C,D)** Relative abundance difference analysis of cecal bacterial species at the phylum level at 28 and 84 d of age, respectively. "*" indicated statistically significant difference from the Con group ($^{*}p < 0.05$ and $^{**}p < 0.01$). "#" indicated a statistical difference between the two groups ($^{\#}p < 0.05$ and $^{\#\#}p < 0.01$).

Bacteroides_fragilis, *Helicobacter_pullorum*, *Candidatus_Arthromitus_sp.*, *Barnesiella_intestinihominis*, *uncultured_bacterium_g_Romboutsia* and *Lactobacillus_reuteri* (Figure 6A). Notably, *Lactobacillus_gallinarum* was the most dominant bacterium, accounting for more than 19% of the total microbial community detected. Compared to the Con group, the GUE, Lac and GL groups all significantly increased the relative abundance of *Lactobacillus_gallinarum* and significantly decreased that of *Helicobacter_pullorum*. Additionally, the GL group exhibited a significant increase in the relative abundance of *Bacteroides_fragilis* and *Candidatus_Arthromitus_sp.*, and a decrease in *Alistipes_sp.*, *Barnesiella_viscericola* and *Barnesiella_intestinihominis* compared to the GUE or Lac groups (Supplementary Table S1).

At 84 d of age, the most abundant taxa in the Con, GUE, Lac, and GL groups were *Bacteroides_dorei*, *uncultured_bacterium_g_Barnesiella*, *Alistipes_sp.*, *Escherichia_coli*, *Megamonas_funiformis*, *uncultured_bacterium_g_[Ruminococcus]_torques_group*, *Barnesiella_viscericola*, *Bacteroides_vulgatus*, *uncultured_bacterium_f_Firmicutes_bacterium_CAG_822* and *Phascolarctobacterium_sp* (Figure 6B). Compared with the Con group, the relative abundance of *Alistipes_sp* was significantly higher, while that of *Bacteroides_dorei* and *Bacteroides_vulgatus* was significantly lower in the supplemented groups. Additionally, the GL group displayed a notable increase in the relative abundance of *Megamonas_funiformis* and *uncultured_bacterium_g_[Ruminococcus]_torques_group*, along with a decrease in *Phascolarctobacterium_sp* compared to the GUE or Lac groups (Supplementary Table S2). The relative abundance of other species in the top 20 taxa also exhibited variability across groups.

3.5 Differences between groups in microbial diversity

The Linear discriminant analysis (LDA) combined LDA effect size (LEfSe) method was used to further analyze the differential marker species in the cecal samples in groups. At 28 d of age, 4 significant biomarkers were enriched in the GL group, namely *f_Clostridiaceae_1*, *g_Candidatus_Arthromitus*, *s_Candidatus_Arthromitus_sp.*, and *f_Moraxellaceae* (Figures 7A,B). At 84 d of age, 23 biomarkers were significantly enriched in the GUE, Lac and GL groups. Specifically, there were 8 species in the GUE group, including

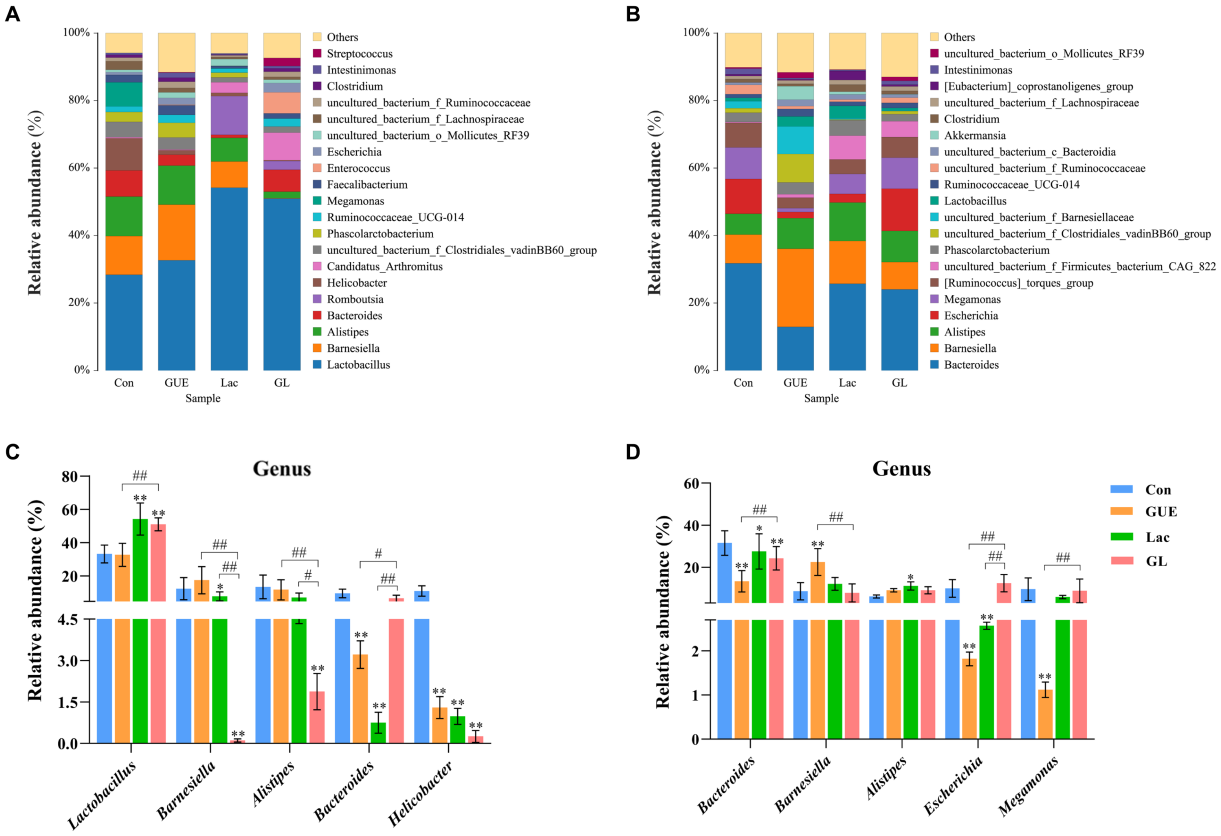

FIGURE 5
The relative abundance of cecal microbiota at the genus level. **(A,B)** Relative abundance taxa at 28 and 84 d of age. Relative abundances in the top 20 taxa were shown, and other taxon were combined as "Others." **(C,D)** Relative abundance difference analysis of cecal bacterial species at the genus level at 28 and 84 d of age, respectively. "*" indicated statistically significant difference from the Con group ($*p < 0.05$ and $**p < 0.01$). "#" indicated a statistical difference between the two groups ($^{\#}p < 0.05$ and $^{\#\#}p < 0.01$).

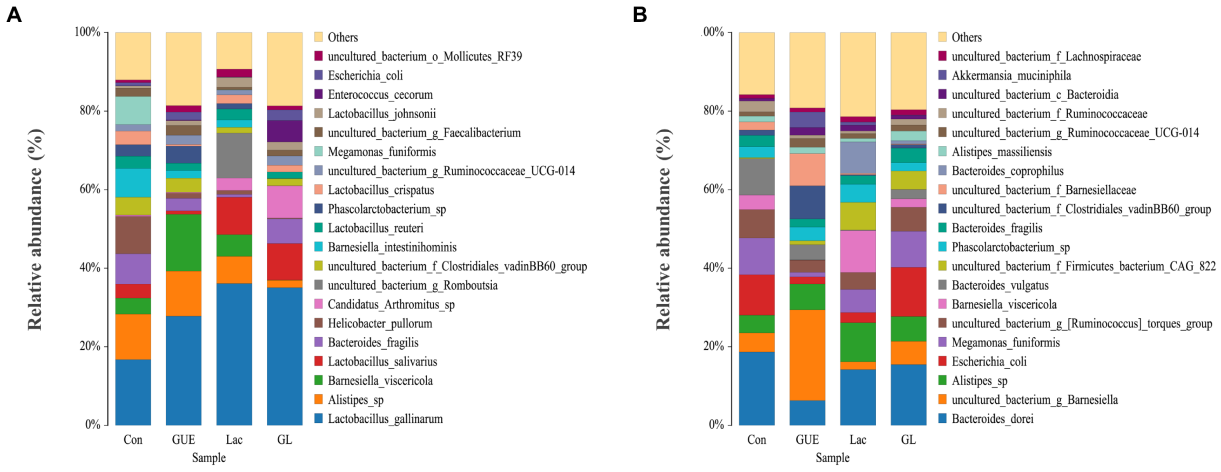

FIGURE 6
The relative abundance taxa of cecal microbiota in broiler chickens at the species level. **(A,B)** Relative abundance taxa at 28 and 84 d of age. Relative abundances in the top 20 taxa were shown, and other taxon were combined as "Others".

f_Barnesiellaceae, *s_Lactobacillus_oris*, *f_Clostridiales_vadinBB60_group*, 2 uncultured genera, and 3 unnamed species. The Lac group exhibited 5 species, including *s_Barnesiella_visceriricola*, *g_Eubacterium_coprostanoligenes_group*, *g_Eisenbergiella*, and 2 unnamed species. The GL group presented 10 species, including *s_Escherichia_coli*, *g_Escherichia*, *f_Enterobacteriaceae*,

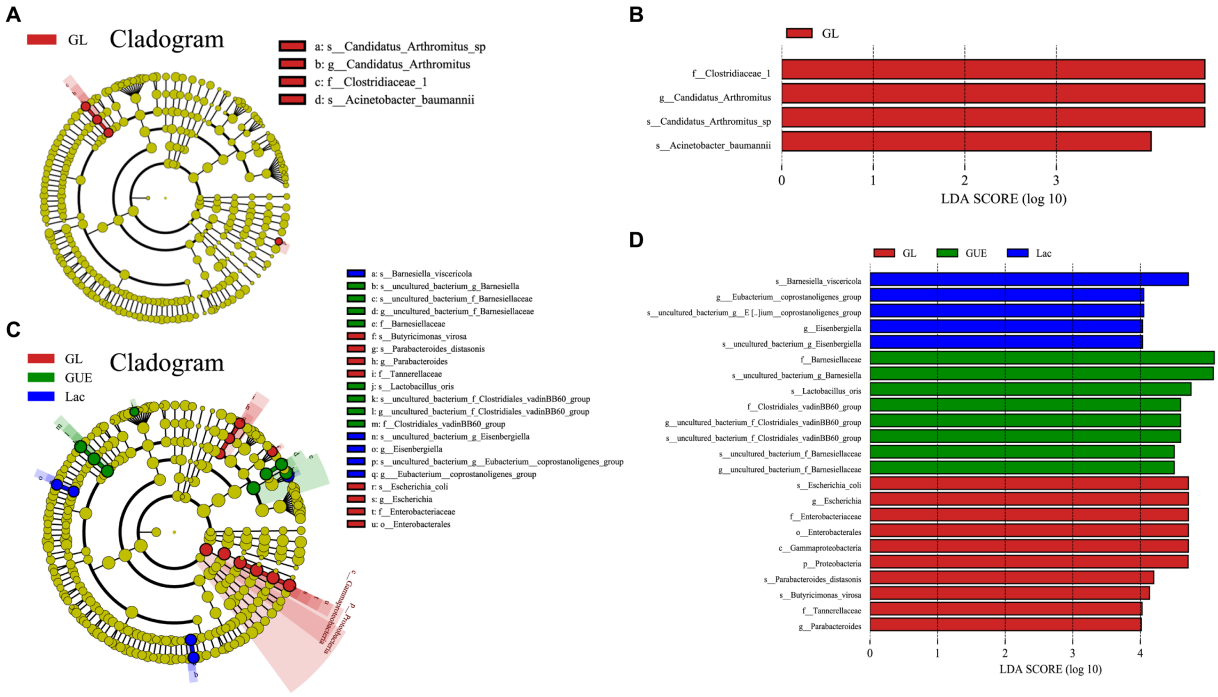

FIGURE 7
LEfSe taxonomic cladogram analysis and LDA score of cecal microbiota. **(A,B)** LEfSe taxonomic cladogram and LDA score at 28 d of age. **(C,D)** LEfSe taxonomic cladogram and LDA score at 84 d of age. The circles radiated from the center to the outer edges of the evolutionary branch map represent the classification level from the phylum to the species. The yellow nodes represent taxonomic units that show no significant differences between groups, and the size of the circle is positively correlated with relative abundance. Different colors indicate different groups. The length of the LDA histogram represents the LDA score, and the difference is significant when LDA > 4.0. p, phylum; c, class; o, order; f, family; g, genus; s, species.

o_Enterobacterales, *c_Gammaproteobacteria*, *p_Proteobacteria*, *s_Parabacteroides_distasonis*, *s_Butyricimonas_virosa*, *f_Tannerellaceae*, and *g_Parabacteroides* (Figures 7C,D).

4 Discussion

4.1 Growth performance

Probiotics, certain medicinal plants and their extracts are being viewed as promising alternatives to in-feed antibiotics due to their unique functions, including preventing intestinal diseases, enhancing overall health and performance of poultry. Numerous studies have reported that dietary supplementation with *Lactobacillus acidophilus* (31) and *Lactobacillus plantarum* (27) could enhanced the growth performance of broilers by improving intestinal health. Additionally, supplementation with GUE maintained intestinal barrier integrity and improved growth performance of broilers by up-regulating the expression of the intestinal barrier function factors junctional adhesion molecule (JAM) and mucin 2 (MUC-2) (26), and increased weight gain in the late growth and throughout the growth period of broilers reared at high-density (32).

In the present study, it was shown that supplementation with GUE, Lac, and their combination significantly boosted the BW of broilers at 84 d of age, and increased the ADG from d 29 to 84 and throughout the entire experiment, while no significant impact on chickens aged 1 to 28 d were observed. Additionally, the combination GUE and Lac had a higher ADG and a lower F/G in broilers aged 1 to 84 days compared to their individual supplementation. These findings suggested that both GUE and Lac enhanced the growth performance of broilers, and the combination of GUE and Lac had a noticeable synergistic effect.

4.2 Intestinal antioxidant and sIgA

As a channel for animals to communicate with the external environment, the intestine is susceptible to oxidative stress caused by various factors. Zhang et al. (33) found that dietary *Glycyrrhiza* polysaccharide significantly increased the expression of NF-E2-related factor 2 (Nrf-2), SOD1 and CAT in the jejunum mucosa, and improved the antioxidant capacity of piglets. Probiotics have the effect of resisting oxidative stress in the intestine, and dietary *Lactobacillus plantarum* could regulate the expression and production of antioxidant enzymes, thereby activating antioxidant defense system (34). Our results indicated that supplementation with GUE and Lac increased the activity of antioxidant enzyme SOD and GSH-Px and reduced MDA content in the jejunum mucosa of 28- and 84-d-old broilers. Furthermore, there was a synergistic effect of their combination. These results suggested that both GUE and Lac could enhance intestinal antioxidant capacity by regulating antioxidant enzyme activity.

SIgA is present in the intestinal mucosa, protects the intestine from pathogens, and regulates the intestinal microbiota throughout

animal development (35). The deficiency of sIgA could lead to incomplete intestinal barrier function, resulting in decreased productivity and even death of poultry (36). As the predominant Ig isotype in the intestinal mucosa, sIgA inhibited abnormal epithelial cell translocation and preventing excessive inflammatory responses induced by lipopolysaccharide of Shigella (37). The increase of sIgA secretion contributed to the improvement of intestinal mucosal health, which led to the enhanced nutrient absorption and improved growth performance in broilers (38).

The present study found that GUE increased sIgA levels in the jejunum mucosa, indicating a potential enhancement of intestinal health in broilers, consistent with findings from Wu et al. (39). Probiotics, defined as "living microorganisms," are known for their immunomodulatory and sIgA-inducing effects (40, 41). Probiotic supplementation enhanced the intestinal sIgA response, leading to changes in the overall intestinal microbiota structure and its interaction with sIgAs, thereby affecting host health (42). In this study, we observed that Lac supplementation notably raised sIgA levels in the jejunum mucosa of broilers, indicating its potential to enhance the intestinal health. Moreover, the combination of GUE and Lac exhibited a stronger effect in enhancing sIgA secretion compared to their individual supplementation.

4.3 Variation in cecal microbiota diversity

In this study, the full-length 16S rRNA gene of the cecal microbiota in broilers was sequenced on the Sequel II platform. Although previous study showed that supplementation of probiotic *Lactobacillus* increased intestinal microbial diversity under heat stress condition (43), our study found that dietary Lac or GUE had no significant effect on the α diversity based on the OTU level of 28- and 84-d-old broilers. Interestingly, the combination of GUE and Lac exhibited a significant synergistic effect on the ACE index.

Beta diversity analysis primarily describes variations in composition among microbiota (44). We observed that dietary GUE, Lac and their combined supplementation did not affect the structure of the cecal microbiota in broilers at 28 d of age, but had a distinct separation among groups at 84 d of age. Microbiota colonization in the cecum is a dynamic process influenced by various factors such as diet, disease defense, and host interactions. There were differences in microbiota composition at different growth stages of broiler (45). Moreover, microbial colonization in the intestine is affected by genetic background of the host, too. *Liangfenghua* broiler, a medium-speed growth broiler (46), might require an extended period for the colonization and accumulation of microbiota in the intestine.

In addition, LEfSe analysis indicated that only GL supplementation enriched 4 differential bacteria markers at 28 d of age, which were mainly included in *Firmicutes* and *Proteobacteria*. At 84 d of age, GUE enriched 8 specific bacteria, primarily within *Bacteroidetes* and *Firmicutes*, Lac enriched 5 differential bacteria, mainly involved in *Bacteroidetes* and *Proteobacteria*, and GL enriched 10 differential bacteria, primarily within *Proteobacteria*, *Firmicutes* and *Bacteroidetes*. From the above, each supplement had its unique microbial populations, suggesting that supplementation with GUE and Lac increased the abundance of cecal specific microbiota, with a synergistic effect from their combination. Furthermore, these also reflected that the diversity of intestinal microbiota in broilers increased with age until it stabilized, consistent with the findings of beta diversity.

4.4 Variation in cecal microbiota composition

The species annotation results were analyzed to understand the growth-promoting mechanisms of GUE, Lac, and their combination through intestinal microbiota. In this study, *Firmicutes* and *Bacteroidetes* were the most predominant, followed by *Proteobacteriau* and *Tenericutes*, with only a few of bacterial sequences in other phyla, which was consistent with Liu et al.'s research on broiler (47). Similar results were found in the study on the intestinal microbiota composition of Muscovy ducks (48), indicating that the intestinal microbiota composition of poultry is relatively stable. This study also observed that *Firmicutes* and *Bacteroidetes* were the two most dominant phyla in the cecal microbiota of 28-d-old broilers, while *Bacteroidetes* and *Firmicutes* predominated in that of 84-d-old broilers. These suggests that as broilers grow, their intestinal microbiota becomes more diverse, eventually establishing a complex and dynamic microbiome (49, 50). Furthermore, *Firmicutes* and *Bacteroidetes* collectively impacted the host's energy absorption and storage, and the *Firmicutes* to *Bacteroidetes* (F / B) ratio in the GIT influenced the host's ability to obtain energy from feed (51). A higher F / B ratio was often linked to enhanced growth performance (52). In this research, dietary Lac and the combination of GUE and Lac increased the F / B ratio in 28-d-old broilers, suggesting that these supplements could improve the weight gain by influencing cecal microbiota composition in broilers. Qiao et al. (53) found that adding compound polysaccharides derived from *Astragalus* and *Glycyrrhiza* to the diet improved broiler weight gain by raising the intestinal F / B ratio. Additionally, GUE, Lac, and their combination increased the relative abundance of *Tenericutes* and decreased that of *Epsilonbacteraeota*. In the research by Yang et al. (54), lentinan ameliorated intestinal microbiota dysbiosis in high-fat diet mice by decreasing the abundance of *Epsilonbacteraeota*.

Based on the analysis of cecal microbial composition at the genus and species levels, we found that Lac and the combination of GUE and Lac significantly increased the abundance of *Lactobacillus* and *Lactobacillus gallinarum* in the cecum of broilers at 28 d of age. Gut-residing *Lactobacillus* not only communicated with each other but also with the intestinal epithelial lining to balance the intestinal barrier integrity and mucosal barrier defense, and ameliorate the host immune responses (55). Under production conditions, *Lactobacillus* could colonize the GIT of broilers soon after hatching, and their metabolic activity reduced the pH of the chyme, which helped prevent the growth of harmful intestinal bacteria (56). *L. gallinarum* is beneficial to intestinal health, modulating intestinal microbial composition, secreting protective metabolites (57), improving the intestinal absorption capacity (58), and inhibiting the colonization of *Salmonella* in GIT (59). Conversely, *Helicobacter* and *Escherichia* easily colonize the intestines of humans and animals, causing various diseases by modulating the production of intestinal inflammatory factors and disrupting intestinal mucosal permeability, damaging the intestinal barrier (60–62). In this study, both GUE and Lac reduced the abundance of *Helicobacter* and *Helicobacter pullorum* in 28-d-old

broilers, and *Escherichia* and *Escherichia coli* in 84-d-old broilers. The combination of GUE and Lac had a synergistic effect on reducing the abundance of *Helicobacter* and *Helicobacter pullorum*.

In this study, we also observed the GUE, Lac and their combination remarkably decreased the abundance of genus *Bacteroides*, specifically *Bacteroides_fragilis* and *Barnesiella_intestinihominis* in 28-d-old broilers, and *Bacteroides_dorei* and *Bacteroides_vulgatus* in 84-d-old broilers. *Bacteroides* was positively correlated with serum inflammatory cytokines TNF-a, IL-1β, and IL-6, and dietary supplementation with *Glycyrrhiza* polysaccharides suppressed the proliferation of *Bacteroides* (1). *Bacteroides*, a Gram-negative anaerobic bacterium, primarily achieved mutualism with the host through utilizing polysaccharides (63). The composition of *Bacteroides* is diverse and complex, playing a crucial role in various metabolic activities in animals (64). Certain *Bacteroides* species have pathogenic potential, promoting intestinal bacterial penetration and causing diarrhea by producing enterotoxins on the surfaces of intestinal epithelial cells (65). While some members of the phylum are part of the normal GIT microbiota, they may cause opportunistic infections if the intestinal mucosal barrier integrity is disrupted (66). This infection is typically triggered by various microorganisms, with *B. fragilis* being the most prevalent, found in the GIT of healthy individuals and associated with anaerobic bacteremia (67). *B. dorei* can cause intestinal inflammation and is recognized as a bacterial pathogen. Lan et al. (68) reported that α-glycerol monolaurate regulated the cecal microbiota of broilers at late growth stage by reducing the relative abundance of opportunistic pathogens such as *B. dorei*. Additionally, Bamba et al. (69) proposed a potential association between *B. vulgatus* and ulcerative colitis. The results mentioned above indicated that GUE and Lac could regulate the balance of intestinal microecology by increasing beneficial bacteria and reducing harmful bacteria in the cecum of broilers.

5 Conclusion

In summary, dietary GUE and *L. acidophilus* improved the growth performance of *Liangfenghua* broiler chickens, especially during the growing-finishing period, and enhanced intestinal health. Moreover, they increased the richness and diversity of cecal microbiota, and modulated the balance of intestinal microecology by increasing beneficial bacteria and reducing harmful bacteria in the cecum. The combined use of GUE and Lac had synergistic effects on growth performance, intestinal health, and microbiota composition. These findings suggest that the combination of GUE and *L. acidophilus* as feed additives has better application prospects in the poultry industry. However, further study is needed to understand the mechanism by which the combined supplementation of GUE and Lac affects the intestinal microbiota.

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 approved by Northwest Minzu University Animal Care and Use Committee. The study was conducted in accordance with the local legislation and institutional requirements.

Author contributions

XL: Conceptualization, Data curation, Formal analysis, Investigation, Software, Validation, Writing – original draft, Writing – review & editing. JLi: Methodology, Resources, Validation, Writing – original draft. HY: Data curation, Methodology, Validation, Writing – original draft. YC: Software, Visualization, Writing – original draft. SL: Data curation, Methodology, Writing – original draft. SJ: Resources, Software, Writing – original draft. YZ: Formal analysis, Visualization, Writing – original draft. GZ: Conceptualization, Funding acquisition, Investigation, Supervision, Writing – review & editing. JLu: Conceptualization, Project administration, Supervision, Writing – review & editing.

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Conflict of interest

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

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Supplementary material

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Influence of the rearing system on the ileum microbiome, metabolome, and transcriptome in meat rabbits

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The rearing system of livestock plays a vital role in animal production, meat quality, and overall welfare. This study aimed to assess the influence of cage-rearing system and forest-rearing system on the ileum microbiota, metabolome, and ileal mucosa transcriptome in meat rabbits. Moreover, 16S rDNA sequencing revealed significant differences in the ileal microbiome composition: caged rabbits exhibited a higher abundance of the genera uncultured *Erysipelotrichaceae* and *Delftia*, whereas the levels of *Muribaculaceae*, unclassified *Burkholderiales*, and uncultured *Eubacteriaceae* were lower compared to rabbits reared in the forest. Metabolome analysis identified 372 differentially accumulated metabolites in the ileum content, which were predominantly mapped to amino acid metabolism, nucleotide metabolism, and energy metabolism pathways. The cage-rearing system was found to positively correlate with the efficient utilization of nutrient sources. Additionally, transcriptome analysis of the ileal mucosa revealed 984 differentially expressed genes, predominantly involved in metabolic pathways, signal transduction pathways, and immune response processes. Through Pearson correlation analysis, we were able to elucidate the metabolic pathway, immune responses, and disease resistance mechanisms were affected by the rearing system. Overall, the findings suggested that metabolic adaptation, nutrient utilization, and immune response play crucial roles in how rabbits adjust to different rearing systems. While the cage system may enhance nutrient efficiency, it appears to suppress immune function and disease resistance.

KEYWORDS

rabbit, rearing system, ileal microbiome, metabolome, ileal mucosa transcriptome

1 Introduction

Rabbit meat consumption and production are not widespread globally, but they have developed into a highly specialized livestock industry in some Asian countries and most Mediterranean countries, particularly in China, Italy, France, and Spain (1). Among these countries, China alone accounts for approximately 60% of the world's total production, with Europe being the second-largest producer (1, 2). Rabbits are considered ideal for meat production due to their many advantageous qualities, such as a short vital cycle and gestation period, significant daily weight gain, and high fertility. As highly specialized monogastric herbivores, rabbits possess a digestive system well-adapted to a high-fiber diet, enabling them to have remarkable feed conversion efficiency.

Rabbit meat is also lean, rich in essential amino acids, and contains highly unsaturated fats (3). It provides moderate energy levels and low cholesterol content (1). Despite these beneficial qualities, rabbit meat consumption is declining in Western countries, largely due to concerns over animal welfare and consumer preferences (4).

In recent years, there has been growing consumer interest in animal welfare, organic farming, meat nutrition and human health. Many consumers prefer to buy meat products from outdoor rearing systems due to their superior sensor qualities compared to those from conventional housing systems (5, 6). For rabbits, the rearing system is one of the factors that moderately affects growth performance, behavior, immunity, oxidative stress, and carcass and meat quality (7–9). For instance, outdoor rearing systems are associated with the expression of more natural behaviors and a lower incidence of digestive disorders (10). Digestive disorders are a major cause of welfare impairment, with a high occurrence rate in conventional housing systems.

The ileum, the terminal part of the small intestine, harbors trillions of microbes that intimately interact with the host (11). The composition of the resident microbiome is influenced by the host's physiological condition and, in turn, impacts overall health (12). Some studies have shown that the intestinal microbiota can directly interact with intestinal epithelial cells and further modulate the intestinal immune system (13), epithelium differentiation (14), and immune system-mediated mucosal protection (15).

Currently, the effects of rearing systems on intestinal histomorphology and gut microbial composition have been extensively explored in pigs (18), chickens (19), and geese (20). Similarly, previous studies conducted on rabbits have concluded that different rearing systems significantly affect growth performance, slaughter yield, and meat composition (21) while also reducing the incidence of digestive disorders (10). Importantly, integrative analysis of the microbiome, transcriptome, and metabolome provides novel insight into how host-microbiota interactions affect animal performance and their overall welfare (16, 17). To date, the complex interactions between intestinal microbiota and host genetic responses in meat rabbits reared under different rearing systems remain largely unexplored.

Therefore, to address this knowledge gap, a multi-omics approach was employed to explore the ileal bacterial composition, metabolome, and host gene expression in meat rabbits reared either in cages or in a forest environment. This approach aims to identify key microbiota, uncover regulatory metabolic pathways, and clarify the molecular mechanisms underlying physiological responses. Ultimately, these results are expected to identify host-microbe associations and provide a comprehensive view of the biological systems involved, offering valuable insights into how rearing systems contribute to the welfare of rabbit farming.

2 Materials and methods

2.1 Experiment design and animal treatment

A total of 30 healthy male New Zealand rabbits from a purebred line were used in this study. From 18 to 20 days of age, young animals were gradually introduced to solid feed, alongside breast milk, to help them adapt to pelleted food. At 30 days of age, all rabbits were weaned and provided with commercial pelleted food (the diet ingredients are shown in Supplementary Table 1), fed *ad libitum* three times daily at 8:00, 13:00, and 18:00, respectively.

At 40 days of age, the rabbits were randomly assigned to two groups based on their rearing systems: cage-rearing system (RC) and forest-rearing system (RF). The RC group was individually housed in stainless steel cages with a density of 0.2 m^2/head under standard conditions with temperatures between 15°C and 23°C. In the RF system, a forest area of approximately 200 m^2 was enclosed by a 3-m high metal fence, with a calculated stocking density of 13 m^2/head. Both groups were fed the same commercial pelleted food three times daily *ad libitum*, and water was freely available through valve self-drinkers throughout the 50-day experimental period.

2.2 Sample preparation

At 90 days of age, six rabbits with similar body weights (2,180.5 ± 102.5 g) from each group were randomly selected. The selected animals were subjected to electrical stunning, followed by exsanguination, skinning, and evisceration procedures. Immediately after slaughter, ileum content samples were collected aseptically, snap-frozen in liquid nitrogen, and stored at −80°C for subsequent microbial and metabolomic analyses. At the same time, sections of the ileum were collected, and the digesta was washed away from the epithelial lining using ice-cold sterile phosphate-buffered saline (PBS). Subsequently, the ileum mucosa was gently scraped after being washed three times with PBS and then quickly stored in liquid nitrogen for RNA sequencing.

2.3 Analysis of ileum content-associated microbiota by *16S rRNA* gene sequencing

The bacterial DNA extraction, amplification, library construction, and sequencing were conducted as previously described (22). Briefly, frozen ileum content samples were subjected to microbial genomic DNA extraction using the QIAamp DNA Stool Mini Kit (Qiagen, Shanghai, China) according to the manufacturer's protocol. The DNA concentration and purity were evaluated using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Montchanin, DE, USA). The V3–V4 hypervariable region of the bacterial *16S rRNA* gene was amplified by PCR using specific primers 338F and 806R. After PCR amplification, all qualified amplicons were further subjected to library construction and subsequently sequenced on the Illumina HiSeq 2500 platform, generating 250-bp paired-end reads.

Raw reads were filtered and analyzed using QIIME2 software (23). Tags were clustered into operational taxonomic units (OTUs), and then, the taxonomic assignment was conducted using the SILVA v138 database (silva-138–99-nb-classifier.qza) with the classify-sklearn algorithm. Alpha and beta diversities were

calculated using the Kruskal–Wallis test and the PERMANOVA method, respectively (23, 24). Statistical analyses were conducted using R software (v4.1.3) (https://www.r-project.org/). The criterion of significance was determined at a *P*-value of <0.05, and the values were presented as means. Finally, PICRUST2 (v1.7.3) (25) was utilized to predict the functional profiles of the *16S rRNA* gene data, and pathways were predicted using the KEGG database.

2.4 Analysis of ileum content-associated metabolomics by LC-MS

Liquid chromatography-mass spectrometry (LC-MS/MS) technology was used to analyze the metabolic profiling of intestinal content, following a previously described method (26). Briefly, metabolites were extracted using a 400 μL methanol solution (4:1, v/v) with 0.02 mg/mL L-2-chlorophenylalanin as an internal standard. The mixture was sonicated at 40 kHz for 30 min at 5°C, followed by protein precipitation at −20°C for 30 min. Subsequently, the supernatant was obtained by centrifugation at 13,000 rpm for 10 min at 4°C. Finally, the supernatant was evaporated to dryness under a gentle stream of nitrogen for LC-MS analysis, and a pooled quality control sample (QC) was prepared by mixing equal amounts of metabolites from each sample to ensure data consistency.

The LC-MS analysis was conducted using the UHPLC-Q Exactive HF-X system from Thermo Fisher Scientific, following conditions outlined in our previous study (27). For liquid chromatographic separation, a flow rate of 0.25 mL min^{-1} was maintained, and the column temperature was set to 40°C. Each sample was equilibrated, and a 2 μL volume was injected for analysis. Mass spectral data were acquired using spray voltages of 3.8 kV for positive ion mode (ESI+) and −2.5 kV for negative ion mode (ESI^-).

Finally, the raw LC-MS data were processed using Progenesis QI software (Waters Inc., Milford, MA, USA). Metabolite identification was conducted by searching the reference standard MS/MS spectral libraries or databases such as the HumanMetabolome Database (HMDB, http://www.hmdb.ca), Metlin (http://metlin.scripps.edu), and mzCloud (https://www.mzcloud.org) database. Differentially accumulated metabolites (DAMs) were identified based on a variable importance in projection (VIP) threshold >1.0 in the OPLS-DA model and a *p*-value of <0.05 in a student's *t*-test. Functional enrichment analysis of the DAMs was conducted using the Kyoto Encyclopedia of Genes and Genomes (KEGG) database.

2.5 Analysis of the ileal mucosa transcriptome profiling by RNA sequencing

Total RNA was extracted from the ileal mucosa using TRIzol Reagent (TaKaRa, Dalian, China), and DNA was removed using the RNeasy Midi Kit (Qiagen, Valencia, CA, USA) according to the manufacturer's protocols. RNA quality was assessed with a 5,300 Bioanalyser (Agilent Technologies, USA) and quantified using the NanoDrop ND-2000 spectrophotometer (NanoDrop Technologies).

The RNA-seq transcriptome library was constructed using the NEBNext Ultra™ RNA Library Prep Kit of Illumina (NEB, USA) according to the manufacturer's instructions. The library sequencing was conducted on the Illumina NovaSeq™ X Plus platform, generating 150 bp paired-end reads.

The raw reads were subjected to adaptor removal and quality control, with low-quality reads filtered as described in our previous report (28). The cleaned data were then mapped to the latest rabbit reference genome (*OryCun*2.0.110 in Ensembl) using HISAT2 software (v2.2.1) with default parameters (29). Gene expression was quantified using featureCounts (v2.0.1) (30), which counted the number of mapped reads for each gene. Differentially expressed genes (DEGs) between the two groups were analyzed using the DESeq2 R package (v3.2.3) (31), with significant DEGs defined by an adjusted *P*-value (Padj) of <0.05 and a |log2 (FoldChange)| of >1.

Finally, the DAVID (v6.8) (32) software was used to analyze the statistical enrichment of Gene Ontology (GO) terms and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways, with an FDR threshold of <0.05 indicating significance.

2.6 Statistical analysis

All results from six replicates per group are presented as mean value ± standard deviation (SD). An unpaired Student's *t*-test was used to compare the two groups using SPSS 21.0 (IBM Corp., New York, USA), with statistical significance defined at a *P*-value of < 0.05. The Spearman's correlation coefficient and cluster analysis were conducted using the R package (v4.2.0), and all results were visualized using ggplot2 (v3.3.6) in the R package (33). Pearson's correlation coefficient was used to identify significant correlations in the multi-omics data, with *P*-values of < 0.05 considered statistically significant.

3 Results

3.1 Rearing system induced a shift in the ileum content microbiota composition

A total of 1,119,688 bacterial sequences with an average length of 448 bp were retained and categorized into 396 operational taxonomic units (OTUs) using the DEBLUR program. There were no significant differences in alpha diversity indices between the two rabbit groups, as indicated by Chao1 richness (Figure 1A) and the Shannon diversity (Figure 1B) index ($P > 0.05$). However, a notable shift in beta diversities was observed through principal coordinates analysis (PCoA) based on Bray–Curtis and unweighted unifrac methods. PCoA plots revealed that the ileum content microbiota of RF animals clustered together and were clearly separated from those of RC rabbits (Figures 1C, D), indicating

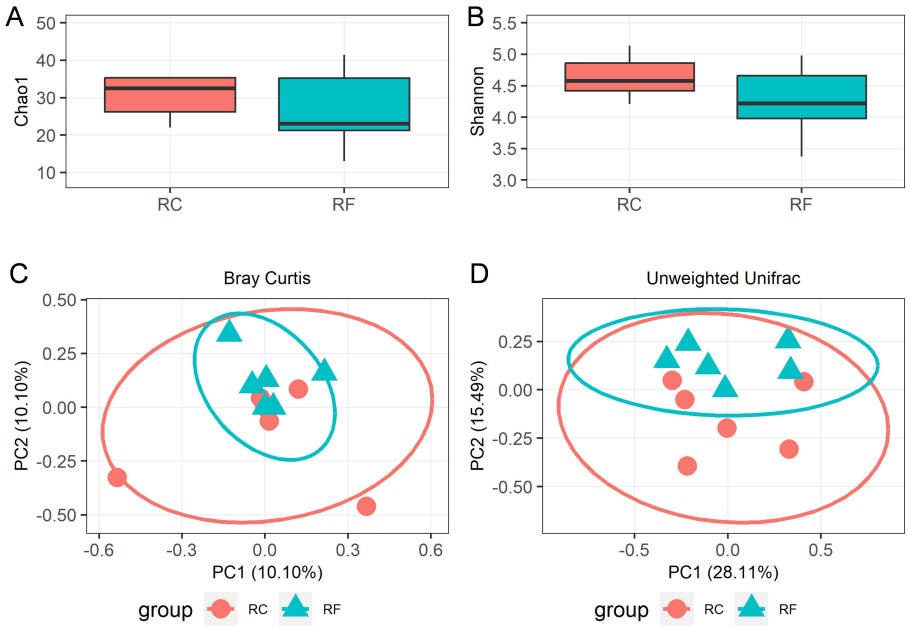

FIGURE 1
Alpha diversity and beta diversity indices of ileal content microorganisms in RF and RC. Comparison of the richness (Chao1) **(A)** and diversity (Shannon) **(B)**. The overall microbiota structures were shown by principal coordinate analysis (PCoA) of Bray-Curtis distances **(C)** and unweighted UniFrac distances **(D)**. RF, forest rearing system; RC, cage rearing system.

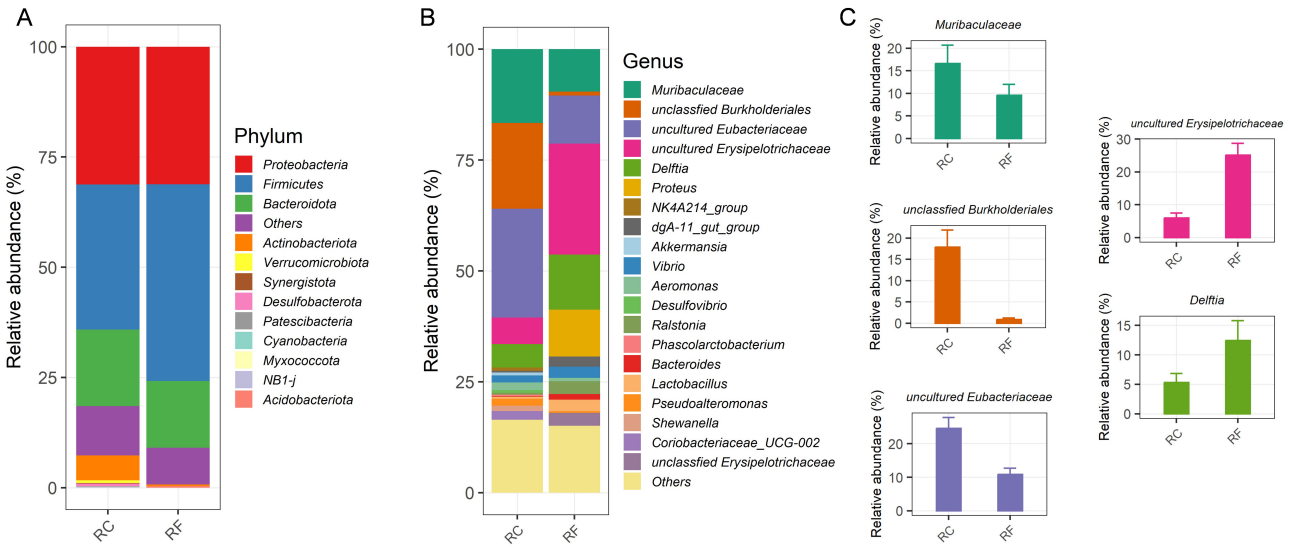

FIGURE 2
Different rearing systems altered the specific ileal bacterial compositions of rabbits. Relative abundance of the ileal phylum taxonomic level **(A)**. Relative abundance of the top 20 bacteria at the genus taxonomic level **(B)**. Most changed bacteria genera **(C)**. RF, forest rearing system; RC, cage rearing system.

that the bacterial communities were positively correlated with the rearing system.

At the phylum level, the ileum content microbiota was predominantly composed of four major phyla in both rabbit groups: *Proteobacteria*, *Firmicutes*, *Bacteroidota*, and *Actinobacteriota*, which together accounted for 89.24% of the OTUs (Figure 2A).

Significant differences were observed between the groups in the relative abundance of *Firmicutes* and *Actinobacteriota*, with *Firmicutes* being more abundant in RF rabbits and *Actinobacteriota* more prevalent in RC rabbits (Figure 2A). The average bacterial community compositions of the top 20 genera are shown in Figure 2B. Furthermore, significant differences were noted in the abundance of the five most prevalent genera between RF

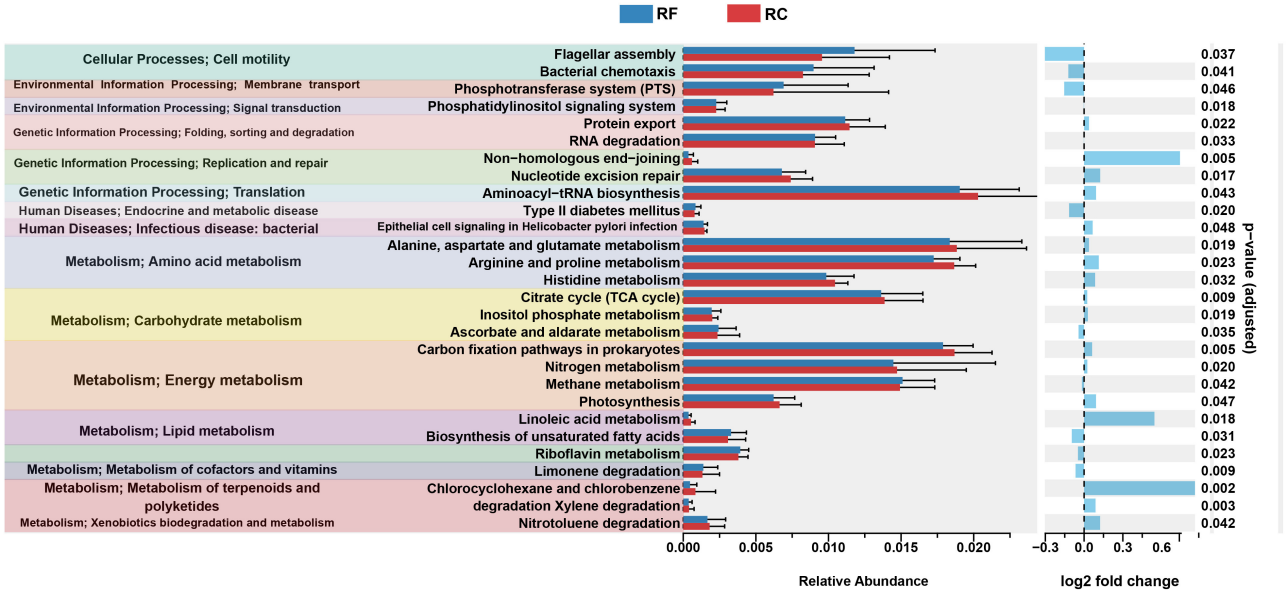

FIGURE 3
Significantly differing ileal microbiota pathways between RF and RC rabbits. All OTUs were used to predict their functions against the KEGG database through PICRUST2 software (v1.7.3). Difference values are presented as the difference from RF to RC group. RF, forest rearing system; RC, cage rearing system.

and RC rabbits. RF rabbits exhibited increased colonization of several genera, including uncultured *Erysipelotrichaceae*, *Delftia*, and *Proteus*. However, the RC rabbits had higher abundances of *Muribaculaceae*, unclassified *Burkholderiales*, and uncultured *Eubacteriaceae* in their ileum content (Figure 2C).

3.2 Predicted functions of the ileum content microbiota

Using PICRUST2 analysis, the functional profile of the ileum content microbiota was inferred based on the rearing system, revealing 28 significantly enriched KEGG pathways between the two groups. In rabbits reared in the forest system, pathways related to lipid metabolism, such as the biosynthesis of unsaturated fatty acids, riboflavin metabolism, and limonene degradation, were highly represented (Figure 3).

In addition, pathways associated with cellular processes, including flagellar assembly and bacterial chemotaxis, as well as pathways involved in the phosphotransferase system, type II diabetes mellitus, and ascorbate and aldarate metabolism, were more prominent in RF rabbits (Figure 3).

In contrast, rabbits in the cage-rearing system showed higher expression of pathways related to amino acid metabolism, including histidine, alanine, aspartate, glutamate, arginine, and proline metabolism. Energy metabolism pathways, such as carbon fixation in prokaryotes, nitrogen metabolism, and photosynthesis, were also more pronounced in the RC rabbits compared to the RF group (Figure 3).

3.3 The rearing system induced a shift in the ileum content metabolomic profile

A total of 1,174 metabolites were detected in the ileum content, with 627 identified in positive ion mode and 547 in negative ion mode. The OPLS-DA analysis indicated a clear separation between the two rabbit groups (Figures 4A, B), indicating distinct metabolic profiles between them with stable and reliable models. Based on the thresholds of a VIP of $>$ 1.00 and *P*-value of $<$ 0.05, 372 DAMs were identified, of which 181 were upregulated and 191 were downregulated (Supplementary Table 2).

These DAMs were classified into 13 categories, such as 118 lipids and lipid-like molecules, 59 organic acids and derivatives, 47 organoheterocyclic compounds, 44 phenylpropanoids and polyketides, 27 organic oxygen compounds, and 22 benzenoids, among others. Notably, lipids and lipid-like molecules, organic acids and derivatives, organoheterocyclic compounds, and phenylpropanoids and polyketides accounted for 33.62%, 16.81%, 13.39%, and 12.54% of the DAMs, respectively (Supplementary Figure 1). Then, a cluster heatmap analysis of the top 30 metabolites further confirmed that RF and RC rabbits could be distinctly separated based on their metabolomic profiles (Figure 4C).

The KEGG pathway enrichment analysis of DAMs from RF and RC rabbits showed that the DAMs were primarily enriched in 10 significant KEGG pathways, among which eight pathways were downregulated and two pathways were upregulated in RF rabbits compared to RC rabbits. The enriched pathways included lipid metabolism, amino acid metabolism, energy metabolism, nucleotide metabolism, and other amino acid

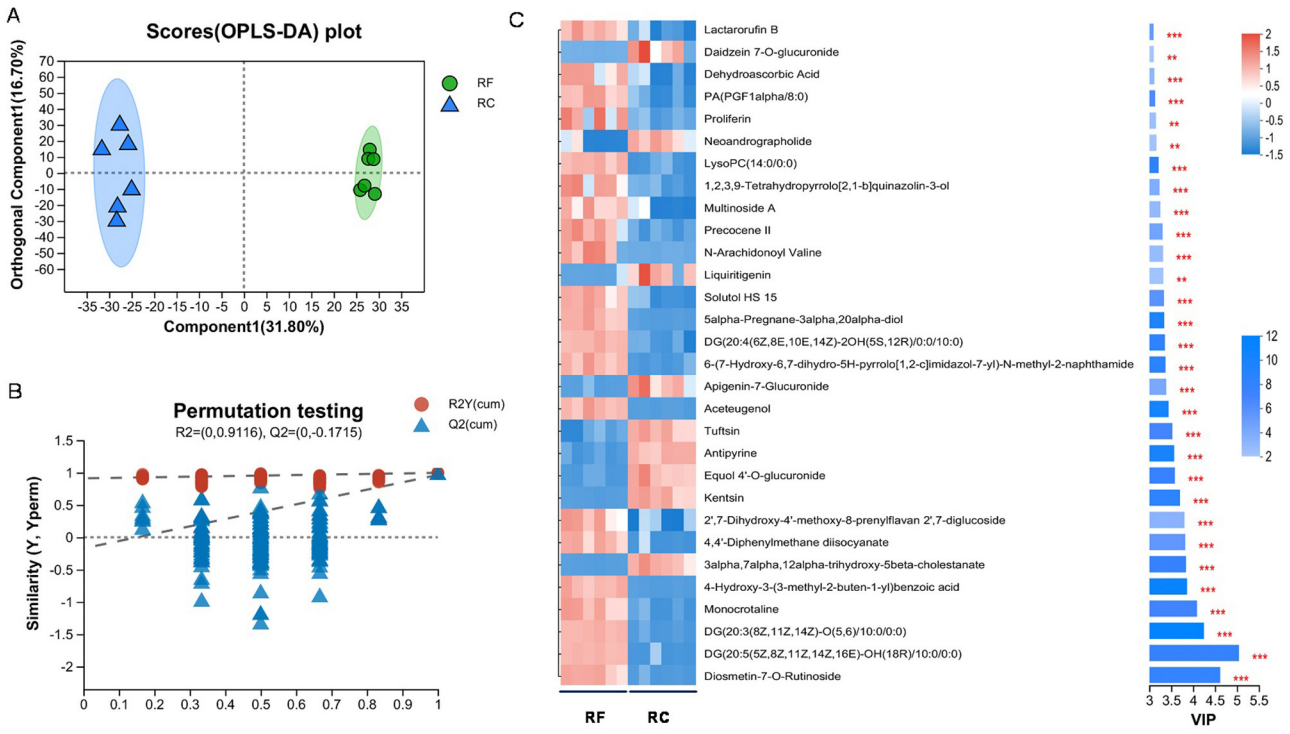

FIGURE 4
Metabolome analysis of ileum content samples from different rearing systems. Orthogonal partial least squares discriminant analysis (OPLS-DA) score plots **(A)** and permutation tests were obtained for RF and RC groups **(B)**. Top 30 differentially accumulated metabolites in the ileum content of rabbits identified by OPLS-DA **(C)** (*0.01 < $P \leq 0.05$; **0.001 < $P \leq 0.01$; ***$P \leq 0.001$). RF, forest rearing system; RC, cage rearing system.

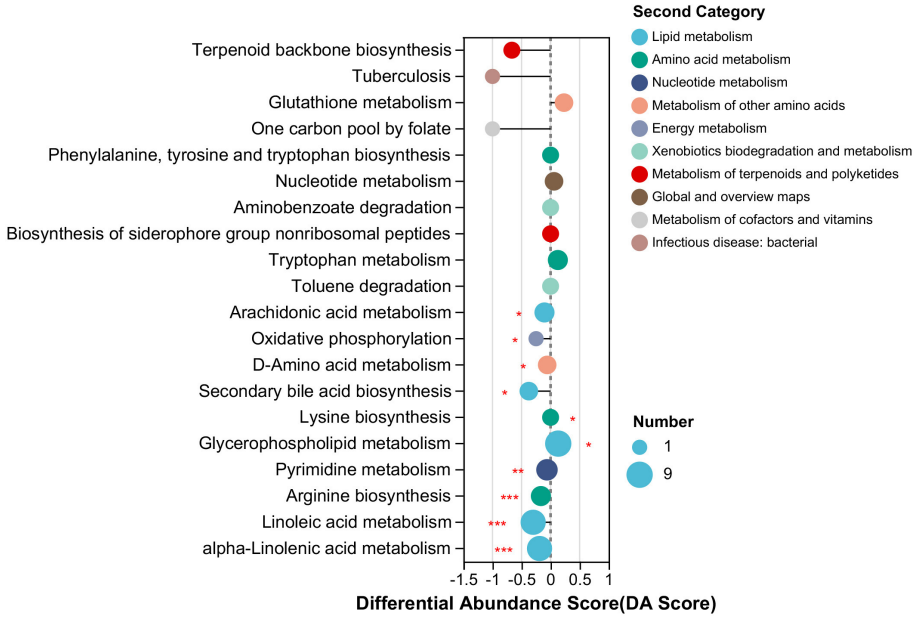

FIGURE 5
Significant differing ileal metabolomic pathways based on differentially accumulated metabolites from RF and RC rabbits. The length of the line segment represents the absolute value of the differential abundance score, and the size of the dots refers to the number of metabolites in the pathway (*0.01 < $P \leq 0.05$; **0.001 < $P \leq 0.01$; ***$P \leq 0.001$). RF, forest rearing system; RC, cage rearing system.

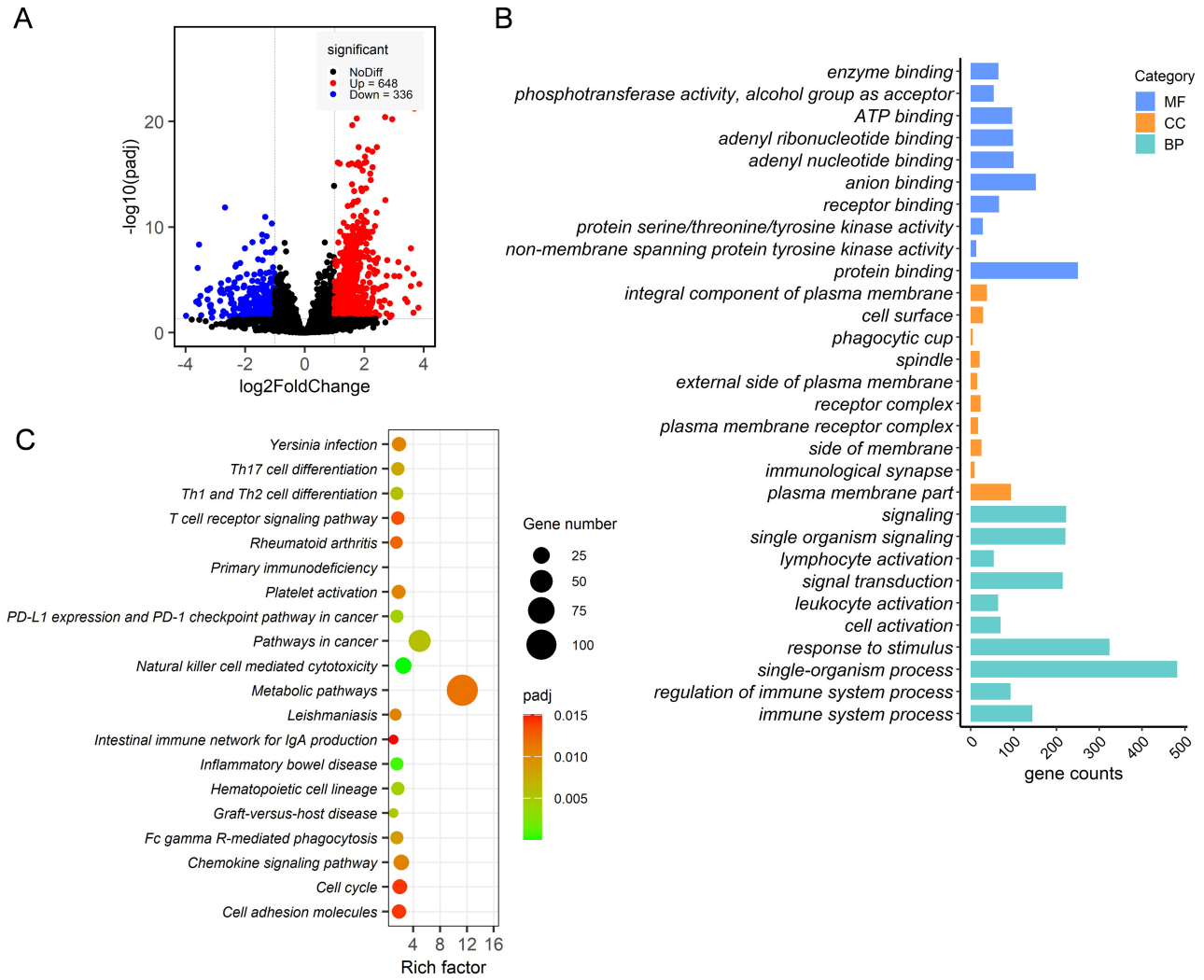

FIGURE 6
Transcriptome analysis of ileal mucosa samples from RF and RC rabbits. Volcano plot of differentially expressed genes **(A)**. The top 10 highly significant GO terms in each group include MF, CC, and BP **(B)**. Significantly enriched KEGG pathways are potentially related to metabolism, disease resistance and the immune response process **(C)**. RF, forest rearing system; RC, cage rearing system.

metabolism pathways (Figure 5). Notably, the majority of the altered metabolites exhibited a higher abundance in RC rabbits, with the majority being involved in lipid metabolism pathways, such as alpha-linolenic acid metabolism, linoleic acid metabolism, secondary bile acid biosynthesis, and arachidonic acid metabolism (Figure 5).

3.4 Gene expression profile of ileal mucosa under different rearing systems

A total of 567,304,736 raw reads were generated from 12 ileum epithelial samples. After filtering adaptor sequences and low-quality reads, a total of 562,212,578 clean reads of 150 base pairs were retained. Over 82.72% of these clean reads were successfully mapped to the rabbit genome using HISAT2 software. After filtering genes with no more than 10 raw count reads in at most two samples, a total of 9,734 annotated genes were identified, representing 46.05% of the 21,140 gene set. Overall, 984 DEGs were detected in the ileum epithelia, with 648 DEGs upregulated and 336 DEGs downregulated in RF rabbits compared to the RC group (Figure 6A). These DEGs were defined using a threshold of $|\log2(\text{FoldChange})| > 1$ and Padj of < 0.05.

GO and KEGG pathway enrichment pathways were conducted to assess the biological processes and pathways associated with these DEGs. The GO enrichment analysis revealed that the DEGs were enriched in 335 GO terms, with the 10 most significant terms summarized for each category.

In the biological process (BP) category, the DEGs were primarily associated with cell activation, immune system processes, and their regulation. In the molecular function (MF) category, the DEGs were majorly associated with kinase activity and binding. For the cellular component (CC) category, the DEGs were mainly enriched in processes related to signaling at the immunological synapse, receptor complexes, phagocytic cup

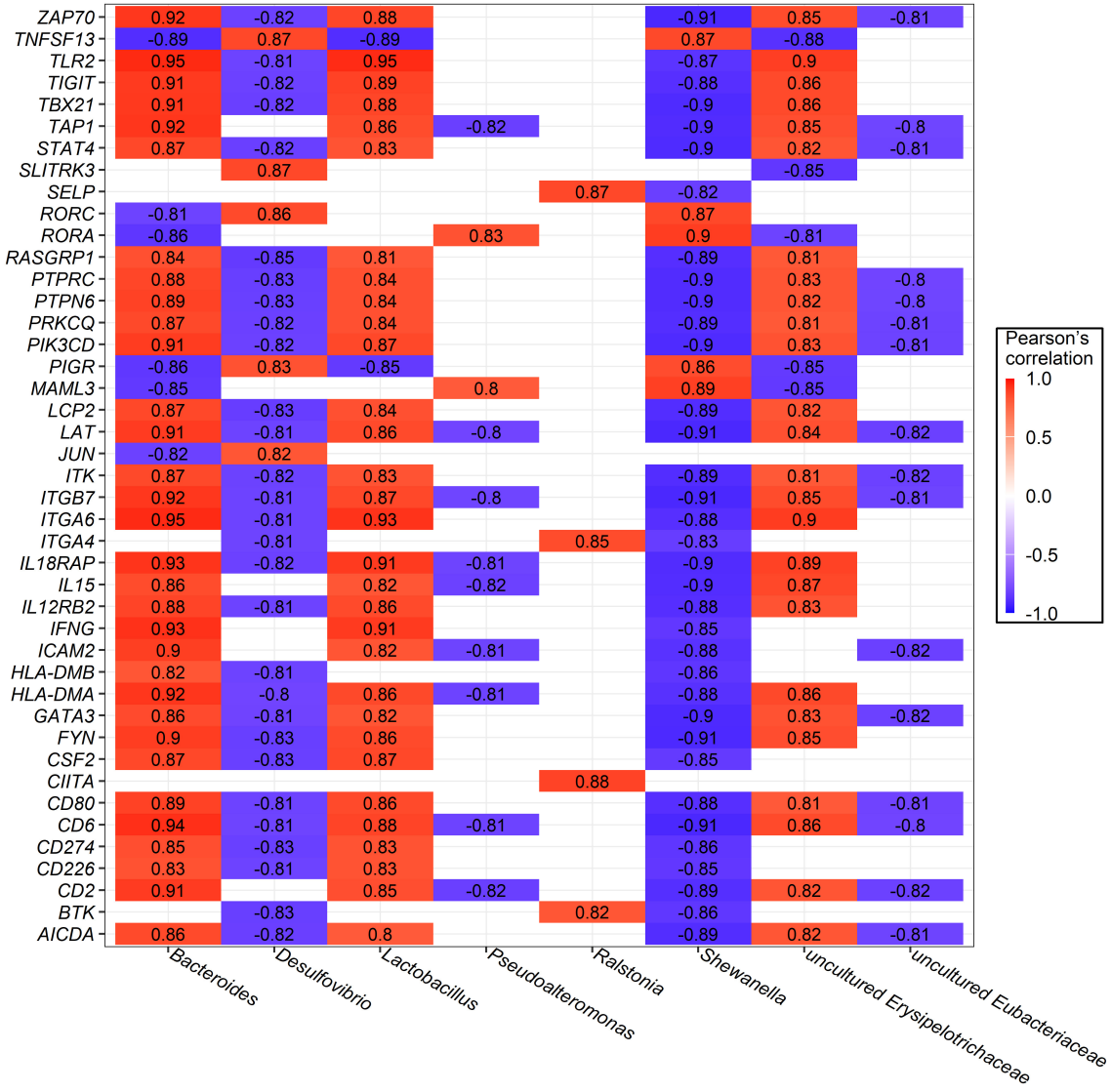

FIGURE 7
Significant Pearson's correlations between ileum bacterial genera and ileal mucosa genes involved in immune response and metabolic pathway. Red represents a positive correlation, while blue represents a negative correlation.

formation, and the plasma membrane (Figure 6B). KEGG pathway analysis showed that DEGs were significantly enriched in metabolic pathways, signal transduction pathways, including the chemokine signaling pathway and the T-cell receptor signaling pathway, and immune response processes, such as graft-vs.-host disease, Th1 and Th2 cell differentiation, Th17 cell differentiation, Fc gamma R-mediated phagocytosis, and primary immunodeficiency (Figure 6C).

3.5 Correlation analysis of ileum bacteria, metabolites and host gene expression

The influence of the rearing system on the three omics-microflora, metabolome, and host gene expression—was explored. For metabolome features, 78 annotated differentially accumulated metabolites (DAMs) enriched in lipid, amino acid, and energy metabolism were included.

Additionally, the top 10 most abundant bacterial genera and a subset of 43 DEGs enriched in immune response processes and metabolic pathways were selected for Pearson's correlation analysis. Only coefficients with $|r| > 0.8$ and P-values of < 0.05 are shown in Figure 7. Significant correlations were found between bacteria and host gene expression, with *Bacteroides* strongly and positively correlated with 32 genes, *Lactobacillus* with 31 genes, and uncultured *Erysipelotrichaceae* with 26 genes. In contrast, unclassified *Burkholderiales* were negatively correlated with 36 genes, while *Shewanella*, and *Desulfovibrio* were negatively correlated with 35 and 29 genes, respectively.

Furthermore, the relationships between bacterial communities and metabolites provided insights into the functions of key

microbiota and metabolites under different rearing systems. Correlations with coefficients $|r| > 0.8$ and P-values of < 0.05 are shown in Supplementary Figure 2. Among them, *Bacteroides* showed a broad range of strong and significant correlations, with 25 positive and 28 negative correlations with metabolites. In addition, uncultured *Erysipelotrichaceae* was positively correlated with 12 metabolites and negatively correlated with 8 metabolites. Interestingly, we also observed that the genera *Lactobacillus* was positively correlated with 14 metabolites, while uncultured *Eubacteriaceae* was positively correlated with 12 metabolites.

Taken together, these results indicate that specific bacteria, specifically *Bacteroides*, uncultured *Erysipelotrichaceae*, and *Lactobacillus*, might play important roles in interacting with numerous metabolites and host genes, influencing the adaptation of the rearing system by modulating host immune responses and metabolic pathways.

4 Discussion

To meet the growing consumer demand for sustainable purchase choices, previous studies have examined the effects of rearing systems on the growth performance and welfare of livestock. In particular, the gut microbiome is a crucial determinant of animal health and productivity, and its composition is well known to be associated with the rearing environment. For example, Lin et al. (34) demonstrated that geese reared indoors exhibited a higher abundance of pathogenic genera and lower levels of commensal genera compared to those raised outdoors.

Similarly, for broiler chickens, floor-reared birds showed a relatively higher abundance of potentially pathogenic and litter-associated bacteria (35), which could be due to increased exposure to environmental microbes. This exposure may enhance immune function and disease resistance (35, 36). Despite these insights into poultry, there remains a gap in understanding the impact of rearing systems on the gut microbiota of rabbits and the potential links between bacterial genera, host gene expression patterns, and the metabolome. Our 16S rDNA sequencing results revealed that the dominant phyla in both forest-reared (RF) and cage-reared (RC) rabbits were *Proteobacteria, Firmicutes, Bacteroidota*, and *Actinobacteriota*. Although relative abundances differed, these dominant phyla align with previous studies on the cecal microbiota of New Zealand White rabbits (37).

While overall bacterial communities were mostly similar, key taxa differences may reflect adaptations to different rearing environments. *Muribaculaceae*, unclassified *Burkholderiales*, and uncultured *Eubacteriaceae* were reduced, while uncultured *Erysipelotrichaceae* and *Delftia* were significantly increased in RF rabbits compared to RC rabbits. *Muribaculaceae* is considered beneficial and linked to pathways involving cytokines and short-chain fatty acids (15), with its abundance varying seasonally in rabbits (38). *Burkholderiales* include bacteria with diverse metabolic functions (39), while uncultured *Eubacterium*, a member of *Eubacteriaceae,* can produce butyrate, which plays a critical role in energy homeostasis, colonic motility, immunomodulation, and suppression of inflammation in the gut (40, 41).

The bacterial taxa *Erysipelotrichaceae* have been recurrently associated with dyslipidemic phenotypes in hosts, including mice and humans, particularly in the context of obesity, metabolic syndrome, and hypercholesterolemia (42). Furthermore, PICRUST2 analysis of these key taxa indicated that pathways related to lipid metabolism and disease resistance, such as the phosphotransferase system and type II diabetes mellitus, were more prominent in forest-reared (RF) rabbits. In contrast, functions associated with amino acid and energy metabolism were more prevalent in cage-reared (RC) rabbits. These findings suggest that the shifts in gut microbiota composition may have led to substantial changes in host metabolism and disease resistance, potentially explaining why cage-reared rabbits demonstrated favorable growth performance (21). This is also consistent with previous findings on rabbits (20, 43) and broiler chickens (35) reared in cage systems.

The intestine is not only the primary digestive organ but also an important immune organ in animals. It plays a major role in the digestion and absorption of nutrients from ingested food, while the intestinal mucosa functions as a key component of the physical and chemical barriers, as it can recognize and combat pathogen infections, maintaining homeostasis between the host and the commensal gut microflora (44). Numerous studies have demonstrated the influence of gut microbiota composition on host intestinal epithelium gene expression and intestinal mucosal immune function (45, 46).

In the present study, we explored the effects of different rearing systems on gene expression in the ileal mucosa using RNA-seq analysis. We identified a total of 984 DEGs between rabbits reared in the RF and those in the RC group. These genes were found to be implicated in multiple biological processes and pathways, with many involved in immune system processes and their regulation. Moreover, KEGG enrichment analysis revealed that these DEGs were significantly enriched in metabolic pathways, signal transduction pathways, and immune response processes, such as Th1 and Th2 cell differentiation, Th17 cell differentiation, Fc gamma R-mediated phagocytosis, and primary immunodeficiency. Previous studies have reported that compared to caged chickens, ground-floor-reared birds exhibited higher levels of IL-1β and IFN-γ mRNA in the ileum (47).

Similarly, our transcriptome and metagenome results indicated that rabbits reared in the forest exhibited stronger intestinal mucosal immune function. Notably, immune-related genes such as *IL9*, *IL15*, *IL2RG*, *IL12RB2*, *IL1RN*, *IL18RA*, *IF2A*, *IRF1*, *IFNG*, *TLR2*, and *TLR8* were significantly upregulated in RF rabbits compared to those reared in cages. In line with our findings, Inman et al. (48) reported that piglets raised in an isolator had significantly increased *IL-2* levels produced by mucosal T cells and significantly reduced *IL-4* levels compared to piglets raised outdoors, further supporting the notion that rearing conditions can impact the immune response. These results suggest that changes in rearing conditions can lead to enhanced immune responses in the rabbit ileal mucosa at the transcriptome level.

The intestinal content serves as a valuable indicator of gut microbial activity and host metabolism. To investigate the metabolic response to changes in the rearing system, we compared the ileal content metabolome between RF and RC

rabbits. The differentially accumulated metabolites between the two groups were primarily associated with lipid metabolism, amino acid metabolism, energy metabolism, and nucleotide metabolism (Figure 5). Notably, these pathways were more abundant in the cage-reared rabbits, suggesting a positive correlation between the cage-rearing system and the efficient utilization of nutrient sources and growth performance. This finding aligns with previous studies demonstrating that animals raised in floor conditions tend to have higher feed efficiency and superior growth performance across various livestock species, including ducks (49), chickens (47), and pigs (50).

Furthermore, a comprehensive correlation analysis across metagenomics, metabolomics, and transcriptomics revealed that specific bacterial genera, such as *Bacteroides*, *Lactobacillus*, and uncultured *Erysipelotrichaceae,* were found to be significantly positively associated with multiple metabolites involved in nutritional metabolism, as well as genes associated with immune response and metabolic pathways. These findings suggest that these bacteria may play an essential role in interacting with ileal metabolites and mucosa genes, thereby influencing the host's adaptation to different rearing systems by modulating immune responses and metabolic processes.

5 Conclusion

The present study demonstrated that the rearing system has a significant impact on the microbial composition, metabolomics of ileal content, and host transcriptomics. Rabbits reared in the forest exhibited the gut microbiome with a lower relative abundance of *Muribaculaceae*, unclassified *Burkholderiales*, and uncultured *Eubacteriaceae*, but a higher relative abundance of uncultured *Erysipelotrichaceae* and *Delftia* compared to those reared in cages.

In addition, the metabolomic profile of ileal content differed significantly between the groups, with changes primarily in pathways related to amino acid metabolism, nucleotide metabolism, and energy metabolism. Notably, the cage-rearing system was positively associated with improved nutrient utilization. However, significant transcriptional changes were also observed in the ileal mucosa, particularly in metabolic pathways, signal transduction, and immune response processes. Overall, while the cage-rearing system enhances nutrient utilization, it appears to be associated with a depressed immune response and reduced disease resistance.

Data availability statement

The RNA-seq data generated in this project are deposited in the Sequence Read Archive (SRA) repository, accession number PRJNA1105390. The 16S rRNA gene sequencing data are deposited in the SRA repository, accession number PRJNA1105432.

Ethics statement

The animal care and experimental procedures involved in this study were approved by the Animal Care and Use Committee of Chengdu University and adhered to the university's guidelines for animal research (SSXY600008). The studies were conducted in accordance with the local legislation and institutional requirements. Written informed consent was obtained from the owners for the participation of their animals in this study.

Author contributions

ZW: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Resources, Software, Supervision, Validation, Visualization, Writing – original draft, Writing – review & editing. MX: Data curation, Formal analysis, Methodology, Validation, Writing – original draft. XLi: Investigation, Methodology, Validation, Writing – original draft. BW: Data curation, Methodology, Validation, Writing – original draft. XF: Data curation, Investigation, Writing – original draft. ZT: Data curation, Writing – original draft. XLiu: Data curation, Writing – original draft. JZ: Data curation, Investigation, Methodology, Resources, Visualization, Writing – original draft.

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Conflict of interest

ZT and XL were employed by Sichuan Aichi Rabbit Food Co., Ltd.

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

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fvets.2024.1456790/full#supplementary-material

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Changes in blood physiological and biochemical parameters and intestinal flora in newborn horses and mares with angular limb deformities

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Introduction: Angular limb deformities (ALDs) are a common skeletal development disorder in newborn foals. This condition affects the growth and development of foals and severely impacts their future athletic performance and economic value, causing significant financial losses to the horse industry. Placentitis, metritis, and severe metabolic diseases during mare pregnancy are significant causes of ALDs in newborn foals. It has been established that intestinal flora disorders can easily lead to inflammatory and metabolic diseases in the host. However, the incidence of ALDs in foals in Zhaosu County, Xinjiang, a key production area of China's horse industry, remains unclear. Additionally, the relationship between functional changes in foals with ALDs and their mares and changes in their intestinal flora is not well-understood.

Methods: This study investigated the status of ALD in newborn foals through clinical observation and imaging examinations. In addition, molecular biological methods were applied to examine the effects of ALDs foals and their mares on physiological and biochemical markers and gut microbiota.

Results: The results showed that the incidence of ALD in Zhaosu area of China was 4.13%. In addition, by comparing and correlating the physiological and biochemical indicators and intestinal flora of foals and mares with ALD with those of healthy horses, it was found that foals and mares with ALD may promote the occurrence and development of the disease through the "blood marker changes-intestinal flora-ALDs" axis. In addition, by comparing the physiological and biochemical indicators and intestinal flora of foals and mares with ALD with the intestinal flora of healthy horses, it was found that the physiological and biochemical indicators and intestinal flora structure and metabolic pathways of foals and mares with ALD had significant changes.

Discussion: The diversity, species composition, and function of the intestinal flora of ALDs and their mares were significantly altered. These findings provide a scientific basis for understanding the etiology of ALDs in foals and offer new perspectives for diagnosing and treatment ALDs in newborn foals.

KEYWORDS

angular limb deformities, equine, intestinal flora, skeleton, metabolism

1 Introduction

Angular limb deformities (ALDs) are common skeletal deformities in horses, manifesting as valgus or varus deformities, often accompanied by some axial rotation (1). Initially, these deformities are postural. However, prolonged external pressure on the bone can increase bone density and hardness over time. The uneven growth of the epiphyseal

growth plate can lead to permanent angulation deformities (2). Therefore, early detection and intervention are crucial to prevent these temporary postural changes from developing into permanent structural deformities.

The causes of ALDs are complex and can be summarized into perinatal and developmental factors (3). Perinatal factors include adverse effects on the foal during late pregnancy and delivery. Developmental factors encompass various influences causing limb axis abnormalities in foals during growth. These influencing factors are closely related to the nutritional status of the mare, leading to changes in conventional blood markers such as blood physiological and biochemical indicators, enzymes and metabolites, thus affecting the growth and development and nutritional metabolism of the offspring (4, 5). Intestinal flora plays a key role in the early growth and development of horses, promoting the proliferation of intestinal cells and providing energy by decomposing cellulose to produce short-chain fatty acids such as propionic acid, acetic acid, and butyric acid (6). Intestinal microbes help build an immune barrier to resist the invasion of foreign pathogens by activating immune cells under the host's intestinal mucosa. Specific intestinal microbes can also promote the formation of immune tolerance and reduce the risk of allergic and autoimmune diseases (7). Additionally, intestinal flora is crucial for bone growth, development, and metabolism by regulating the absorption of essential nutrients like calcium and phosphorus (8). For example, it regulates intestinal pH levels, optimizes the depth ratio of intestinal villi to crypts, and inhibits programmed cell death of epithelial cells, ensuring effective absorption of key nutrients such as calcium and phosphorus (9). Studies have found an association between gut microbiota and the development of inflammatory bowel diseases, which may interfere with the intestinal absorption of calcium and vitamin D, indirectly affecting bone tissue metabolism and increasing the risk of bone health issues (10). These all indicate that intestinal flora is closely related to inflammatory and metabolic diseases of the host, but it is not clear whether the intestinal flora of newborn foals with ALDs and their mares are different from those of normal foals and their mares.

Therefore, this paper intends to investigate ALDs and then explore the differences in blood physiological and biochemical indicators and intestinal flora between ALDs foals and their mares and healthy newborn foals and their mares, in order to clarify the relationship between the occurrence and development of ALDs and intestinal flora and provide new insights into the prevention and treatment of ALDs.

2 Materials and methods

2.1 Experimental animals

This study investigated the epidemiology of ALDs in 460 newborn foals of Ili horses, purebred horses, and crossbred horses (Foals, 2–8 days old, mares, 5–14 years old) in the Zhaosu area (Xinjiang, China) from April to June 2023. The study included foals of both genders. The horse owners and stables approved all investigations, and informed consent was signed. The Animal Welfare and Ethics Committee of Xinjiang Agricultural University approved the experimental design and animal ethics (2023033).

2.2 ALDs prevalence survey

2.2.1 Diagnosis

ALDs was diagnosed using morphological observation and clinical portable X-ray imaging (YZB0671-2007, Dandong Keda Instrument Co., Ltd., China). This combined approach allowed for the accurate determination of deformity types and severity and the evaluation of bone symmetry and joint integrity.

2.2.2 Morphological examination

Clinical observations were first conducted on the newborn foals. The foal was positioned on a flat surface and observed by a veterinarian from the front, sides, and back, with particular attention to the morphological symmetry of the wrist and tarsal joints. The foal was driven to walk slowly and quickly for gait examination to observe limb coordination and claudication. Suspected morphologically abnormal joints were then examined in detail. The veterinarian held the metacarpal bones and attempted to gently bend the wrist or tarsus to be parallel to the radius or tibia. Finally, the site of the deformity was evaluated by comparing it with the contralateral limb. Clinical observations were performed by two veterinarians blinded of the study details.

2.2.3 Imaging examination

In foals with suspected limb deformities identified during morphological examination, X-ray imaging was performed to confirm ALDs. The primary projection positions for imaging were dorsolateral and dorsomedial. The X-ray was centrally aligned to examine the middle of the joint, including the long bones above and below the joints. On the X-ray film, two straight lines were drawn from the long bones above and below the joint along their long axes. The intersection point of these lines indicated the location of the deformity, and the angle formed represented the degree of deviation (indicating the severity of the deformity).

2.3 Blood physiological and biochemical indicators and intestinal microbiome detection

2.3.1 Sample collection

Horses were divided into four groups: healthy foals, healthy mares, ALDs foals, and ALDs mares. Five milliliter of blood was collected through the jugular vein using a vacuum blood collection needle for determination of blood physiological and biochemical indicators of the foals. Fresh fecal samples from each group were collected using sterile cotton swabs (pre-moistened with normal saline) that were slowly inserted 2–3 cm into the horses' anus. The samples were placed into sterile frozen storage tubes and stored in liquid nitrogen until analysis.

2.3.2 Blood physiological and biochemical indices detection

Blood physiological and biochemical parameters of normal foals and ALDs foals and normal mares and ALDs mares were measured using BC-5300Vet Mindray fully automatic five-category animal blood cell analyzer and BS-240VET Mindray fully automatic biochemical analyzer (Shenzhen, China), respectively.

2.3.3 DNA extraction and PCR amplification

DNA was extracted using the CTAB method (Phusion® High-Fidelity DNA polymerase, New England Biolabs, USA). The purity and concentration of the DNA were then measured. The V3 + V4 variable region of the DNA was amplified by PCR using specific primers (Table 1).

2.3.4 Purification and mixing of PCR products

Aliquots were mixed based on concentration. After thorough mixing, the products were purified using 2% agarose gel electrophoresis. The universal DNA purification recovery kit (TianGen, China) was used to recycle the bands of interest.

2.3.5 Library construction and sequencing

The NEB Next® Ultra DNA Library Prep Kit (New England Biolabs) was used for library construction. The quality of the prepared libraries was checked and quantified using the Agilent 5400 Fragment Analyzer (USA). After confirming the library met the quality standards, high-throughput sequencing was performed using the Illumina platform.

Potential contaminating sequences, such as mitochondrial and chloroplast sequences, were excluded using the Feature-table plugin of QIIME2 to ensure result accuracy. Differences in the abundance of the microbiota among the different samples were identified using two statistical methods: analysis of variance (ANOVA) and linear discriminant analysis effect size (LefSe). Correlation matrices for alpha diversity and beta diversity were calculated using the core-diversity plugin of QIIME2.

TABLE 1 Primer information.

Primer	Primer sequence	Product length/bp
341F	5′-CCTAYGGGRBGCASCAG-3′	468
806R	5′-GGACTACNNGGGTATCTAAT-3′	

2.4 Data processing and statistical analysis

Statistical analyses were performed using statistical package for social sciences (SPSS) software (version 19.0 for Windows, SPSS, Chicago, IL, United States). The results are presented as mean ± standard deviation. For two-group comparisons, data were analyzed using a two-tailed Student's *t*-test. For multiple group comparisons, statistical analysis was performed using ANOVA followed by the least significant difference test. $P < 0.05$ was considered statistically significant.

3 Results

3.1 Typical foals with ALDs

Morphological examination revealed that Foal A, a 3-day-old male crossbred foal (its mare was a 9-year-old crossbred horse that had given birth five times), had bilateral tarsal distortion (Figure 1A). Foal B, a 14-day-old male purebred foal (its mare was a 12-year-old purebred horse that had given birth nine times), had lateral fetlock distortion on the left forelimb (Figure 1B). X-ray imaging diagnosed Foal A with varus of the left hind limb tarsal joint and valgus of the right hind limb tarsal joint, indicating a windswept deformity (Figure 1A). Foal B was diagnosed with a valgus deformity of the left forelimb fetlock (Figure 1B).

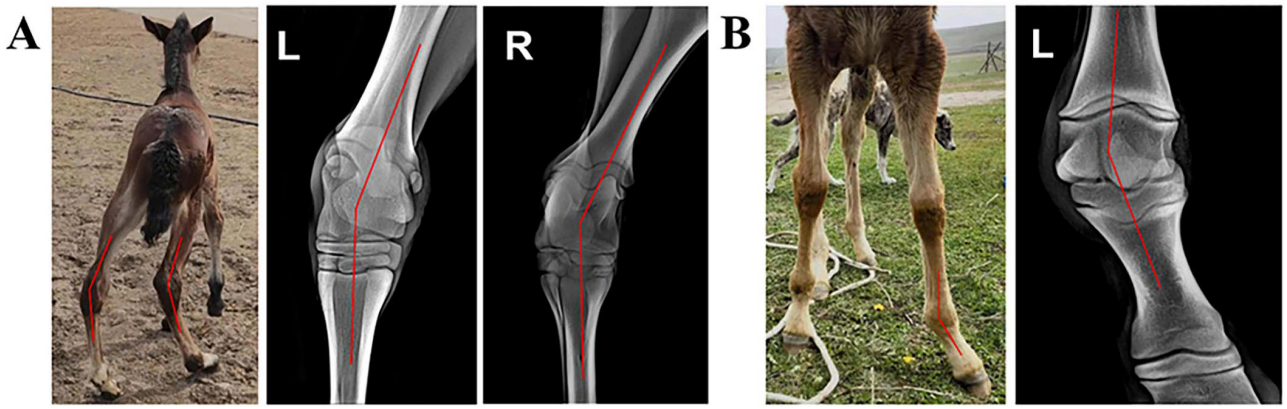

FIGURE 1
Deformities of different foals. **(A)** A 14-day-old purebred male foal with a valgus deformity of the left forelimb fetlock. The dorsal view of the foal is shown in the middle, and dorsal metacarpal radiographs of the tarsal joint area of both hind limbs are on the left and right. **(B)** A 3-day-old male crossbred foal with a windswept deformity. The frontal view of the foal is on the left, and the dorsal metacarpal radiograph of the fetlock area of the left forelimb is on the right.

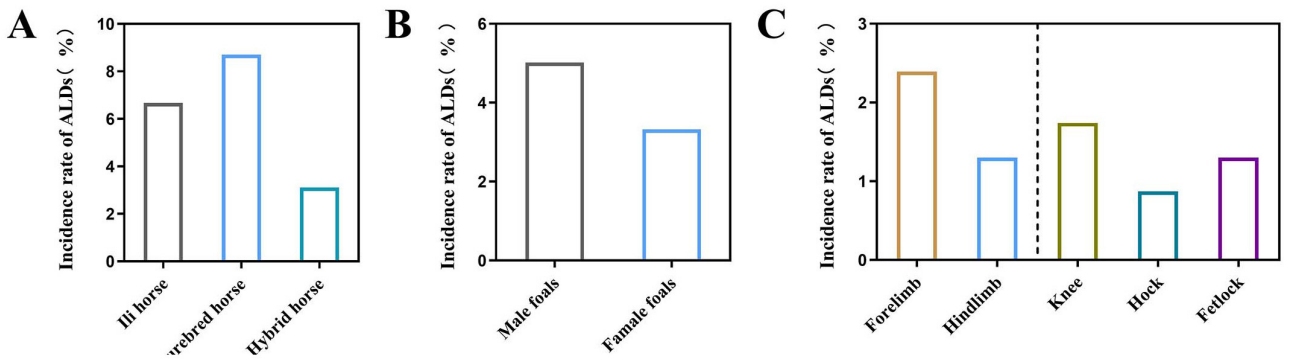

FIGURE 2
Influence of different factors on the prevalence of ALDs in foals. **(A)** The incidence of ALDs in different breeds of foals. **(B)** The incidence of ALDs in foals of different sexes. **(C)** The incidence of ALDs in foals with different malformed parts.

3.2 The influence of different factors on the prevalence of ALDs

Among the 460 newborn foals surveyed, 19 were diagnosed with ALDs (4.13%). The incidence in various breeds was found to be 6.67% (4/60) in Ili horses, 8.70% (4/46) in purebred horses, and 3.11% (11/354) in hybrid horses (Figure 2A). The results showed that 5.02% (11/219) of male foals and 3.32% (8/241) of female foals were diagnosed with ALDs (Figure 2B). Among the 19 diagnosed ALD foals, the deformities involved the forelimbs and hindlimbs, specifically the wrist joint, tarsal joint, and fetlock. The proportion of forelimb deformities was 2.39% (10/460), while hindlimb deformities was 1.30% (6/460). Wrist deformities accounted for 1.74% (7/460), tarsal joint deformities for 0.87% (4/460), and fetlock deformities for 1.3% (5/460) (Figure 2C).

3.3 Differential blood physiological and biochemical indices of ALDs

The results of blood physiological indices of normal foals and ALDs foals are shown in Figure 3A. Monocyte (Mon), Eosinophil (Eos), Eosinophil% (Eos%), Basophil% (Bas%), and Erythrocyte distribution width CV (RDW-CV) of ALDs foals were significantly higher than those of normal foals ($P < 0.05$). The results of blood biochemical indexes of normal foals and ALDs foals are shown in Figure 3B. The levels of Calcium (Ca), Phosphorus (P), Glucose (Glu), and Creatinine (CREA) of ALDs foals were significantly lower than those of normal foals ($P < 0.05$), and the levels of High density lipoprotein cholesterol (HDL-C), Alkaline phosphatase (ALP), Total protein (TP), and Albumin (ALB) of ALDs foals were significantly higher than those of normal foals ($P < 0.05$).

3.4 Hormone and inflammatory cytokine levels in ALDs foals

There was no significant change in blood inflammatory factors between ALDs foals and normal foals ($P > 0.05$). The levels of PTH and 1,25-(OH)2D3 in ALDs foals were significantly or extremely significantly higher than those in normal foals ($P < 0.05$) (Figure 4).

3.5 Intestinal flora diversity and LefSe analysis

The Chao1, Faith_pd, Observed_features, Shannon_entropy, and Simpson index of the intestinal flora of normal foals and ALDs foals, as well as normal mares and ALDs mares, were analyzed. No significant differences were found between the two groups of normal foals and ALD foals, neither between the two groups of normal mares and ALD mares ($P > 0.05$) (Figure 5A).

Bray-Curtis distance analysis was used to perform principal coordinates analysis comparative analysis on the intestinal flora structure of normal foals, ALDs foals, and normal mares and ALDs mares. The results showed that samples within normal and ALD foals groups clustered together while the groups were separated. An inter-group Anosim test revealed that the composition of the intestinal flora of normal foals and ALD foals was significantly different ($R^2 = 0.054$, $P = 0.017$). The samples from normal mares and ALDs mares were clustered together, and the groups were obviously separated. The inter-group Anosim test showed that the intestinal flora composition of normal mares and ALDs mares was extremely significantly different ($R^2 = 0.045$, $P = 0.002$) (Figure 5B).

LefSe analysis results indicated that the absolute value of LDA was log $\geq$ 1.0. In normal foals, there were 32 dominant bacterial groups at the genus level (such as *Faecalitalea*, *Anaerofilum*, and *Lachnospiraceae*_NK4A136_group), and 17 in ALDs foals (e.g., *Mycoplasma*, *Faecalibacterium*, and *Nautilia*). In normal mares, the dominant bacterial flora consisted of 30 genera (such as *Saccharopolyspora*, *Alteromonas*, and *Lachnospiraceae*_NC2004_group), while in ALDs mares, it consisted of 23 genera (such as *Escherichia_Shigella*, *Streptococcus*, and *Bacteroides*). This suggests clear differences in the gut microbiome composition between normal and ALD status (Figure 5C).

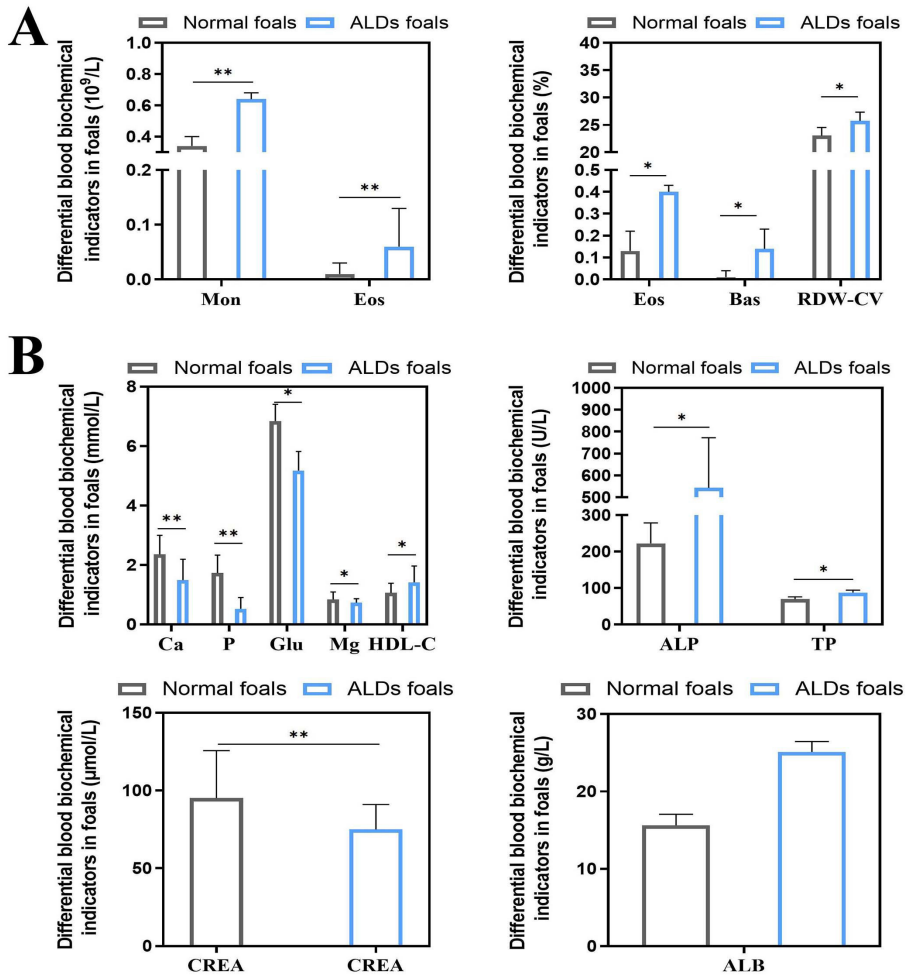

FIGURE 3
Differential changes in blood indices between ALDs foals. **(A)** Differential changes in blood physiological parameters in ALDs foals. **(B)** Differences in blood biochemical parameters in ALDs foals. $P < 0.05$ was considered statistically significant. $^{*}P < 0.05$; $^{**}P < 0.01$.

3.6 Analysis of the composition and relative abundance of intestinal flora

The top 0.1% of the average relative abundance of intestinal flora was selected for further analysis. Among foals, the dominant phyla were *Proteobacteria*, *Firmicutes*, *Bacteroidota*, *Verrucomicrobiota*, and *Fusobacteriota*, with others including *Actinobacteriota*, *Halobacterota*, unclassified, *Campilobacterota*, and *Euryarchaeota* (Figure 6A). In mares, the dominant phyla were *Firmicutes*, *Proteobacteria*, *Bacteroidota*, *Actinobacteriota*, *Verrucomicrobiota*, and *Patescibacteria*, with others including *Spirochaetota*, *Fusobacteriota*, *Cyanobacteria*, *Fibrobacterota*, Desulfobacterota, *Synergistota*, and *Campilobacterota* (Figure 6B).

Using an independent sample *T*-test, the relative abundance of *Verrucomicrobiota* and *Actinobacteriota* in the intestinal flora of ALD foals was significantly increased compared to normal foals ($P < 0.05$) (Figure 6A). In ALD mares, the relative abundance of *Verrucomicrobiota* and *Fusobacteriota* was significantly increased ($P < 0.05$). In contrast, the relative abundance of *Campylobacterota*, *Cyanobacteria*, and *Proteobacteria* was significantly decreased ($P < 0.05$) compared to normal mares (Figure 6B).

3.7 Kyoto encyclopedia of genes and genomics function prediction of intestinal flora and differential analysis of three-level metabolic functions

KEGG function prediction of intestinal flora showed six biological metabolic functional pathways at the first-level functional level. The highest average relative abundance was metabolism at 71.8%, followed by genetic information processing at 10.0%, cellular processes at 6.2%, human diseases at 5.7%, environmental information processing at 4.1%, and organic systems at 2.3%. At KEGG level 2, a total of 47 metabolic pathways were detected. The top 10 in terms of average relative abundance were carbohydrate metabolism (11.7%), cofactors and vitamins metabolism (9.6%), amino acid metabolism (9.2%), other amino

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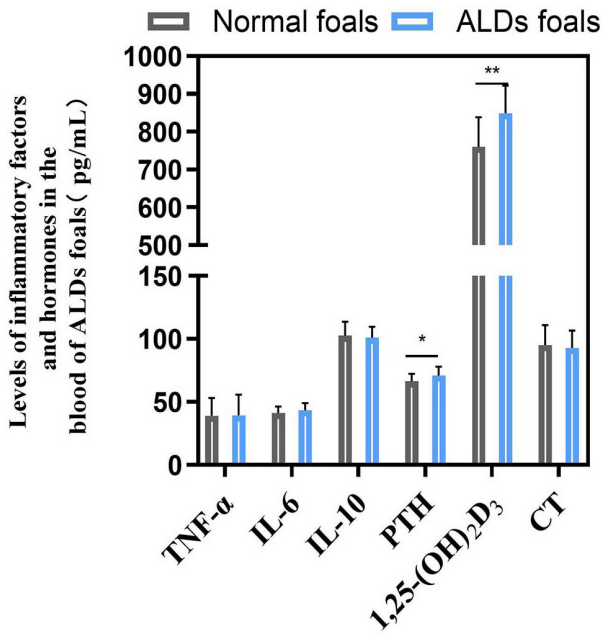

FIGURE 4
Blood inflammatory factors and hormone levels in ALDs foals. $P < 0.05$ was considered statistically significant. $^{*}P < 0.05$; $^{**}P < 0.01$.

acids metabolism (7.5%), other secondary metabolites biosynthesis (5.1%), global and overview maps (5.1%), glucose biosynthesis and metabolism (5.0%), lipid metabolism (4.8%), replication and repair (4.5%), and energy metabolism (4.2%) (Figure 7A).

Based on KEGG level 3 analysis, 12 metabolic pathways showed differences. In ALD foals, the relative abundance of D-alanine metabolism and biosynthesis of amino acids pathways was significantly increased ($P < 0.05$) compared to normal foals. In contrast, the relative abundance of vitamin B6 metabolism, pyruvate metabolism, phenylalanine metabolism, pentose and glucuronate interconversions, nitrogen metabolism, lipopolysaccharide biosynthesis, glyoxylate and dicarboxylate metabolism, glutathione metabolism, flavone and flavonol biosynthesis, and arginine and proline metabolism pathways was significantly decreased ($P < 0.05$) (Figure 7B). In ALD mares, four differential metabolic pathways were identified (Figure 7C). Compared to normal mares, the relative abundance of biotin metabolism, vitamin B6 metabolism, folate biosynthesis, and nicotininate and nicotinamide metabolism pathways were significantly decreased ($P < 0.05$).

3.8 Correlation analysis of differential physiological and biochemical indicators, intestinal bacteria genera and KEGG differential functions

Through Spearman correlation analysis, the results showed that there were multiple significant correlations between the physiological and biochemical indicators of foals (Figure 8A) and the differential bacterial genera [($P < 0.05$), red indicates positive correlation, blue indicates negative correlation]. There was a significant correlation between the differential physiological and biochemical indicators of foals (Figure 8B) and the KEGG tertiary metabolic functions [($P < 0.05$), red indicates positive correlation, blue indicates negative correlation]. Interestingly, there was also a significant correlation between the differential bacterial genera of foals (Figure 8C) and the KEGG tertiary metabolic functions [($P < 0.05$), red indicates positive correlation, blue indicates negative correlation].

4 Discussion

ALDs are common bone development disorders in newborn foals, significantly impacting their future athletic performance and economic value. Inflammations during pregnancy, such as placentitis and metritis, as well as severe metabolic diseases, are significant causes of ALDs in newborn foals (11). The intestinal flora is closely linked to host health and disease (12). This study first investigated the current status of ALDs in newborn foals and, based on these findings, evaluated the changes in blood physiological and biochemical parameters of ALDs foals, used 16sRNA gene sequencing technology to analyze changes in the composition and function of their intestinal flora to reveal the relationship between intestinal flora and ALDs.

A survey of 460 newborn foals revealed that 19 foals (4.13%) were diagnosed with ALDs. Analyzing the incidence of ALDs among different breeds showed that purebred horses had the highest incidence (8.70%), followed by Ili horses (6.67%), with hybrid horses having a relatively low incidence (3.11%). Studies have shown that the incidence of ALDs in purebred horse populations can be as high as 11% (12). These findings suggest that horse breeds have varying susceptibility to ALDs, likely related to genetic factors. Due to long-term selective breeding, purebred horses may have accumulated more genetic variants that cause ALDs (13). In contrast, hybrid horses benefit from diverse genetic backgrounds, which reduces the risk of certain genetic diseases (14). These data emphasize the importance of personalized management and prevention. Purebred horses require more sophisticated management and monitoring, but hybrid horses should not be overlooked. Gender also influences the incidence of ALDs. The incidence rate in males (5.02%) is higher than in females (3.32%), which may be related to genetic factors, hormone levels, and metabolic processes (15). Genes on sex chromosomes (such as X and Y chromosomes) are associated with sex-specific disease susceptibility (16). If ALDs are related to genes on the Y chromosome, males (XY) and females (XX) would have different genetic susceptibilities (17). Additionally, male and female foals experience various levels of exercise, nutrition, and other management methods, indirectly affecting the development of ALDs. Studies have shown that male foals exercise more frequently in their early years, impacting limb development (18). The location of limb deformities is also related to the prevalence of ALDs. Results showed a high incidence of forelimb deformities (2.39%), with wrist deformities being the most common (1.74%). This may be related to the physiological structure and movement pattern of horses. When horses exercise, their forelimbs bear the most weight and impact,

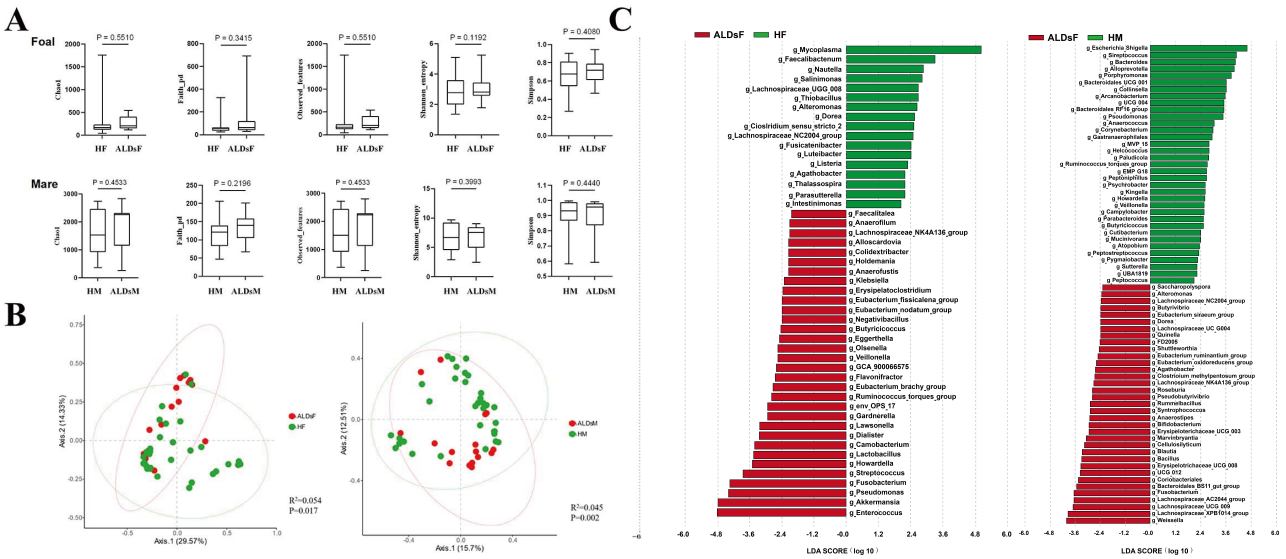

FIGURE 5
LefSe analysis of gut microbiota. **(A)** Comparison of the alpha diversity index of the intestinal flora in normal and ALDs foals (top) and in normal and ALDs mares (bottom). **(B)** Principal coordinate analysis of normal foals and ALDs foals (left), as well as normal mares and ALDs mares (right) based on Bray-Curtis distance. **(C)** LDA plots of LefSe analysis of normal foals and ALDs foals (left) and normal mares and ALDs mares (right). HF, healthy foals; ALDsF, ALDs foals; HM, healthy mares; ALDsM, ALDs mares.

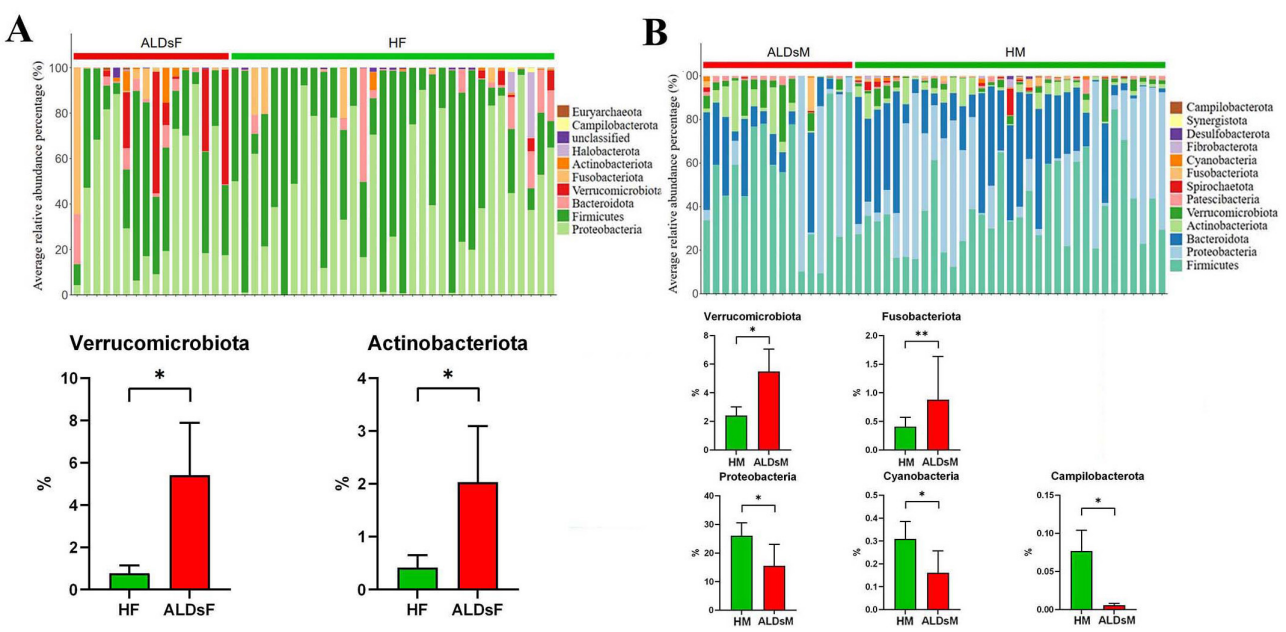

FIGURE 6
Analysis of microphyla composition and relative abundance of gut microbiota. **(A)** Bacterial phyla with an average relative abundance > 0.1% in normal and ALDs foals (above). Microphyla with significantly increased relative abundance in ALDs foals (below). **(B)** Bacterial phyla have an average relative abundance > 0.1% in normal and ALDs mares (above). Microphyla has significant increases and decreases in relative abundance in ALDs mares (below). HF, healthy foals; ALDsF, ALDs foals; HM, healthy mares; ALDsM, ALDs mares. $P < 0.05$ was considered statistically significant. $^{*}P < 0.05$; $^{**}P < 0.01$.

making them more deformable (19). Additionally, the anatomical structure of the forelimbs, such as the position and function of the wrist joint, is more vulnerable (20).

ALDs can be diagnosed based on clinical observation and imaging changes. The purpose of testing the physiological and biochemical indicators of ALD foals is to assist in observing the body state of ALD foals through changes in these indicators, so as to evaluate the physiological function and immune function of the foals. In this study, the white blood cell indexes of ALDs foals, such as Mon, Eos, Eos%, and Bas, were significantly higher than those

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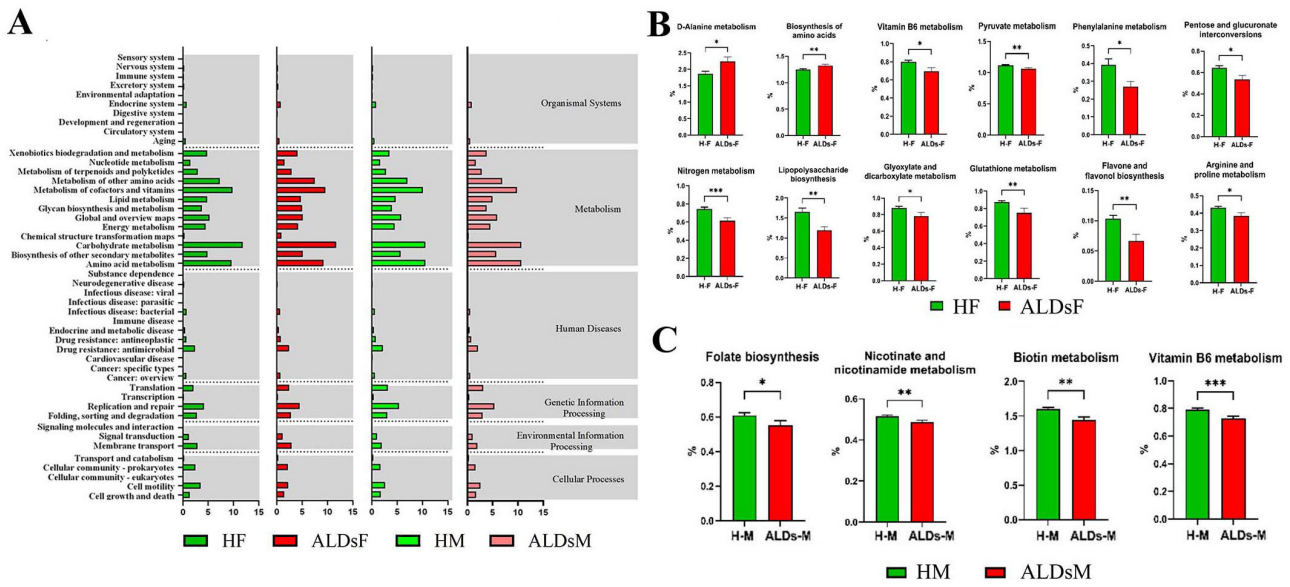

FIGURE 7
KEGG function of gut microbiota and differential analysis of tertiary metabolic function. **(A)** KEGG function prediction. **(B)** Comparison of metabolic functions of gut microbiota in normal foals and ALDs foals. **(C)** Comparison of metabolic functions of intestinal flora in normal mares and ALDs mares. HF, healthy foals; ALDsF, ALDs foals; HM, healthy mares; ALDsM, ALDs mares. $P < 0.05$ was considered statistically significant. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.

of normal foals. This result suggests that ALDs foals induce stress response due to limb abnormalities. GLU in the blood is essential for the synthesis of non-essential amino acids, which are further involved in the process of protein synthesis. In this study, we found that the GLU level of ALDs newborn foals was significantly lower than that of normal foals, indicating that the energy and nutritional level of ALDs newborn foals was insufficient. TP and ALB levels reflect the protein synthesis capacity of the liver and the maintenance of plasma osmotic pressure, and are important indicators of animal liver function and energy metabolism. ALP elevation is associated with changes in liver function and abnormal bone growth. The ALP level of ALDs foals was significantly higher than that of normal foals, indicating that ALDs foals had abnormal bone development. The role of calcium and phosphorus in bone formation and bone growth and development cannot be ignored. The serum Ca and P content of LDs newborn foals was significantly lower than that of normal foals, and the Ca and P levels were lower than the normal reference range, indicating that the deficiency of these mineral elements may directly affect the bone growth and development of foals. Combined with the above experimental results, this reflects that ALDs foals are suffering from malnutrition and mineral deficiency, which is speculated to be related to the occurrence of ALDs in foals. Although the use of hematological indicators alone cannot provide a specific early diagnosis of ALDs, changes in hematological indicators of ALDs foals can reflect the nutritional status of foals in areas with high incidence of ALDs. By judging the changes in the body state of ALDs foals, ALDs can be prevented and treated from multiple aspects and angles, providing a theoretical reference for nutritional supplementation and healthy breeding of newborn foals.

The intestinal flora comprises a complex ecosystem of microorganisms that convert nutrients entering the intestine into essential substances for normal bodily growth and development (21). It is crucial in maintaining normal digestion, nutrient absorption, energy conversion, and animal metabolic functions (22). In recent years, more and more attention has been paid to the effect of intestinal flora on animal bone development and its mechanism. Many studies have demonstrated the role of intestinal flora in nutrient absorption, immunity, endocrine function and metabolite production, especially on bone metabolism, providing a new way to prevent and treat animal bone diseases (23). Regarding the alpha diversity index, encompassing Chao1, Faith_pd, Observed_features, Shannon_entropy, and Simpson index, no significant differences were found between normal foals and those with ALDs, nor between normal mares and ALDs mares. This indicates comparable richness and uniformity in intestinal flora among the four groups at the alpha diversity level. Beta diversity, a critical indicator for assessing microbial composition differences across samples and communities, revealed distinct intestinal microbiota structures between ALD and normal foals and between normal mares and ALD mares (24). While the total count and distribution of microbiota were similar, specific microbial abundances differed between normal and ALD states, suggesting a potential link between intestinal microbiota and ALD occurrence. LefSe analysis identified 17 dominant microbiota in ALD foals, including mycoplasma, known to induce various diseases in animals and humans (25). Mycoplasma's presence may indirectly impact bone development by triggering chronic inflammation or affecting the host's immune response (26). *Faecalibacterium prausnitzii*, a beneficial bacterium within the *Faecalibacterium* genus, is associated with anti-inflammatory effects (27). This suggests a protective role in combating ALD-related inflammationby increasing anti-inflammatory microbiota (28). In ALD mares, 23 dominant bacterial groups were identified,

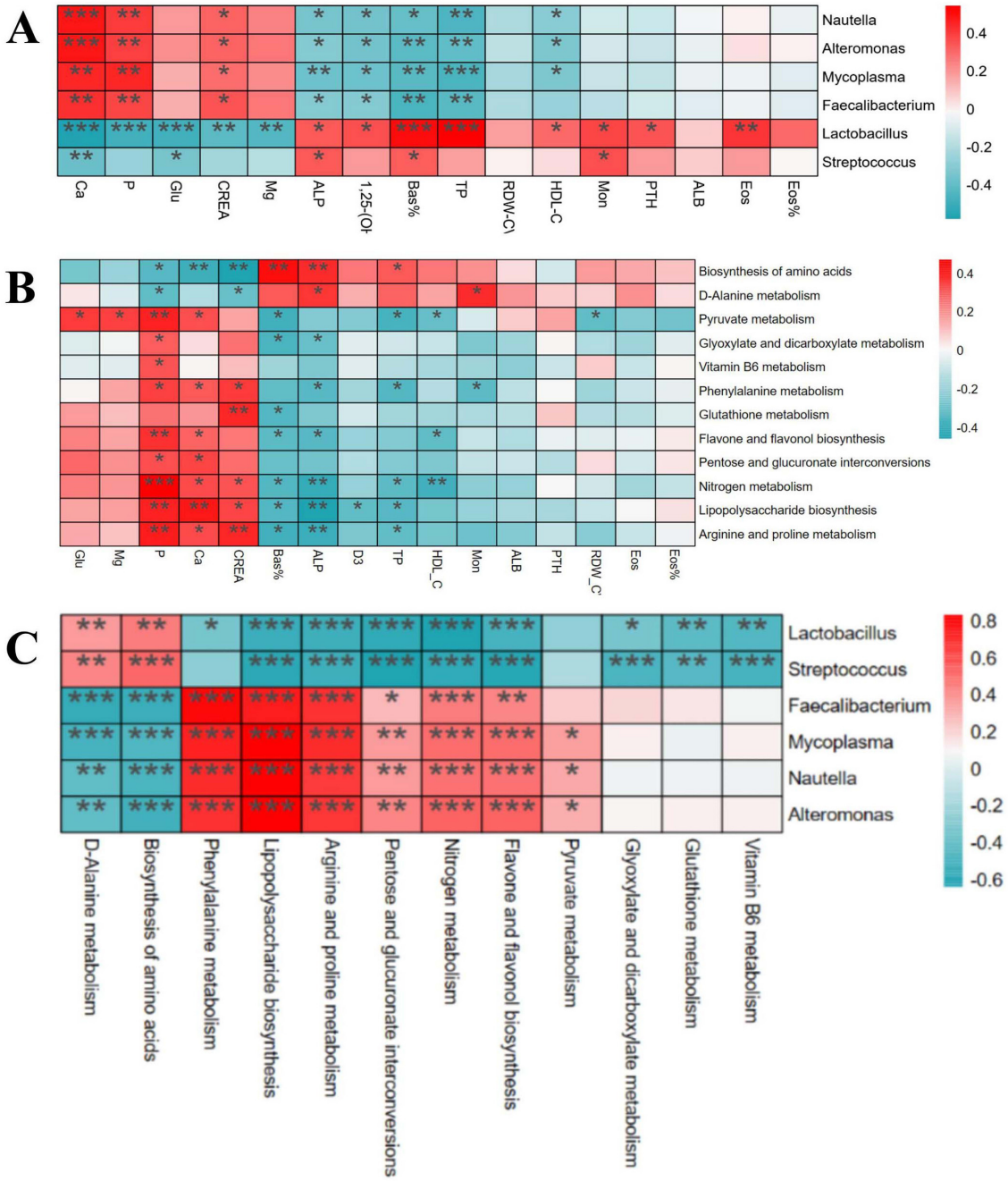

FIGURE 8
Correlation analysis of differential physiological and biochemical indexes, intestinal bacteria genera and KEGG differential function in foals. **(A)** Correlation analysis between different strains and different physiological and biochemical indexes. **(B)** Correlation analysis between different physiological and biochemical indexes and KEGG tertiary metabolic function. **(C)** Correlation analysis between different strains and KEGG tertiary metabolic function.

such as *Escherichia_Shigella* and *Streptococcus*, known pathogens capable of causing intestinal and systemic infections (29). Their dominance in ALD mares may correlate with their health status (30). Notably, ALD foals and mares exhibited more pathogenic bacteria in their intestines than their normal counterparts. *Streptococcus*, uncommon in normal intestinal flora, may play a significant role in the unique intestinal environment of ALD mares, potentially promoting ALD development. This similarity in the intestinal environment between ALD foals and mares suggests a potential influence of genetic or environmental factors on the occurrence of ALDs.

Further analysis of the phylum structure of the intestinal flora revealed that in both normal and ALD foals, the average relative abundance of *Proteobacteria*, *Firmicutes*, *Bacteroidota*, *Verrucomicrobiota*, and *Fusobacteriota* exceeded 1%, classifying them as high-abundance phyla. Similarly, in normal and ALDs

mares, *Firmicutes*, *Proteobacteria*, *Bacteroidota*, *Actinobacteriota*, *Verrucomicrobiota*, and *Patescibacteria* were high-abundance phyla. Notably, the abundance of *Verrucomicrobiota* and *Actinobacteriota* increased significantly in ALD foals. *Akkermansia muciniphila*, a bacterium in the phylum *Verrucomicrobia*, enhances intestinal barrier function, modulates immune responses, and is associated with reduced inflammation (31). The phylum *Actinobacteriota* includes many antibiotic-producing bacteria, such as *Bifidobacterium*, which can modulate the immune system and suppress inflammation (32). The increase in these bacteria in ALD foals suggests that the intestinal microbiome responds to the disease state by increasing beneficial bacteria to alleviate inflammation or mucosal damage caused by ALDs. Conversely, the phylum *Fusobacteria,* associated with various inflammatory diseases, increased, indicating an aggravated intestinal inflammatory state in ALD mares and a potential link to ALDs in foals (33).

Numerous recent studies have increasingly revealed the intricate link between intestinal microorganisms and various diseases (34). Particularly, the "gut-bone axis" concept underscores the complex interaction between intestinal flora and bone health. For example, intestinal flora regulates bone metabolism and health by influencing nutrient absorption, immune regulation, and hormone levels through its metabolic activities (23). Microbial metabolites, such as short-chain fatty acids (SCFAs), have positively impacted bone health by promoting calcium absorption and increasing bone density (35). Moreover, microorganisms can mitigate bone health issues by reducing inflammatory responses (36). These studies all demonstrate a close link between gut microbiota and bone metabolism. The results show that a total of 12 metabolic pathways were found to significantly differ between ALD and normal foals. Among these pathways, many can affect bone metabolism. D-alanine metabolism regulates bone energy metabolism by modulating insulin secretion and participating in blood glucose regulation in mammals (37). Abnormal nitrogen metabolism and the interconversion pathways of pentose and glucuronic acid, closely tied to energy metabolism, affect protein synthesis and degradation, thereby influencing bone health (37). Additionally, the downregulation of glutathione metabolism can induce oxidative stress, pivotal in the pathogenesis of growth and developmental diseases (38). These results are consistent with previous studies highlighting the critical role of the gut microbiome in host immune and metabolic processes, which directly affect bone development and health (39, 40). Similarly, ALD mares exhibit significantly distinct metabolic pathways compared to normal mares, particularly in biotin metabolism, vitamin B6 metabolism, folate biosynthesis, niacin, and nicotinamide metabolism. Biotin metabolism, crucial for synthesizing and degrading key biological molecules, influences fatty acid synthesis and amino acid metabolism, essential for normal bone development (41). Dysfunction in the folate biosynthesis pathway, essential for energy metabolism, can lead to various health issues, including congenital disabilities and osteoporosis (42). Vitamin B6 metabolism affects intestinal inflammation levels and the host immune response, which is critical for maintaining normal metabolism, especially anti-inflammatory responses (43). Niacin and nicotinamide metabolism are linked to energy metabolism and DNA repair, which are vital for maintaining bone cell health and function (44). Further, through correlation analysis, we found that *Lactobacillus* was negatively correlated with Ca, P, and Glu indices in ALDs foals, and was positively correlated with 1,25-(OH)2D3 and PTH. *Faecalibacterium* was positively correlated with Ca, P, and Glu indices, and negatively correlated with 1,25-(OH)2D3 and PTH. *Lactobacillus* and *Streptococcus* were positively correlated with Biosynthesis of amino acids, negatively correlated with Arginine and proline metabolism, and negatively correlated with Glutathione metabolism. This indicates that intestinal flora imbalance in foals is associated with the physiological and biochemical indicators of ALDs foals. These results suggest that mares may mediate skeletal development in foals by modulating their gut microbiota to influence their own nutrient absorption, immune regulation, and metabolite production. Therefore, it is speculated that gut microbiota may be involved in the pathogenesis of ALDs through its role in the "gut-bone axis".

In summary, blood markers such as calcium, phosphorus, and blood glucose were altered in ALDs foals, and the diversity, species composition, and function of the intestinal flora of ALDs and their mares were significantly altered. Compared with ALD foals, normal foals showed significant differences in *Lactobacillus* and *Faecalibacterium*, which could become potential markers of ALDs. Compared with ALD mares, normal mares showed significant differences in *Escherichia-Shigella* and *Pseudomonas*, which could become potential markers of ALDs. We speculate that there may be a pathogenic mechanism of ALDs in foals through the "blood marker changes-gut microbiota-ALDs" axis, but further mechanistic studies are needed in the future. In conclusion, these findings provide new insights into the interaction between gut microbiota and bone health and provide possible targets for the development of early diagnosis techniques and prevention and treatment strategies.

Data availability statement

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

Ethics statement

The animal studies were approved by the Animal Welfare and Ethics Committee of Xinjiang Agricultural University. The studies were conducted in accordance with the local legislation and institutional requirements. Written informed consent was obtained from the owners for the participation of their animals in this study.

Author contributions

YM: Conceptualization, Data curation, Funding acquisition, Writing – original draft. YL: Data curation, Formal analysis,

Writing – original draft. HL: Formal analysis, Methodology, Writing – original draft. KY: Writing – review & editing. GY: Writing – review & editing.

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Conflict of interest

YM was employed by Xinjiang Zhaosu County Xiyu Horse Industry Co., Ltd.

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

Generative AI statement

The authors declare that no Generative AI was used in the creation of this manuscript.

Publisher's note

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Effect of fructo-oligosaccharides on growth performance and meat quality in broilers

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This study investigated the fructo-oligosaccharides (FOS) on growth performance and meat quality in broilers. Total 160 Xianghuang broilers aged 2 months were randomly assigned into 2 groups, CON (control), FOS (supplemented 0.5% fructo-oligosaccharides in diet). After 38 days, the breast, thigh muscle and liver samples were collected for further analysis. Results showed that no significant effect of 0.5% FOS on growth performance such as average daily gain (ADG), average daily feed intake (ADFI) or feed-to-gain ratio (F:G) were observed ($P > 0.05$). Broilers in FOS group had a yellower breast than that in CON group ($P < 0.05$). Breast pH_{45min} and thigh pH_{24h} value of FOS group were greater than that in CON group ($P < 0.05$). Max shear force and work of shear of cooked breast (*pectoralis major*) muscle was lower in FOS group compared with CON group ($P < 0.05$). Hardness ($P = 0.065$), fracturability ($P = 0.063$), gumminess ($P = 0.079$), chewiness ($P = 0.080$) of cooked thigh meat tended to be higher in FOS group compared to the CON group. Addition of 0.5% FOS resulted in lower thigh total superoxide dismutase (T-SOD) activity compared to CON group ($P < 0.05$). The malonaldehyde (MDA) concentration ($P = 0.066$) of breast muscle tended to be lower in FOS group compared with CON group. There was an increasing trend for total antioxidant capacity (T-AOC) activity of thigh muscle in FOS group compared to CON group ($P = 0.053$). Relative mRNA expression of breast catalase (CAT), superoxide dismutase 1 (SOD1), thioredoxin reductase 1 (TXNRD) were up-regulated by FOS supplementation compared with CON group ($P < 0.05$). In conclusion, FOS can be utilized at 0.5 % to improve meat quality such as elevating pH value, yellowness and decreasing max shear force of muscle through enhancing the antioxidant activity in broilers.

KEYWORDS

fructo-oligosaccharides, growth performance, texture characteristics, myofibrillar morphology, muscle

1 Introduction

Fructo-oligosaccharide is water-soluble dietary fiber which formed by D-fructose and sucrose binding by β-1,2 glycosidic bonds (1). It exists in wheat, potatoes, onion, garlic, bananas and other plants. FOS was reported as involved in the fat metabolism through mobilizing the intestinal bacteria and their metabolites. Supplementation of 1 g FOS per liter of water increased the mRNA expression of genes related to fat digestion and absorption, leucine and isoleucine biosynthesis in ileal mucosa of Taiping chickens (2). Supplemented with

5 g/kg FOS significantly inhibited cecal *E.coli* growth in 3-and 5-wk-old broilers (3), increased microbial diversity of ileal mucosa in 21-day-old broilers when compared with wheat-corn-soybean meal based diet (4). Cecal abundance of *Escherichia coli* decreased but *Bifidobacterium* spp. and *Lactobacillus* spp. increased after supplementation of FOS and beneficial microorganisms (*Bifidobacterium animalis, Enterococcus faecium, Lactobacillus reuteri, Pediococcus acidilactici*) in heat-stressed broilers (5). Increasing colonization of *B. subtills* in broilers' gastrointestinal tract would be beneficial to their musculoskeletal health (6). Visual appearance of broilers' thigh muscles was improved by *Lactobacillus* through increasing xanthophyll accumulation in soft tissues (7). *B. subtilis*-fed broilers had greater water holding capacity, better taste (flavor, texture, preference, and general aspect) in leg muscle, and these probiotic effects were greater in 0.5 g/kg group than in the 0.25 g/kg group (6). Further, broilers muscle is rich in polyunsaturated fatty acids (8), which makes it sensitive to oxidative deterioration. Due to the effect on bacterial fermentation in the intestine, mineral absorption increased when broilers supplemented with 0.4% (9) or 0.5% (10) FOS (11). Supplemented with coated trace minerals (Cu, Fe, Mn, Zn, Se) in broilers' diet could decrease both serum and muscle MDA levels and then reduce drip loss of meat (12). Mineral element Zn and Cu is essential for SOD activity. Antioxidant enzymes such as SOD and glutathione peroxidase are able to protect polyunsaturated fatty acids in chicken muscle from free radicals and reactive oxygen species damage. Whether meat quality even meat texture could be improved by this 0.5% relatively high dosage FOS supplementation in broilers is still not well known.

There was positively correlation between the ratio of type I myofiber and antioxidized activities, pH value postmortem, intramuscular fat and saturated fatty acid (SFAs) content in Yak beef *Semitendinosus* muscles (13). Type IIB myofiber was fast glycolytic myofiber, it contained two-thirds of myoglobin as type I fibers (14), leading to a paler meta color. Compared to glycolytic-type fiber (Type IIX and IIB), oxidative-type muscle fibers (Type I and IIA) had smaller diameters and higher density (15), which contributes to decrease in shear force and increase in meta tenderness (16). Xianghuang broiler is a slower growing breed. Results showed that the breast (*pectoralis major*, PM) muscle only made up of type IIB fibers in slow-growing Xueshan chicken and fast-growing Ross 308 broiler (17) or Japanese quail (18) but little type I fibers could be found in thigh (*gastrocnemius*, GAS) muscle of Xueshan and Ross 308 broilers (17). If breast muscle and thigh muscle of Xianghuang broilers respond different to this relatively high dosage of FOS still need to further study. Therefore, we performed a comparative analysis of the effect of 0.5% of dietary FOS on breast and thigh muscle. The objectives of the current work were to evaluate the effect of dietary FOS on growth performance and meat quality in Xianghuang broilers. We hypothesized that high dosage FOS supplementation would improve meat quality through affecting muscle metabolic and antioxidant function in broilers.

2 Materials and methods

2.1 Animal ethic statement

Animal work was approved by the Animal Care and Use Committee of Hengyang Normal University, protocol HNUACUC-B202201005.

2.2 Animals and experimental treatments

A total of 160 male Xianghuang broilers (0.876 ± 0.149 kg, 2 months old) were randomly assigned to 2 treatments. Each treatment had 8 replicates with 10 broilers per replicate cage. Broilers were fed a corn-soybean meal-based diet (Table 1) that met the nutritional recommendations for yellow-feathered broilers (19), but with or without 0.5% fructo-oligosaccharides, and named as FOS or CON, respectively. FOS was kindly provided by Shandong Longli Biological Technology Co., Ltd. (Shandong, China). Broilers were raised in floor commercial pens (about 0.1 m^2/bird) with free access to semi-powder semi-pellet feed and water over the total period of 38 days. Room temperatures were maintained at 22°C by indoor air conditioning. Light was provided for 16 h at 10 lux throughout the experimental period.

2.3 Sample collection

All birds were weighed every week per replicate cage. Feed intake/leftover was recorded every day. Body weight gain and feed conversion ratio were calculated. On d 38, all birds were weighed individually and 2 medium-weight birds per cage were randomly taken and euthanized by carbon dioxide and then cervical dislocation. Liver, boned right breast and thigh muscle were weighed and their percentage were calculated as hot tissue weight/live body weight × 100%. After weighing the eviscerated carcasses, the giblets were removed and the head and toes of the chicken were preserved. Left breast and thigh muscles of 5 cm length were removed along the breastbone and placed in 4% paraformaldehyde for histological analysis. Residual muscle and

TABLE 1 Calculated ingredient composition of Xianghuang broilers' diets (%, as-fed basis).

Formular		Nutrient levels[1]	
Name	Content	Name	Content
Ingredients		ME, kcal/kg	2,800
Corn	69.42	CP	15.50
Soybean meal (43% CP)	22.37	Ca	0.90
Wheat bran	2.96	P	0.60
Limestone	1.39	Digestible P	0.41
$CaHPO_4$	1.55	NaCl	0.30
Vitamin premix[2]	1.00	Lys	0.73
Mineral premix[2]	1.00	Met+Cys	0.55
NaCl	0.27	Thr	0.64
Met	0.04	Trp	0.20
Total	100.00		

[1]Nutrient levels were all calculated values, and amino acids were standardized ileal digestible amino acids.

[2]Provided the following quantities of vitamins and micro-minerals per kilogram of complete diet: vitamin A as retinyl acetate, 4,000 IU; vitamin D_3 as cholecalciferol, 800 IU; vitamin E as DL-alpha tocopheryl acetate, 8 IU; vitamin K as menadione dimethylpyrimidinol bisulfite, 0.5 mg; thiamin as thiamine mononitrate, 1.0 mg; riboflavin, 1.8 mg; pyridoxine as pyridoxine hydrochloride, 3.0 mg; vitamin B_{12}, 3.0 μg; D-pantothenic acid as D-calcium pantothenate, 10.0 mg; niacin, 11.0 mg; folic acid, 0.25 mg; biotin, 0.1 mg; choline chloride 900 mg; Cu, 8 mg as copper sulfate; Fe, 80 mg as ferrous sulfate; I, 0.35 mg as ethylenediamine dihydride; Mn, 60 mg as manganese sulfate; Se, 0.3 mg as sodium selenite; and Zn, 60 mg as zinc sulfate.

liver were collected and stored frozen (−80°C) until gene analyses and enzymes analyses.

2.4 Meat quality and nutrient measurements

Meat color such as lightness (L*), redness (a*), yellowness (b*) were determined at 45 min and 24 h postmortem on left 3 cm thick deboned muscle sample using colorimeter (CR-410, Kinica Minolta Sensing Inc., Osaka, Japan). The evaluation was carried out three times on the posterior surface of the skinless breast and thigh muscle. The pH measurement was taken from three different regions of each muscle with portable pH probe (Matthaus pH Star, Germany). Drip loss of muscle was measured as follows, approximately 2 g of left fillet was weighed and suspended on a barbless hook in an inverted plastic cup, suspended for 24 h at 4°C before being removed from the hook, and reweighed. Approximately 5 g of right muscle was weighed, cooked on a steamer, boiling water (95°C) vapor in the bottom of the steamer rise and through the pore to boil the meat for 30 min until the inner temperature reached to 70°C, they were reweighed after these cooked samples cooled to room temperature, and cooking loss was expressed as percentage loss during cooking. Cooked samples were placed in silver paper and held at −20°C until texture profile analysis (TPA) and shear force analysis. Muscles and liver were freeze-dried for 72 h (YAMATO DC801, Japan). Crude fat content was extracted by petroleum ether under Soxhlet extraction method (20). Crude protein content were determined by Kjeldahl method (20).

2.5 Myofibrillar morphology

Muscle samples from the 4% polyformaldehyde were washed in running water overnight, treated with increasing concentrations of ethanol, transparence with xylene and embedded in solid paraffin. Slides of 5 μm thick were obtained on rotary microtome (Leica RM2135, Leica Microsystems, Wetzlar, Germany), and then hematoxylin and eosin staining. Images were recorded by Leica inverted microscope (Leica DM500) with camera (Leica MC170 HD). Fiber diameter, cross-sectional area and density were analyzed from 80 fibers per broiler using Image-Pro Plus software (Media Cybernetics Inc., Silver Spring, MD).

2.6 Shear force and texture parameters

Raw and cooked breast and thigh muscle were cut into 1.5 cm × 1.5 cm × 0.5 cm (height) parallel to the muscle fiber orientation 1 day postmortem. Shear force of muscle or meat were measured using Warner-Bratzler HDP/BSW under toughness program fitted with a 50-kg load cell on Texture Analyzer (TA. XT. Plus. Stable Micro systems, United Kingdom). Test settings included a button type trigger, 62 mm travel distance, 2 mm/s test speed, and 10 mm/s post-test speed (21). Max shear force (kg) and total shear energy (work of shear, kg.sec) were recorded.

Texture profile analysis (TPA) of muscle and meat was measured on Texture Analyzer (TA. XT. Plus. Stable Micro systems, United Kingdom) using probe P36R under TPA program. Testing conditions were as follows, holding time was 2 s, trigger force was 0.1 g, test speed was 5.0 mm/s (pre-test), 1 mm/s (test), and 5.0 mm/s (post-test) to reach a 50% compression (22). TPA parameters including hardness, fracturability, adhesiveness, springiness, cohesiveness, gumminess, chewiness, resilience were calculated from the Texture Expert version 1.0 software. Measurements were performed in triplicate for each meat sample and the average value was used for statistical analysis.

2.7 Antioxidant status measurement

Approximately 0.5 g fresh muscle or liver were homogenized in 4.5 mL of 0.9% NaCl solution using tissue grinder (SCIENTZ-12, Xinzhi Biotech logy, Ningbo, China), and then centrifuged (2,500 r/min (1845 g), 15 min, 4°C) to collect supernatant. Activities of total antioxidant capacity (T-AOC), superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GSHPX) and MDA concentration were tested according method mentioned in Tan et al. (23). Briefly, activity of T-AOC (mmol/L) was analyzed using its OD 593 nm value compared with standard curve of $FeSO_4$. The Unit of CAT activity was defined as mg of hydrolyzed H_2O_2 in 1 min per mg protein of sample. One Unit of SOD enzyme was defined as the amount of enzyme that inhibits 50% of lighting reaction of nitroblue tetrazolium. Supernatant was extracted in 10% trichloroacetic acid and then was used to test MDA concentration. Protein concentrations were determined using Bradford method with bovine albumin as the standard.

2.8 Gene expression analysis

Total RNA from muscle and liver was extracted using Trizol reagent (Takara, Dalian, China). Sample concentration and quality were determined on BioSpec-nano (Shimadzu, Japan). 1.0 μg of total RNA was reverse-transcribed into cDNA using the Reverse Transcription Reagent Kit (Aikerui, Changsha, China). The mRNA expression levels of genes (Table 2) were determined using Real-time PCR performed on an QuantStudio 3 (Applied Biosystems, Branchburg, NJ) using SYBR Green quantitative PCR mix (Aikerui, Changsha, China). The $2^{-\triangle\triangle Ct}$ method (24) was used to calculate the gene expression relative to β-actin which was used as housekeeping gene.

2.9 Statistical analysis

Pen was considered as the experimental unit. All experimental data were analyzed by One-way ANOVA procedure of SAS 8.2 software package (SAS Inst. Inc., Cary, NC). Differences between the means were determined with t tests. Data were presented as mean ± standard error. A value of $P < 0.05$ was considered significant and $0.05 < P < 0.10$ was reported as a trend.

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

Name[1]	Sequence (5–3′)	Product length	NCBI reference sequence
β-actin	F: CATTGTCCACCGCAAATGCT	108	NM_205518. 1
	R: AGCCATGCCAATCTCGTCTT		
HMOX1	F: ACACCCGCTATTTGGGAGAC	167	NM_205344.1
	R: AAGGGCATTCATTCGGGACC		
NFE2L2	F: ATGTCACCCTGCCCTTAGAG	189	NM_205117.1
	R: TGCAGAAGAGGTGATGACGG		
CAT	F: GCCACATGGTGACTACCCTC	107	NM_001031215.2
	R: TGTTGCTAGGGTCATACGCC		
SOD1	F: CACGGTGGACCAAAAGATGC	123	NM_205064.1
	R: GATGCAGTGTGGTCCGGTAA		
NQO1	F: GAGCGAAGTTCAGCCCAGTAT	151	NM_001277619.1
	R: CATGGCGTGGTTGAAAGAGG		
TXNRD1	F: ATCGCTATGGCTGACCTGTG	136	NM_001030762.3
	R: GGTGGCTAACTCCCCTCTTG		
IL1β	F: TGCCTGCAGAAGAAGCCTCG	204	NM_204524.1
	R: GACGGGCTCAAAAACCTCCT		
IL8L2	F: CCTAACCATGAACGGCAAGC	174	NM_205498.1
	R: CTTGGCGTCAGCTTCACATC		
TNFα	F: GGGACGGCCTTTACTTCGTA	113	MF000729.1
	R: GTCTTTGGGGTACTCCTCGG		

[1]HMOX1, heme oxygenase 1; NFE2L, nuclear factor, erythroid 2 like 2; CAT, catalase; SOD1, superoxide dismutase 1; NQO1, NAD(P)H quinone dehydrogenase 1; TXNRD, thioredoxin reductase 1; IL1β, interleukin 1, beta; IL8L2, interleukin 8-like 2; TNFa, tumor necrosis factor alpha.

3 Results

3.1 Growth performance

Growth performance such as ADFI, ADG or F:G was not affected by dietary FOS treatment ($P > 0.05$) (Table 3).

3.2 Carcass traits

Dietary FOS supplementation did not affect breast or thigh muscle yield, liver weight and percentage, eviscerated carcass yield of Xianghuang broilers ($P > 0.05$) (Table 4).

3.3 Meat quality

Breast filets from FOS birds had higher b^*_{24h} value than that in CON group ($P < 0.05$) (Table 5). The pH_{45min} value of breast fillet and pH_{24h} of thigh fillet were significantly higher for FOS broilers when compared to CON broilers ($P < 0.05$). For breast muscle, no significant difference was observed on drip loss, cooking loss, pH_{24h}, meat color (L*, a*, b*) at 45 min, L* and a* index at 24 h between treatments ($P > 0.05$). There was no significant difference in drip loss, pH_{45min} value, meat color at 45 min and 24 h of thigh muscle ($P > 0.05$). Cooking loss of thigh fillet tended to be affected by diet ($P = 0.071$), with decreased value occurring in FOS birds compared to CON group. Crude protein and fat content of breast and thigh muscle or liver were not affected by FOS supplementation ($P > 0.05$) expect that FOS group had lower crude protein content in liver compared to CON group ($P < 0.05$).

3.4 Myofibrillar morphology

Dietary FOS supplementation did not affect fiber diameter (Figure 1A), cross-sectional area (Figure 1B) of breast (Figures 2A,B) or thigh muscle (Figures 2C,D) ($P > 0.05$). Muscle breast fiber density in FOS group tended to be higher than that in CON group (Figure 1C) ($P = 0.078$), but the fiber density of thigh muscle was the same between FOS and CON group ($P > 0.05$).

3.5 Textural parameters

For toughness parameters, there were no significant differences between groups in regards to max shear force and work of shear of fresh breast (*pectoralis major*, PM) and thigh (*gastrocnemius*, GAS) muscle in broilers ($P > 0.05$) (Figure 3A). Max shear force and work of shear of cooked breast muscle was lower in FOS group compared with CON group ($P < 0.05$) (Figure 3B).

Fresh breast muscle in FOS group showed higher resilience compared to the CON group (Table 6). Adhesiveness of fresh thigh muscle tended to decrease ($P = 0.080$) in FOS group compared with CON group. Hardness ($P = 0.065$), fracturability ($P = 0.063$), gumminess ($P = 0.079$), chewiness ($P = 0.080$) of cooked thigh meat tended to be higher in FOS group compared to the CON group. Whereas, no

TABLE 3 Effect of fructo-oligosaccharides on growth performance of Xianghuang broilers[1].

Items[1]	Control	FOS	*P*-value
Initial BW, day 85, kg	0.85 ± 0.03	0.90 ± 0.13	0.495
Final BW, day 123, kg	1.20 ± 0.04	1.34 ± 0.13	0.084
First 10 days			
ADFI, g/d	69.75 ± 4.50	66.38 ± 2.25	0.228
ADG, g/d	8.69 ± 1.39	9.13 ± 2.32	0.758
F:G, g/g	8.15 ± 1.07	7.65 ± 2.01	0.682
Week 2			
ADFI, g/d	69.78 ± 5.15	64.64 ± 6.74	0.271
ADG, g/d	9.11 ± 3.12	10.36 ± 1.96	0.523
F:G, g/g	8.52 ± 3.37	6.33 ± 0.73	0.252
Week 3			
ADFI, g/d	82.66 ± 3.83	82.29 ± 4.87	0.908
ADG, g/d	8.48 ± 4.78	5.63 ± 1.73	0.304
F:G, g/g	12.03 ± 5.74	15.51 ± 3.84	0.352
Week 4			
ADFI, g/d	79.8 ± 6.05	77.13 ± 9.19	0.645
ADG, g/d	11.88 ± 1.38	11.16 ± 2.53	0.638
F:G, g/g	6.82 ± 1.19	7.17 ± 1.66	0.740
Week 5			
ADFI, g/d	80.07 ± 14.34	89.07 ± 9.04	0.329
ADG, g/d	8.04 ± 3.00	22.5 ± 20.59	0.214
F:G, g/g	7.24 ± 2.71	6.78 ± 1.72	0.783
Total 38 days			
ADFI, g/d	75.89 ± 3.51	75.15 ± 1.56	0.714
ADG, g/d	9.19 ± 0.42	11.55 ± 3.81	0.266
F:G, g/g	8.26 ± 0.30	8.44 ± 1.5	0.817

[1]BW, body weight; ADG, average daily gain; ADFI, average daily feed intake; F:G, ratio of feed to gain. FOS 0.5%.

TABLE 4 Effect of fructo-oligosaccharides on carcass parameters in Xianghuang broilers.

Items	Control	FOS	*P*-value
Weight, g			
Body weight, Kg	1.15 ± 0.18	1.16 ± 0.09	0.886
Right breast muscle	71.89 ± 13.68	67.95 ± 7.93	0.523
Right thigh muscle	77.82 ± 15.90	78.94 ± 15.16	0.895
Liver	19.64 ± 5.58	17.08 ± 2.90	0.302
Full net chamber[1]	882.56 ± 119.08	849.75 ± 104.94	0.594
Ratio to body weight, %			
Right breast muscle	6.23 ± 0.44	5.87 ± 0.79	0.315
Right thigh muscle	6.73 ± 0.42	6.79 ± 1.18	0.892
Liver	1.69 ± 0.25	1.47 ± 0.21	0.100
Eviscerated carcass yield	77.3 ± 8.66	73.15 ± 7.76	0.365

[1]Head and chicken toe were preserved when eviscerated carcass were weighed.

TABLE 5 Effect of fructo-oligosaccharides on meat quality in Xianghuang broilers.

Items	Control	FOS	*P*-value
Breast muscle			
Drip loss, %	1.93 ± 0.54	1.78 ± 0.53	0.613
Cooking loss, %	32.13 ± 2.36	32.18 ± 1.27	0.957
$pH_{45\ min}$	6.21 ± 0.21^{B}	6.48 ± 0.24^{A}	0.041
$pH_{24\ h}$	5.74 ± 0.09	5.78 ± 0.10	0.481
Lightness $(L^*)_{45\ min}$	53.01 ± 4.2	53.87 ± 1.33	0.615
Redness $(a^*)_{45\ min}$	2.92 ± 1.91	2.78 ± 0.89	0.863
Yellowness $(b^*)_{45\ min}$	9.44 ± 1.99	10.84 ± 1.21	0.137
$L^*_{24\ h}$	60.92 ± 3.28	59.00 ± 3.60	0.317
$a^*_{24\ h}$	1.86 ± 1.48	2.1 ± 0.86	0.712
$b^*_{24\ h}$	10.24 ± 2.04^{B}	13.15 ± 2.30^{A}	0.032
Crude fat[1], %	2.25 ± 0.78	3.13 ± 1.17	0.126
Crude protein[1], %	22.41 ± 2.09	22.96 ± 2.45	0.661
Thigh muscle			
Drip loss, %	1.82 ± 0.59	2.11 ± 0.73	0.432
Cooking loss, %	42.7 ± 5.73	35.85 ± 5.97	0.071
$pH_{45\ min}$	6.55 ± 0.24	6.58 ± 0.11	0.755
$pH_{24\ h}$	6.02 ± 0.17^{B}	6.22 ± 0.12^{A}	0.003
$L^*_{45\ min}$	51.71 ± 1.4	50.4 ± 2.76	0.286
$a^*_{45\ min}$	5.12 ± 1.48	5.63 ± 0.78	0.430
$b^*_{45\ min}$	7.86 ± 1.22	7.79 ± 3.80	0.962
$L^*_{24\ h}$	54.58 ± 6.48	50.72 ± 3.3	0.186
$a^*_{24\ h}$	5.98 ± 1.92	6.88 ± 4.32	0.625
$b^*_{24\ h}$	10.29 ± 6.07	9.94 ± 2.94	0.893
Crude fat[1], %	2.44 ± 1.08	2.61 ± 0.72	0.729
Crude protein[1], %	21.49 ± 3.74	19.83 ± 4.32	0.458
Liver			
Crude fat[1], %	3.90 ± 0.58	3.16 ± 1.16	0.155
Crude protein[1], %	28.65 ± 2.35^{A}	25.06 ± 1.19^{B}	0.004

[1]Crude protein and crude fat concentrations were given by fresh muscle or liver sample. Values in a row without common superscripts differ significantly at $P < 0.05$.

significant differences were observed in the other TPA parameters such as adhesiveness, springiness, cohesiveness, resilience between the FOS and CON groups within fresh and cooked muscle ($P > 0.05$).

3.6 Antioxidant function

The MDA concentration and T-SOD activity of breast muscle tended to be lower in FOS group compared with CON group ($P = 0.066$) (Table 7). Activities of T-AOC, CAT, GSHPX of breast muscle did not differ significantly from each other ($P > 0.05$). Broilers in FOS group showed lower T-SOD activity in thigh muscle compared with CON group ($P < 0.05$). There was an increasing trend for T-AOC activity of thigh muscle in FOS group compared to CON group ($P = 0.053$). There were no significant differences in concentration of MDA and activities of CAT, GSHPX of thigh

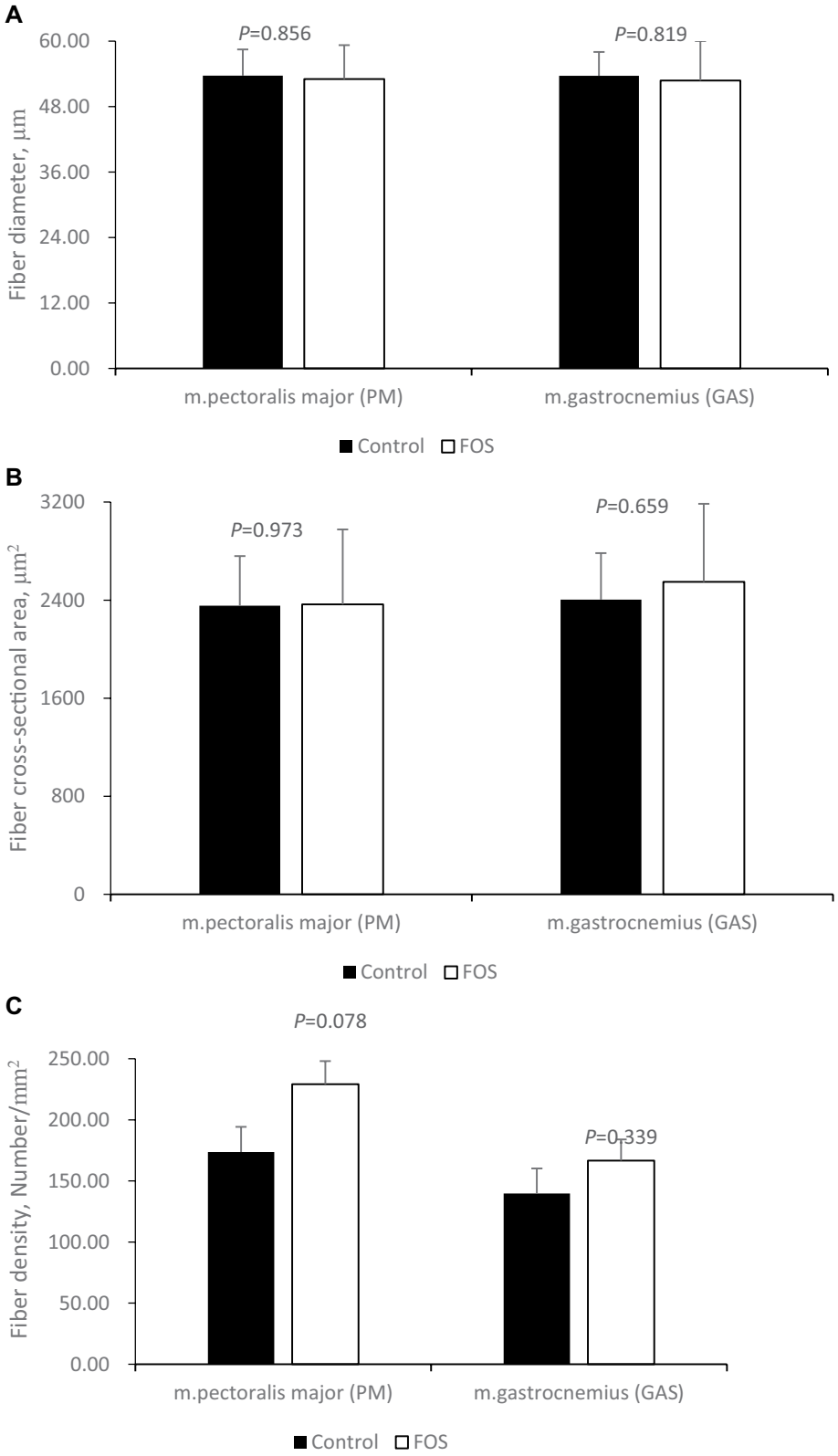

FIGURE 1
Muscle fiber morphology traits in breast (*pectoralis major*, PM) and thigh (*gastrocnemius*, GAS) muscle from control or 0.5% fructo-oligosaccharide (FOS) groups of Xianghuang broilers. Fiber diameter (**A**, μm), fiber cross-section area (**B**, μm^2), fiber density (**C**, Number/mm^2).

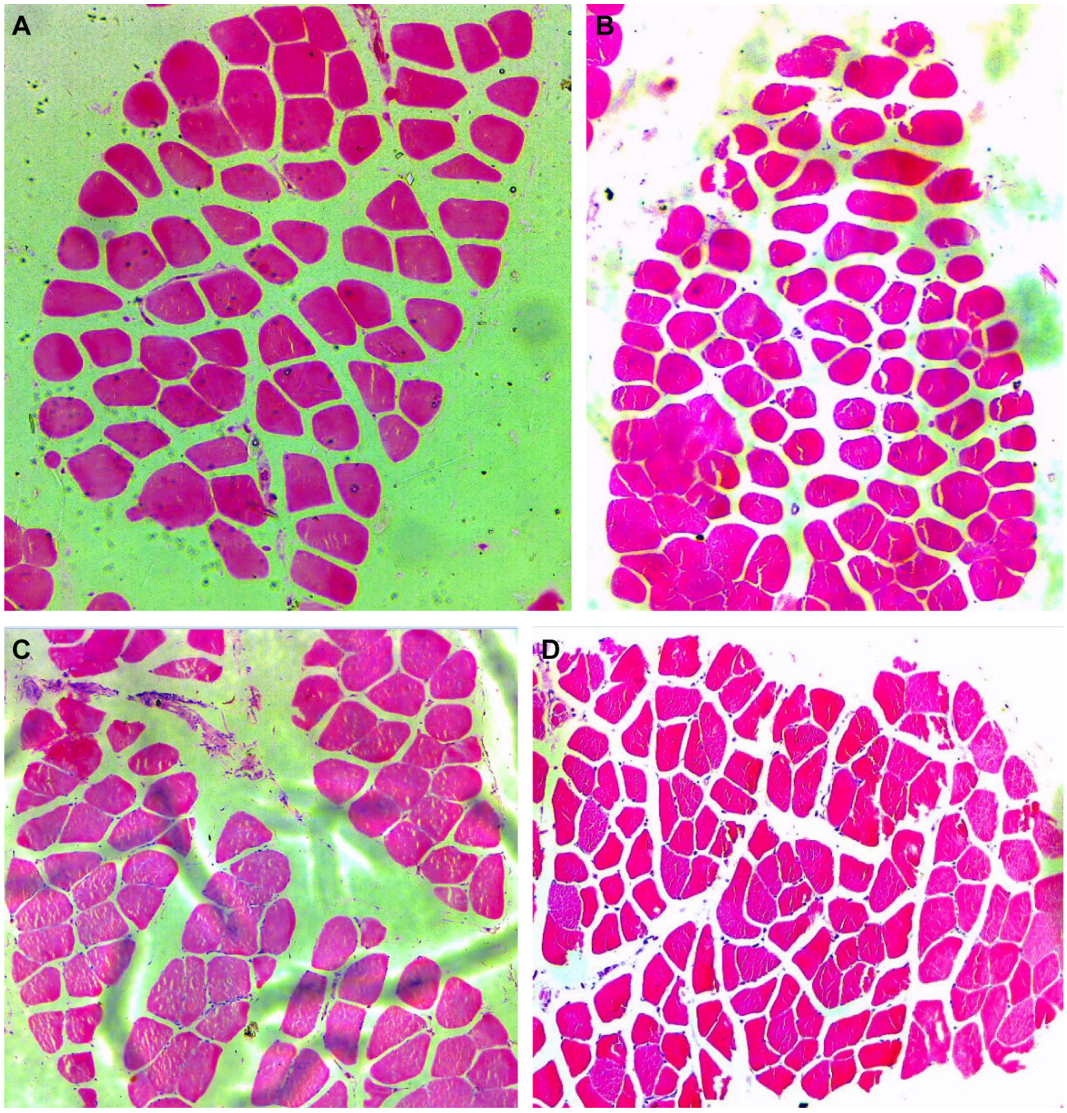

FIGURE 2
Hematoxylin and eosin staining in breast (*pectoralis major*, PM) and thigh (*gastrocnemius*, GAS) muscle from control or 0.5% fructo-oligosaccharide (FOS) groups of Xianghuang broilers. Magnification of 10*10 was used. PM in control group **(A)**, PM in FOS group **(B)**, GAS in control group **(C)**, GAS in FOS group **(D)**.

muscle between FOS and CON groups ($P > 0.05$). Dietary FOS supplementation did not affect MDA concentration and activities of T-AOC, T-SOD, CAT in liver of broilers ($P > 0.05$).

3.7 Gene expression

Expression of genes related to inflammation and antioxidant function in muscle and liver were shown in Table 8. Hepatic genes' mRNA expression such as heme oxygenase 1 (HMOX1), nuclear factor, erythroid 2 like 2 (NFE2L), CAT, SOD1, NAD(P)H quinone dehydrogenase 1 (NQO1), thioredoxin reductase 1 (TXNRD), interleukin 1, beta (IL1β), interleukin 8-like 2 (IL8L2), tumor necrosis factor alpha (TNFa) were not affected by dietary FOS supplementation ($P > 0.05$). The mRNA expression of NFE2L, CAT, SOD1, NQO1,TXNRD were higher in FOS-fed broiler breast compared to the CON diet ($P < 0.05$). There was no significant difference in HMOX1, IL1β, IL8L2, TNFa mRNA expression in breast muscle between FOS and CON groups ($P > 0.05$). In thigh samples, expressions of HMOX1, TXNRD, SOD1 were down-regulated by FOS supplementation compared to the control group ($P < 0.05$). Birds from FOS group expressed higher IL1β in thigh muscle than that in CON group ($P < 0.05$). Gene expression of NFE2L, CAT, NQO1, and IL8L2 in thigh muscle were not affected by dietary FOS treatment ($P > 0.05$).

4 Discussion

Dietary FOS supplementation at 0.5% (5 g/kg) did not affect growth performance during late-growing period. This was the same that FOS did not affect ADG of broilers at 0.5% when compared with control group (25). Our earlier report showed that 200 mg/kg FOS had positive effect on ADG during first 5 weeks in chicken (26). No significant differences in breast, thigh yields were reported after

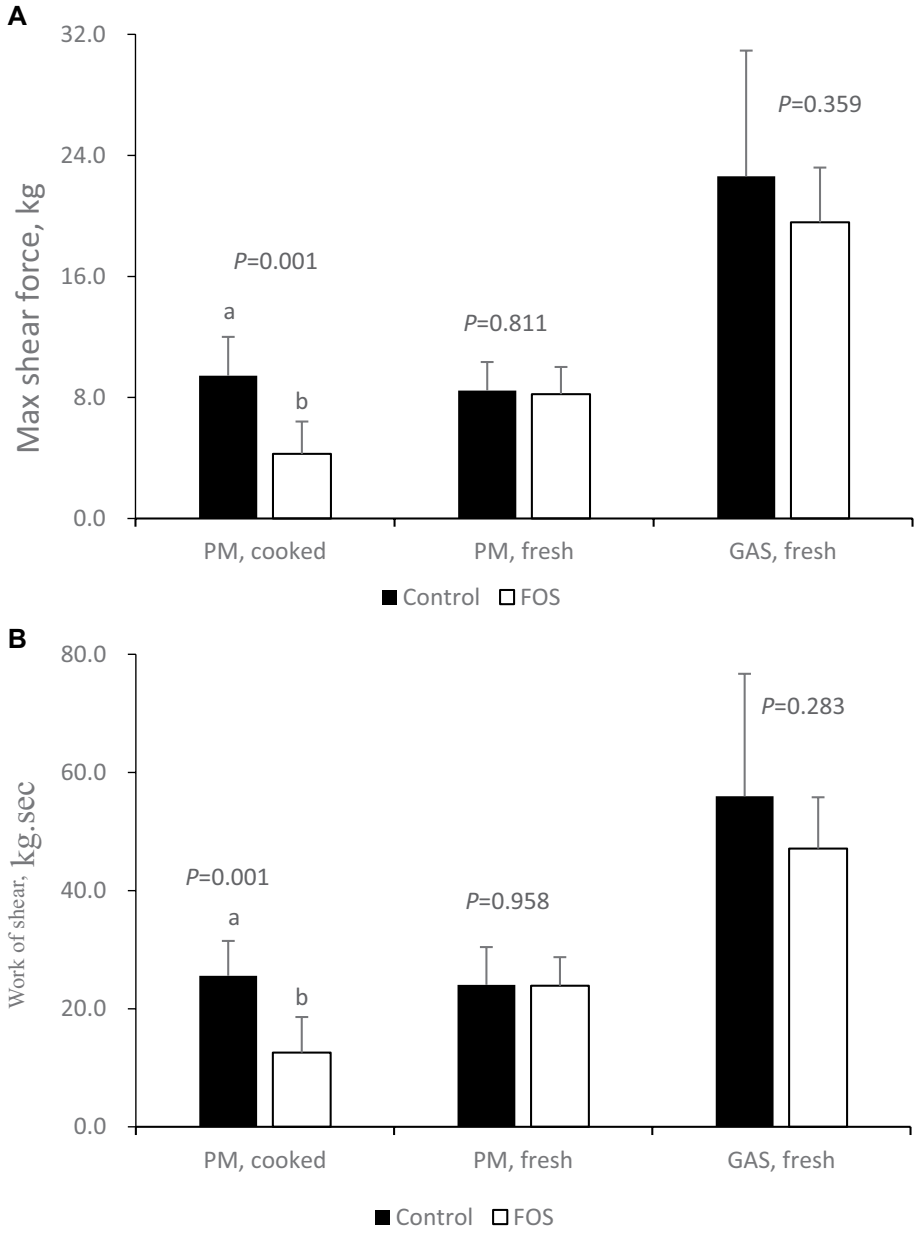

FIGURE 3
Toughness of cooked or fresh breast (*pectoralis major*, PM) and thigh (*gastrocnemius*, GAS) muscle from control or 0.5% fructo-oligosaccharide (FOS) groups of Xianghuang broilers. Probe: HDP/BSW. Max shear force **(A)**, Work of shear **(B)**. Data were presented as mean ± standard error of the mean. a, b Differs significantly at $P < 0.05$.

dietary inclusion of 0.2 or 0.4% fructo-oligosaccharides (27). Earlier published studies also showed different results when considering of effects of FOS on growth performance of poultry. Birds given 0.6 g/kg fructo-oligosaccharides had lower ADFI and ADG compared with wheat based control group (28). Feeding 1.2 g/kg of inulin or 1.5 g/kg of FOS had a positive effect on ADFI and ADG of Archer Abro broilers aged 21 to 42 days (29). Study showed that trimmed asparagus by-products which contain 1.84% fructo-oligosaccharide led to higher ADFI, ADG at 30 and 50 g/kg but not 10 g/kg in Ross broiler chicks during first 0–25 days compared with control group (30). Synbiotic which containing probiotic and fructo-oligosaccharides showed an increasing effect on body weight of 42-day-old broilers subjected to daily cyclic heat stress episodes (31). Inulin which consists of fructose and glucose appeared to change the intestinal microbiota and showed a negative effect on growth performance before day 21 but positive effect subsequently up to day 42 (32). It seems that dosage of FOS and the age of broiler would affect the effect of FOS on growth performance.

In the present study, dietary inclusion of 0.5% FOS showed an increase in pH_{45min} value of breast muscle. If inclusion proportion was as low as 0.1 or 0.2%, FOS supplementation will not influence pH and water holding capacity (WHC) of chicken meat (27). Higher muscle pH could reflect slower speed of muscle glycogen degradation after slaughter (33). High pH in FOS could be a result by enhancing Bifidobacterium growth in small intestinal and cecal digesta which

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TABLE 6 Effect of fructo-oligosaccharides on texture properties of muscle in Xianghuang broilers.

Items[1]	Control	FOS	*P*-value
Breast muscle, fresh			
Hardness, g	193.82 ± 76.58	151.6 ± 71.11	0.306
Fracturability, g	151.95 ± 60.3	119.06 ± 55.75	0.310
Adhesiveness, g.sec	−11.55 ± 2.77	−13.93 ± 4.29	0.241
Springiness	0.98 ± 0.01	0.98 ± 0.01	0.943
Cohesiveness	0.56 ± 0.04	0.58 ± 0.03	0.263
Gumminess	107.86 ± 38.5	88.57 ± 40.57	0.379
Chewiness	105.52 ± 36.97	86.57 ± 39.03	0.370
Resilience	0.11 ± 0.06^{B}	0.15 ± 0.08^{A}	0.023
Thigh muscle, fresh			
Hardness, g	251.93 ± 134.91	242.12 ± 145.7	0.898
Fracturability, g	209.96 ± 115.07	201.21 ± 119.55	0.891
Adhesiveness, g.sec	−2.16 ± 1.11	−2.77 ± 1.43	0.080
Springiness	0.98 ± 0.00	1.00 ± 0.18	0.812
Cohesiveness	0.63 ± 0.03	0.65 ± 0.03	0.243
Gumminess	157.57 ± 85.2	154.45 ± 89.16	0.948
Chewiness	154.78 ± 83.58	152.21 ± 87.28	0.956
Resilience	0.33 ± 0.07	0.36 ± 0.13	0.539
Breast meat, cooked			
Hardness, g	1253.91 ± 255.51	1203.95 ± 364.11	0.771
Fracturability, g	1143.52 ± 235.03	1100.03 ± 339.71	0.785
Adhesiveness, g.sec	−0.24 ± 0.22	−0.30 ± 0.26	0.668
Springiness	1.2 ± 1.02	1.11 ± 0.66	0.855
Cohesiveness	0.71 ± 0.03	0.71 ± 0.03	0.748
Gumminess	895.38 ± 191.95	855.20 ± 277.33	0.758
Chewiness	1153.24 ± 1231.48	896.42 ± 372.41	0.607
Resilience	0.45 ± 0.29	0.35 ± 0.03	0.375
Thigh meat, cooked			
Hardness, g	466 ± 290.55	617 ± 321.22	0.065
Fracturability, g	416 ± 268.6	556 ± 292.78	0.063
Adhesiveness, g.sec	−0.24 ± 0.20	−0.43 ± 0.23	0.479
Springiness	1.44 ± 0.67	0.98 ± 0.29	0.772
Cohesiveness	0.65 ± 0.11	0.62 ± 0.18	0.423
Gumminess	303.58 ± 197.93	400.72 ± 215.74	0.079
Chewiness	303.37 ± 197.75	400.37 ± 215.6	0.080
Resilience	0.55 ± 0.42	0.41 ± 0.17	0.608

[1]Values in a row without common superscripts differ significantly at $P < 0.05$.

TABLE 7 Effect of fructo-oligosaccharides on lipid peroxidation and antioxidant activity in Xianghuang broilers.

Items[1]	Control	FOS	*P*-value
Breast muscle			
MDA, mmol/g prot	107.89 ± 40.97	67.82 ± 32.81	0.066
T-AOC, mmol/g prot	0.42 ± 0.03	0.45 ± 0.05	0.172
T-SOD, U/mg prot	21.84 ± 3.16	19.2 ± 1.37	0.066
CAT, U/mg prot	5.64 ± 3.91	7.22 ± 5.33	0.540
GSHPX, U/g prot	83.62 ± 42.02	95.82 ± 13	0.477
Thigh muscle			
MDA, mmol/g prot	81.62 ± 37.48	73.48 ± 33.92	0.678
T-AOC, mmol/g prot	0.36 ± 0.03	0.39 ± 0.01	0.053
T-SOD, U/mg prot	23.17 ± 1.28^{A}	17.39 ± 4.30^{B}	0.005
CAT, U/mg prot	13.39 ± 11.09	13.3 ± 6.44	0.986
GSHPX, U/g prot	132.57 ± 71.32	103.52 ± 73.36	0.467
Liver			
MDA, mmol/g prot	21.59 ± 19.69	15.44 ± 11.18	0.486
T-AOC, mmol/g prot	0.30 ± 0.06	0.27 ± 0.02	0.165
T-SOD, U/mg prot	6.62 ± 3.22	6.83 ± 1.03	0.873
CAT, U/mg prot	30.54 ± 7.7	25.67 ± 6.16	0.215

[1]MDA, malonaldehyde; T-AOC, total antioxidant capacity; T-SOD, total superoxide dismutase; CAT, catalase; GSHPX, glutathione peroxidase. Values in a row without common superscripts differ significantly at $P < 0.05$.

confirmed by early report (4.0 g/kg FOS) (11). Oxidative stress after slaughter could speeds up pH drop (34). A higher ultimate pH value in the breast or thigh muscle may be related to less oxidative stress. The decrease tendency in MDA accumulation of breast muscle indicated that lipid peroxidation of meat decreased in FOS group. Lipid, protein carbonyls, and endogenous reducing sugars may promote the initiation of Maillard reactions, and lead to formation of compounds, this oxidation reaction might reduce protein solubility and enhance denaturation and aggregation (35). pH value exhibited significant negative correlation with yellowness and Warner Bratzler shear force (36). Our result confirmed that broilers from FOS treatment showed lower toughness in breast muscle compared with control group.

Supplementation 0.5% FOS resulted in lower T-SOD activity and lower SOD1 gene expression of thigh muscle compared with control group in present study. The effect of FOS on antioxidant function could be different when dosage was not the same. Report showed that inclusion of 0.1 or 0.2% FOS in broilers' diet showed no significant difference in free radical inhibition percentage expressed by ABTS (2,2 azino-bis-3-ethyl benzothiazoline-6-sulfonic acid) values and DPPH (2,2-diphenyl-1-picrylhydrazyl) values in fresh meat (27). But serum T-AOC increased and hepatic MDA reduced when FOS was at 0.3, 0.5, or 0.7% in broilers' diet (25). Inulin could protect breast muscle by elevating SOD activity when birds challenged with *Clostridium perfringens* (37). Preventing myoglobin from being oxidized could improve the meat color. The b* value of thigh muscle was the same in FOS and CON. Though interleukin 1 beta gene expression increased and heme oxygenase 1, thioredoxin reductase 1 mRNA expression decreased, the muscle percentage and MDA concertation of thigh muscle were not affected by 0.5% FOS supplementation in this study. FOS (3.5 g of fiber/100 g of the mixture) could decrease the firmness of low-fat meatballs when compared with the control (38). A higher level of pH value in thigh muscle may indicate better tenderness, meat color and water holding capacity (39). But hardness of cooked meat tended to be higher in broilers from FOS group compared with control group. Breast muscle had lower fiber cross-sectional area and higher fiber density than those of thigh muscle (17). Fiber type composition can influence postnatal meat quality. The freezing storage conditions of test cooked meat samples prior to texture analysis might also contribute

TABLE 8 Effect of fructo-oligosaccharides on gene relative mRNA expression in liver and muscle of Xianghuang broilers.

Items[1]	Control	FOS	*P*-value
Breast muscle			
HMOX1	1.34 ± 1.19	3.29 ± 2.65	0.093
NFE2L	2.03 ± 1.63^{B}	7.39 ± 6.72^{A}	0.049
CAT	2.97 ± 2.68 ^{B}	8.89 ± 6.68 ^{A}	0.034
SOD1	0.68 ± 0.55 ^{B}	23.14 ± 18.32 ^{A}	0.004
NQO1	1.50 ± 0.79 ^{B}	5.64 ± 3.21 ^{A}	0.008
TXNRD	0.76 ± 0.49 ^{B}	14.22 ± 11.53 ^{A}	0.006
ILIβ	1.05 ± 0.32	0.68 ± 0.6	0.131
IL8L2	2.10 ± 2.62	2.43 ± 2.97	0.820
TNFa	0.85 ± 0.52	1.76 ± 1.33	0.099
Thigh muscle			
HMOX1	1.34 ± 0.94^{A}	0.32 ± 0.20 ^{B}	0.010
NFE2L	3.37 ± 5.31	0.27 ± 0.19	0.148
CAT	3.26 ± 3.52	2.34 ± 0.95	0.516
SOD1	1.25 ± 0.67 ^{A}	0.47 ± 0.26 ^{B}	0.008
NQO1	1.08 ± 0.43	0.88 ± 0.91	0.603
TXNRD	1.23 ± 0.86 ^{A}	0.25 ± 0.24 ^{B}	0.012
ILIβ	1.59 ± 1.53 ^{B}	4.03 ± 1.63 ^{A}	0.008
IL8L2	1.60 ± 1.66	1.28 ± 0.69	0.646
TNFa	2.99 ± 3.65	8.02 ± 5.53	0.050
Liver			
HMOX1	1.86 ± 2.11	2.63 ± 2.92	0.524
NFE2L	1.03 ± 0.56	1.42 ± 0.93	0.312
CAT	10.72 ± 10.96	12.76 ± 12.14	0.714
SOD1	2.31 ± 2.95	0.63 ± 0.27	0.130
NQO1	1.45 ± 1.28	1.22 ± 0.79	0.661
TXNRD	3.24 ± 4.74	0.60 ± 0.30	0.137
ILIβ	0.80 ± 0.32	1.04 ± 0.49	0.246
IL8L2	2.36 ± 2.99	1.69 ± 1.35	0.564
TNFa	0.55 ± 0.58	0.67 ± 0.25	0.576

[1]HMOX1, heme oxygenase 1; NFE2L, nuclear factor, erythroid 2 like 2; CAT, catalase; SOD1, superoxide dismutase 1; NQO1, NAD(P)H quinone dehydrogenase 1; TXNRD, thioredoxin reductase 1; IL1β, interleukin 1, beta; IL8L2, interleukin 8-like 2; TNFa, tumor necrosis factor alpha. Values in a row without common superscripts differ significantly at $P < 0.05$.

textural differences between treatments. And it seems that breast and thigh muscle respond differently to dietary FOS especially on firmness.

5 Conclusion

In conclusion, 0.5% of FOS supplementation did not affect growth performance of slower-growing Xianghuang broilers. Furthermore, FOS at 0.5% in diet might help to mitigate oxidate stress and then improve meat quality traits through increasing pH value, yellowness and tenderness of muscle.

Data availability statement

The datasets presented in this article are not readily available because no. Requests to access the datasets should be directed to Can Yang, yangcansky@163.com.

Ethics statement

Experimental procedure in this study was reviewed and approved by the Animal Care and Use Committee of Hengyang Normal University, protocol HNUACUC-B202201005. Animal production test were conducted in Yimin Ecological Agriculture Development Co., Ltd. The studies were conducted in accordance with the local legislation and institutional requirements. Written informed consent was obtained from the owners for the participation of their animals in this study.

Author contributions

ZHY: Data curation, Formal analysis, Investigation, Methodology, Project administration, Supervision, Writing – original draft. XWT: Funding acquisition, Investigation, Methodology, Project administration, Supervision, Validation, Visualization, Writing – original draft. RTW: Data curation, Formal analysis, Investigation, Methodology, Software, Supervision, Writing – review & editing. CY: Conceptualization, Formal analysis, Funding acquisition, Investigation, Project administration, Resources, Supervision, Validation, Visualization, Writing – original draft, Writing – review & editing. YMJ: Data curation, Formal analysis, Investigation, Methodology, Project administration, Software, Supervision, Validation, Writing – review & editing. XW: Data curation, Formal analysis, Investigation, Methodology, Software, Writing – review & editing. QHT: Writing – review & editing. YLH: Writing – review & editing. LLW: Writing – review & editing. ZJ: Funding acquisition, Resources, Writing – review & editing.

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Conflict of interest

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

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Effects of supplementation with vitamin D_3 on growth performance, lipid metabolism and cecal microbiota in broiler chickens

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Lower intramuscular fat (IMF) and excessive abdominal fat reduce carcass quality in broilers. The study aimed to investigate the effects of dietary VD_3 on growth performance, lipid metabolism and cecal microbiota in broilers over an 84-d feeding experiment. One-day-old male *Luhua* broilers (210) were randomly assigned to control (basal diet) and VD group (basal diet supplemented with 3,750 IU/kg VD_3). Samples were collected after a 12-h fasted feeding on days 28, 56, and 84. Supplementary VD_3 significantly enhanced average daily gain (ADG) in broilers aged 57-84 d and 1-84 d, and increased leg muscle rate and fat content in breast and leg muscles and reduced abdominal fat rate of broilers at 84 d. VD_3 increased TG and glycogen content in the liver of 28- and 84-d-old broilers, serum TG and VLDL-C content at 56 and 84 d, and TC, HDL-C and LDL-C at 84 d. VD_3 increased mRNA expressions of genes related to *de novo* lipogenesis (DNL) (*mTOR*, *SREBP-1c*, *FAS* and *ACC*), lipid oxidation (*AMPK*, *PPARα*, *CPT-1α* and *ACO*) and lipid transport (*ApoB* and *MTTP*), and FAS, ACC and CPT1 enzyme activities in the liver. However, mRNA levels of genes involved in DNL and cellular lipid uptake (*LPL* and *FATP1*) and LPL activity were decreased in abdominal adipose tissue, and that of genes involved in lipid oxidation and lipolysis (*HSL* and *ATGL*) was increased by VD_3. *LPL* and *FATP1* expression in breast and leg muscles was increased by VD_3. Moreover, VD_3 increased the abundance of cecum *Bacteroides* at 28 and 84 d, *Rikenellaceae_RC9_gut_group* and *Faecalibacterium* at 56 and 84 d, and *Lachnoclostridium* at 84 d. These bacteria were correlated with increased DNL, lipid oxidation and lipid transport in liver, and cellular lipid uptake in muscle, as well as decreased DNL and cellular lipid uptake, and increased lipid oxidation and lipolysis in abdominal adipose tissue. Altogether, supplementary VD_3 in basal diet improved growth performance, increased IMF, and reduced abdominal fat rate, which is significant for enhancing feed utilization and improving the carcass quality of broilers. The regulation of VD_3 on lipid metabolism could was associated with variation in cecal microbiota composition.

KEYWORDS

broiler chicken, vitamin D_3, growth performance, lipid metabolism, cecal microbiota

1 Introduction

Chicken meat is the second most-consumed meat worldwide by humans. With continuous advancements in breeding and high-density feeding, the chicken production has significantly increased. However, a relatively lower intramuscular fat (IMF) content negatively impacts the meat quality (1), while excessive accumulation of abdominal fat reduces the carcass quality and feed conversion rate in broiler chickens (2), presenting a challenging issue in modern broiler production systems. Many factors, such as genetics, rearing systems, and dietary energy balance, influence fat deposition in broilers. The regulation of micronutrients on lipid metabolism is recognized as a crucial pathway for controlling body fat deposition and energy distribution in animals.

Vitamin D (VD) is a class of steroid derivatives that exist in multiple forms due to variations in their side chain structures. VD exhibits the functional characteristics of both vitamins and hormones, with ergocalciferol (vitamin D_2, VD_2) and cholecalciferol (vitamin D_3, VD_3) serving as the primary functional substances in animals. VD_3 is the primary form of VD used by chickens, as they cannot efficiently metabolize plant-derived VD_2 (3). VD_3 can be synthesized endogenously by the skin at sufficient UV-B exposure, but its main source is dietary intake (4, 5). It is well known that VD plays a key regulatory role in the absorption, utilization and metabolic homeostasis of calcium and phosphorus, as well as in bone development and health of animals (3, 6, 7). However, recent studies have shown that adipose tissue, as a main storage site for VD, also expresses VD hydroxylase, catalyzing the generation of the active form of VD, 1,25-dihydroxyvitamin D_3 [1,25 $(OH)_2D_3$] (8). This active form could influence biological functions, such as adipogenesis, in non-classical target tissues through autocrine, paracrine, or endocrine pathways. VD regulated various biological processes in its active form in adipose tissue through the mediation of the vitamin D receptor (VDR), including the proliferation and differentiation of adipocyte, lipogenesis, and the expression of adipokines and inflammatory responses (9, 10).

VD_3 has been reported to improve glucose homeostasis and maintain metabolic balance in humans and rats (11, 12). VD_3 enhanced glucose uptake in 3 T3-L1 cells by promoting AMPK phosphorylation and the expression and translocation of glucose transporter 4 (GLUT4), which was supported by *in vivo* experiments showing that VD_3 increased glucose uptake in both adipocytes and adipose tissue of mice (13). VD_3 reduced triglyceride (TG) levels in liver by inhibiting lipogenesis through the downregulation of sterol regulatory element binding protein 1-c (SREBP1-c) and fatty acid synthase (FAS) expression, while simultaneously enhancing β-oxidation of fatty acids by increasing the expression of carnitine palmitoyl transferase 1α (CPT-1α) and peroxisome proliferator-activated receptor (PPAR) α in mice (14). Supplementation of VD_3 alleviated liver steatosis in high-fat-induced obese mice (12) and mitigated hepatic steatosis by regulating fatty acid uptake and β-oxidation through PPARα signaling pathway in mice (15). $1,25(OH)_2D_3$ reduced lipid storage and lipid droplet aggregation by decreasing the expression of *FAS* and *PPARγ*, while increasing the expression of *CPT-1α*, *PPARα* and hormone-sensitive lipase (*HSL*) in 3 T3-L1 adipocytes (16). On the contrary, VD_3 deficiency significantly decreased the expression of β-oxidation-related genes, such as *CPT1α*, PPARγ coactivator-1 α (*PGC1α*), and *PPARα*, as well as protein level of AMP-activated protein kinase (AMPK) in obese rats (17). Additionally, VD_3 influenced gut microbiota to modulate the host's metabolism through VDR, which was abundantly expressed in the gut (18). Dietary supplementation with VD_3 reduced serum levels of HDL-C, TG, and TC, increased the abundance of gut microbiota, and improved carbohydrate and amino acid metabolism in mice (19). Zhang et al. (20) showed that VD_3 promoted the restoration of gut microbiota dysbiosis and inhibited lipid accumulation caused by a high-fat diet by increasing the abundance of *Lactobacillus* while decreasing that of *Oscillibacter* and *Flavonifractor* in the gut of rats.

Previous studies have shown that VD_3 regulated glucose and lipid metabolism in humans and rodents, and this effect was enduring and persistent (21, 22). It has also been demonstrated that VD_3 was beneficial for bone quality (7), immunity (9), and antioxidants (23), which could be related to the gut microbiota. While VD_3 supplementation improved growth and carcass performance, affected meat color, and decreased abdominal fat percentage (24, 25), current studies have not clarified the effect of VD_3 on lipid metabolism and its relationship with the gut microbiota in broilers. In this study, we investigated the effects of VD_3 supplementation on a basal diet on the growth performance and carcass quality in *Luhua* broilers, a medium-growing strain that is popular locally because of the chewy texture of the meat. Due to its lengthy growth period and significant fat deposition capability, this broiler is an excellent subject for studying fat deposition. Furthermore, we systematically explored the mechanism by which supplementary VD_3 affected fat accumulation by investigating variations in lipid metabolism in the liver and adipose tissue, lipid transport, and cecal microbiota in the broilers.

2 Materials and methods

2.1 Ethics statement

All procedures involving in animals were performed following the Regulations for the Administration of Affairs Concerning Experimental Animals (Ministry of Science and Technology, China, 2004) and were approved and supervised by the Northwest Minzu University Animal Care and Use Committee (Permit No. xbmu-sm-20210130).

2.2 Experimental design and animal management

A total of 210 healthy one-day-old male *Luhua* chicken broilers with similar body weight were randomly divided into two groups, each with 7 replicates and 15 chickens per replicate. The broilers in the control group (CON group) were fed a basal diet, while the broilers in the VD_3 group (VD group) received the basal diet supplemented with 3,750 IU/kg of VD_3 (24). The VD_3 product was supplied by Guangzhou Applon Biotechnology Co., Ltd. (Guangzhou, China). The basal diet was formulated according to the broiler Feeding Standard in China (GB/T 5916-2020) and was detailed in Table 1.

The trial was conducted at ShunHe Broiler Breeding Farm (Lanzhou, China). The broilers were raised in flat net-rearing system. Each replication was reared separately in a single pen (70 cm from the concrete floor) consisting of a stainless-steel frame with a flat wire net

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TABLE 1 Formulation and proximate composition of the basal diets (as-fed basis, %).

Items	1–28 days of age	29–56 days of age	57–84 days of age
Ingredients			
Corn	51.8	50.7	58.7
Soybean oil	2.5	4.6	5.2
Soybean meal	28	26	23
Cottonseed meal	8	7.6	7
Rapeseed meal	0	8	3.36
Corn gluten meal	6	0	0
$CaHPO_4$	1.5	0.82	0.6
NaCl	0.35	0.34	0.3
L-Lys HCL	0.2	0.36	0.2
DL-Met	0.1	0.1	0.1
Cys	0.08	0.08	0.03
Premix[1]	1.47	1.4	1.51
Total	100	100	100
Nutrient levels[2]			
ME/(MJ/kg)	18.53	16.91	14.65
CP	21.00	18.00	17.00
Ca	1.04	1.10	0.86
TP	0.89	0.85	0.62
Lys	1.024	1.054	0.900
Met	0.435	0.349	0.292

[1]The premix provided the following per kg of diets: 1–28 days of age, VA 12,000 IU, VD_3 3,500 IU, VE 60 IU, VK_3 4 mg, VB, 2.5 mg, VB_1 10 mg, VB6 6 mg,VB_6 6 mg, VB_{12} 8 μg, D-pantothenic acid 40 mg, nicotinic acid 75 mg, folic acid 10 mg, biotin 0.8 mg, choline 700 mg, Zn 90 mg, Fe 110 mg, Cu 20 mg, Mn 100 mg, I 0.5 mg, Se 0.3 mg; 29–56 days of age, VA 11,000 IU, VD_3 3,300 IU, VE 55 IU, VK_3 3.5 mg, VB_1 6 mg, VB_2 10 mg, VB_6 5 mg, VB_{12} 6 μg, D-pantothenic acid 30 mg, nicotinic acid 70 mg, folic acid 9 mg, biotin 0.7 mg, choline 600 mg, Zn 80 mg, Fe 100 mg, Cu 17 mg, Mn 90 mg, I 0.5 mg, Se 0.3 mg; 57–84 days of age, VA 10,000 IU, VD_3 3,000 IU, VE 50 IU, VK_3 3.0 mg, VB1 2 mg, VB_2 14 mg, VB_6 5 mg, VB_{12} 4 μg, D-pantothenic acid 20 mg, nicotinic acid 60 mg, folic acid 7 mg, biotin 0.6 mg, choline 600 mg, Zn 70 mg, Fe 100 mg, Cu 15 mg, Mn 85 mg, I 0.5 mg, Se 0.3 mg.
[2]Nutrient levels were all calculated values.

covered. Each pen was 200 × 100 cm with a round feeder pan and nipple drinkers (5 nipple drinkers in middle of pen). All broilers were housed in a windowed house with a controlled ventilation regime and a wet curtain cooling system. The temperature inside the house was maintained at 35 ~ 37°C with a relative humidity (RH) of 50% during the first week, gradually decreasing by 2°C each week until the coop temperature reached 24°C with RH at 45%. The lighting regime was 23-h light/1-h dark daily for chicks aged 1–28 days, and 16-h light/8-h dark daily for broilers aged 29–84 days. The experiment lasted 84 days. The broilers had ad libitum access to feed and water. The broilers received the Newcastle disease vaccine and the infectious bursal polyvalent vaccine on day 7 and 14 of the experiment, respectively.

2.3 Growth performance and carcass traits

The body weight (BW) of broilers was measured every 14 days after fasting for 12 h, and the feed intake on a pen basis was recorded daily. The average daily gain (ADG), average daily feed intake (ADFI) and feed conversion rate (FCR, feed/gain) were calculated. Mortality was recorded as it occurred.

On the last day of the experiment (day 84), two broilers with a BW close to the average in each replicate were selected and killed after fasting for 12 h. Following bleeding and plucking, the carcass was individually weighed. Subsequently, the birds were eviscerated, and weights were measured. The half-eviscerated carcass weight was calculated by excluding the trachea, esophagus, intestines, spleen, pancreas, gallbladder, reproductive organs, and gizzard contents and corneum from the carcass. The eviscerated weight was calculated by removing the heart, liver, proventriculus, gizzard, lungs, and abdominal fat from the half-eviscerated carcass. The rates of dressing, half-eviscerated carcass, eviscerated carcass, breast muscle, thigh muscle (thigh and drumstick), abdominal fat (fat around the abdomen), and liver were calculated as relative weight to the live BW.

2.4 Sample collection

On the morning of the last day of the starter stage (days 1 to 28), the grower stage (days 29 to 56), and the finisher stage (days 57 to 84), that was on days 28, 56, and 84, after a 12-h fasted feeding, two broilers in each pen (i.e., 14 birds per group) were randomly selected for sampling. 5 mL of blood samples were collected from the wing vein of each individual, and serum was collected through centrifuging at 3,000 rpm for 10 min. Afterwards, the broilers were slaughtered and quickly dissected, and approximately 5 g samples of liver, abdominal adipose tissue, and leg and breast muscle were collected. The cecal contents were carefully collected, homogenized using a sterile spatula, and transferred to CryoPure Tubes (Sarstedt AG + Co., Nümbrecht, Germany). The samples collected above were snap-frozen in liquid nitrogen and stored at −80°C until analysis.

2.5 Determination of fat content

Approximately 2.0 g of tissue samples were freeze-dried using a vacuum freeze dryer and then ground to a homogeneous powder. The fat (ether extract, EE) in the tissue samples was extracted using a Soxhlet extractor (SOX406, Hanon Advanced Technology Group Co., Ltd., Shandong, China) (GB 5009.6–2016, China). Fat content was expressed as a percentage of fat in fresh tissue samples.

2.6 Biochemical parameter analysis

Nine times the cooled physiological saline was added to 1 g of liver and adipose tissue samples, which were ground in a glass homogenizer to prepare a tissue homogenate. The homogenate was centrifuged at 4°C at 3000 × *g* for 10 min, and the supernatant was collected. The supernatant and serum samples were analyzed for triglyceride (TG), total cholesterol (TC), high-density lipoprotein cholesterol (HDL-C), low-density lipoprotein cholesterol (LDL-C), very low-density lipoprotein cholesterol (VLDL-C), free fatty acid (FFA), glycogen and glucose content using an automatic biochemical analyzer (BS-200; Shenzhen Mindray Bio-Medical Electronics Co.,

Ltd., Shenzhen, China). The contents of insulin and VD, and the activities of FAS, acetyl-CoA carboxylase (ACC), CPT-1α and lipoprotein lipase (LPL), were determined using chicken-specific enzyme-linked immunosorbent assay (ELISA) kits following the manufacturer's protocol (Solarbio Science & Technology Co., Beijing, China).

2.7 Real-time quantitative polymerase chain reaction

Total RNA were extracted using TRIzol reagent and cDNA was synthesized with a reverse transcription kit according to the manufacturer's protocol (TaKaRa Biotechnology, Dalian, China). Real-time RT-PCR was performed on a fluorescent quantitative detection system (FQD-96A; Hangzhou Bioer Technology Co., China). Amplification was conducted in a total volume of 10 μL containing 5 μL of SYBR Green PCR Master Mix (TaKaRa Biotechnology, Dalian, China), 0.4 μL of each forward and reverse primer, 1 μL of cDNA, and 3.2 μL of ddH_2O. PCR reaction procedure: pre-denaturation at 95°C for 30 s; followed by 40 cycles of 95°C for 5 s, 60°C for 30 s, and 72°C for 30 s. The relative expression of each target gene was expressed using comparative threshold cycle ($2^{-\Delta\Delta Ct}$) method and β-*actin* as an internal control. The specificity of the PCR amplification was verified with melting curve analysis. The information of primers used in this study was listed in Table 2.

2.8 Cecal microbial diversity analysis

Bacterial genomic DNA was isolated from the cecal contents using the TGuide S96 kit (DP812; Tiangen Biotech Co., Beijing, China) following the manufacturer's instructions. The purity and quality of the DNA were verified in 0.8% agarose gels. Subsequently, the full-length 16S rRNA gene was amplified using the primers (27F: AGRGTTTGATYNTGGCTCAG and 1492 R: TASGGHTACCTTGTTASGACTT). The sequencing libraries (SMRT Bell) were constructed using purified PCR products and sequenced on PacBio Sequel II platform (Biomarker-Technologies Co., China). SMRT-Link v8.0 was used to correct the original subreads to obtain Circular Consensus Sequencing (CCS) sequence. The CCS sequences were identified using Lima v1.7.0 software and filtered with cutadapt 1.9.1 software, and the non-chimeric CCS were obtained by the DADA2 method of QIIME2 2020.6 software. BMK Cloud[1] was used for Alpha diversity, Beta diversity, and microbial composition analysis.

2.9 Statistical analysis

Statistical analysis was performed using a two-tailed Student's *t*-test in the SPSS software (version 26.0; IBM Corp., Armonk, NY, USA), with the results expressed as± Standard Error of Means (SEM). Statistical significance was established at $p < 0.05$. Spearman correlation analysis was performed using R version 3.5.1 to evaluate the correlations between cecal biomarker bacterial genera and significantly different lipid metabolism indicators. GraphPad Prism 8.02 (GraphPad, Inc. La Jolla, CA, USA) was used for data mapping charts.

3 Results

3.1 Growth performance, carcass traits and fat deposition

The broilers remained in good health throughout the experiment. The effect of the supplementary VD_3 on growth performance was presented in Table 3. There was no significant difference ($p > 0.05$) in the BW of broilers at 1, 28, and 56 days of age among the groups; however, the BW of broilers at 84 days of age significantly increased in the VD group compared to the CON group ($p < 0.05$). VD_3 supplementation did not significantly affect ($p > 0.05$) the ADFI and FCR (feed/gain) of broilers at any growth stages, nor the ADG of broilers aged 1–28 days and 29–56 days. However, supplementary VD_3 significantly increased ($p < 0.05$) the ADG in broilers aged 57–84 days and 1–84 days.

The carcass performance of broilers at 84 days of age and the fat deposition at 28, 56 and 84 days of age were evaluated (Table 4). Broilers fed a diet supplemented with VD_3 exhibited a significantly higher ($p < 0.05$) leg muscle rate and liver organ index compared to the CON group at 84 days of age. However, there were no significant differences ($p > 0.05$) in the other tested traits.

Compared to the CON group, the supplementary VD_3 significantly ($p < 0.05$) reduced the abdominal fat rate and increased the fat content in the liver, breast muscle, and leg muscle of broilers at 84 days of age, with no significant effects observed at 28 and 56 days of age ($p > 0.05$). This indicated that supplementation with VD_3 in diet had varying effects on fat deposition in different tissues of broilers.

3.2 Parameters related to lipid metabolism in liver and serum

The supplementary VD_3 significantly increased ($p < 0.05$) the content of TG and glycogen while decreasing ($p < 0.05$) the TC content in the liver of 28- and 84-day-old broilers, and also reduced ($p < 0.05$) the FFA content in the liver of 84-day-old broilers (Figures 1A–D). However, supplementary VD_3 did not affect ($p > 0.05$) these parameters in the liver of 56-day-old broilers. Notably, all these parameters were significantly higher ($p < 0.05$) in 84-day-old broilers compared to those that were 28 and 56 days old.

The supplementary VD_3 significantly increased ($p < 0.05$) the content of TG, glucose, insulin and VD in serum of 28-, 56- and 84-day-old broilers, as well as the content of TC, HDL-C and LDL-C in serum of 84-day-old broilers, and the VLDL-C content in serum of 56- and 84-day-old broilers (Figures 1E–L). This indicates that at different growth stages, VD_3 increased serum lipid levels and promoted lipid transport in the blood, especially in the middle and late stages of growth.

1 https://www.biocloud.net

TABLE 2 Primer sequences for RT-PCR.

Gene	Primer sequences, 5′–3′	Gene Bank No.
β-actin	F: TCCACCGCAAATGCTTCTAA	NM_205518.2
	R: AAGCCATGCCAATCTCGTCT	
Mammalian target of rapamycin, mTOR	F: CCCGCTGTCCAAGGTTTCT	XM-040689168.2
	R: CTATTTGGATTGCCTTGACCC	
Sterol regulatory element binding protein 1-c SREBP-1c	F: GTCCCGAGGGAGACCATCTA	AY029224
	R: CAACGCATCCGAAAAGCA	
Fatty acid synthase, FAS	F: AGCTGAAGGCTGCTGACAAG	NM_205155
	R: CCTCCAATAAGGTGCGGTSA	
Acetyl-CoA Carboxylase, ACC	F: TCCTGATTCCCATTTACCACC	NM_205505
	R: TTTCCAGTCCAGAATGTCCGT	
AMP-activated protein kinase, AMPK	F: CGAAGTGGCATTTGGGGATA	NM_001039603
	R: CCGTCGAACACGCAAGTAGTA	
Peroxisome proliferator-activated receptor α, PPARα	F: TTTTGTCGCTGCCATCATTT	NM-001001464.1
	R: GGAGAAGTTTCGGGAAGAGGA	
Carnitine palmitoyltransferase 1α, CPT-1α	F: TAGAGGGCGTGGACCAATAA	NM_001012898.1
	R: GAGCAGGATGGCATGGATAA	
Acyl-CoA oxidase, ACO	F: AATGCTGGTATTGAGGAATGTCG	NM_001006205.1
	R: TGCAGGATGGGGTGAACGT	
Apolipoprotein B, ApoB	F: GCTTAGAATAGATGTGCCGTTTG	NM-001044633.2
	R: CCCATTTCCTGGTGCCTTGT	
Microsomal triglyceride transfer protein, MTTP	F: AGTTTTCACAGTACCCCTTCCTAG	NM-001109784.3
	R: TCCAACATTTCTGCTTTCCCT	
Sterol regulatory element binding protein 2, SREBP2	F: CGAAGTCCCTGGAGATGTCTG	XM-015289037.4
	R: GCACCGCTGCTCATGTTGA	
Cholesterol 7α-hydroxylase, CYP7A1	F: TCTGTTGCCAGGTGATGTTTG	NM-001001753.2
	R: TGGGCACTCTTGAATAGATGGATAG	
Lipoprotein lipase, LPL	F: GAAGGGTTTGAAGGTAGGCATT	NM-205282.2
	R: ACCACCTCCACATTTTGTCTTG	
Fatty acid transport protein1, FATP1	F: CAGCAATCGCAGATCCTAAAA	NM-001398142.1
	R: CAACCTGGGGTGAAAGACG	
Hormone-sensitive lipase, HSL	F: GATTTCTCAGCCTTTCCCCTCT	XM-040657096.1
	R: CCATCCCCATAGCACCCAAT	
Adipose triglyceride lipase, ATGL	F: GCTCAGGTAAAGAAAGTGCAGGTC	NM-001113291.2
	R: GCAAGAACGTCAAGGAAATTGTG	

3.3 The expressions of genes and the activities of enzymes related lipid metabolism in the liver

Considering that the liver is the primary site for lipid biosynthesis in poultry, the expression of genes and the activity of enzymes involved in lipid metabolism in the liver were detected (Figure 2). For broilers at 28, 56 and 84 d of age, supplementary VD_3 increased significantly ($p < 0.05$) the mRNA expression of the lipogenic gene *mTOR*, *SREBP-1c*, *FAS* and *ACC*, the lipid oxidizing gene *AMPK*, *PPARα*, *CPT-1α* and *ACO*, the VLDL assembly protein encoding-gene *ApoB* and *MTTP*, the cholesterol catabolism gene *CYP7A1*. Conversely, the expression of the cholesterol synthesis gene *SREBP2*, and the lipolytic gene *HSL* and *ATGL* was significantly decreased ($p < 0.01$) by the supplementary VD_3 (Figure 2A). Furthermore, the supplementary VD_3 significantly increased ($p < 0.01$) the activity of FAS, ACC and CPT-1α in the liver of broilers at 28, 56 and 84 days of age (Figure 2B).

3.4 The expressions of genes and the activity of enzyme related lipid metabolism in extrahepatic tissues

To understand the effects of supplementary VD_3 on lipid metabolism in extrahepatic tissues, the mRNA expression of lipid metabolism genes in abdominal adipose tissue, breast and leg muscle tissues was detected (Figure 3). Supplementary VD_3 decreased significantly ($p < 0.01$) the expression of lipogenic gene *mTOR*,

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TABLE 3 Effects of VD_3 supplementation on growth performance of broilers.

	CON Group	VD Group	SEM	*p*-value
Day 1–28				
BW (1 d), g	34.87	34.98	0.11	0.630
BW (28 d), g	704.2	712.86	3.83	0.276
ADFI, g/d	44.53	43.68	0.41	0.308
ADG, g/d	23.90	24.21	0.14	0.279
FCR (feed/gain)	1.86	1.80	0.02	0.142
Day 29–56				
BW (56 d), g	2076.28	2126.83	14.33	0.108
ADFI (g/d)	133.61	128.24	2.12	0.234
ADG (g/d)	49.01	50.50	0.46	0.140
FCR (feed/gain)	2.73	2.54	0.05	0.072
Day 57–84				
BW (84 d), g	3472.07	3585.83*	21.24	0.023
ADFI (g/d)	167.70	171.27	2.85	0.545
ADG (g/d)	49.85	52.11*	0.45	0.030
FCR (feed/gain)	3.37	3.29	0.17	0.574
Day 1–84				
ADFI (g/d)	115.28	114.39	1.43	0.767
ADG (g/d)	40.92	42.27*	0.25	0.024
FCR (feed/gain)	2.82	2.71	0.04	0.168

Values with superscripts "*" indicate significant difference ($p < 0.05$).

TABLE 4 Effects of VD_3 supplementation on the carcass performance and fat deposition in broilers (%).

Items	CON Group	VD Group	SEM	*p*-value
Day 28				
Liver organ index	26.48	28.66	0.51	0.054
Abdominal fat rate	2.41	2.26	0.06	0.406
Fat content in liver	4.80	4.82	0.13	0.245
Fat content in breast muscle	1.70	1.88	0.06	0.211
Fat content in leg muscle	3.71	4.08	0.12	0.188
Day 56				
Liver organ index	17.55	19.41	0.44	0.067
Abdominal fat rate	2.01	1.93	0.05	0.295
Fat content in liver	5.55	5.83	0.19	0.175
Fat content in breast muscle	2.52	2.75	0.08	0.174
Fat content in leg muscle	5.77	6.17	0.11	0.122
Day 84				
Dressing rate	92.78	93.73	0.39	0.285
Half-eviscerated rate	84.55	86.06	0.56	0.203
Eviscerated rate	66.19	66.39	0.61	0.870
Breast muscle rate	15.39	15.41	0.21	0.950
Leg muscle rate	23.57	26.98*	0.49	0.004
Liver organ index	15.36	18.76*	0.63	0.020
Abdominal fat rate	3.05	2.57*	0.08	0.010
Fat content in liver	8.71	9.74*	0.18	0.019
Fat content in breast muscle	4.07	5.29*	0.15	0.017
Fat content in leg muscle	10.08	11.13*	0.14	0.022

Values with superscripts "*" indicate significant difference ($p < 0.05$).

SREBP-1c, *FAS* and *ACC*, lipid uptake gene *LPL* and *FATP1*, and increased ($p < 0.05$) the expression of the lipolytic gene *HSL* and *ATGL*, and lipid oxidizing gene *PPARα* and *CPT-1α* in abdominal adipose tissue of broilers at 28, 56 and 84 days of age, as well as *AMPK* and *ACO* in broilers at 56 and 84 days of age (Figure 3A). Additionally, the LPL activity in the abdominal adipose tissue of broilers at 84 days of age was significantly decreased ($p < 0.05$) by supplementary VD_3 (Figure 3D).

The supplementary VD_3 significantly increased ($p < 0.01$) the *LPL* expression in breast muscle of broilers at 56 and 84 days of age, and the expression of *LPL* in leg muscle and *FATP1* in both breast muscle and leg muscle of broilers at 28, 56 and 84 days of age (Figures 3B,C).

3.5 Variation in cecal microbiota diversity

3.5.1 Alpha diversity and beta diversity

A total of 15,411 operational taxonomic units (OTUs) were identified in 36 cecal content samples of broilers from the CON and VD groups with 97% confidence interval (Figure 4A). The Rarefaction curves and Shannon curves of cecal samples flattened out with the increasing numbers of sequences sampled, indicating that the sample size was reasonable, and the sequencing depth was sufficient for all samples based on a saturated trend (Figures 4B,C).

Alpha diversity analysis was used to assess the richness and diversity of gut microbiota (26). Both the ACE and Chao1 indexes represent the richness of the microbial community. Dietary VD_3 supplementation significantly increased ACE and Chao1 indexes in 28-day-old broilers compared to the CON group, while showing no effects in 56- and 84-day-old broilers (Figures 4D,E). Both Shannon and Simpson indexes were significantly higher in the VD group than in the CON group at 28 and 56 days of age ($p < 0.05$), while there was no significant difference at 84 days of age (Figures 4F,G). These results indicated that VD_3 influenced α-diversity of the cecum microbiota in broiler chickens during the early growth stage.

Beta diversity, a measure of variance in taxa composition between sampling sites (27), was visualized by plotting the distances between samples using principal coordinates analysis (PCoA) and non-metric multi-dimensional scaling (NMDS) biplot. The PCoA and the NMSD analysis exhibited that at 28 days of age, there was not a complete separation in the cecal microbial community between the CON and VD groups (Figures 4H,K). At 56 and 84 days of age, a distinct separation between the groups was observed, with samples clustering within each group (Figures 4I,J,L,M). These results indicated that supplementary VD_3 had an impact on the composition of cecal microbiota in broilers.

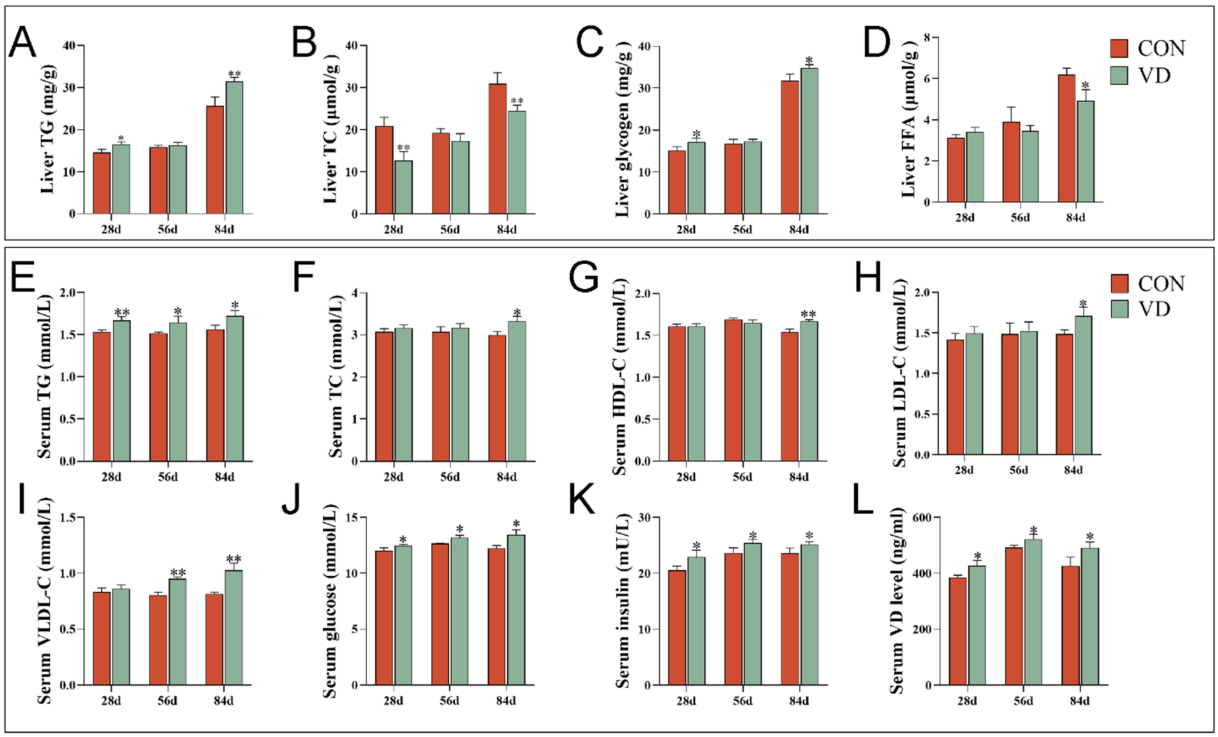

FIGURE 1
Effect of dietary VD_3 supplementation on the liver and serum parameters in broilers. **(A)** The content of TG in the liver; **(B)** TC content in the liver; **(C)** liver glycogen content in the liver; **(D)** FFA content in the liver; **(E)** TG content in serum; **(F)** TC content in serum; **(G)** HDL-C content in serum; **(H)** LDL-C content in serum; **(I)** VLDL-C content in serum; **(J)** glucose content in serum; **(K)** insulin content in serum; **(L)** VD content in serum. Data are presented as mean ± SEM, $n = 14$. *$p < 0.05$; **$p < 0.01$.

3.5.2 Variation in cecal microbiota composition at the phylum level

Based on the results of OUT delineation and taxonomic status identification, the specific composition of each sample at each taxonomic level could be obtained. At the phylum level, a total of 19 microbial phyla were identified in the cecal contents of broilers from the two groups (Figures 5A–C). We selected the dominant bacterial phyla that exhibited relatively high abundance across all three growth stages of broilers for analysis, resulting in the identification of six phyla: *Bacteroidota*, *Firmicutes*, *Proteobacteria*, *Verrucomicrobiota*, *Deferribacterota*, and *Desulfobacterota* (Figures 5D–I).

At 28 days of age, compared to the CON group, supplementary VD_3 increased significantly ($p < 0.05$) the relative abundance of *Firmicutes* (38.49% vs. 46.92%), while reduced ($p < 0.05$) that of *Proteobacteria* (1.04% vs. 0.74%), *Deferribacterota* (1.70% vs. 0.24%), and *Desulfobacterota* (1.72% vs. 1.21%). Additionally, the ratio of the *Firmicutes* / *Bacteroidota* (F/B; 0.72 vs. 0.95) was significantly increased ($p < 0.05$; Figure 5J), while there was no significant change in the relative abundance of *Bacteroidota* (53.75% vs. 49.53%). At 56 day of age, supplementary VD_3 increased significantly ($p < 0.01$) the relative abundance of *Bacteroidota* (42.41% vs. 52.33%), and reduced ($p < 0.01$) that of *Deferribacterta* (3.74% vs. 0.33%), *Desulfobacterota* (1.90% vs. 1.15%), and *Verrucomicrobiota* (7.74% vs. 2.84%). The ratio of the F / B (0.93 vs. 0.73) was significantly decreased ($p < 0.05$), while there was no significant change in the relative abundance of *Firmicutes* (39.33% vs. 38.27%). At 84 days of age, supplementary VD_3 increased significantly ($p < 0.05$) the relative abundance of *Bacteroidota* (43.49% vs. 48.62%), and reduced ($p < 0.01$) that of *Proteobacteria* (1.15% vs. 0.72%), *Deferribacterota* (0.95% vs. 0.27%), and *Verrucomicrobiota* (7.18% vs. 2.46%). However, there were no significant changes in the F / B ratio. Obviously, the VD_3 supplementation had a notable impact on the cecal microbiota composition at the phylum level in broilers.

3.5.3 Variation in cecal microbiota composition at the genus and species levels

The top 15 genera by relative abundance in the cecal microbiota were presented in Figure 6. At 28 days of age, the most dominant bacterial genera in the CON and VD groups were *Bacteroides*, *Megamonas*, *Rikenellaceae_RC9_gut_group*, *[Ruminococcus]_torques_group* and *Barnesiella*. The relative abundance of *Bacteroides* significantly increased ($p < 0.05$), while that of *Megamonas*, *Rikenellaceae_RC9_gut_group* and *Barnesiella* significantly decreased ($p < 0.05$) in the VD group (Supplementary Table S1). At 56 days of age, the most dominant genera were *Bacteroides*, *Rikenellaceae_RC9_gut_group*, *uncultured_Verrucomicrobia_bacterium*, *Parabacteroides*, and *unclassified_Prevotellaceae* in the CON and VD groups. The relative abundance of *Rikenellaceae_RC9_gut_group* and *unclassified_Prevotellaceae* significantly increased ($p < 0.05$), while that of *uncultured_Verrucomicrobia_bacterium* significantly decreased ($p < 0.05$) in the VD group (Supplementary Table S2). At 84 days of age, the most dominant genera were *Bacteroides*, *Rikenellaceae_RC9_gut_group*, *uncultured_rumen_bacterium*, *uncultured_ Verrucomicrobia_bacterium*, and *Parabacteroides*. The relative abundance of *Bacteroides* and

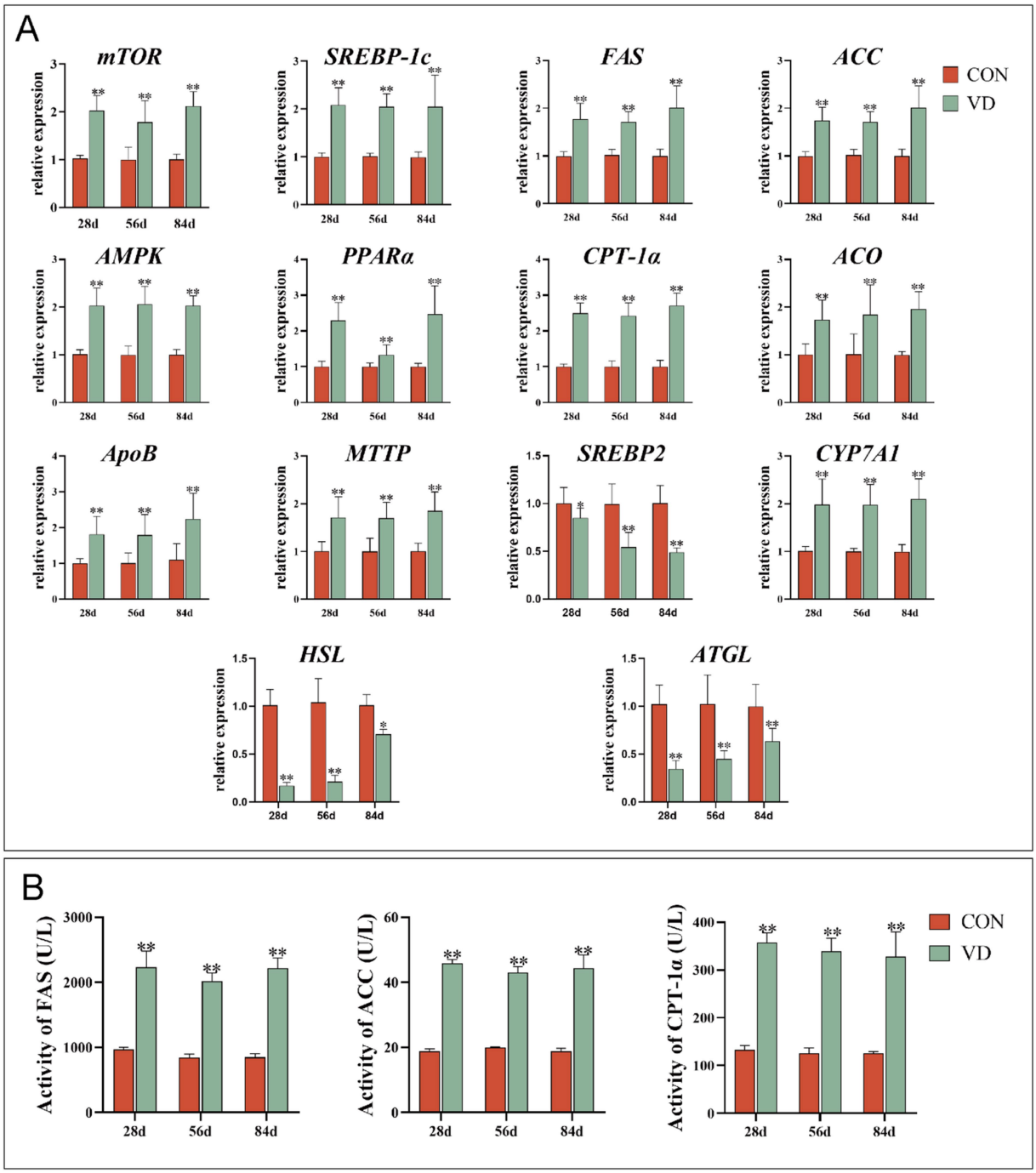

FIGURE 2
Effect of dietary VD_3 supplementation on the expression of genes and the activity of enzymes involved in lipid metabolism in the liver. **(A)** The expression of lipid metabolism genes. **(B)** The activity of FAS, ACC and CPT-1α. Data are presented as mean ± SEM (n = 14). *p < 0.05; **p < 0.01.

Rikenellaceae_RC9_gut_group significantly increased ($p < 0.05$), while that of *uncultured_rumen_bacterium* and *uncultured_Verrucomicrobia_bacterium* reduced significantly ($p < 0.05$) in the VD group (Supplementary Table S3). Moreover, supplementary VD_3 also enhanced ($p < 0.05$) the relative abundance of *Ligilactobacillus* at day 28, *Faecalibacterium* and *Phascolarctobacterium* at day 56, and *Faecalibacterium* and *Lachnoclostridium* at day 84.

The Linear Discriminant Analysis (LDA) combined with LDA Effect Size (LEfSe) analysis was employed to further examine the changes in the cecum microbiota at both the genus and species levels in broilers (LDA scores >3.5). At 28 days of age, there were 11 biomarkers enriched in the VD group, such as *g_Bacteroides*, *g_Ligilactobacillus*, *s_Ligilactobacillus_salivarius*, *s_Lactobacillus_intestinalis*, and *g_Veillonella*, and 4 biomarkers enriched in CON group, such as *g_Blautia*, and *g_Fusobacterium* (Figures 7A,B). At 56 days of age, *g_Rikenellaceae_RC9_gut_group* was identified as biomarker in the VD group (Figures 7C,D). At 84 days of age, there were 10 biomarkers in the VD group, including *g_Bacteroides*, *g_Lachnoclostridium*, *g_Faecalibacterium* and

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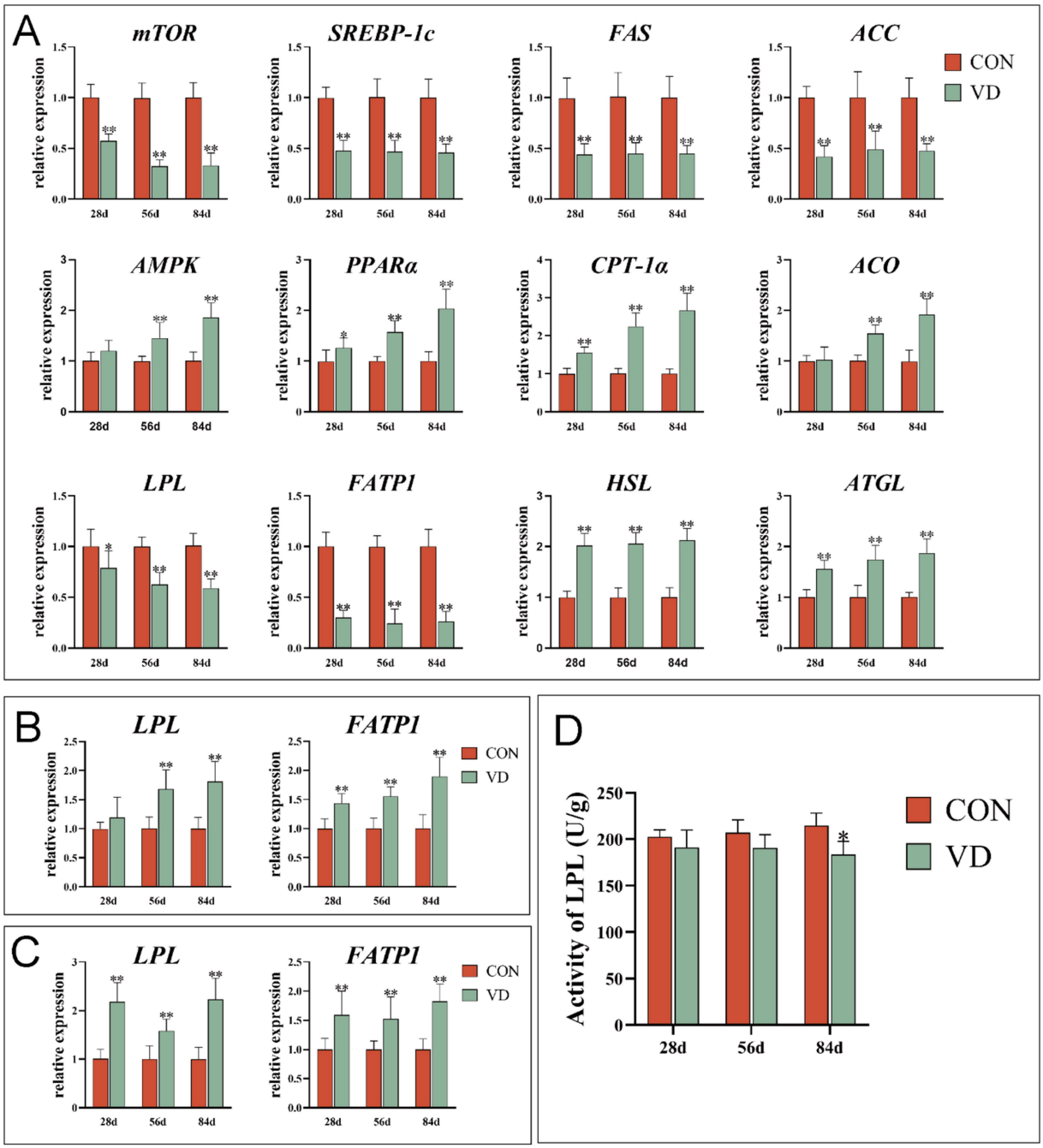

FIGURE 3
Effect of dietary VD_3 supplementation on gene expression and enzyme activity related to lipid metabolism in extrahepatic tissues. **(A)** The expression of genes in abdominal adipose tissue. **(B)** The expression of genes in breast muscle. **(C)** The expression of genes in leg muscle. **(D)** The LPL activity in abdominal adipose tissue. Data are presented as mean ± SEM (n = 14). *p < 0.05; **p < 0.01.

g_Ruminococcus_torques_group, and the CON group was enriched with *g_Prevotellaceae_UCG_001*, *g_Prevotellaceae_Ga6A1_group*, *g_Mucispirillum* (Figures 7E,F).

3.5.4 Correlation of microbiota with indicators of lipid metabolism

To investigate the relationships between intestinal microbiota and lipid metabolism in broilers, we performed a Spearman correlation analysis (Figure 8) based on the aforementioned cecal biomarker microbiota at the genus level, lipid metabolism indicators, and gene expression data to determine the microbes linked to the regulatory effects of VD_3 on lipid metabolism. At 28 days of age, the content of TG, glucose, and insulin in the serum, glycogen content and the expression of the mTORC1/SREBP-1c pathway (*mTOR*, *SREBP-1c*, and *ACC*), AMPK/PPARα/CPT1 pathway (*AMPK*, *PPARα*, *CPT-1α*, and *ACO*), *MTTP* and *CYP7A1* in the liver, as well as the expression of lipolytic pathway (*HSL* and *ATGL*) in the abdominal adipose tissue, and *FATP1* in

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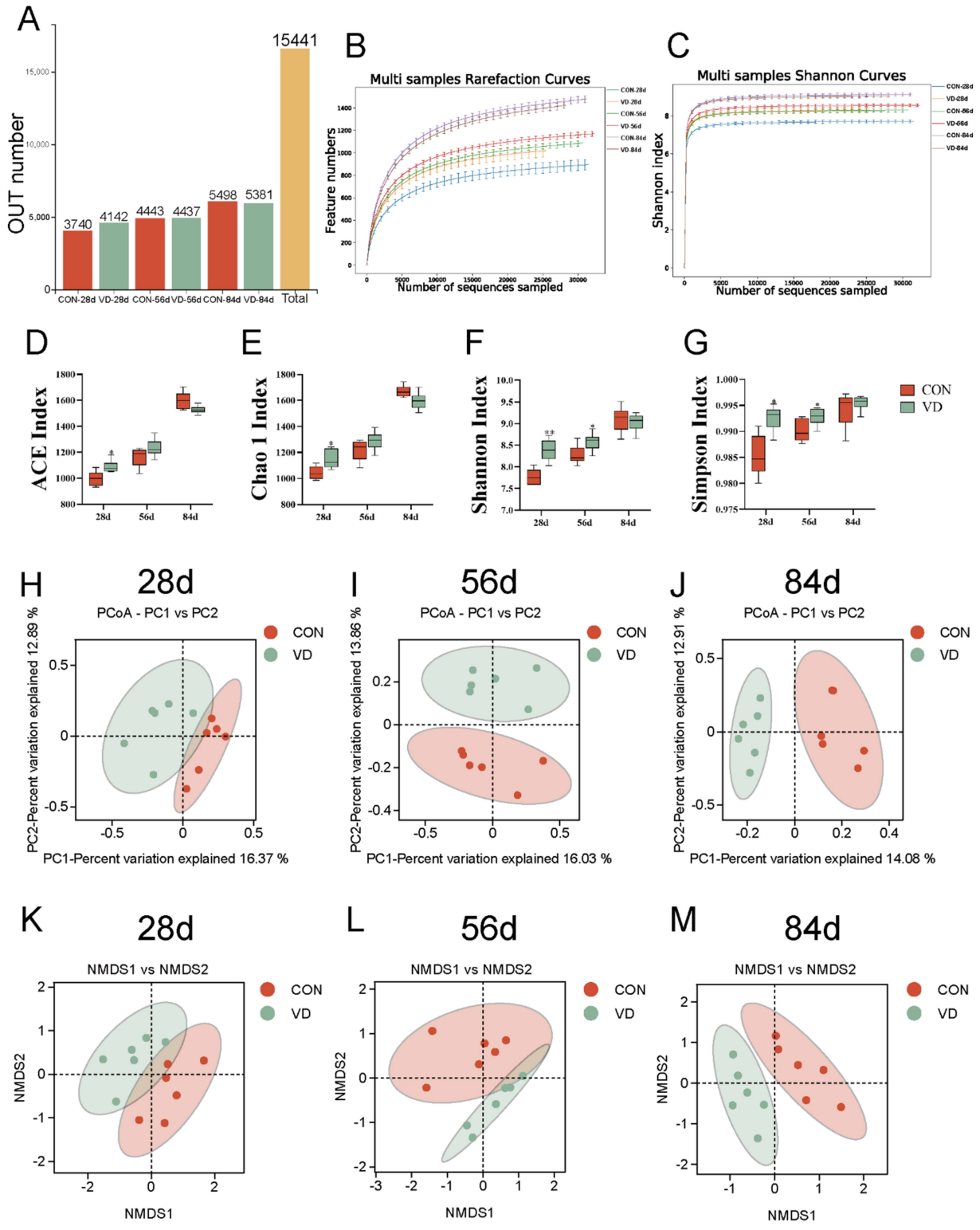

FIGURE 4
Effect of dietary VD_3 supplementation on cecal microbiota diversity in broiler. **(A)** OUT number. **(B)** Rarefaction curve. **(C)** Shannon-Wiener curve. **(D)** ACE index. **(E)** Chao1 index. **(F)** Shannon index. **(G)** Simpson index. **(H–J)** PCoA based on OTUs at 28, 56 and 84 days of age, respectively. The percent variation explained by each principal coordinate is indicated on the axes. (**K–M**) NMDS based on OTUs at 28, 56 and 84 days of age. *$p < 0.05$; **$p < 0.01$.

breast muscle, *LPL* in leg muscle were positively correlated with the relative abundance of *Bacteroides*, *Ligilactobacillus* and *Veillonella*. Moreover, the liver TC content, and the expression of *mTOR*, *SREBP-1c* and *FATP1* in the abdominal adipose tissue were negatively correlated with *Bacteroides* and *Ligilactobacillus* (Figure 8A).

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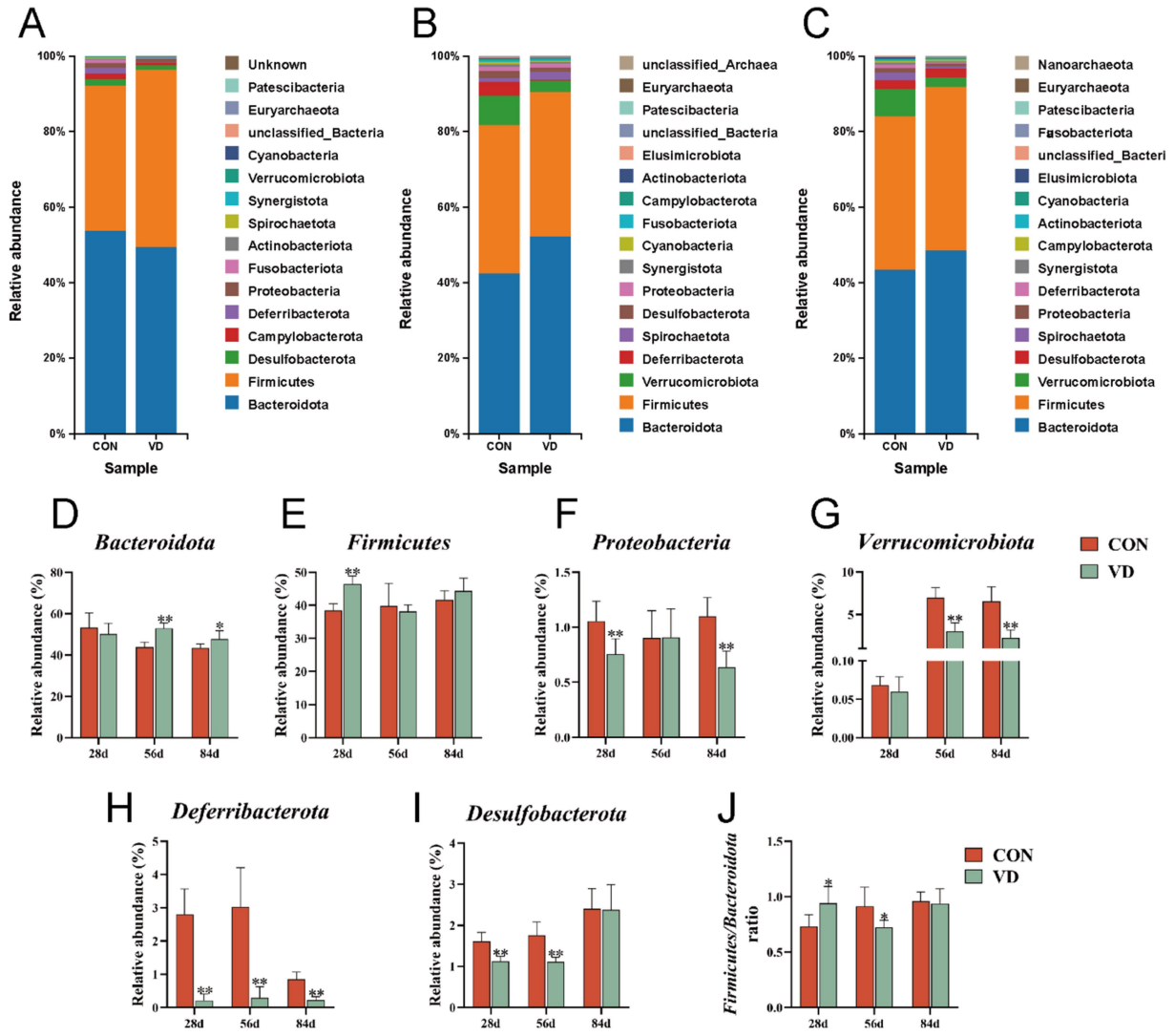

FIGURE 5
The relative abundance taxa of cecal microbiota in broilers at the phylum level. **(A–C)** Relative abundance taxa at 28, 56 and 84 days of age. **(D–I)** The relative abundance of *Bacteroidota*, *Firmicutes*, *Proteobacteria*, *Verrucomicrobiota*, *Deferribacterota* and *Desulfobacterota*, respectively. **(J)** The ratio of *Firmicutes/ Bacteroidota*. In panels **(D–J)**, data are presented as mean ± SEM (n = 6). *p < 0.05; **p < 0.01.

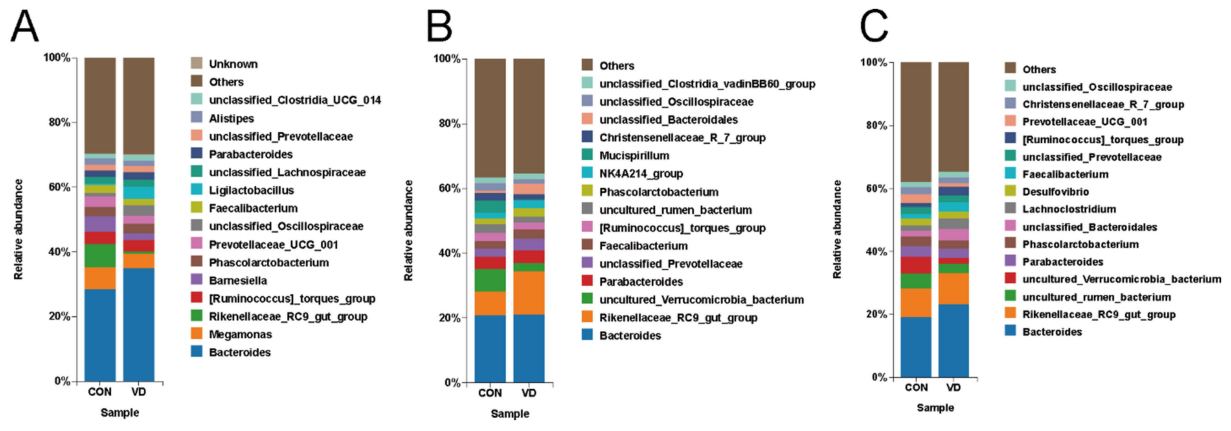

FIGURE 6
The relative abundance of cecal microbiota at the genus level. **(A–C)** Relative abundance taxa at 28, 56, and 84 days of age. The top 15 genera by relative abundance were shown, while the remaining genera were categorized as "Others".

At 56 days of age, *Rikenellaceae_RC9_gut_group* was positively correlated with the expression of *ApoB* in the liver, as well as *PPARα*, *HSL* and *ATGL* in abdominal adipose tissue, exhibiting a negative correlation with the expression of *SREBP2* in the liver, *mTOR* and *FAS* in the abdominal adipose tissue (Figure 8B).

At 84 days of age, *Lachnoclostridium* and *Faecalibacterium* exhibited a positive correlation with the fat content in the liver and

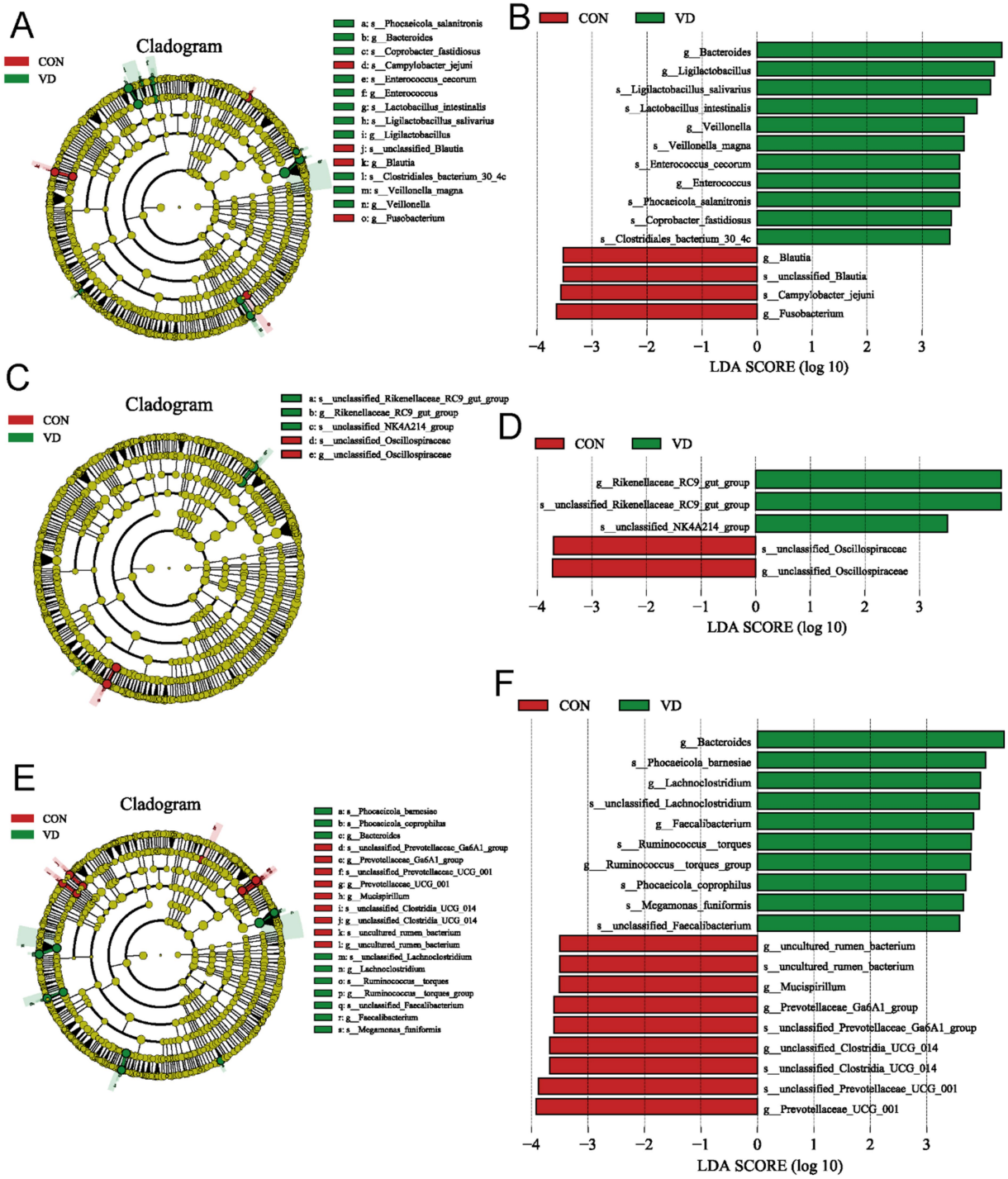

FIGURE 7
LEfSe taxonomic cladogram and LDA score of cecal microbiota. **(A,B)** LEfSe taxonomic cladogram and LDA score at 28 days of age. **(C,D)** LEfSe taxonomic cladogram and LDA score at 56 days of age. **(E,F)** LEfSe taxonomic cladogram and LDA score at 84 days of age. The circles radiating from the center to the outer edges of the evolutionary branch map represent classification levels from phylum to species, with the circle's diameter proportional to the taxon's abundance. The yellow nodes represent taxonomic units that exhibit no significant differences between groups. The LDA histograms display the LDA scores, and the difference is significant when LDA >3.5. g, genus; s, species.

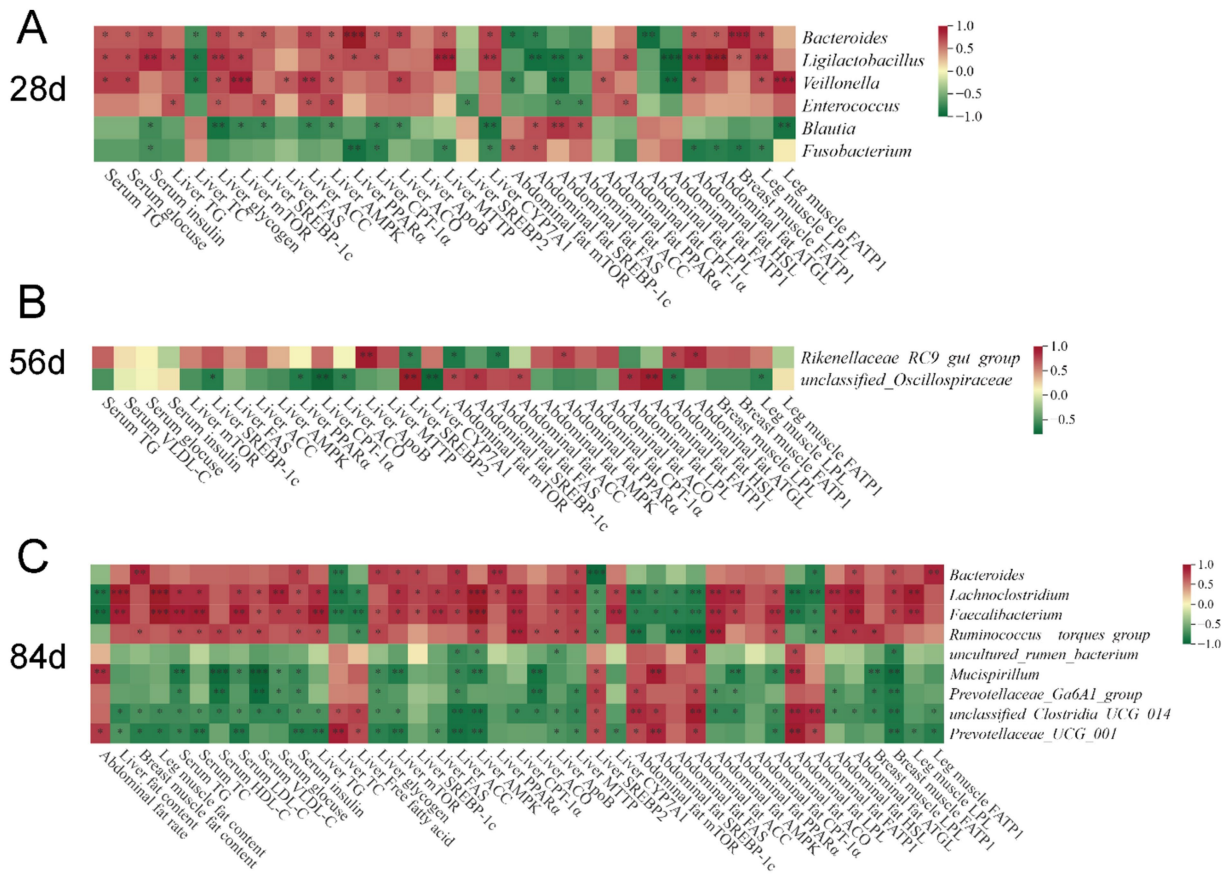

FIGURE 8
Correlation heatmap of cecal biomarker bacteria and differential lipid metabolism indicators of broilers. **(A)** 28 days of age; **(B)** 56 days of age; **(C)** 84 days of age. Spearman's correlations were calculated between cecal biomarker bacterial genera and all significantly different fat content, gene expression in tissues, and serum parameters. Colors of squares represent r values of Spearman's correlation coefficient, with red indicating a positive correlation and green signifying a negative correlation. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$.

leg muscle, serum metabolism parameters (TG, TC, LDL-C, VLDL-C, glucose, and insulin), liver TG, the expression of mTORC1/SREBP-1c pathway (*mTOR*, *SREBP-1c*, *FAS*, and *ACC*) and AMPK/PPARα/CPT1 pathway (*AMPK*, *PPARα*, and *CPT1α*), VLDL assembly (*ApoB* and *MTTP*), and *CYP7A1* in liver, and AMPK/PPARα/CPT1 pathway (*AMPK*, *PPARα*, and *ACO*), lipolytic pathway (*HSL* and *ATGL*) in the abdominal adipose tissue, and extrahepatic lipid uptake (*FATP1* in breast muscle and *LPL* in leg muscle), while exhibiting a negative correlation with other indicators, such as the abdominal fat rate, the TC and FFAs content and the expression of *SREBP2* in the liver, and the expression of mTORC1/SREBP-1c pathway (*mTOR*, *SREBP-1c*, *FAS*, and *ACC*), *LPL*, and *FATP1* in the abdominal adipose tissue (Figure 8C). However, contrasting results were observed for *Prevotellaceae_UCG_001* in comparison to these two genera. Additionally, *Bacteroides* was positively correlated with fat content in breast muscle, serum insulin and liver glycogen content, the expression of *mTOR*, *SREBP-1c*, *ACC*, *PPARα* and *MTTP* in the liver, and *ATGL* in abdominal adipose tissue, and *FATP1* in breast and leg muscle, while showing a negative correlation with liver TC, the expression of *SREBP2* in the liver, and *FATP1* in abdominal adipose tissue. However, the opposite result to *Bacteroides* was observed for *Prevotellaceae_UCG_001*.

3.5.5 Functional projections

To gain deeper insights into the variations in function of cecal microbiota between the CON and VD group, we performed KEGG enrichment prediction analysis on 16S rDNA sequencing data using the Integrated Microbial Genomes (IMG) database[2] by PICRUST2. At 28 days of age, the relative abundance of pathways related to cellular processes (such as mismatch repair, pyrimidine metabolism and homologous recombination) and glycolysis / gluconeogenesis was significantly higher, while there was a decrease ($p < 0.05$) in the abundance of secondary metabolite biosynthesis in the VD group (Figure 9A). At 56 days of age, although several glucose metabolism and amino acid biosynthesis pathways were enriched, none were statistically significant ($p > 0.05$) between groups (Supplementary Figure S1). At 84 days of age, the relative abundance of energy metabolic pathways, such as fructose and mannose metabolism, pentose phosphate pathway, starch and sucrose metabolism, were significantly increased, while that of phenylalanine, tyrosine and tryptophan biosynthesis was decreased ($p < 0.05$) in the VD group (Figure 9B).

2 https://img.jgi.doe.gov/cgi-bin/m/main.cgi/m/main.cgi

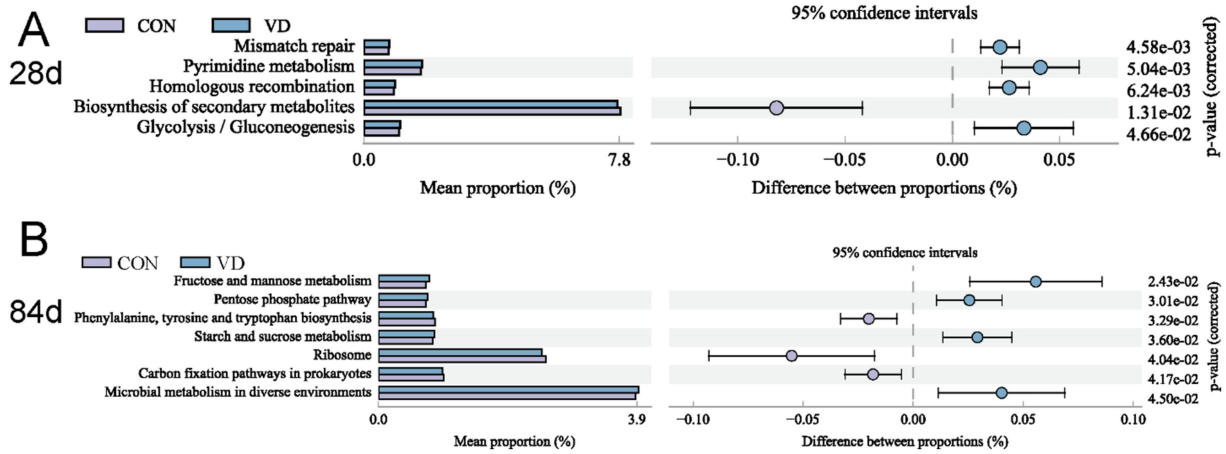

FIGURE 9
Differential KEGG level 3 metabolic pathways between groups. **(A)** 28 days of age; **(B)** 84 days of age. The *p*-value on the right is derived from G-test in the statistical analysis of taxonomic and functional profiles (STAMP) software, where *p*-value <0.05 indicates a significant difference.

4 Discussion

4.1 Growth performance and carcass traits

In contemporary broiler production, excessive accumulation of abdominal fat is a significant factor influencing the carcass quality and feed conversion of broiler chickens. Increasing evidences have shown that VD_3 regulated lipid metabolism in humans and animals (12, 28), and that there was a significant association between VD_3 levels, gut microbiota and lipid metabolism (18–20). However, the effect of VD_3 on fat accumulation and lipid metabolism in broilers has been less frequently reported. In this study, supplementation with VD_3 increased ADG of broilers aged 57–84 and 1–84 days, and the final body weight and leg muscle rate at 84 days of age. However, the ADFI during each growth period was not significantly influenced by VD_3 supplementation, resulting in a non-significant FCR. Additionally, the supplementary VD_3 significantly reduced abdominal fat rate and increased the fat content in liver, leg and breast muscle of broilers at 84 days of age, while having no effects on those of broilers at 28 and 56 d of age. Consistent with this, it was reported that VD_3 promoted growth performance and bone development by exerting a series of regulatory effects in broilers (24, 29). Considering that serum VD_3 levels were continuously elevated by supplemental VD_3, we proposed that the VD_3 added to the basal diet enhanced growth performance, as well as improved carcass quality by differentially influencing fat accumulation in various tissues of broilers, with these effects mainly occurring during the finishing period.

Additionally, it should be noted that VD_3 stored in edible parts, such as muscle and adipose tissue, did not present food safety issues under the supplementary dosage of 3,750 IU/kg of VD_3 in this study. VD_3 mainly store in fat-rich tissues and organs due to its fat-soluble property. The liver and adipose tissue of humans and animals expressed and secreted 24-hydroxylase CYP24A1 (30). CYP24A1 catalyzed the breakdown of $25(OH)D_3$ and $1,25(OH)_2D_3$ into calcitroic acid and other inactive metabolites, and $1,25(OH)_2D_3$ induced CYP24A1, promoting its own degradation and ultimately self-regulating through inactivation pathways to maintain homeostasis (31). Morrisey et al. (32) evaluated VD_3 toxicity through renal histopathological examination, a sensitive method for detecting VD toxicity, and showed that a dose of 400,000 IU/kg VD_3 resulted in calcification of renal tubules in chickens, whereas 40,000 IU/kg VD_3 exhibited no toxicity, which was determined to be the maximum food safety level. Additionally, a VD_3 addition level of 12,000 IU/kg showed no adverse effects on laying hens and their eggs (33).

4.2 Lipid metabolism in the liver

The lipid metabolism in the animals is in dynamic equilibrium, with lipid levels in the liver, peripheral tissues, and blood being interconnected and mutually affecting each other (34). Liver lipid levels served as a crucial indicator of the overall lipid metabolism in broilers (35). The supplementary VD_3 increased the liver TG and glycogen levels, as well as the liver organ index, while reducing TC levels in broilers at 28 and 84 days of age, along with a decrease in FFA levels at 84 days of age. The increased relative liver weight indicated a rise in lipid content rather than liver enlargement, as it has been established that VD_3 doses below 40,000 IU/kg are non-toxic to broiler chickens (32).

Poultry primarily synthesize lipids in the liver, and substantial evidence has shown that the level of liver lipid metabolism depends on *de novo* lipogenesis (DNL), β-oxidation, and VLDL export (36, 37). To gain a deeper understanding of the effect of VD_3 on liver lipid metabolism, we analyzed the expression of genes and the activity of enzymes related to lipid metabolism. The supplementary VD_3 significantly up-regulated the expression of *mTOR*, *SREBP-1c*, *FAS* and *ACC*, and increased the activity of FAS and ACC enzymes in the liver. In the same way, the expression of *AMPK*, *PPARα*, *CPT-1α* and *ACO* and the activity of CPT-1α enzyme in the liver were significantly increased by the supplementary VD_3. The mTORC1/SREBP-1c pathway was closely related to fat accumulation in animals (38). The transcription factor SREBP-1c regulated glycolysis and lipogenesis by modulating the expression of its target genes, such *ACC* and *FAS* (39, 40). The mTOR pathway was activated by signals like insulin, which led to the process of SREBP-1c from its precursor to a mature protein

and its translocation to the nucleus, thereby promoting the transcription of lipogenic genes (39). PPARα, a type of nuclear receptor, is activated by free fatty acids and various lipid molecules, stimulating to the transcription of genes involved in fatty acid transport, β-oxidation, and lipoprotein metabolism (41). AMPK activated PPARα signaling pathways, promoting fatty acid oxidation (42). AMPK activation enhanced lipid metabolism by switching from carbohydrate metabolism to lipid oxidation, leading to a reduction of both liver TG and obesity in mice (43). Both *CPT-1α* and *ACO*, as the target genes of PPARα, were involved in β-oxidation of fatty acids. CPT-1α was the rate-limiting enzyme for fatty acid oxidation and served as the delivery of long-chain fatty acids from the cytoplasm into the mitochondria (44, 45). It has been demonstrated that the activation of the AMPK/PPARα/CPT1 pathway stimulated the lipid metabolism and reduced lipid accumulation in obese mice (46). Additionally, the supplementation with VD_3 reduced the expression of *SREBP2* and increased the expression of *CYP7A1* in the liver. The transcription factor SREBP2 stimulated cholesterol biosynthesis, while the CYP7A1 enzyme was responsible for converting cholesterol into bile acids in the liver of poultry (47). Therefore, it is reasonable to assume that supplementation with VD_3 could enhance glucose metabolism, DNL, and lipid oxidation, all of which contributed to an increase in TG and glycogen levels in the liver of broilers at 28 and 84 days of age.

This was also supported by elevated insulin concentration, and reduced the level of FFAs and the expression of lipolytic gene *HSL* and *ATGL* in liver. The main function of insulin in hepatic lipid metabolism is to regulate lipid storage by exerting control over DNL, FFA oxidation and VLDL export (48). Hormone-sensitive lipase (HSL) and adipose triglyceride lipase (ATGL) are key enzymes in the lipolytic pathway, hydrolyzing stored triglycerides to release FFAs for β-oxidation (49, 50). Insulin profoundly inhibited lipolysis primarily by suppressing the activity of HSL and stimulated glycogen accumulation through a coordinated increase in glucose transport and glycogen synthesis (51).

Conversely, VD_3 could decrease TC levels by inhibiting cholesterol synthesis and promoting the conversion of cholesterol to bile acid efflux in the liver. However, the alterations in gene expression and enzyme activity related to lipid metabolism in the liver of broilers at 56 days of age did not result in significant changes in TG content, which could be attributed to the fact that this stage is in a period of rapid growth and requires increased energy to support growth.

4.3 Lipid transport in the blood

The lipids produced in the liver were transported by the bloodstream to extrahepatic tissues for storage or oxidation. Serum TG and TC levels are important indicators of lipid transport in the body. HDL, LDL and VLDL are all types of lipoproteins responsible for transporting lipids through the bloodstream, which can provide an insight into overall lipid metabolism. HDL is involved in cholesterol removal and transport triglycerides back to the liver, LDL is responsible for delivering cholesterol to cells, and VLDL transports triglycerides from the liver to other tissues (52). Lipoproteins are composed of a lipid core encased in a layer of phospholipids and apolipoproteins, with the liver serving as a crucial site for the synthesis of apolipoproteins. Both Apolipoprotein B (ApoB) and Microsomal Triglyceride Transfer Protein (MTTP) are critical components involved in the assembly of lipoproteins (53). In this study, the supplementary VD_3 elevated serum TG and VLDL levels, indicating that it could enhance the transport of triglycerides to peripheral tissues. Additionally, the elevated serum HDL levels at 84 days of age indicated an increase in lipid transport back to the liver, which contributed to a rise in liver TG levels, attributed to the rise in body fat mass during the later stage of growth. These results were also supported by the expression of *ApoB* and *MTTP* in the liver, which was elevated by the supplementary VD_3 in this study.

4.4 Lipid metabolism in the adipose tissue and muscle

The accumulation of triglycerides within lipid droplets was one of the causes of adipose tissue expansion, which was positively correlated with abdominal fat mass (54). The sources of fat in animal tissues include cellular uptake and DNL. DNL in the liver supplied the necessary fatty acids to other tissues by cellular uptake (55). However, the adipose tissue was also an important site for DNL in broilers (56). Fatty acids for cellular uptake are derived from the hydrolysis of blood triglycerides mediated by lipoprotein lipase (LPL) (54). Triglycerides produced in the liver were transported by apolipoproteins through the bloodstream to extrahepatic tissues (such as adipose tissue and muscle) (57). LPL catalyzed the hydrolysis of the triglycerides in VLDL to release FFAs, and promoted the uptake of fatty acids by tissues (54). FATP1 promoted cellular fatty acid uptake and adipocyte hypertrophy by mediating the synthesis of fatty acids into triglycerides (58). The supplementary VD_3 suppressed the expression of mTOR/SREBP-1c pathway related genes (*mTOR*, *SREBP-1c*, *FAS* and *ACC*), while increased the expression of AMPK/PPARα/CPT-1α pathway related genes (*AMPK*, *PPARα*, *CPT-1α* and *ACO*) and lipolytic enzyme-encoding genes (*HSL* and *ATGL*), suggesting that VD_3 could inhibit lipogenesis, and promote lipolysis and fatty acid β-oxidation in abdominal adipose tissue. Moreover, we found also that VD_3 significantly downregulated the expression of *LPL* and *FATP1*, and decreased the activity of LPL enzyme in abdominal adipose tissue of broilers at 84 days of age, suggesting that VD_3 suppressed the uptake of lipids transported from the liver by abdominal adipocytes in broilers. Consequently, the supplementary VD_3 decreased the abdominal fat rate by inhibiting DNL and cellular uptake pathways while enhancing the lipolysis and β-oxidation pathway.

Unlike abdominal fat reduction, the supplementary VD_3 significantly increased fat content in breast and leg muscles. Elevated liver TG levels, blood lipid transport, and the expression of *LPL* and *FATP1* in breast and leg muscles due to VD_3 could result in an increased cellular lipid uptake, which partially accounted for the rise in muscle fat content. However, muscle tissue mainly consists of myocytes, adipocytes, and connective tissue cells. Unlike abdominal fat, the formation of IMF is more complex due to the interference of myocytes (56). Thus, our experimental evidence was insufficient to fully clarify the promotion of muscle fat deposition by supplementary VD_3 in broilers.

The supplementary VD_3 differentially influenced lipid metabolism and fat deposition in the liver, muscle and adipose tissue of broilers, which could reflect tissue-specific effects of VD_3. As previously discussed, VD_3 enhanced both the mTORC1/SREBP-1c and AMPK/PPARα/CPT-1α signaling pathways while decreasing the expression of lipolytic genes in the liver. Conversely, in adipose tissue, the expression of the mTOR/SREBP-1c pathway was suppressed, whereas the

expression of the AMPK/PPARα/CPT-1α pathway and lipolytic genes was elevated by supplementary VD_3. Researches have confirmed that nutrients affect lipid metabolism and fat distribution by influencing the expression of related genes in the liver, muscle, and adipose tissues of animals in a tissue-specific manner (59, 60). VD_3 could regulate lipid metabolism through multiple pathways, and its genomic and epigenetic effects that depend on VDR, as well as its non-genomic effects, have been reviewed in detail (61). The finding of VD_3 that elevated insulin levels in this study could provide anther cue for understanding the regulation of VD on lipid metabolism, but further research is needed.

4.5 Variation in cecal microbiota composition and its effect on lipid metabolism

VD_3 supplementation significantly altered α-diversity of cecal microbiota. We also observed that VD_3 significantly affected the structure of cecal microbiota in broilers, especially in the grower and finisher stage. PICRUSt2 functional prediction for cecal microbiota in broilers at 28 days of age indicated that VD_3 increased the abundance of metabolic pathways involved in the cellular processes and energy metabolism. The age of 1–28 days (early growth stage) is a critical period for shaping gut microbiome composition, and the enhancement of VD_3 on the cellular processes could contribute to a healthier microbial population, reducing the risk of mutations and promoting overall microbiome stability. For broilers at 84 days of age, VD_3 increased the abundance of energy metabolic pathways, while decreasing the biosynthesis of amino acids, all of which contributed an increased capacity of the microbiota to utilize energy substances. This indicated that VD_3 could improve the availability of nutrients and energy storage by modulating the metabolic activities of the cecal microbiota in the late growth stage of broilers.

The cecal microbiota significantly contributed to fat deposition and could independently account for 21% of the variance in the abdominal fat mass after correcting for host genetic effects (62), and the enterohepatic axis had a significant effect on lipid metabolism in poultry (63). Previous study found that VD_3 reduced the expression of lipogenic genes in the liver by regulating the gut microbiota in mice (64). Here, we primarily discussed the potential effects of VD_3 on lipid metabolism by influencing cecal microbiota composition in broilers.

The composition of cecal microbiota at the phyla varied significantly across different growth stages in broilers, but *Firmicutes* and *Bacteroidetes* were always most predominant, which was consistent with previous study on broilers (26). The relative abundance of *Firmicutes* at 28 days of age and that of *Bacteroidota* at 56 and 84 days of age were increased by the supplementary VD_3, while showing inconsistent changes in F/B ratio. *Firmicutes* and *Bacteroidetes* in the gut collectively influenced the host's energy absorption and storage, and the F/B ratio affected the host's ability to obtain energy from feed (65). Both *Firmicutes* and *Bacteroidetes* promoted fat deposition in animals, and *Firmicutes* exhibited a stronger promotion compared to *Bacteroidetes* (66). Nakamoto et al. (67) found that both *Firmicutes* and *Bacteroidetes* were associated with liver lipid metabolism and stimulated the expression of lipogenic enzyme-encoding gene such as *FAS*, stearoyl-CoA desaturase-1 (*SCD1*), and diacylglycerol O-acyltransferase 2 (*DGAT2*) in mice. An increase in *Firmicutes* enhanced carbohydrate metabolism and energy absorption (68), while *Bacteroidetes* fermented indigestible polysaccharides in the gut, providing energy to the host (69). Gut *Firmicutes* and *Bacteroidetes* have been shown to influence lipid metabolism and obesity in animals by producing SCFAs (70). Additionally, we also observed that VD_3 reduced the relative abundance of these results suggested that the supplementary VD_3 could enhance energy utilization and lipogenesis in broilers by affecting the composition of gut microbiota.

Based on the analysis of cecal microbial composition at the genus levels, we found that *Bacteroides*, *Rikenellaceae_RC9_gut_group* and *Prevotellaceae_UCG_001* were all dominant bacterial genera in broilers at three different ages, all of which belong to the *Bacteroidetes*. VD_3 significantly increased the abundance of *Bacteroides* in the cecum of broilers at 28 and 84 d of age, and LEfSe analysis also showed that *Bacteroides* was a biomarker for the VD group. *Bacteroides* species utilized various plant polysaccharides and host-derived glycans to produce beneficial end products, fostering the mutualistic relationship between humans and their resident intestinal *Bacteroides* (71). *Bacteroides* supplementation improved glucose tolerance and intestinal barrier structure, and reduced the adiposity in female mice fed a high-fat diet (HFD) (72). Moreover, *Bacteroides* reduced body weight gain, and cholesterol and triglyceride levels in the liver and serum in HFD-fed mice (73). In this study, we found that *Bacteroides* was positively correlated with glycogen content and the expression of the mTOR/SREBP-1c and AMPK/PPARα/CPT1 pathways in the liver of 28- and 84-day-old broilers. However, *Bacteroides* was negatively correlated with the mTOR/SREBP-1c pathway, while showing a positive correlation with the lipolysis pathway in the abdominal adipose tissue of 28-day-old broilers.

Rikenellaceae_RC9_gut_group produced SCFAs and was correlated with a lot of lipid metabolism pathways (74). It was found that the abundance of *Rikenellaceae_RC9_gut_group* was low in obese mice and negatively correlated with serum glucose, TG, TC, LDL-C, insulin, and hepatic TC (75), and positively correlated with fecal butyrate levels (76). In contrast, the reduction of *Rikenellaceae_RC9_gut_group* in the gut was accompanied by a decrease in serum levels of TC, TG, and LDL-C in weaned piglets (77). In this study, the supplementary VD_3 significantly increased the abundance of *Rikenellaceae_RC9_gut_group* at 56 and 84 days of age, while decreasing it at 28 days of age, suggesting that this genus could be relevant one in VD_3 regulation. Furthermore, *Rikenellaceae_RC9_gut_group,* identified as a marker genus of VD group at 56 days of age, showed a positive correlation with the expression of *PPARα*, *HSL* and *ATGL,* while exhibiting a negative correlation with the expression of *mTOR* and *FAS* in the abdominal adipose tissue.

Prevotellaceae_UCG_001, a member of the family *Prevotellaceae*, generated enzymes that degrade cellulose and xylan to produce SCFAs, which play a protective role against gut inflammation (78). The abundance of *Prevotellaceae_UCG_001* was positively correlated with the AMPK signaling pathway that promoted glycolysis and fatty acid oxidation in mice (79). In this study, the abundance of *Prevotellaceae_UCG_001* in broilers at 84 days of age was reduced by the supplementary VD_3, and its correlation with lipid metabolism pathways was contrary to that of *Bacteroides*, except for a positive correlation with the abdominal fat rate. *Bacteroides* and *Prevotellaceae* have been reported to exhibit mutual antagonism or competition in the distal gut (69). The decrease in the abundance of *Prevotellaceae_UCG_001* might be related to the increase in the abundance of *Bacteroides* by supplementation with VD_3 in this study. These results indicated that the regulation of VD_3 on lipid metabolism, including the enhanced lipid metabolism in the liver, and the reduced lipogenesis and the enhanced lipolysis and lipid oxidation

in adipose tissue, was linked to an increased abundance of *Bacteroides* and *Rikenellaceae_RC9_gut_group*, along with a decreased abundance of *Prevotellaceae_UCG_001* in the cecum of broilers.

In this study, we also observed that VD_3 increased the abundance of *Lachnoclostridium* at 84 days of age and that of *Faecalibacterium* at 56 and 84 days of age. These two bacterial genera belong to the phylum *Firmicutes* and contribute to the production of acetate and butyrate (80), which serve as energy sources for gut epithelial cells, and influence host's lipid metabolism by impacting glucagon-like peptide-1 (GLP-1) and peptide YY (PYY). Although SCFAs are not considered a major energy source for broilers, current evidence suggested that gut microbiota, including *Faecalibacterium* and *Lachnoclostridium*, affected overall lipid metabolism through the gut-brain axis in humans (81). SCFAs affected adipose tissue biology and physiology, potentially influencing lipid metabolism (82). Studies have demonstrated that the acetate inhibited body fat accumulation and hepatic lipid metabolism in mice by enhancing the expression of *PPARα*, *CPT-1* and *ACO* in the liver (83). An increased abundance of gut *Lachnoclostridium* was associated with enhanced SCFAs production and reduced blood glucose levels in rats (84). However, Nogal et al. (85) reported that a higher abundance of *Lachnoclostridium* decreased circulating acetate levels, resulting in an increase in visceral fat in humans. Additionally, *Faecalibacterium prausnitzii*, the most recognized species in *Faecalibacterium*, enhanced fatty acid oxidation and adiponectin signaling in the liver and visceral adipose tissue, while reducing adipocyte size in HFD-fed mice (86). The correlation analysis revealed that both *Lachnoclostridium* and *Faecalibacterium* were positively correlated with the fat and TG content, the mTOR/SREBP-1c and the AMPK/PPARα/CPT1 pathway in the liver, as well as serum parameters related to lipid metabolism and VLDL assembly. However, they exhibited a negative correlation with abdominal fat rate, mTOR/SREBP-1c pathway, and cellular lipid uptake in abdominal adipose tissue, while showing a positive correlation with lipolysis and AMPK/PPARα/CPT1 pathway. Conversely, these two bacterial genera were positively correlated with the fat content and cellular lipid uptake in breast and leg muscles. These results indicated that VD_3 could differentially regulate lipid metabolism in different tissues by increasing the abundance of *Lachnoclostridium* and *Faecalibacterium* in the cecum of broilers, including that enhanced the lipid metabolism and transport in the liver and fat accumulation in muscles, as well as reduced fat deposition in adipose tissue by inhibiting DNL and lipid uptake and promoting lipolysis and fatty acid oxidation.

To sum up, VD_3 could differentially regulate lipid metabolism and fat deposition in different tissues by influencing the composition of intestine microbiota in broilers. The increased abundance of *Bacteroides*, *Rikenellaceae_RC9_gut_group*, *Lachnoclostridium* and *Faecalibacterium* was linked to enhanced lipid metabolism and transport in the liver, as well as fat deposition in muscles. However, the increased abundance of *Bacteroides* and *Rikenellaceae_RC9_gut_group*, and the decreased abundance of *Prevotellaceae_UCG_001*, was associated with the reduced lipogenesis and enhanced lipolysis and lipid oxidation in adipose tissue.

5 Conclusion

In summary, the addition of VD_3 on the basal diet enhanced growth performance, increased IMF in breast and leg muscles, and reduced the abdominal fat rate leading to an improvement in carcass quality by differentially regulating lipid metabolism in various tissues of broilers, with these effects primarily occurring during the finishing period. Moreover, the regulation of supplementary VD_3 on lipid metabolism could was closely associated with an increased abundance of *Firmicutes* including *Lachnoclostridium and Faecalibacterium*, and *Bacteroidetes* including *Bacteroides* and *Rikenellaceae_RC9_gut_group*.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of therepository/repositories and accession number(s) can be found at: https://www.ncbi.nlm.nih.gov/, PRJNA1195667.

Ethics statement

The animal studies were approved by the Northwest Minzu University Animal Care and Use Committee. The studies were conducted in accordance with the local legislation and institutional requirements. Written informed consent was obtained from the owners for the participation of their animals in this study.

Author contributions

JLi: Conceptualization, Data curation, Formal analysis, Investigation, Software, Validation, Writing – original draft, Writing – review & editing. XL: Methodology, Resources, Validation, Writing – original draft. JT: Data curation, Methodology, Validation, Writing – original draft. LX: Software, Visualization, Writing – original draft. YC: Data curation, Methodology, Writing – original draft. SJ: Resources, Software, Writing – original draft. GZ: Conceptualization, Funding acquisition, Investigation, Supervision, Writing – review & editing. JLu: Conceptualization, Project administration, Supervision, Writing – review & editing, Methodology.

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Conflict of interest

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

Generative AI statement

The authors declare that no Gen AI was used in the creation of this manuscript.

Publisher's note

Supplementary material

The Supplementary material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fvets.2025.1542637/full#supplementary-material

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Dietary supplementation with *Bacillus subtilis PB6* alleviates diarrhea and improves growth performance and immune function in weaned piglets fed a high-protein diet

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This study aimed to evaluate the effects of dietary supplementation with *Bacillus subtilis PB6* on growth performance, diarrhea scores, nutrient digestibility, immune function, and gut microbiota in weaned piglets fed a high-protein diet. A total of 96 weaned piglets were randomly divided into three groups in a randomized complete block design and received a low-protein diet (LP, 18.27% crude protein), a high-protein diet (HP, 20.97% crude protein), or a high-protein diet supplemented with probiotics (HPPRO, *B. subtilis PB6* spores 2×10^{11} CFU/ kg) for 21 days. Each group had eight replicates with four piglets per replicate. The results showed that piglets fed the HPPRO diet had significantly higher average daily gain and average daily feed intake during days 8–14, days 15–21, and throughout the experimental period than piglets fed the LP diet ($p < 0.01$). In parallel, piglets fed the HPPRO diet had lower feed-to-gain ratio (F:G) values during days 8–14 than piglets fed the LP diet ($p < 0.05$). Piglets fed the HP diet had increased diarrhea scores than piglets fed the LP diet ($p < 0.01$), but the diarrhea scores of piglets fed the HPPRO diet were lower than those of the HP piglets ($p < 0.01$), which had the lowest nutrient digestibility. Moreover, piglets fed the HPPRO diet had lower plasma concentrations of haptoglobin than HP piglets ($p < 0.05$) and lower pig major acute-phase protein levels than HP and LP piglets ($p < 0.05$). The downregulation of genes (toll-like receptor-4, tumor necrosis factor-α [TNF-α], and TNF receptor-associated factor-6) in the ileal tissue associated with inflammation was observed in HPPRO-fed piglets compared to LP- and HP-fed piglets ($p < 0.05$). Piglets fed the HPPRO diet had increased relative abundance of genera related to proteolysis, such as g_*Fusobacterium* and g_*Acidaminococcus*, and genera related to butyrate production, such as g_*Anaerostipes* and g_*Megasphaera*. Furthermore, piglets fed the HPPRO diet had a higher concentration of butyrate in the colonic digesta than piglets fed the LP diet ($p < 0.05$). In conclusion, piglets fed the high-protein diet supplemented with 300-mg/kg *B. subtilis PB6* had better growth performance, which was associated with relatively higher nutrient digestibility, an improved intestinal bacterial profile, and a lower inflammatory response.

KEYWORDS
probiotics, nutrient digestibility, inflammation, weaning stress, gut microbiota

1 Introduction

After weaning, the nutrient source of piglets shifts from highly digestible liquid milk to a solid dry diet, which often leads to diarrhea due to undigested proteins, antinutritional factors in the feed (1), and the immature digestive system of piglets (2). In the past two decades, it has become common practice to reduce the dietary protein level of the feed while supplementing essential amino acids to alleviate digestive dysfunction. Currently, in commercial feed, the crude protein (CP) level for weaned piglets is typically below 20%. However, it should be noted that the primary nutrient source for suckling piglets, the milk protein level of sows, is more than 25% dry matter (DM) (3), indicating that weaned piglets are capable of digesting more protein if it is of high protein quality. In contrast, the lower amount of intact protein in the low-protein diet supplemented with crystalline amino acids may be insufficient to maximize the growth potential of piglets (4).

For weaning piglets, however, the practical conflict between high intact protein requirements and diarrhea induced by high dietary protein levels cannot be ignored and needs to be resolved. It should be noted that the undigested and unabsorbed proteins reach the hindgut and are fermented by the distal intestinal flora to generate toxic nitrogen metabolites, such as ammonia, amines, hydrogen sulfide, and *N*-nitroso compounds (5), increasing the risk of postweaning diarrhea in piglets. In parallel, gut microbiota dysbiosis, induced by abrupt dietary transition and environmental changes at weaning, is also recognized as one of the keys leading to the etiology of postweaning diarrhea and enteric infections (6). Protein fermentation in the hindgut may also be associated with an increased abundance of pathogenic bacteria (7).

As a Gram-positive bacterium that produces spores and displays resistance to various environmental stresses, *Bacillus subtilis* preserves excellent viability and stability during gastrointestinal transit (8). In addition, *B. subtilis* has the potential to release exoenzymes to digest dietary protein (8). Meanwhile, the metabolites of *B. subtilis* stimulate the biosynthesis of protease in the pancreas or hepatopancreas, inducing the secretion of endogenous digestive enzymes in the gastrointestinal tract and activating protease activities and pancreatic trypsin (9). It has been suggested that B. subtilis increased the expressions of intestinal tight junction proteins, indicating the role in maintaining intestinal barrier function (10). In this study, therefore, we aimed to evaluate the effects of a high-protein diet supplemented with *B. subtilis PB6* on growth performance, diarrhea scores, nutrient digestibility, immune function, and gut microbiota in weaned piglets.

2 Materials and methods

2.1 Ethical approval

The experiment was performed following the animal protection law (ethic approval code: SICAU 2023314113) and was performed in accordance with the Guide for Animal Care and Use approved by the Sichuan Agricultural University Institutional Animal Care and Use Committee.

2.2 Animals and experimental design

A total of 96 piglets (Duroc × Landrace × Yorkshire), weaned at 21 ± 2 days of age with an initial body weight (BW) of 6.12 ± 0.30 kg, were randomly assigned to 3 dietary treatments in a randomized complete block design with BW as a block. Each dietary treatment had eight replicates with four piglets per replicate. The piglets received a low-protein diet (LP, 18.27% CP), a high-protein diet (HP, 20.97% CP), and a high-protein diet supplemented with 300 mg/kg probiotics (HPPRO) for 21 days. The probiotics used in this study, provided by Kemin (Zhuhai, China) Technologies Co. Ltd., contained *B. subtilis PB6* spores at 2×10^{11} CFU/kg. All the protein-containing ingredients were analyzed for gross energy and crude proteins (Table 1). The LP diet was formulated to be similar to the typical CP level of commercial feed for weaned piglets. The CP level of the HP diet was calculated based on the average nitrogen requirements of growing pigs at 5–7 kg and 7–11 kg, as estimated by the National Research Council (11). The two diets were designed to be iso-energetic, and the CP levels of the diets were analyzed after feed preparation. All piglets were housed in an environmentally controlled room, and the temperature was maintained between 26 and 28°C. Piglets had free access to feed and water throughout the experimental period.

2.3 Growth performance and diarrhea scores

The BW and feed consumption of piglets were recorded weekly to calculate the average daily gain (ADG), average daily feed intake (ADFI), and the ratio of ADFI to ADG (F:G). Diarrhea scores were visually assessed 3 times a day as described in a previous study (12). Briefly, firm and well-formed feces were scored as 0; soft and formed feces were scored as 1; fluid and usually yellowish feces were scored as 2; and watery and projectile feces were scored as 3. The average diarrhea score = the sum of diarrhea scores/(number of piglets per pen × experimental days × assessed times per day).

2.4 Sample collection

The piglets were weighed individually on the morning of day 8 (6 a.m.) after an overnight fast. One pig per pen, closest to the pen mean, was then selected for blood sampling ($n = 8$). In total, 10 milliliters of blood samples from the jugular vein were collected into sodium heparinized tubes. The plasma samples were obtained by centrifuging blood samples at 3,000 *g* for 15 min and then stored at −20°C for later analysis.

To determine the apparent total tract digestibility (ATTD) of nutrients (gross energy, CP, DM, and amino acids), chromium oxide

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TABLE 1 Composition of ingredients and nutrient levels of the diet (as-fed basis).

Ingredients[a], %	Low-protein diet	High-protein diet
Corn (7.32% CP)	26.67	21.92
Extruded corn (7.79% CP)	26.00	22.00
Dehulled soybean meal (44.14% CP)	6.10	15.30
Enzymatic soybean (38.5% CP)	5.00	5.00
Extruded soybean (34.99% CP)	5.00	5.00
Low-protein whey powder (2.00% CP)	10.00	10.00
Soybean concentrate protein (66.43% CP)	4.00	4.00
Fish meal (68.75% CP)	4.00	4.00
Whole milk powder (23.57% CP)	4.00	4.00
Soybean oil	0.85	1.00
Sucrose	4.00	4.00
$_{L}$-Lysine·HCl (98%)	0.70	0.45
$_{DL}$-Methionine (98.5%)	0.28	0.20
$_{L}$-Threonine (98%)	0.29	0.17
$_{L}$-Tryptophan (98%)	0.10	0.05
Choline chloride (50%)	0.16	0.16
Calcium carbonate	0.86	0.82
Dicalcium phosphate monohydrate	0.84	0.78
Sodium chloride	0.40	0.40
Acidifier[b]	0.50	0.50
Mineral premix[c]	0.20	0.20
Vitamin premix[d]	0.05	0.05
Total	100.00	100.00
Calculated nutrient levels		
Digestible energy, MCal/kg	3.58	3.58
Crude protein, %	18.01	21.00
Ca, %	0.83	0.83
Available P, %	0.43	0.43
SID Lys, %	1.43	1.43
SID Met, %	0.56	0.52
SID Met+Cys, %	0.78	0.78
SID Thr, %	0.84	0.84
SID Trp, %	0.27	0.27
Analyzed		
Gross energy, MCal/kg	3.98	4.00
Crude protein, %	18.27	20.97

[a]The crude protein values of corn, extruded corn, dehulled soybean meal, enzymatic soybean, extruded soybean, low-protein whey powder, soybean concentrate protein, fish meal, and whole milk powder were analyzed values.
[b]The acidifier (ACID LAC™) was provided by Kemin (China) Technologies Co., Ltd.
[c]Mineral premix provided per kilogram of feed: Fe, 100 mg; Cu, 6 mg; Mn, 4 mg; Zn, 100 mg; I, 0.14 mg; and Se, 0.3 mg.
[d]Vitamin premixes provided per kilogram of diet: vitamin A, 15,000 IU; vitamin D_3, 5,000 IU; vitamin E, 40I U; vitamin K_3, 5 mg; vitamin B_1, 5 mg; vitamin B_2, 12.5 mg; vitamin B_6, 6 mg; vitamin B_{12}, 0.06 mg; D-biotin, 0.25 mg; D-pantothenic acid, 25 mg; folic acid, 2.5 mg; and nicotinamide, 50 mg.

was added to the diets at 0.3% as an indigestible marker in the second week of the experiment. The first 4 days of this week were considered an adaptation period; therefore, fresh fecal samples were collected only during the last 3 days and pooled. The collected samples of feed and feces were stored at −20°C for later analysis.

After the 21-day dietary intervention, the piglets were weighed individually in the morning (6 a.m.) of day 22 after an overnight fast. Similarly, the piglet with BW closest to the average BW of this pen received an intramuscular injection of anesthetic (Shu Mianling II Injection, 0.1 mL/kg BW; Laboratory of Animal Disease, Chengdu,

China) and was then euthanized. The ileal tissue samples of approximately 4 cm in length were opened longitudinally and were washed with physiological saline to remove chyme and stored immediately in liquid nitrogen. The colonic chyme was collected into sterile tubes and stored immediately in liquid nitrogen. All the samples stored in liquid nitrogen were then transferred to a − 80°C refrigerator for long-term storage.

2.5 Chemical analysis

Feed and fecal samples were dried at 65°C for 72 h, ground through a 0.42-mm sieve, and analyzed for DM according to AOAC (13) methods. CP was determined using the copper catalyst Kjeldahl method, and gross energy was determined using an automatic adiabatic oxygen bomb calorimeter (Parr 6,400, Parr Instrument Co., Moline, IL, United States). Amino acids, except tryptophan, were measured using an automatic amino acid analyzer (L-8900, Hitachi, Tokyo, Japan) after acidolysis for 24 h. Chromium was determined using a flame atomic absorption spectrophotometer (ContrAA 700, Analytik Jena, Jena, Germany). The ATTD was calculated according to the following equation: $ATTD_{nutrient} = 1 - (Cr_{diet} \times Nutrient_{feces})/(Cr_{feces} \times Nutrient_{diet})$.

2.6 Plasma metabolites

The frozen plasma samples were thawed on the ice and centrifuged at 3000 *g* for 5 min. The supernatant was collected to determine the concentration of haptoglobin and pig major acute-phase protein (Pig-MAP) using enzyme-linked immunosorbent assay (ELISA), according to the kit instructions (Nanjing Jiancheng Bioengineering Institute, Nanjing, China).

2.7 RNA extraction and real-time quantitative PCR

The total RNA from the ileal tissue was extracted using a Trizol reagent (TaKaRa Biotechnology, Dalian, China). Then, the concentration and purity of the extracted RNA were measured using a nucleic acid analyzer (Beckman DU-800; Beckman Coulter, Inc., Brea, CA). Reverse transcription and real-time quantitative polymerase chain reaction (RT-qPCR) were performed according to the kit instructions (Vazyme, Nanjing, China). The total PCR reaction system was 20-μL, consisting of 0.4-μl forward primer, 0.4-μl reverse primer, 2.0-μl cDNA, 7.2-μl ddH_2O, and 10.0-μl Master Mix (Nanjing, China). *β*-Actin was treated as a housekeeping gene to normalize the expression of target genes according to the $2^{-\Delta\Delta Ct}$ method. The PCR primers used in this study are listed in Supplementary Table S1.

2.8 Sequencing of gut microbiome

Genomic DNA was extracted from the colonic chyme samples using the Mo Bio Power DNA Isolation Kit (Mo BIO, San Diego, USA). Then, 1% agarose gels were used to monitor DNA concentration and purity. The v4 hypervariable regions of 16S rRNA were amplified using primers 515F (GTGCCAGCMGCCGCGGTAA) and 806R (GGACTACHVGGGTWTCTAAT), and the amplicon pyrosequencing was carried out on an Illumina HiSeq PE250 platform (Illumina) by Novogene (Novogene, Beijing, China). Sequencing libraries were generated using the Ion Plus Fragment Library Kit 48 rxn (Thermo Scientific, Massachusetts, USA). The Ribosomal Database Project Classifier (version 2.2) was used to assign taxonomic rank. Operational taxonomic units (OTUs) were clustered at 97% sequence identity (sequences with ≥97% similarity were assigned to the same OTU). The relative abundance of each OTU was examined at different taxonomic levels. Diversity within communities (*α*-diversity) calculations and taxonomic community assessments were performed using Mothur 1.30.2 and Qiime 1.9.1. Principal coordinates analysis (PCoA) plots were produced using the weighted UniFrac metrics. The linear discriminant analysis (LDA) effect size (LEfSe) method was performed to elucidate the difference between treatments with LDA scores above 2.5.

2.9 Quantification of short-chain fatty acids

The concentrations of short-chain fatty acids (SCFAs, such as acetate, propionate, and butyrate) in the colonic digesta were determined by gas chromatography (Varian CP-3800). Approximately 0.5 g of chyme matter was diluted with 1.5 mL of ultrapure water and centrifuged at 10,000 *g* for 15 min. The 1.0 mL of supernatant was mixed with 0.2 mL of 25% metaphosphoric acid solution and 23.3 μL of 210 mmol/L crotonic acid, and then placed at 4°C for 30 min before centrifuging at 10,000 *g* for 10 min. The 300 μL of supernatant was mixed with 900 μL of methanol (1:3 dilution), centrifuged at 10,000 *g* for 15 min, and filtered using a 0.22-μm filter (Millipore Co., Bedford, MA, United States) before being manually applied onto the gas chromatograph for quantification.

2.10 Statistical analysis

The homogeneity of variance and normality of the data were evaluated using the Shapiro–Wilk test and Levene's test procedures of the SAS 9.4 (SAS Institute, Inc., Cary, NC, United States) software package. For growth performance, diarrhea scores, and ATTD, pens served as the experimental unit, and piglet data were reported as the mean of the pen. The data were analyzed using the PROC "MIXED" procedure, with dietary treatment as a fixed effect and initial BW as a random effect, using the following statistical model:

$$Y = \mu + \alpha_i + \upsilon_j + \varepsilon_{ij}$$

where Y is the parameter to be tested, μ is the mean, α_i is the effect of the diet ($i = 1, 2$), υ_j is the random effect of the BW, and ε_{ij} is the error term. The differences in the abundance of microbial taxa among the three groups were performed using the Kruskal–Wallis test. The differential bacterial taxa among the groups were identified using linear discriminant analysis (LDA) by LEfSe. Pearson correlation analysis was applied to explore the potential association between SCFA concentration and immune-related gene expressions. Spearman correlation was applied to the microbiota data and individual SCFA

data. For all statistical analyses, the statistical significance was declared at $p < 0.05$ and trends at $0.05 \leq p < 0.10$.

3 Results

3.1 Growth performance

As shown in Table 2, piglets fed the HPPRO diet had elevated ADG and ADFI during the second and third weeks of the experiment and throughout the experimental period ($p < 0.05$), compared to piglets fed the LP diet. Similarly, piglets fed the HPPRO diet had increased BW on day 15 of the experiment (+7.4%) and at the end of it (+12.2%) than those fed the LP diet ($p < 0.01$). In parallel, piglets fed the HPPRO diet had a lower F:G during the second week ($p < 0.05$) and tended to have a lower F:G throughout the experimental period ($p = 0.05$) than the LP piglets. The growth performance of piglets fed the HP diet showed no significant differences throughout the experiment from that of piglets fed the LP and HPPRO diets ($p > 0.05$).

3.2 Diarrhea scores

The results of the diarrhea scores are shown in Figure 1. The HP and HPPRO diets markedly increased the diarrhea scores of the piglets during the second and third weeks of the experiment and throughout the experimental period, as compared to the LP diet ($p < 0.01$). In addition, the piglets fed the HPPRO diet had lower diarrhea scores than those fed the HP diet during the second and third weeks of the experiment and throughout the experimental period ($p < 0.01$).

3.3 ATTD of nutrients and amino acids

As shown in Table 3, the piglets fed the LP and HPPRO diets had elevated ATTD of gross energy, DM, and total amino acids compared to the piglets fed the HP diet ($p < 0.01$). Meanwhile, the ATTD of CP in piglets fed the HPPRO diet was higher than in piglets fed the LP and HPPRO diets ($p < 0.01$). Specifically, the ATTD of Lys, Met, Thr, Ile, Val, and Ala in the LP and HPPRO groups was higher than that of the HP group ($p < 0.01$). The ATTD of Phe, Asp., Glu, Pro, Ser, and Tyr in the HPPRO group was higher than that of the LP and HP groups ($p < 0.01$). The ATTD of Cys in the LP group was higher than that of the HP group ($p < 0.05$). The ATTD of Arg in the LP and HPPRO groups tended to be higher than that of the HP group ($p = 0.09$).

3.4 Stress-related indicators

Piglets from the HPPRO group had lower plasma concentrations of haptoglobin than HP piglets (Figure 2A; $p < 0.05$). Meanwhile, piglets fed the HPPRO diet had decreased plasma concentrations of

TABLE 2 Growth performance in weaned piglets.

	LP	HP	HPPRO	SEM	*p*-value
BW, kg					
Day 1	6.13	6.12	6.12	0.17	0.81
Day 8	6.44	6.45	6.48	0.16	0.79
Day 15	7.18^{b}	7.44^{ab}	7.71^{a}	0.20	<0.01
Day 22	8.64^{b}	9.22^{ab}	9.69^{a}	0.27	<0.01
ADG, g/d					
Days 1–7	44	46	52	4.42	0.69
Days 8–14	106^{b}	141^{ab}	176^{a}	10.07	<0.01
Days 15–21	208^{b}	255^{ab}	283^{a}	13.40	0.04
Days 1–21	119^{b}	148^{ab}	170^{a}	7.38	0.01
ADFI, g/day					
Days 1–7	139	130	141	5.19	0.62
Days 8–14	264^{b}	315^{ab}	348^{a}	14.72	0.01
Days 15–21	397^{b}	459^{ab}	509^{a}	18.28	<0.01
Days 1–21	266^{b}	301^{ab}	333^{a}	11.63	0.01
F: G					
Days 1–7	2.94	2.84	2.76	0.20	0.90
Days 8–14	2.59^{a}	2.38^{ab}	2.00^{b}	0.10	0.02
Days 15–21	1.92	1.83	1.86	0.05	0.74
Days 1–21	2.24	2.07	1.98	0.05	0.05

LP, low-protein diet; HP, high-protein diet; HPPRO, high-protein diet with probiotics; ADG, average daily gain; ADFI, average daily feed intake; F:G, the ratio of ADFI to ADG (feed to gain ratio). Values within a row with different superscripts differ significantly at $p < 0.05$. $n = 8$.

Pig-MAP compared to those fed the LP and HP diets (Figure 2B; $p < 0.01$).

3.5 Gene expression

As shown in Figure 3, compared to those fed the LP and HP diets, piglets fed the HPPRO diet had lower mRNA expressions of *TNF-α*, *TLR-4*, and *TRAF-6* in the ileum ($p < 0.01$).

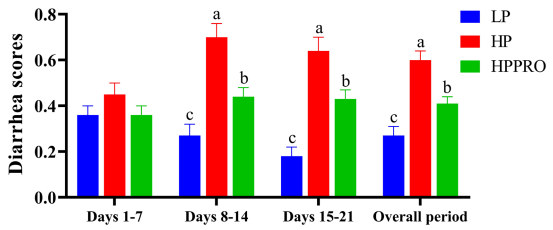

FIGURE 1
Diarrhea scores in weaned piglets. LP, low-protein diet; HP, high-protein diet; HPPRO, high-protein diet with probiotics. [a,b,c] Values without a common letter differ significantly ($p < 0.05$). $n = 8$. Diarrhea score: 0, normal; 1, soft feces; 2, mild diarrhea; and 3, severe diarrhea.

3.6 Gut microbiota of colonic digesta

Across all 24 colonic chyme samples, a total of 1,933,314 high-quality sequences were classified as being bacteria, with an average of 80,554 ± 906 sequences per sample. A total of 5,259 OTUs were identified by a nucleotide sequence identity of 97% between reads. A Venn diagram was used to demonstrate the results of shared richness between samples. As shown in Figure 4A, 1236 OTUs were shared in three groups, while 788 were unique in the LP group, 954 were unique in the HP group and 1,048 were unique in the HPPRO group. To demonstrate the separation of bacterial community composition in two groups, unweighted-UniFrac dissimilarities were calculated using the first two principal component scores of PC1 and PC2 (24.79 and 10.64%, respectively) of the explained variance and finally displayed by PCoA (Figure 4B).

No significant difference was found among the groups in *α*-diversity (Supplementary Table S2). The microbiota data cover 39 phyla and were further divided into 718 genera. At the phylum level, *Firmicutes* and *Bacteroidetes* were the two predominant bacterial taxa in the gut microbiota (Figure 5A). HPPRO piglets had a higher relative abundance of *Firmicutes*, but a lower relative abundance of *Bacteroidota* than LP piglets ($p < 0.05$). The top 15 genera are shown in Figure 5B. The LEfSe approach was conducted to discover high-dimensional biological markers among the groups. Seven taxa exhibited higher abundance in the LP group, while 4

TABLE 3 Apparent total tract digestibility of nutrients and amino acids in weaned piglets.

	LP	HP	HPPRO	SEM	*p*-value
Gross energy	83.17[a]	80.86[b]	85.07[a]	0.51	<0.01
Dry matter	83.89[a]	81.73[b]	85.29[a]	0.44	<0.01
Crude protein	72.81[b]	71.37[b]	76.97[a]	0.80	<0.01
Total amino acids	77.61[a]	74.53[b]	79.96[a]	0.70	<0.01
Essential amino acids					
Lys	84.28[a]	78.23[b]	83.51[a]	0.71	<0.01
Met	81.94[a]	74.81[b]	79.27[a]	0.82	<0.01
Thr	76.76[a]	70.65[b]	76.93[a]	0.83	<0.01
Leu	73.37[ab]	70.43[b]	76.36[a]	0.81	<0.01
Ile	69.57[a]	65.82[b]	72.80[a]	0.95	<0.01
Val	69.92[a]	65.90[b]	72.83[a]	0.94	<0.01
His	85.25[ab]	84.17[b]	87.36[a]	0.48	0.01
Phe	72.45[b]	69.96[b]	76.07[a]	0.82	<0.01
Non-essential amino acids					
Ala	69.12[a]	63.80[b]	71.16[a]	1.00	<0.01
Asp	74.87[b]	72.39[b]	78.74[a]	0.79	<0.01
Arg	87.68	87.17	89.31	0.42	0.09
Cys	82.58[a]	76.88[b]	81.64[ab]	1.04	0.04
Glu	82.78[b]	81.03[b]	85.52[a]	0.57	<0.01
Gly	71.23[ab]	68.02[b]	74.55[a]	0.85	<0.01
Pro	81.26[b]	79.75[b]	83.73[a]	0.59	<0.01
Ser	75.98[b]	74.00[b]	79.87[a]	0.75	<0.01
Tyr	68.59[b]	68.24[b]	74.58[a]	1.00	<0.01

LP, low-protein diet; HP, high-protein diet; HPPRO, high-protein diet with probiotics. Values within a row with different superscripts differ significantly at $p < 0.05$. $n = 8$.

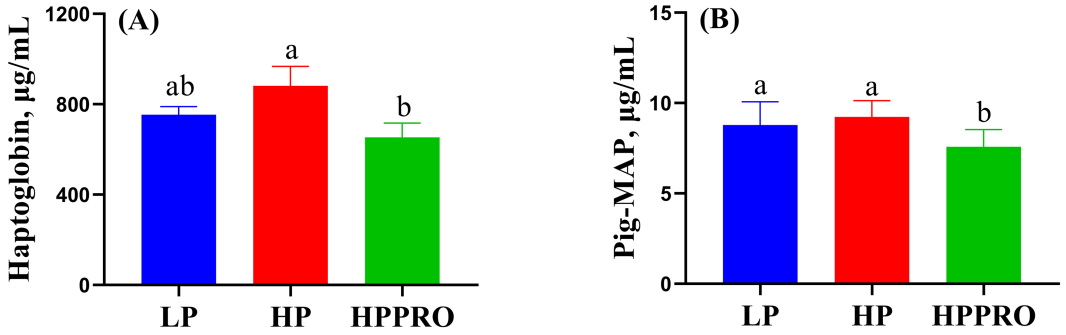

FIGURE 2
The stress-related indicators in weaned piglets. LP, low-protein diet; HP, high-protein diet; HPPRO, high-protein diet with probiotics; pig major acute-phase protein. [a,b] Values without a common letter differ significantly ($p < 0.05$). $n = 8$.

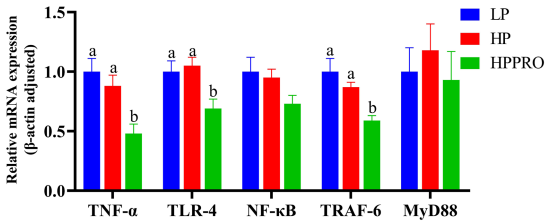

FIGURE 3
Gene expression in the ileum of weaned piglets. LP, low-protein diet; HP, high-protein diet; HPPRO, high-protein diet with probiotics; *TNF-α*, tumor necrosis factor-α; *TLR-4*, toll-like receptor-4; *NF-κB*, nuclear factor kappa B; *TRAF-6*, TNF receptor-associated factor-6; *MyD88*, Myeloid differentiation-88. [a,b,c] Values without a common letter differ significantly ($p < 0.05$). $n = 8$.

taxa and 17 taxa were enriched in the HP and HPPRO groups, respectively (Figure 5C).

3.7 Concentrations of SCFA in colonic digesta

The piglets fed the HPPRO diet had increased levels of butyric acid (Figure 6C, $p < 0.05$) and tended to have higher levels of total SCFA (Figure 6D, $p = 0.09$) in the colonic digesta compared to the piglets fed with the LP diet. The levels of acetic acid and propionic acid in the colonic digesta did not markedly differ among groups (Figures 6A,B, $p > 0.05$).

3.8 Correlations

Pearson correlation analysis was used to find the association between SCFA concentration and immune-related gene expressions (Figure 7A). The butyric acid was negatively correlated with the mRNA expressions of TNF-α, TLR-4, and TRAF-6.

Correlations between the abundance of specific taxa and SCFA using Spearman correlation analysis were shown as heatmaps (Figure 7B). Multiple taxa were correlated with SCFA in abundance. Specifically, *Campylobacter* was negatively correlated with concentrations of acetic acid, propionic acid, and butyric acid, while *Terrisporobacter* was positively correlated with concentrations of acetic acid and butyric acid. *Clostridia_UCG_014* and *Muribaculaceae* showed a positive correlation with acetic acid concentration. In addition, *T34* was negatively correlated with propionic acid concentration. *Actinobacillus* was negatively correlated with butyric acid concentration, while *Agathobacter* was positively correlated with butyric acid concentration.

4 Discussion

Dietary protein and its metabolites, small peptides, and amino acids play an important role in cell proliferation and tissue synthesis. Insufficient protein intake compromises the growth performance of weaned piglets. Low-protein diets supplemented with synthesized amino acids have been widely used to reduce postweaning diarrhea in piglets and the use of protein ingredients in feed. Although the amino acid pattern has been considered and crystalline amino acids are added to satisfy the requirement of amino acids, the reduced intact proteins (protein-bound amino acids) and small peptides are inevitable in a low-protein diet due to the reduced dietary protein. Our previous study indicated that intact protein is superior to free amino acids for whole-body protein synthesis (14). Compared to free amino acids, the intact protein or protein-bound amino acids elevate the nitrogen retention rate and protein homeostasis, which is been associated with amino acid availability in the form of di- and tripeptides digested from intact protein (14). Although no statistically significant difference was observed between LP and HP piglets in growth performance, the HP piglets had numerical increases in ADG (+24%) and ADFI (+13%). In the present study, the energy and essential amino acid levels between the low-protein and high-protein diets were comparable, implying that the better growth performance of piglets in the HP and HPPRO groups may be attributed to the higher content of intact protein, which is required for maintaining the optimal growth and immune function in pigs (4).

Despite this, it should be stressed that the digestibility of gross energy, DM, CP, and amino acids in the HP group was lower than that in the LP group, indicating that more undigested protein is generated due to the higher dietary protein content. Nevertheless, the decreased nutrient digestibility of the HP diet was reversed by *B. subtilis PB6*

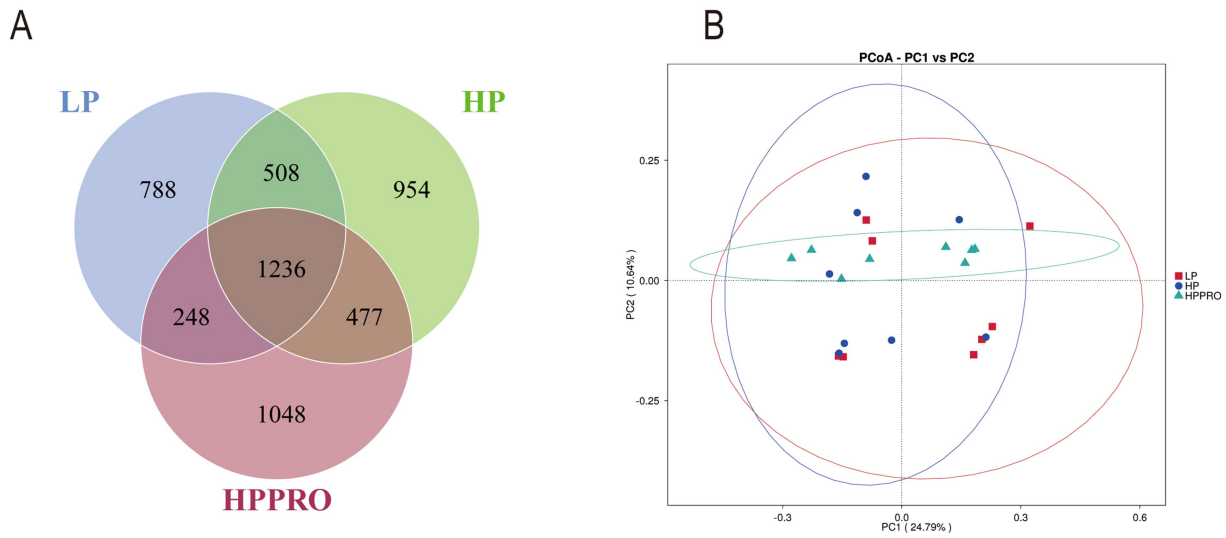

FIGURE 4
Gut microbiota of the colonic chyme in weaned piglets fed different diets. **(A)** Venn diagram based on OTUs; **(B)** β-diversity using PCoA. $n = 8$.

supplementation. Consistent with this, a previous study reported that dietary *B. subtilis* DSM32315 increased the digestibility of CP, DM, and gross energy in weaned piglets (15). Supplementation of *B. subtilis* in the diet could reduce the pH value of jejunal and ileal digesta (15), creating an acidic environment conducive to protein degradation and absorption. Meanwhile, the protease could enhance the deposition of both protein and energy by hydrolyzing the less digestible protein (16), which may explain the higher energy digestibility in HPPRO piglets. In addition, we have previously found that dietary *B. subtilis PB6* supplementation increased the activities of disaccharidases (maltase and sucrase) in the jejunum of piglets (17), indicating better digestive capability in response to *B. subtilis PB6* supplementation. These results indicate that *B. subtilis PB6* improves nutrient availability in weaned piglets.

In this study, we observed that the piglets on the HP and HPPRO diets had more severe diarrhea compared to the LP piglets, implying an impaired intestinal mucosal barrier and increased intestinal permeability due to higher protein intake. However, it should be noted that HPPRO piglets had markedly lower diarrhea scores than HP piglets, further suggesting the beneficial role of *B. subtilis PB6* in maintaining intestinal homeostasis in piglets fed a high-protein diet. The tight junction proteins, such as zonula occludens-1 (ZO-1), occludin, and claudin-1, modulate intestinal barrier function. Our previous study reported that *B. subtilis* BP6 increased the relative protein expression of ZO-1 and claudin-1 in the ileum of pigs (17). Similarly, *B. subtilis* treatment reduced plasma diamine oxidase activity (10), a sensitive indicator of intestinal permeability. Therefore, *B. subtilis PB6* could potentially improve the integrity of the intestinal barrier, which may contribute to alleviating diarrhea.

The profound link between diet and gut microbiota has been widely reported. It is accepted that diet plays a vital role in shaping the microbial communities of the host (18). Conversely, the gut microbiota is also involved in the retention of nutrients (19). In this study, we performed 16S rRNA sequencing to investigate the gut microbiota responses to dietary treatments. The diversity and richness of the gut microbiota did not markedly differ among the groups. A previous study reported that the ratio of *Firmicutes* to *Bacteroidetes* was positively correlated with an increased body weight gain (20). In the current study, consistently, the increased abundance of *Firmicutes*, decreased abundance of *Bacteroidetes*, and the highest *Firmicutes*/*Bacteroidetes* ratio among the three groups explained the highest BW and ADG in HPPRO piglets. It is reported that gut microbiota mediate dietary protein metabolism and nitrogen recycling in the intestine (21). To elucidate the differences in gut microbiota composition between treatments, the LEfSe method was conducted to analyze the enriched bacteria in each group. The genera of *Bacteroides*, *Fusobacterium*, *Clostridium*, and *Lactobacillus* possess proteolytic activity in the hindgut (21, 22). In addition, the g_*Acidaminococcus* and s_*Veillonella magna*, which are enriched in the colonic chyme of HPPRO and LP piglets respectively, are classified as *Clostridium* (23). In this study, the increased abundances of s_*Bacteroides_plebeius*, g_*Fusobacterium*, and g_*Acidaminococcus* in the HPPRO group may favor the digestion and absorption of protein, which was consistent with the highest CP digestibility in HPPRO piglets. In contrast, HP piglets had an enriched abundance of s_*Eubacterium_coprostanoligenes*, which ferment sulfur-containing amino acids to generate hydrogen sulfide and impair the gut barrier (24). Collectively, the altered gut microbiota may influence protein retention and ultimately impact piglet growth.

Acute-phase proteins (APPs), the proteins that respond to inflammation caused by stress or infection, are commonly used as markers of inflammatory problems or disease (25). In pigs, haptoglobin and Pig-MAP are the two significant APPs that are increased in experimental acute-phase models (25). In this study, HPPRO piglets had the lowest haptoglobin and Pig-MAP levels among the groups, indicating that the comprehensive stress induced by weaning and diet was alleviated by *B. subtilis PB6* supplementation. The activation of the TLR-4-mediated nuclear factor kappaB (NF-κB)–TNF-α signaling pathway in immune cells stimulates APP production (26, 27). The recruitment of MyD88 to intracellular fragments activates TLR-4 (26), which, via TRAF-6, an essential adapter protein that mediates the transduction of TLR-4/Myd88 signals, stimulates the NF-κB signaling cascade and translocases in

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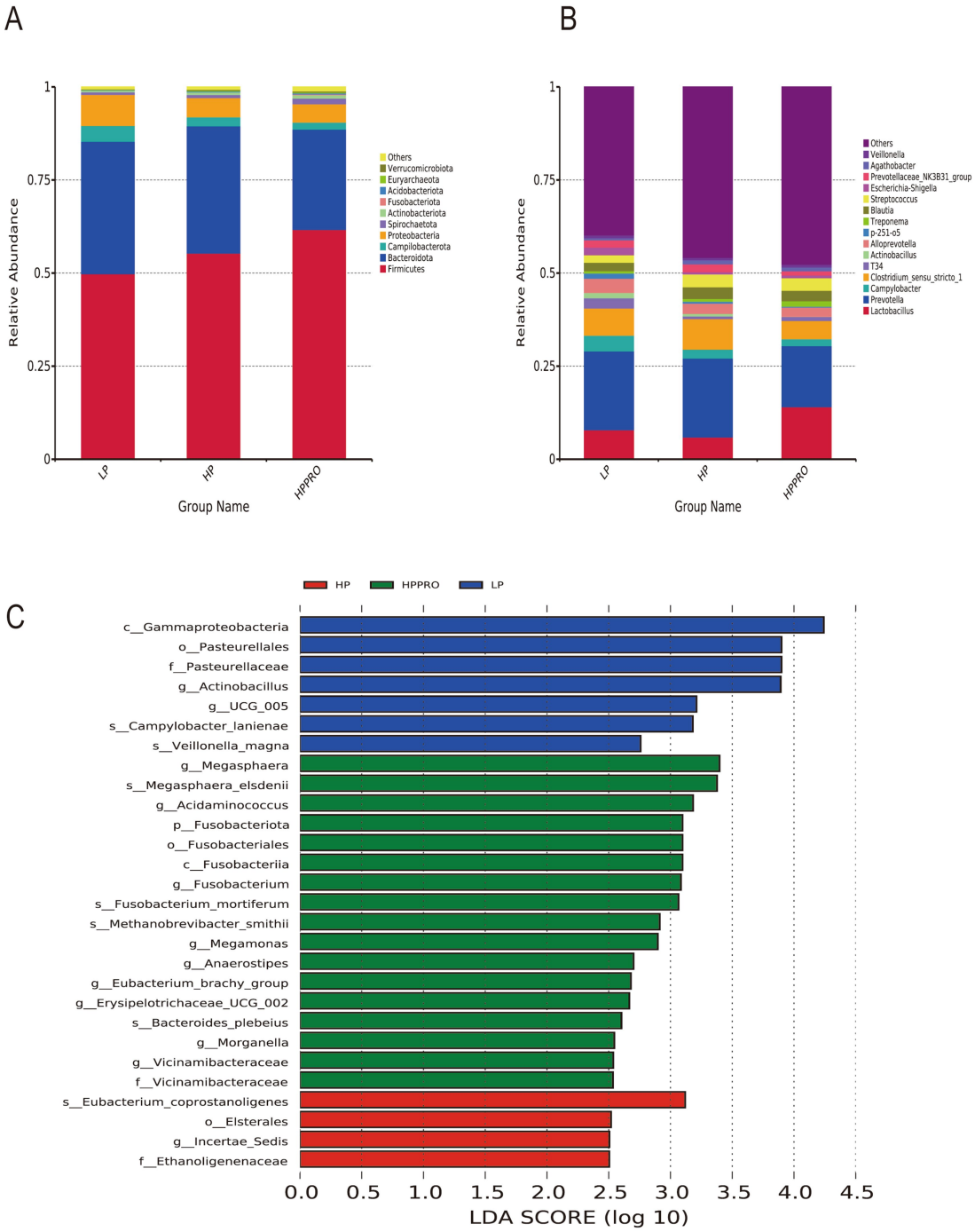

FIGURE 5
Gut microbiota of the colonic chyme in weaned piglets. Relative abundance at the phylum level **(A)** and genus level **(B)**; **(C)** Differentially abundant taxa in weaned piglets based on LEfSe analysis (LDA score > 2.5). LP, low-protein diet; HP, high-protein diet; HPPRO, high-protein diet with probiotics. $n = 8$.

the nucleus (28), and thereby modulates the expression of TNF-α. In this study, the decreased mRNA expressions of TLR-4, TRAF-6, and TNF-α in the HPPRO group further indicated that dietary supplementation with *B. subtilis PB6* ameliorated potential inflammatory lesions in weaned piglets. Short-chain fatty acids (SCFAs) have been widely reported to regulate host immunity and intestinal health (29). In this study, HPPRO piglets had higher butyric acid levels than LP piglets. The genera g_*Anaerostipes* and g_*Megasphaera*, which produce SCFAs, especially butyric acids (20, 30), showed increased abundance in HPPRO piglets. Notably, a previous study demonstrated that butyrate inhibits intestinal inflammatory responses and reduces proinflammatory factor production by inhibiting innate immune responses and inhibiting macrophage TNF-α (31). In support, our correlation analysis showed that the concentration of butyric acid was negatively correlated with the mRNA expressions of TLR-4, TRAF-6, and TNF-α, further confirmed the beneficial effect of butyric acid on the regulation of inflammation.

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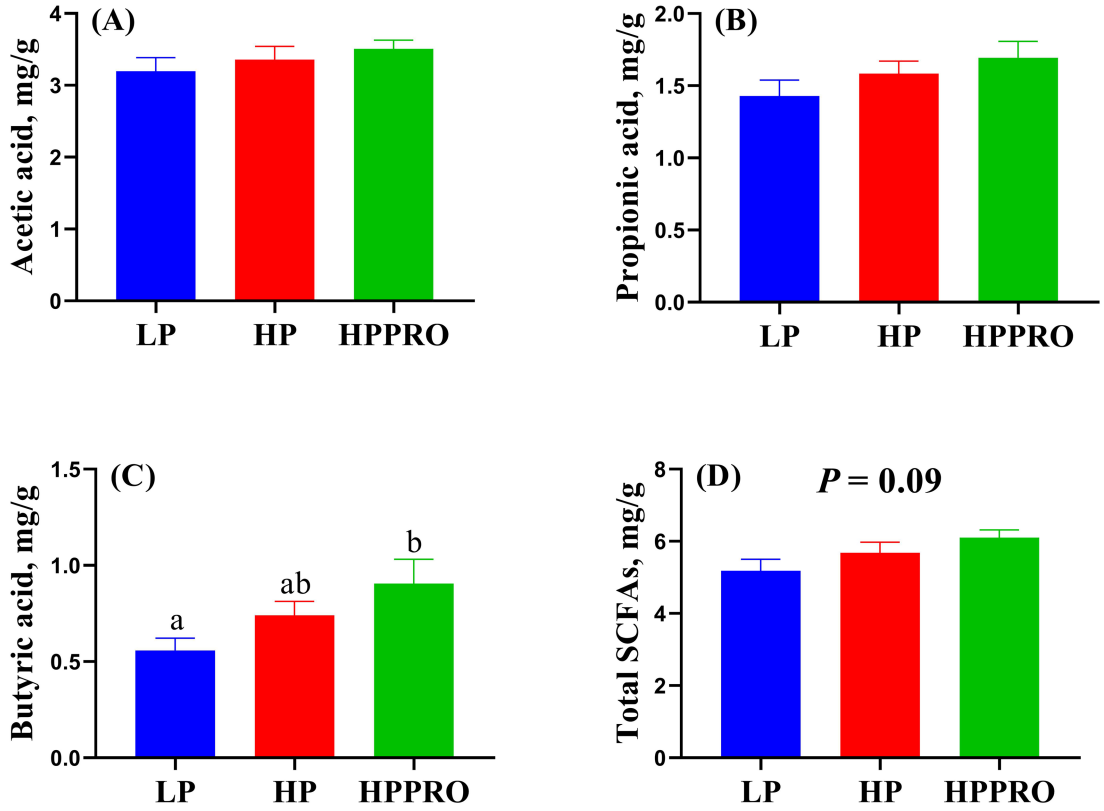

FIGURE 6
Short-chain fatty acids in the colonic digesta of weaned piglets. Total SCFAs are the sum of acetic acid, propionic acid, and butyric acid. LP, low-protein diet; HP, high-protein diet; HPPRO, high-protein diet with probiotics. n = 8. [a,b] Values without a common letter differ significantly ($P < 0.05$).

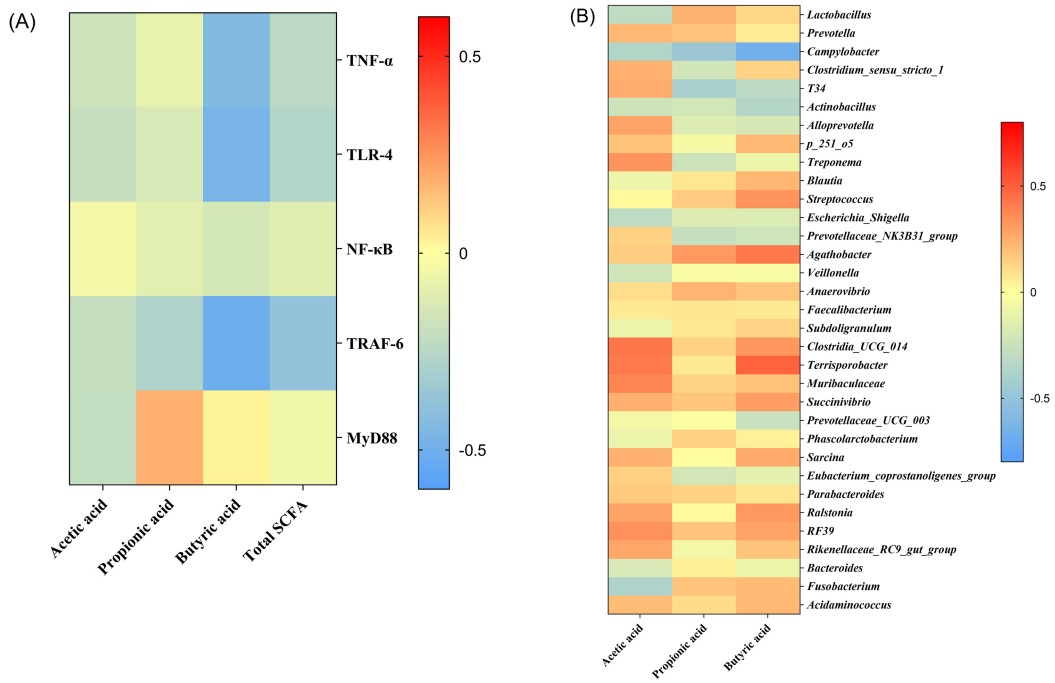

FIGURE 7
Heatmaps of the correlation analysis. Associations between short-chain fatty acids and immune-related gene expressions based on Pearson correlation analysis **(A)**; Associations between gut microbiota and short-chain fatty acids based on Spearman's correlation analysis **(B)**. Correlation coefficients were colored according to the scale listed on the right.

5 Conclusion

In this study, piglets fed the HP diet had higher diarrhea scores, which may be associated with decreased nutrient digestibility and altered gut microbiota composition; however, the inclusion of 300 mg/kg *B. subtilis PB6* in a high-protein diet improved nutrient digestibility, ameliorated diarrhea, and stress-related indicators, resulting in better growth performance and intestinal health.

Data availability statement

The data presented in the study are deposited in the NCBI repository, accession number PRJNA1215014. The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding author/s.

Ethics statement

The experiment was performed following the animal protection law (Ethic Approval Code: SICAU 2023314113) and was performed in accordance with the Guide for the Animal Care and Use approved by Sichuan Agricultural University Institutional Animal Care and Use Committee. The study was conducted in accordance with the local legislation and institutional requirements.

Author contributions

YLiu: Conceptualization, Data curation, Writing – original draft. LCa: Investigation, Methodology, Writing – review & editing. CY: Methodology, Writing – review & editing. QZ: Conceptualization, Methodology, Writing – review & editing. HL: Methodology, Supervision, Validation, Writing – review & editing. RZ: Methodology, Supervision, Validation, Writing – review & editing. JT: Methodology, Supervision, Validation, Writing – review & editing. ZZ: Methodology, Supervision, Validation, Writing – review & editing. ZL: Methodology, Supervision, Validation, Writing – review & editing. XJ: Methodology, Supervision, Validation, Writing – review & editing. ZF: Methodology, Supervision, Validation, Writing – review & editing. YLin: Methodology, Supervision, Validation, Writing – review & editing. SX: Methodology, Supervision, Validation, Writing – review & editing. YZ: Validation, Writing – review & editing, Methodology, Supervision. LH: Methodology, Supervision, Validation, Writing – review & editing. DW: Methodology, Supervision, Validation, Writing – review & editing. BF: Conceptualization, Funding acquisition, Supervision, Writing – review & editing. LCh: Conceptualization, Funding acquisition, Supervision, Writing – review & editing.

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Conflict of interest

ZZ and ZL were employed by Kemin (China) Technologies Co., Ltd.

The authors declare that this study received funding from Kemin (China) Technologies Co., Ltd. The funder had the following involvement in the study: study design and decision to publish.

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

The reviewer YP declared a past co-authorship with the author LCa to the handling editor.

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The authors declare that no Generative AI was used in the creation of this manuscript.

Publisher's note

Supplementary material

The Supplementary material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fvets.2025.1525354/full#supplementary-material

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The effects of fermented *Astragalus* polysaccharides on the growth performance, antioxidant capacity and intestinal health of broilers

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This study aimed to investigate the effects of fermented *Astragalus* polysaccharides(FAP) on the growth performance, antioxidant capacity and intestinal health of broilers. A total of 1,080 Cyan-shank Partridge chickens were divided into 4 groups, with 6 replicates per group and 45 chickens per replicate. Add 0% (T1), 0.2% (T5), 0.4% (T6) and 0.6% (T7) of FAP to the basal diet, respectively. The trial lasted for 42 days. The results indicated that, compared to the T1 group, FW and ADG of broilers in each treatment group were significantly increased ($p < 0.05$). The slaughter rates of the T6 and T7 groups were significantly higher compared to the T1 group, meanwhile, the carcass yields of the T5, T6, and T7 groups were notably enhanced ($p < 0.05$). Compared with T1 group, the activities of CAT, GSH-Px and the content of T-AOC in T6 and T7 groups were increased ($p < 0.05$), while the content of MDA was decreased ($p < 0.05$). All groups exhibited significantly VH and VH/CD in the duodenum compared to the T1 group ($p < 0.05$). Compared with the T1 group, the relative mRNA expression levels of *ZO-1* and *Claudin* in the jejunal mucosa of broilers in all groups were significantly up-regulated, while the expressions of *IL-1β*, *IL-6*, *TNF-α*, and *IFN-γ* were down-regulated ($p < 0.05$). 16S rDNA sequencing analysis revealed that at the phylum level, the abundance of Verrucomicrobiota in the T6 group was significantly increased compared to the T1 group ($p < 0.05$). Cyanobacteria, Nitrospirota, Elusimicrobiota, and Acidobacteriota were unique to the T6 group, while Cyanobacteria and Elusimicrobiota were unique to the T5 group compared to the T1 group. At the genus level, the abundance of *Desulfovibrio* was significantly reduced in the T6 group compared to the T1 group ($p < 0.05$). Additionally, fermented *Astragalus* polysaccharides increased the abundance of *Bacteroidota, Campilobacterota*, *Deferribacterota*, *Firmicutes*, *Fusobacteriota*, *Proteobacteria*, and *Spirochaetota* ($p < 0.05$). The LEfSe analysis found that *Clostridia_vadinBB60_group* and *Comamonas* were identified as potential biomarkers. Overall, feeding fermented *Astragalus* polysaccharides can enhance the growth performance, slaughter characteristics, and antioxidant capacity of broiler chickens by modulating the gut microbiota and strengthening intestinal barrier function.

KEYWORDS

fermented *Astragalus* polysaccharides, antioxidant, intestinal barrier, intestinal microbiota, broilers

1 Introduction

Astragalus is a perennial herbaceous legume widely distributed in temperate regions (1). Astragalus polysaccharide (APS) is one of the main active components of *Astragalus* and has been widely used in animals due to its solubility characteristics (2). Studies have shown that APS not only promotes the colonization of beneficial bacteria in the intestinal tract, but also effectively inhibits the growth of harmful pathogens, thus maintaining intestinal health. In addition, APS has the ability to scavenge Reactive Oxygen Species (ROS) and reduce intestinal inflammation, which further improves intestinal barrier function (3). Also, APS can promote the secretion of immunoglobulin A (IgA), which enhances mucosal immunity (4, 5). APS can activate lymphocyte receptor signaling pathway, which enhances the body's adaptive and innate immunity, thus exerting its antimicrobial effects (6). APS if regulates gene expression of *VCAM1, RELA, CDK2* and other genes to treat pulmonary fibrosis effects (7). In addition, incorporation of APS into the diet promotes growth, improves feed conversion efficiency, and enhances the antioxidant capacity of the organism, e.g., by increasing *superoxide dismutase* (SOD) and *catalase* (CAT) activities (8, 9). In mammalian aquaculture, the addition of APS has been shown to inhibit the lipopolysaccharide-induced *mitogen-activated protein kinase* (*MAPK*) and nuclear *factor-κB* (NF-κB) inflammatory pathways, reduce the expression of intestinal inflammatory factors, improve the morphology of intestinal villi, and reduce intestinal damage, thereby enhancing intestinal immunity and productivity (10). Herbal polysaccharides play a major role in metabolic diseases and have wide applicability to livestock health. Therefore, the therapeutic potential of herbs is enormous (11).

A wide variety of strains are available for the fermentation of traditional Chinese medicine, and we evaluated them in terms of the convenience of testing conditions and the broad spectrum of antimicrobial substances. The results showed that *Bacillus licheniformis* had excellent antimicrobial capacity and tolerance compared to other probiotics and was able to maintain activity at room temperature. It was also shown that the products fermented by *Bacillus licheniformis* may be novel candidate food additives with impact on the farming industry (12). Therefore, we chose *Bacillus licheniformis* as herbal fermentation agent. *Bacillus licheniformis* is widely used in farming (13) and has been shown to possess anti-inflammatory properties (14). Studies on porcine intestinal epithelial cells have shown that *B. licheniformis* can improve the barrier function of intestinal epithelial cells, regulate the expression of *interleukin 6* (*IL-6*) and *interleukin 8* (*IL-8*), and reduce intracellular Reactive Oxygen Species (ROS) production, resulting in enhanced intestinal function and conversion efficiency (15). In addition, it increases the abundance of *B. mitoticus* in the feces of weaned piglets, thereby stabilizing the intestinal microbiota (16). In poultry farming, the addition of *Bacillus licheniformis* to feed has been found to enhance growth performance, immune status, and antioxidant capacity, increase short-chain fatty acid production, and modulate the intestinal microbiota in *broilers* (17). Previous studies have found that fermented *astragalus* polysaccharides inhibit the expression of the pro-inflammatory cytokines tumor necrosis factor *α* (*TNF-α*) and *interleukin 6* (*IL-6*), thereby attenuating tissue inflammatory injury (18).

However, within the context of restricted use of antibiotics, the exploration for safe and effective antibiotic substitutes has emerged as a central focus in the livestock and poultry breeding industry. Fermented polysaccharides, as a novel green feed additive, possess broad application prospects. Current research on the application of astragalus polysaccharides has revealed that astragalus polysaccharides can pass through the Reactive Oxygen Species (ROS) pathway in chicken embryonic fibroblasts thereby impeding cadmium-induced autophagic damage and protect chicken peripheral blood lymphocytes through the *MDA5/NF-κB* pathway.In this study (19, 20). We hope to provide a theoretical basis for elucidating the mechanism of action of *Bacillus licheniformis* fermented Astragalus polysaccharide in poultry farming by combining *Bacillus licheniformis* with APS for the first time for fermentation treatment and further exploring its effects on growth performance, antioxidant capacity and intestinal health of broilers based on the results of the previous work.

2 Materials and methods

2.1 Experimental feed

Bacillus licheniformis was inoculated into LB liquid medium and incubated at 37°C for 24 h to obtain activated bacterial liquid. Then, APS (APS: bacterial liquid = 7:3) was added to *Bacillus licheniformis* (108 CFU/mL) and incubated at 37°C for 24 h. The FAP was then lyophilized and stored in well-sealed glass ampoules protected from moisture by silica gel desiccant. The dried FAP was mixed thoroughly with different ratios (0.2, 0.4, 0.6%) of the basal diet (Table 1), and the temperature and pH conditions were strictly controlled throughout the experimental cycle to avoid any imbalance of FAP.

2.2 Experiment design

A total of 1,080 healthy male Cyan-shank Partridge chickens，free of vertically transmitted diseases and in uniform body condition, weighing 3,135–3,335 g, were selected for this study. Divided into four groups, with 6 replicates per group and 45 chickens per replicate. Each replicate was housed in a cage measuring 220 cm × 200 cm × 100 cm. The T1 group received a basal diet, while the T5, T6, and T7 groups received basal diets supplemented with 0.2, 0.4, and 0.6% FAP, respectively. The experimental period lasted for 42 days. The chickens were fed twice daily and had access to natural light, water, and feed throughout the study. The temperature inside the chicken house was maintained between 18°C and 25°C. The experimental design of this study is shown in Figure 1.

2.3 Sample collection

At the end of the experiment, one chicken was randomly selected from each replicate, and 5 mL of blood was collected from the wing vein. Serum was separated by centrifugation for serum antioxidant index analysis. The chickens were then euthanized using carbon dioxide and dissected to measure carcass traits. Samples (approximately 2 cm each) were taken from the middle sections of the duodenum, jejunum, and ileum, rinsed with physiological saline to remove contents, and preserved in 4% paraformaldehyde for intestinal morphology analysis. Jejunal mucosa and cecal content samples were

TABLE 1 Formulation and nutrient composition of basal diets.

Ingredient	Base diet	Nutritional levels[b]	
Corn	60.00	Metabolic energy (MJ/kg)	12.15
Soybean meal	20.20	Crude protein	18.80
Wheat bran	5.00	Calcium	0.95
Shell powder	8.00	Phosphorus	0.65
$Ca(HCO_3)_2$	2.00	Ca	0.73
Lysine	0.10	P	0.37
Methionine	0.17	Lysine	0.99
NaCl	0.33	Methionine	0.44
Soybean oil	1.20		
Premix[a]	3.00		
Total	100		

[a]The premix provides the following nutrients per kilogram of feed: biotin (0.1 mg), pantothenic acid (20 mg), folic acid (1.5 mg), niacin (50 mg), zinc (110 mg), iodine (0.6 mg), copper (9 mg), iron (100 mg), selenium (0.16 mg), manganese (100 mg), vitamin A (15,000 IU), vitamin D3 (2,500 IU), vitamin E (20 mg), vitamin K3 (3 mg), vitamin B1 (3 mg), vitamin B2 (8 mg), vitamin B6 (7 mg), and vitamin B12 (0.03 mg).
[b]All values were measured except for metabolizable energy.

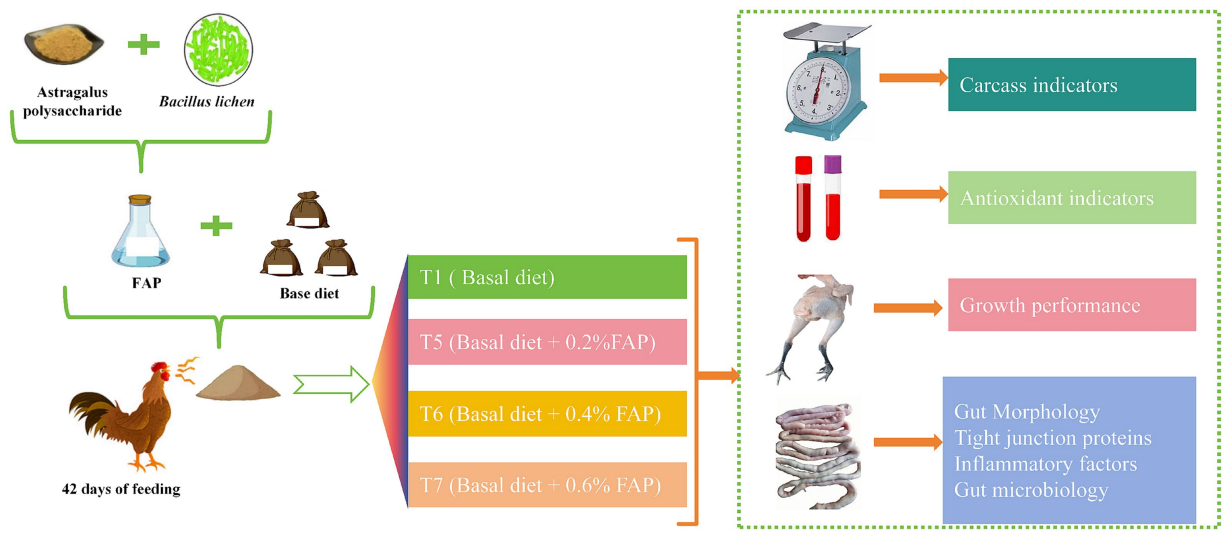

FIGURE 1
Experimental mechanism.

collected, rapidly frozen in liquid nitrogen, and stored at −80°C for further analysis.

2.4 Measurement and methods

2.4.1 Growth performance

Daily feed intake and leftover feed were recorded throughout the experimental period. On days 1 and 42 of the trial, chickens were weighed after a 12-h fasting period to determine initial and final weights. Based on feed consumption, the following parameters were calculated:

Average Daily Feed Intake (ADFI) (g) = Total feed consumption / Number of trial days.

Average Daily Gain (ADG) (g) = (Final weight - Initial weight) / Number of trial days.

Feed Conversion Ratio (F/G) = Total feed consumption / (Final weight - Initial weight).

2.4.2 Carcass quality

At the end of the experiment, one chicken was randomly selected from each replicate, euthanized using carbon dioxide, and quickly dissected to assess slaughter performance. The measured parameters were as follows:

Slaughter Yield (%) = 100% × carcass weight / live weight.

Eviscerated Yield (%) = 100% × eviscerated weight / live weight.

Dressed Yield (%) = 100% × dressed weight / live weight.

Leg Muscle Yield (%) = 100% × leg muscle weight / dressed weight.

TABLE 2 Primer sequences.

Genes	Sequence 5′ – 3′	GenBank number
GAPDH	F: GGAAAGTCATCCCTGAGCTGAAT	NM_204305.1
	R: GGCAGGTCAGGTCAACAACA	
ZO-1	F: AATACCTGACTGTCTTGCAG	XM_015278975.1
	R: TAAAGAAGGCTTTCCCTGAC	
IFN-γ	F: AAAGCCGCACATCAAACACA	NM_205149.1
	R: GCCATCAGGAAGGTTGTTTTTC	
Occludin	F: GCAGATGTCCAGCGGTTACTAC	NM_205128.1
	R: CGAAGAAGCAGATGAGGCAGAG	
Claudin	F: CATACTCCTGGGTCTGGTTGGT	NM_001013611.2
	R: GACAGCCATCCGCATCTTCT	
IL-1β	F: ACTGGGCATCAAGGGCTA	XM_015297469
	R: GGTAGAAGATGAAGCGGGTC	
IL-6	F: GAAATCCCTCCTCGCCAATCT	XM_0152812832
	R: CCTCACGGTCTTCTCCATAAACG	
TNF-α	F: TGTGTATGTGCAGCAACCCGTAGT	NM_204267
	R: GGCATTGCAATTTGGACAGAAGT	

Breast Muscle Yield (%) = 100% × breast muscle weight / dressed weight.

2.4.3 Serum antioxidant capacity

Following the kit instructions (Jiancheng Biotech Ltd., Nanjing, China), Serum catalase (CAT), glutathione peroxidase (GSH-Px), superoxide dismutase (SOD), total antioxidant capacity (T-AOC), and malondialdehyde (MDA) activities or levels were determined using a Multiskan GO microplate spectrophotometer (Multiskan GO Microplate Spectrophotometer without cuvette, Thermo Fisher Scientific, Japan).

2.4.4 Intestinal morphology

After immersion in 4% paraformaldehyde, the duodenum, jejunum and ileum experienced dehydration, transparency, embedding, and sectioning into 5-micrometer slices. Subsequently, the sections were dewaxed and rehydrated. Hematoxylin and eosin (HE) staining was performed followed by dehydration, clearing, and mounting. Images were captured using an optical microscope. Using Image Pro Plus 6.0 (Media Cybernetics, Inc. Silver Spring, Maryland, USA) software, villus height (VH) and crypt depth (CD) were measured to assess intestinal morphology.

2.4.5 Prediction of action site of Astragalus polysaccharide

The structural formula of astragalus polysaccharide was drawn by Chem Draw. The structure of *Occludin* (*1WPA, Homo sapiens*) and *ZO-1 protein* (*3LH5, Homo sapiens*) were retrieved from PDB database.[1] Molecular docking was performed using Autodock Vina software, and Discovery Studio software was utilized to optimize the docking results output (21).

2.4.6 Gene expression analysis of jejunal mucosa

Jejunal mucosa samples were ground in liquid nitrogen and total RNA was extracted from 50 to 100 mg of tissue according to the manufacturer's instructions TaKaRa MiniBEST Universal RNA Extraction Kit (N0.9769). RNA integrity and concentration were assessed using 1% agarose gel electrophoresis and a micro UV spectrophotometer (Thermo Fisher Scientific, Japan) to ensure that the *OD260/OD280* ratio was between 1.8 and 2.1. cDNA was synthesized from RNA samples according to the manufacturer's protocol (RR047A, TaKaRa PrimeScript™ RT reagent Kit with gDNA Eraser, TaKaRa). cDNA was synthesized using the CFX96 Real-Time PCR (Bio-Rad Laboratories, Inc., Hercules, California, USA). Real-time quantitative PCR amplification was performed using the *CFX96* Real-Time PCR (Bio-Rad Laboratories, Inc., Hercules, California, USA) detection system. The primers are shown in Table 2, and *GAPDH* was used as the reference gene.

2.4.7 Sequencing of cecal contents 16S rDNA

Microbial DNA was extracted from 200 mg of cecal samples from each group using the MagPure Soil DNA LQ kit (Guangdong Magen, China), following the manufacturer's instructions. The V3-V4 hypervariable region of the bacterial 16S rRNA gene was amplified using TksGflflex DNA Polymerase (Takara, R060B) and universal primers 343F (5'-TACGGRAGGCAGCAG-3′) and 798R (5'-AGGGTATCTAATCCT-3′) in a 30 μL reaction mixture. Sequencing was performed on the Illumina NovaSeq6000 platform with paired-end reads of 250 bases per cycle. The sequencing of the 16S rDNA gene amplicon fragments and subsequent bioinformatics analysis were conducted by Shanghai OE Biotech Co., Ltd.

1 https://www.rcsb.org/

TABLE 3 Effects of FAP on growth performance of chickens.

Items	T1	T5	T6	T7	SEM	*P*-value
IW (kg)	1.48	1.47	1.51	1.47	0.02	0.073
FW (kg)	2.92^{b}	3.14^{a}	3.23^{a}	3.19^{a}	0.02	*<0.01*
ADG(g)	34.40^{b}	39.67^{a}	40.77^{a}	40.98^{a}	0.87	0.013
ADFI(g)	156.70^{a}	152.13^{ab}	148.98^{ab}	146.99^{b}	0.95	*<0.01*
F/G	4.60^{a}	3.87^{b}	3.67^{b}	3.61^{b}	0.55	*<0.01*

Means with different lowercase are significantly different ($P < 0.05$, Duncan's multiple comparisons).

2.4.8 Data analysis

Alpha and beta diversity analyses were conducted using QIIME software. The alpha diversity of the samples was assessed using indices including Chao1 and Shannon. Unweighted UniFrac distance matrices calculated in R were used for unweighted UniFrac principal coordinates analysis (PCoA) to evaluate the beta diversity of the samples. Differential analysis was performed using ANOVA/Kruskal Wallis/T test/Wilcoxon statistical methods based on R packages. LEfSe was used for differential analysis of species abundance profiles. The relative expression of target gene mRNA was calculated using the $2^{-\Delta\Delta Ct}$ method. All data were initially processed using Excel and subsequently analyzed using SPSS 23.0. One-way ANOVA was performed to analyze the differences among groups, followed by Duncan's multiple range test for post-hoc comparisons. Differences were considered statistically significant at $p < 0.05$. Results are presented as mean values ± standard error.

3 Results

3.1 Effects of FAP on growth performance of chickens

Table 3 illustrates the impact of the FAP on the growth performance of the chickens. Compared to the T1 group, the addition of FAP significantly enhanced the FW and ADG, while notably reducing the F/G ($p < 0.05$). Furthermore, in comparison to the T1 group, the ADFI in the T7 group was significantly decreased ($p < 0.05$).

3.2 Effects of FAP on slaughter performance of chickens

Figure 2A shows the slaughter experiment process. According to the results presented in Figure 3, FAP had a significant impact on the slaughter performance of the chickens. Compared to the T1 group, the slaughter weight in the T6 and T7 groups was significantly increased ($p < 0.05$). Additionally, the visceral carcass yield in the T5, T6, and T7 groups showed a remarkable increase ($p < 0.05$). Furthermore, the yield of breast meat in the T7 group was also significantly enhanced ($p < 0.05$). However, there were no significant differences in semi-eviscerated carcass yield and thigh yield among the groups ($P > 0.05$) (Figures 2B–F).

3.3 Effects of FAP on serum antioxidant capacity of chickens

As shown in Figure 3, the experimental mechanism diagram illustrates the process of serum biochemical assays (Figure 3A). Compared to the T1 group, the activities of CAT in the T5, T6, and T7 groups were significantly elevated ($p < 0.05$). Additionally, the activities of GSH-Px and T-AOC levels in the T6 and T7 groups also significantly increased ($p < 0.05$). Compared with the T1 group, the MDA levels in all groups were significantly reduced ($p < 0.05$) (Figures 3B–F).

3.4 Effects of FAP on intestinal function in chickens

Figure 4 clearly demonstrates that the supplementation of FAP improved the intestinal villus morphology of broiler chickens. A summary of the effects of FAP on intestinal morphology is presented in the experimental mechanism diagram (Figure 4A). Compared to the T1 group, the VH and VH/CD ratio in the duodenum of the T5, T6, and T7 groups were significantly increased ($p < 0.05$). In the jejunum, VH was significantly increased in the T5, T6, and T7 groups, while CD in the T6 and T7 groups was significantly decreased compared to the T1 group, resulting in an increased VH/CD ratio ($p < 0.05$). Furthermore, in the ileum, both VH and CD were significantly improved in the T5, T6, and T7 groups, with the VH/CD ratio in the T5 and T6 groups also significantly increased ($p < 0.05$) (Figures 4B–D).

As illustrated in Figure 5, the relative mRNA expression levels of *ZO-1* and *Claudin* were significantly up-regulated in T5, T6 and T7 groups compared with T1 group (Figure 5A). In addition, the relative mRNA expression levels of *Occludin* were significantly increased in T5 and T6 groups ($p < 0.05$) (Figure 5A). On the contrary, the relative mRNA expression levels of *IL-1β, IL-6, TNF-α*, and *IFN-γ* were significantly down-regulated ($p < 0.05$) in T5, T6 and T7 groups compared with T1 group (Figure 5B). Molecular docking experiments were then performed, and the three-dimensional structures of *Occludin* protein and *ZO-1* protein are shown in Figure 5C, indicated by blue bands. The enlarged area demonstrates the binding site of APS with it. The prediction using Swiss Target Prediction revealed that APS can bind well with *Occludin* and *ZO-1*. The relationship between APS and *ZO-1* and *Occludin* was verified practically. The binding energy of APS to *ZO-1* was −7.1 Kcal/mol. Meanwhile, the binding energy of APS and *Occludin* was −5.0 Kcal/mol (Figure 5C). In addition, it can be seen that APS (represented as a ball-and-stick model) forms interactions with specific amino acid residues of *Occludin* proteins (e.g., K504, K501, Y493, D488, R497, R500, R454, K443) (Figure 5C), while interacting with specific amino acid residues of the *ZO-1* proteins (e.g., T630, R645, F630 R624, Y535, R522, G540, N799, D714, N717, V798) to form interactions (Figure 5C). The intermolecular dashed lines represent hydrogen bonding or hydrophobic interactions. These results suggest that these residues may be critical sites for APS to exert its drug effects. It is also

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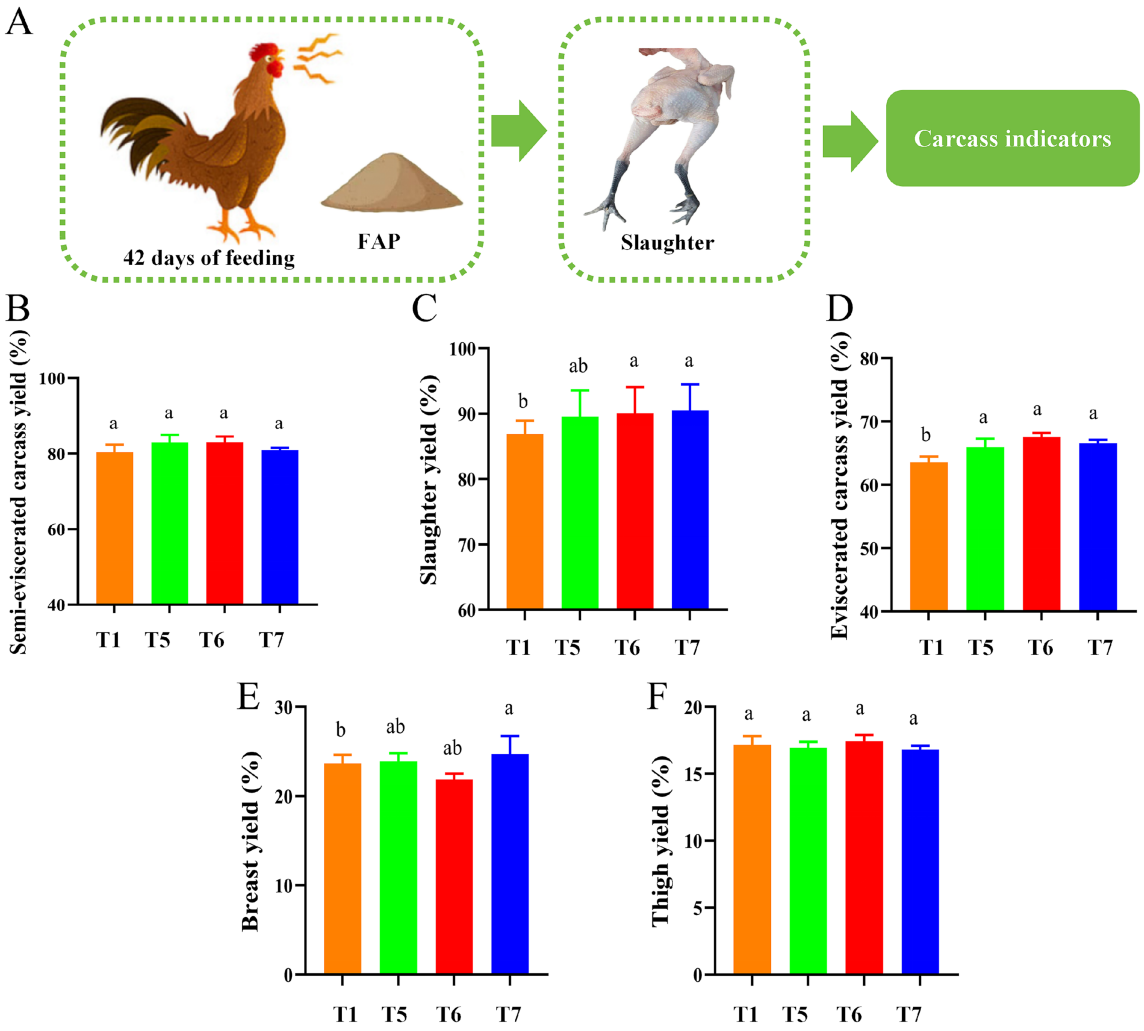

FIGURE 2
Assessment of chicken slaughter performance. **(A)** Diagram of slaughter performance assessment mechanism. **(B)** Semi-skinless carcass yield. **(C)** Slaughter yield. **(D)** Exsanguinated carcass yield. **(E)** Breast yield. **(F)** Thigh yield. Different lowercase letters indicate significant differences ($p < 0.05$, Duncan multiple comparisons).

an important binding site for improving the function of *Occludin* and *ZO-1* proteins.

3.5 The effect of FAP on intestinal microbiota in chickens

This study employed 16S rDNA sequencing to analyze the cecal contents of each group. A total of 2,862 amplicon sequence variants (ASVs) were detected across the four groups, with 569 ASVs found to be shared among all groups. The unique ASVs for the T1, T5, T6, and T7 groups were 454, 419, 387, and 361, respectively (Figure 6A). Bacterial diversity within the cecal microbiota was assessed using Chao1, ACE, Shannon and Simpson indices. Compared to the T1 group, the ACE, Chao1, and Shannon indices of the cecal microbiota in the T5 and T6 groups were significantly lower ($p < 0.05$) (Figures 6B–E). In contrast, there was no statistically significant difference in the Simpson index between groups ($P > 0.05$) (Figure 6D). Beta diversity analysis was conducted using principal coordinates analysis (PCoA) based on weighted UniFrac distance to compare the overall microbial profiles among the groups. The PCoA results indicated that the differences between the two principal coordinates accounted for 37.62%, with PC1 and PC2 explaining 16.52 and 21.1% of the variance in the original dataset, respectively ($p < 0.05$) (Figure 6F).

To further assess differences in microbial community structures, the unweighted UniFrac distance analysis revealed distinct clustering in the unweighted heatmap (Figure 6G). When comparing this with the weighted UniFrac distance, we observed that samples T5, T6, and T7 tended to cluster together, indicating consistency in the treatment effects (Figures 6G,H). Additionally, it was established that abundance information plays a crucial role in evaluating differences among samples.

3.6 Effects of FAP on the structure of chicken intestinal microbiota

To assess the impact of FAP on the cecal microbiota, we analyzed the classification and composition of the cecal microbiota at the

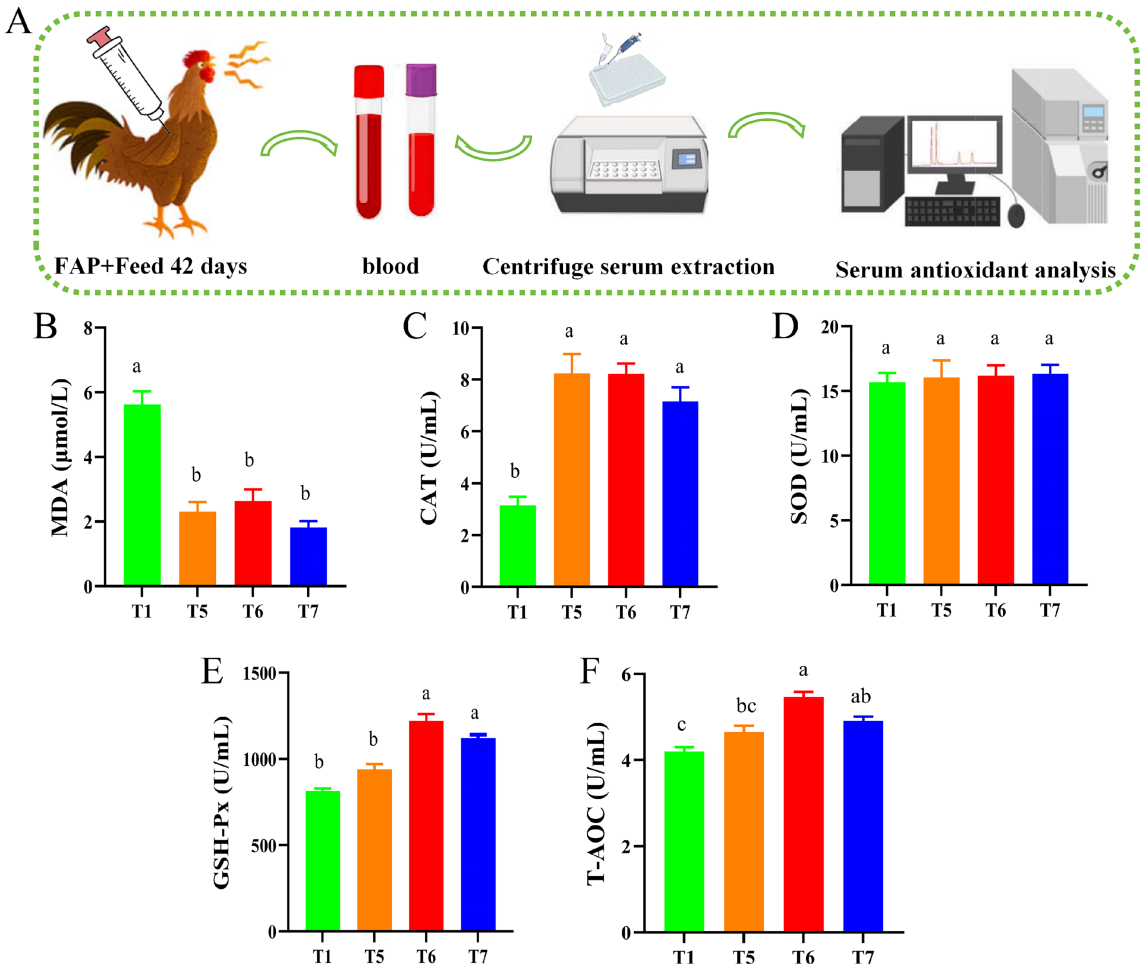

FIGURE 3
Chicken serum antioxidant capacity assays. **(A)** Diagram of serum biochemical assay mechanism. **(B)** MDA content. **(C)** CAT activity. **(D)** SOD activity. **(E)** GSH-Px activity. **(F)** T-AOC level. Different lowercase letters indicate significant differences ($p < 0.05$, Duncan multiple comparisons).

phylum and genus levels (Figures 7A–D). At the phylum level, the composition of the top 15 phyla was analyzed (Figure 8B), and a clustering heatmap of these phyla was generated for multi-factor analysis. The results indicated that Bacteroidota and Firmicutes were the dominant phyla in the cecum of broiler chickens, accounting for over 84% of the total microbial community. Compared to the T1 group, the abundance of Verrucomicrobiota significantly increased in the T6 group (0.10% vs. 0.27%, $p < 0.05$). Cyanobacteria, Nitrospirota, Elusimicrobiota, and Acidobacteriota were unique to the T6 group, while Cyanobacteria and Elusimicrobiota were unique to the T5 group.

At the genus level, the top 15 most abundant genera were identified (Figure 7C), with dominant genera including *Bacteroides*, *Rikenellaceae_RC9 gut group*, *Prevotellaceae_UCG-001*, *Prevotellaceae_Ga6A1 group*, *Clostridium vadinBB60 group*, and *Parabacteroides*. The abundance of *Clostridium vadinBB60 group* significantly increased compared to the T1 group, while *Desulfovibrio* decreased significantly ($p < 0.05$). Additionally, the abundance of *Rikenellaceae_RC9 gut group* decreased in the T6 and T7 groups compared to the T1 group, while the abundance of *Prevotellaceae_Ga6A1 group* and *Parabacteroides* increased ($p < 0.05$). All groups showed a decline in the abundance of *Prevotellaceae_UCG-001* compared to the T1 group ($p < 0.05$). A clustering heatmap for the top 30 genera further confirmed that the prominent dominant genera included *Bacteroides*, *Rikenellaceae_RC9 gut group*, *Prevotellaceae_UCG-001*, and *Prevotellaceae_Ga6A1 group*.

At the class level, the dominant microbial communities were primarily concentrated in *Bacteroidia*, *Clostridia* and *Gammaproteobacteria-γ* (Figure 7E). At the order level, the dominant microbial communities were primarily concentrated in *Bacteroidales*, *Oscillospirales*, *Clostridia_vadinBBB60_group*, and *Erysipelotrichales* (Figure 8F). At the family level, the dominant microbial communities were mainly focused on *Bacteroidaceae*, *Prevotellaceae*, *Ruminococcaceae*, and *Tannerellaceae* (Figure 7G). Finally, at the species level, the dominant microbial communities were concentrated in *Bacteroides barnesiae*, *Bacteroides* caecigallinarum, and *Bacteroides coprophilus* (Figure 7H).

To further explore the evolutionary relationships between the classification units (ASVs) and the impact of FAP on gut health and microbial diversity in chickens, we hypothesized that the addition of FAP influenced the structure and composition of chicken gut microbiota at the phylum level. For instance, higher abundances of *Bacteroidota*, *Campilobacterota*, *Deferribacterota*, *Desulfobacterota*, *Firmicutes*, *Fusobacteriota*, *Proteobacteria*, and *Spirochaetota* were

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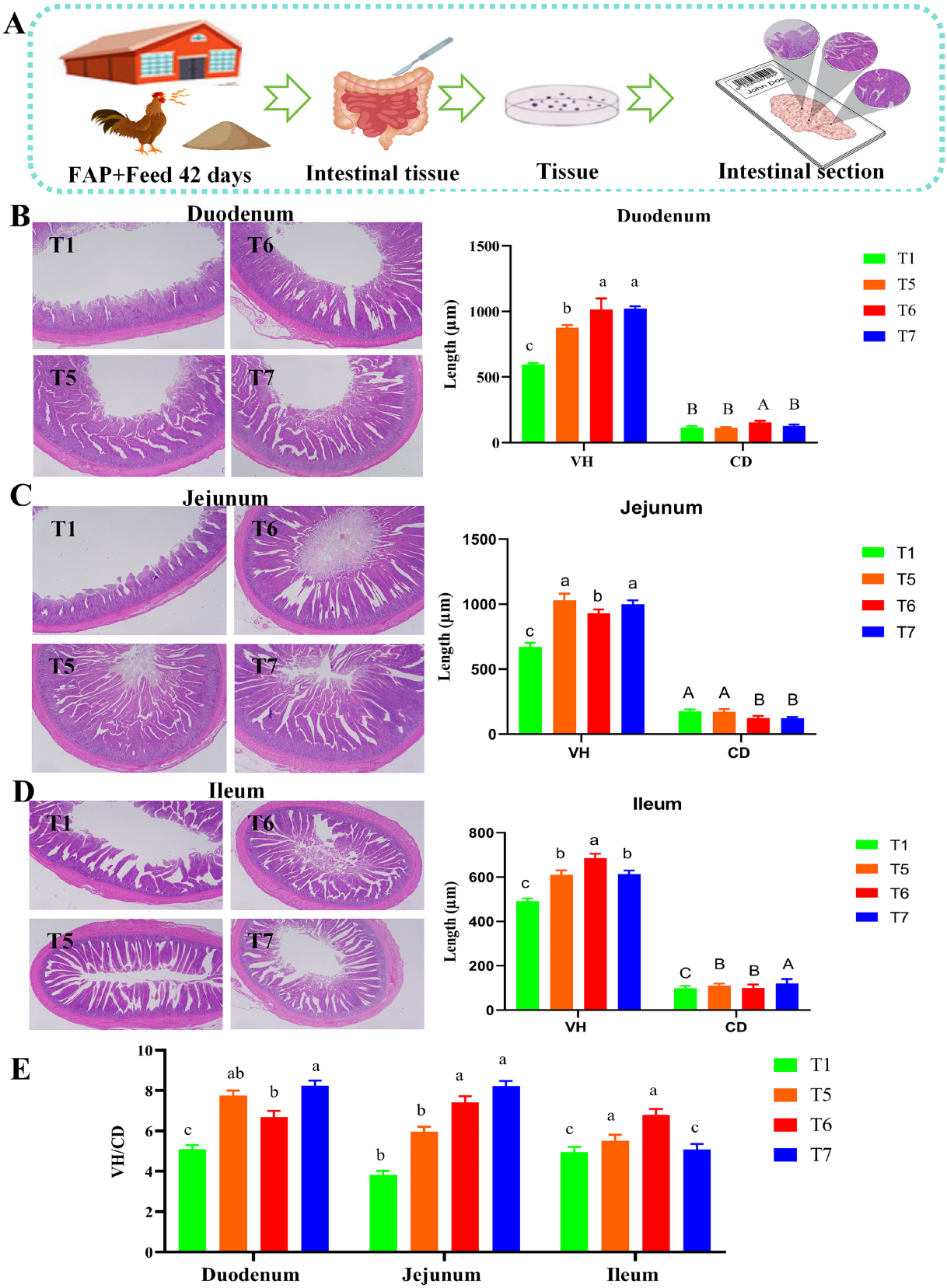

FIGURE 4
Intestinal morphology of chickens. **(A)** Mechanism diagram of section staining assessment. **(B)** Duodenum structural change of tissue sections. **(C)** Jejunum structural change of tissue sections. **(D)** Ileum structural change of tissue sections. **(E)** VH/CD ratio. Different lowercase letters indicate significant differences ($p < 0.05$, Duncan multiple comparisons).

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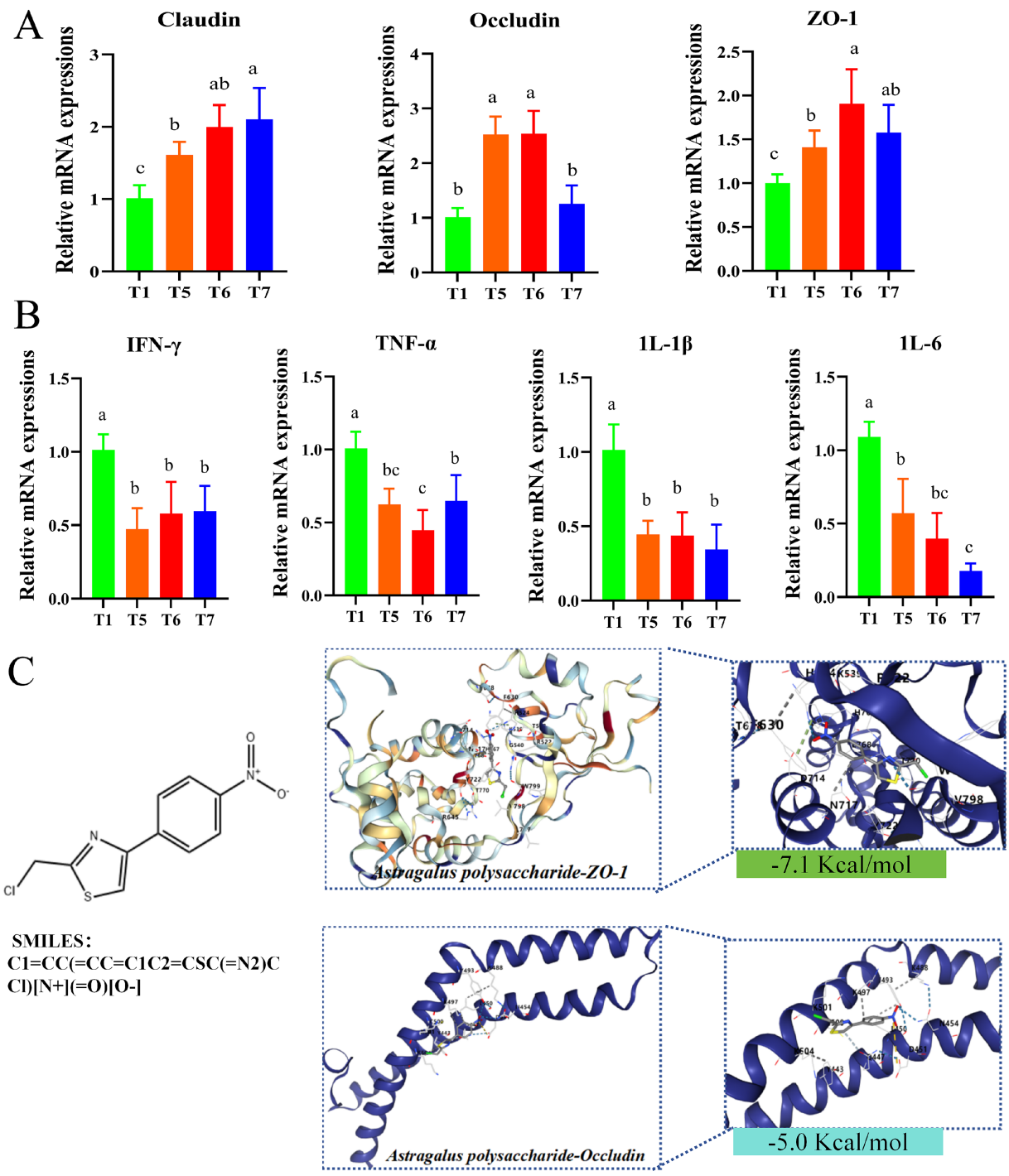

FIGURE 5
Gene expression in chicken intestines. **(A)** Relative expression levels of *Claudin, Occludin* and *ZO-1* genes. **(B)** Relative expression levels of *IL-1β*, *IL-6*, *TNF-α* and *IFN-γ* genes. **(C)** Molecular docking results of core targets and core components. Different lowercase letters indicate significant differences ($p < 0.05$, Duncan multiple comparisons).

observed in the experimental group samples, which may contribute to improving gut health and digestive efficiency in chickens (Figures 7I,J).

To explore the improvement of FAP on specific flora, we performed LDA and LEfSe to visualize the changes in the microbial community structure across different treatment groups. A dendrogram was used to represent the structural and dominant bacteria of the control and experimental groups. The LEfSe analysis (LDA > 3.5, $p < 0.05$) revealed significant differences in microbial taxa among the four groups (Figures 8A,B). The identified biomarkers may hold potential value. In the T1 group, four potential biomarkers were identified: at the phylum level, *Clostridia_UCG_014*; at the family level, *Clostridia_UCG_014*; at the genus level, *Clostridia_UCG_014* and *Desulfovibrio*. In the T6 group, six potential biomarkers were identified: at the order

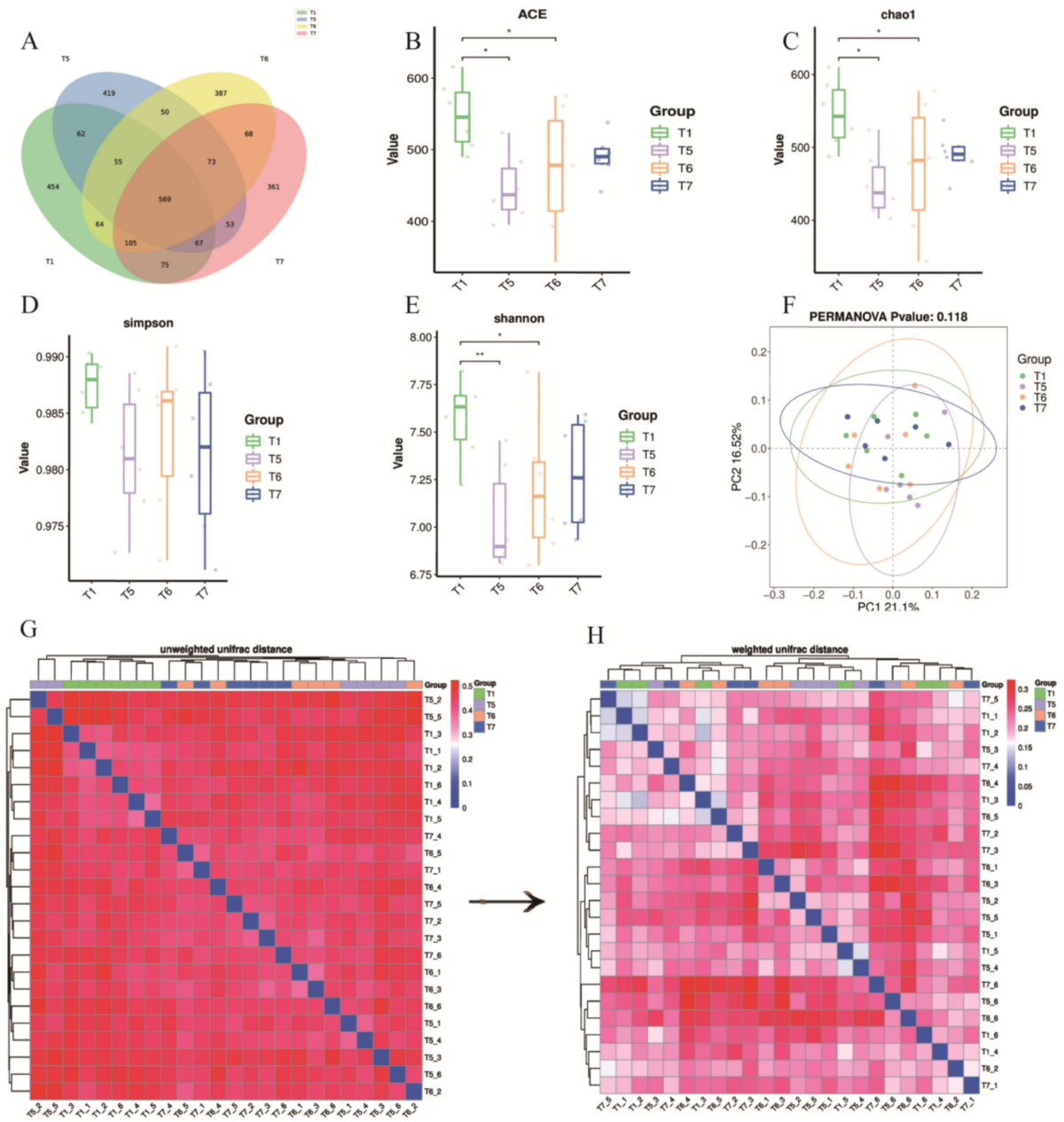

FIGURE 6
Effects of BECF on the cecal microbiota of broiler chickens/Weighted UniFrac. **(A)** Venn diagram. **(B)** ACE index. **(C)** Chao 1 index. **(D)** Simpson index. **(E)** Shannon index. **(F)** PERMANOVA *p*-value: 0.118. **(G)** Unweighted UniFrac distance. **(H)** Weighted UniFrac distance. Differences between groups are statistically significant, indicated by ($p < 0.05$) and ($p < 0.01$).

level, *Clostridia_vadinBB60_group*; at the family level, *Clostridia_vadinBB60_group*, *Bacteroidaceae*; at the genus level, *Clostridia_vadinBB60_group* and *Comamonas*. In the T7 group, four potential biomarkers were found: at the phylum level, *Spirochaetia*; and at the class, order, and family levels, all were *Spirochaetia*.

We further conducted COG analysis, with the top 30 COG categories displayed in Figure 8C. The results revealed that certain COG categories had higher abundance in specific groups, particularly *COG01225, COG00841, COG01998, COG00483*, and *COG00304*, which correspond to proteins involved in viral defense or immune responses, proteins participating in inflammatory responses or immune reactions, enzymes involved in redox reactions, key proteins responsible for intracellular signaling, and enzymes or cofactors involved in cellular metabolism. These were significantly expressed in T5, T6, and T7 (Figure 8C). Based on the Kruskal-Wallis test for COG abundance analysis, we identified *COG1539, COG1995, COG2834, COG0496, COG0605*, and *COG0668* related to metabolic enzymes or proteins, functions related to cell membrane and cell wall synthesis or degradation, proteins related to RNA or DNA processing or modification, processes related to cell proliferation or division, and functions involved in protein degradation (Figure 8D). Compared to the T1 control group, experimental groups showed upregulation,

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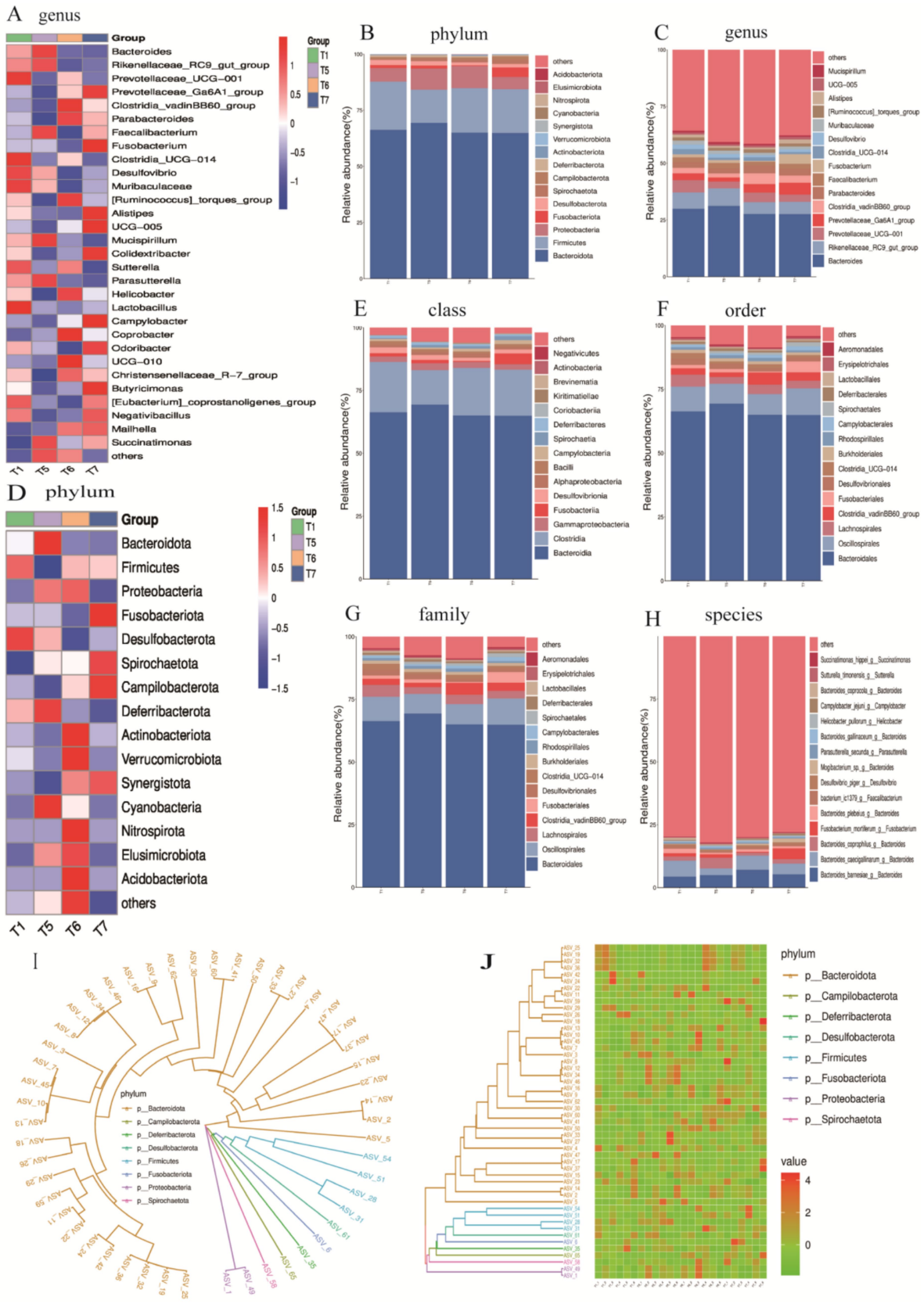

FIGURE 7
Relative abundance expression at various taxonomic levels. **(A)** Clustering heatmap of the top 30 genera. **(B)** Abundance chart of the top 15 phyla. **(C)** Abundance chart of the top 15 genera. **(D)** Clustering heatmap of the top 15 phyla. **(E)** Abundance chart of the top 15 classes. **(F)** Abundance chart of the top 15 orders. **(G)** Abundance chart of the top 15 families. **(H)** Abundance chart of the top 15 species. **(I)** Phylogenetic tree of the top 50 ASVs. **(J)** Heatmap of the top 50 ASVs.

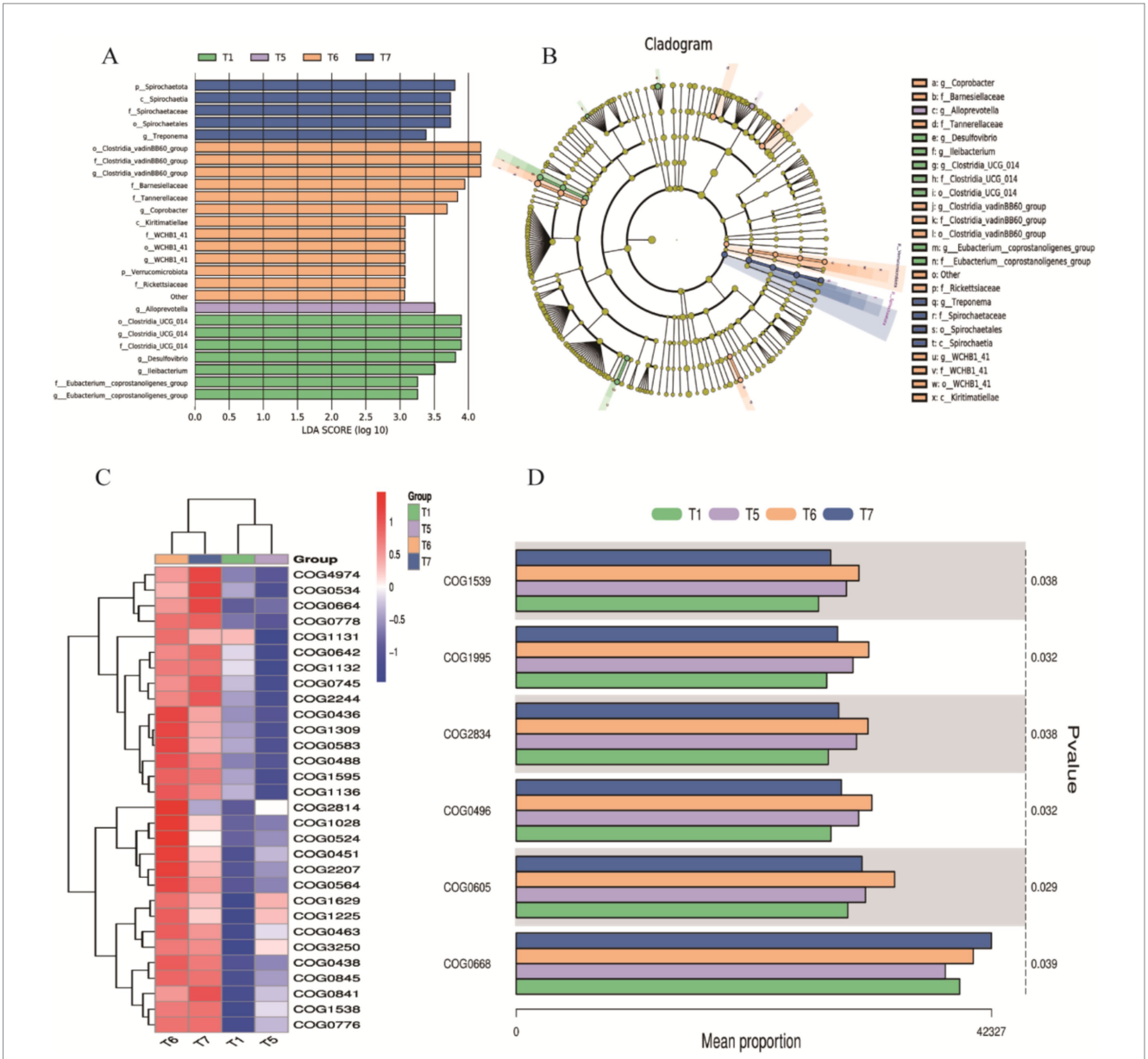

FIGURE 8
LEfSe/COG Analysis. **(A)** Bar chart of LDA value distribution. **(B)** LEfSe phylogenetic tree: Represents the richness of gut microbiota in chickens from the control group and different proportions of herbal diet groups. Different colors indicate enrichment in different groups, with yellow nodes representing no significant differences; each ring corresponds to a taxonomic level. **(C)** Top 30 COG categories. **(D)** COG abundance analysis based on the Kruskal-Wallis tes.

particularly in T6 and T7, where significant statistical differences were observed in the abundance of COG categories. These analytical results indicate that the addition of FAP significantly impacts the composition and function of the gut microbiota in chickens, which may have positive effects on gut health and overall welfare.

3.7 Effects mechanism of FAP on chicken health

In this experiment, FAP was used as a feed additive to explore its mechanism of action on chicken health (Figure 9). It was found that FAP increased the abundance of major bacterial groups such as *Bacteroidetes, Firmicutes* and *Ferri microbiota* by changing the intestinal microbial community in jejunum, thereby improving the damage of intestinal barrier function caused by microorganisms. At the same time, FAP also enhanced the strong intestinal barrier function of jejunum, further reduced the expression of intestinal mucosal cell inflammatory factors *IL-6, IL-1β, TNF-α* and *IFN-γ* induced by intestinal microorganisms, and alleviated the inflammatory damage of intestinal mucosal cells caused by intestinal microorganisms. In addition, improved intestinal barrier function also reduces the entry of bacterial metabolites into the blood and tissues throughout the body, causing oxidative stress in cells. At the same time, the enhancement of intestinal barrier function also improved the antioxidant capacity of serum, increased the activity of

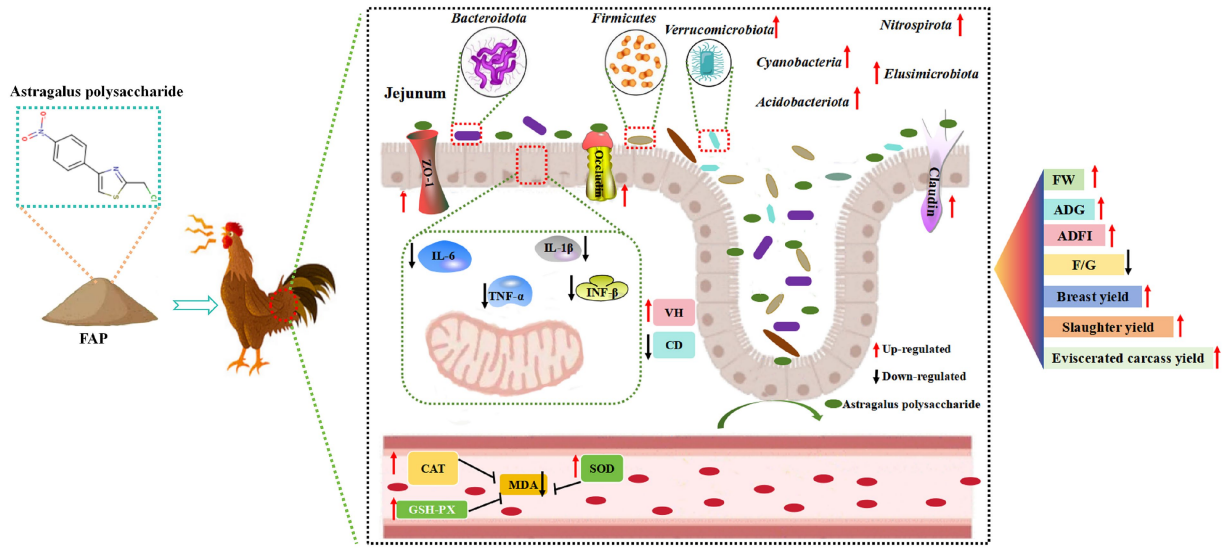

FIGURE 9
Mechanisms of influence of experimental treatments.

CAT and SOD, and decreased the content of MDA. Overall, FAP improves growth performance and health in chickens through the microbial-gut barrier pathway.

4 Discussion

Growth performance index is one of the criteria to evaluate the growth robustness of broilers, and it is also an important index to evaluate the nutritional value and application value of fermented polysaccharide. Slaughter performance accurately reflects the growth condition of animals, with related indicators such as slaughter rate and carcass weight directly indicating the meat production capacity and the economic benefits brought by the animals (22). Previous studies have found that APS can significantly increase ADG in chickens (23). Plant-derived compounds, such as essential oils or polysaccharides, can significantly affect growth performance and immune responses in livestock (24). In addition, Chuanxiong rhizoma is used in the treatment of thromboembolic diseases (25). Adding APS to the diet can significantly reduce F/G (26). Similarly, in this study, it was found that ADG increased and F/G decreased in chickens fed FAP. In addition, this study also found that the addition of FAP can significantly increase slaughter yield, exsanguinated carcass yield and breast yield. Previous studies have also found that APS can improve chicken carcass quality (27, 28). This may be because the FAP improves the intestinal flora structure and increases the abundance of probiotics, thereby improving carcass quality. Although the effect of Cyan-shank Partridge chickens cannot fully represent all other broiler breeds, its effect as one of the broiler breeds can be used as a reference for other breeds to a certain extent. In particular, the impact of the Cyan-shank Partridge chickens is used as a model to provide basic data and theoretical support for further research in other breeds. For example, in order to solve the problem of poor gel performance in the marine organism giant squid (29).

In organisms, free radicals and the antioxidant system exist in a balanced state, which is essential for maintaining animal health. Antioxidant enzymes play a key role in combating oxidative stress, primarily including CAT, SOD, and GSH-Px. These enzymes can neutralize metal ions, free radicals, and reactive oxygen species, preventing peroxide formation and mitigating lipid peroxidation, thereby achieving an antioxidant effect (30, 31). Additionally, MDA serves as an important indicator of oxidative stress, with its concentration reflecting the extent of oxidative damage experienced by the body (32). Studies have shown that APS can increase the activity of SOD and GSH-Px in serum, and reduce the concentration of MDA, so as to improve the antioxidant capacity of the body (33). Similarly, our study found that the addition of fermented Astragalus polysaccharide can increase the activities of CAT and GSH-Px and T-AOC levels in serum of broilers, while decrease the content of MDA in serum, and ultimately enhance the antioxidant capacity of broilers. The results showed that the fermentation of APS had high growth performance.

The small intestine of broilers is a crucial site for nutrient absorption and plays a significant role in the digestion, absorption, and transport of nutrients. The VH and CD of the small intestine are important indicators for assessing its digestive and absorptive capacity. When the intestinal villi lengthen, the contact area between the intestinal mucosa and the intestinal chyme increases, thereby enhancing the small intestine's ability to digest and absorb nutrients (34). The crypt depth reflects the rate of cell proliferation, a shallower crypt depth indicates a higher maturation rate of intestinal epithelial cells and an enhanced ability to absorb nutrients (35). Therefore, the ratio VH/CD can comprehensively reflect the digestive and absorptive functions of the small intestine. An increased ratio indicates enhanced digestive and absorptive functions, whereas a decreased ratio affects the small intestine's ability to digest and absorb nutrients. Related studies have found that dietary supplementation of APS can increase intestinal VH and VH/CD of poultry, and improve intestinal structure and

function (36). Similar to the results of previous studies, the results of this study showed that feeding FAP could increase intestinal VH and CD and improve intestinal morphology of broilers.

Intestinal epithelial cells act as a crucial physical barrier against external environmental stimuli, and tight junction proteins are the primary structural proteins that constitute the intestinal epithelial barrier function (37). These proteins are mainly composed of three types of transmembrane proteins: *ZO-1*, *Claudin*, and *Occludin*. *ZO-1* is a group of scaffold proteins that form part of the tight junction proteins. *Claudin* and *Occludin* are essential components in regulating intestinal epithelial barrier function, sealing intercellular gaps, determining the selective permeability of intestinal epithelial cells, and thereby playing a role in regulating intestinal barrier function and improving intestinal health. Damage to intestinal tight junctions leads to a compromised intestinal barrier structure and increased intestinal permeability (38). Inflammatory responses can increase the basal metabolic rate of animals, elevate protein degradation rates, reduce synthesis rates, and consequently impact animal growth. *IL-6* and *TNF-α* are major pro-inflammatory factors in the body. *IL-1β*, a significant member of the *IL-1* family, plays an important role in inflammation-related diseases. *IL-1β* has strong pro-inflammatory activity and can induce various pro-inflammatory mediators, such as cytokines and chemokines, ultimately leading to an amplified inflammatory response (39). Local activation of *IL-1β* is central to pro-inflammatory responses and can activate secondary inflammatory mediators, including *IL-6*. Similar to I*L-1β*, *TNF-α* is a multifunctional pro-inflammatory cytokine belonging to the *TNF* family. *TNF-α* plays diverse roles in regulating development and immunity, including inflammation, differentiation, lipid metabolism, and apoptosis, and is associated with various diseases (40). *IFN-γ*, a soluble dimeric cytokine, is the sole member of type II interferon, and its overactivation can cause tissue damage, necrosis, and inflammation. The results of this study showed that feeding FAP could up-regulate the relative expression level of tight junction protein-related gene mRNA in jejunal mucosa and down-regulate the relative expression level of jejunal pro-inflammatory factor mRNA in broilers, thereby improving intestinal barrier function. Similar to previous studies on APS (41). It has been shown that FAP can improve intestinal barrier function, enhance intestinal development and maintain intestinal health. In addition, the molecular docking test of *ZO-1* and *Claudin* protein showed that FAP had strong binding properties to *ZO-1* and *Claudin* protein. At the same time, FAP components can combine with *D714* and *N717* amino acid residues in *ZO-1* protein, and FAP components can also bind *K497, Q447* and *L450* residues of Occludin, which may be the key sites for APS to exert its drug function. Therefore, it is speculated that the binding effect of FAP with these residues (*K497, Q447, L450, D714,* and *N717*) is the key to its intestinal barrier function.

The intestinal microbiota of poultry represents a vast microbial community that significantly influences their health and productivity (42). This microbiota plays a critical role in promoting food digestion, maintaining immune balance, and resisting pathogenic invasion. Additionally, it regulates the physiological and biochemical responses in poultry, thereby accelerating the digestion and absorption of nutrients while enhancing immunity (43). Maintaining the equilibrium of intestinal microbiota is crucial for ensuring microbial diversity and stability. However, the composition and ecological succession of the intestinal microbiota in poultry are affected by various factors, including diet composition, environmental conditions, and genetic background (44, 45). Among these factors, diet composition is a key determinant influencing the configuration of the intestinal microbiota (46). After feeding broilers with FAP, the cecal microbiota was analyzed using 16S rDNA sequencing, revealing that the use of FAP significantly reduced the ACE, Chao1, and Shannon diversity indices of the cecal microbiota. This decrease in microbial diversity may be attributed to the colonization of certain dominant bacterial populations, which can hinder the establishment of other bacteria, including harmful species (47). Previous studies have also demonstrated significant reductions in the ACE, Chao1, and Shannon indices of the intestinal microbiota in broilers following the use of fermented feeds (48). At the phylum level, *Bacteroidota* and *Firmicutes* were identified as the predominant phyla, dominating the intestinal microbiota. This suggests that the incorporation of FAP may lead to an increase in their abundance, facilitating improvements in intestinal health and digestive efficiency (49). Notably, the abundance of *Verrucomicrobiota* was significantly elevated in the T6 group compared to the T1 group, and the presence of *Cyanobacteria*, *Nitrospirota*, *Elusimicrobiota*, and *Acidobacteriota* was unique to the T6 group. Some of these bacteria confer benefits to the host, promoting the growth and maintenance of the intestinal mucosal layer, enhancing intestinal barrier function, reducing the growth of harmful bacteria, and lowering intestinal toxin levels (50). These functions help protect intestinal health and support the host's glucose homeostasis, exhibiting anti-inflammatory properties. For instance, bacteria from *Verrucomicrobiota* can degrade polysaccharides through specific enzymes such as fucosidases and sulfatases, thereby improving the host's nutrient absorption capacity (51). Additionally, members of *Elusimicrobiota* can promote intestinal health by producing short-chain fatty acids (SCFAs), which serve as an energy source for the host and possess anti-inflammatory effects. At the class level, *Bacteroidia* and *Clostridia* are two significant classes within the phylum *Firmicutes*. Further classification at the order level revealed an increase in the abundance of *Bacteroidales* and *Oscillospirales* in the poultry intestinal microbiota, aiding in the identification of microorganisms occupying specific ecological niches. Bacteria from these two classes are involved in protein metabolism by hydrolyzing proteins into peptides and amino acids. This process not only facilitates the digestion and absorption of proteins in poultry but also impacts amino acid balance and overall metabolic health (52). Further analysis at the family level indicated an increased abundance of *Bacteroidaceae* and *Prevotellaceae*, which positively influence poultry health and performance through enhanced carbohydrate metabolism, immune modulation, and anti-inflammatory actions (53). For example, bacteria from the *Bacteroidaceae* family can interact with the host's immune system by producing polysaccharides, subsequently promoting the differentiation of regulatory T cells and enhancing host immune tolerance. This interaction helps maintain intestinal immune balance and reduces inflammatory responses (54). This includes the study by Huang et al. who explored the species-specific effects of microbiota on gut health, which is consistent with the microbial changes observed in this study (55). In contrast, bacteria from the *Prevotellaceae* family can mitigate intestinal inflammation by producing anti-inflammatory metabolic products, with the *Prevotella* genus being particularly effective in degrading

complex polysaccharides and promoting energy acquisition (56–58). Overall, the above changes in microbial community abundance reflect functional enhancements in energy metabolism, gut barrier nutrient absorption, and immune regulation, and it is worth noting that the gut flora can communicate bi-directionally with the host's nervous system via the gut-brain axis. May be associated with systemic benefits of improved gut health (59). For example, the growth of cyanobacteria increases the variety and content of neuroactive substances, further enhancing the signaling of the gut-brain axis.

Moreover, a COG functional classification analysis related to intestinal health revealed higher abundances of COGs associated with metabolic enzymes or proteins, including *COG1539, COG1995, COG2834, COG0496, COG0605,* and *COG0668* within the microbial community. The expression of these COGs in the poultry intestinal microbiota may enhance digestive efficiency and immune status. They are also involved in pathways related to viral defense, immune responses, redox reactions, intracellular signal transduction, and cellular metabolism. For example, *COG1539* pertains to key enzymes or proteins in cellular metabolic processes, while *COG0605* relates to the transport and metabolism of inorganic ions, being associated with SOD, an enzyme that plays a crucial role in mitigating oxidative stress and scavenging free radicals. Additionally, LEfSe analysis indicated that groups T1, T6, and T7 contained 4, 6, and 4 potential biomarkers, respectively. These identified biomarkers may possess significant value, but further research is required to elucidate their role in modulating gastrointestinal health in poultry. IIn conclusion, the analysis of the cecal microbiota suggests that the use of FAP may regulate intestinal health by balancing the intestinal microbiota.At the same time, the potential challenges of promoting the use of FAP currently require the investment of a large amount of money for basic research, clinical trials and other aspects of validation, presenting the difficult nature of increased costs, complex and stringent approval processes with uncertainty of benefits. We hope to further address these challenges in the future.

5 Conclusion

This study showed that feeding FAP could improve the growth performance, slaughtering characteristics, immune capacity and antioxidant capacity of broilers by regulating the microbial-intestinal barrier pathway. Then FAP can improve the health condition of chickens.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: https://www.ncbi.nlm.nih.gov/; PRJNA940200.

Ethics statement

The animal study was approved by The experimental protocols were approved by the Animal Ethics Committee of Leshan Academy of Agricultural Sciences (LSNK.No. 20200701). The study was conducted in accordance with the local legislation and institutional requirements.

Author contributions

ZL: Conceptualization, Funding acquisition, Investigation, Methodology, Resources, Supervision, Writing – original draft. HZ: Conceptualization, Investigation, Methodology, Validation, Writing – original draft. XC: Conceptualization, Investigation, Methodology, Validation, Writing – original draft. WY: Conceptualization, Investigation, Methodology, Validation, Writing – original draft. ShL: Conceptualization, Investigation, Methodology, Validation, Writing – original draft. LK: Formal analysis, Investigation, Writing – original draft. SoL: Formal analysis, Investigation, Writing – original draft. YJ: Funding acquisition, Project administration, Supervision, Visualization, Writing – review & editing. XZ: Data curation, Formal analysis, Methodology, Software, Visualization, Writing – review & editing.

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Conflict of interest

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

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The authors declare that no Generative AI was used in the creation of this manuscript.

Publisher's note

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Effects of fermented wheat bran on growth performance, nutrient digestibility and intestinal microbiota of weaned piglets

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The objective of this study was to investigate the effects of fermented wheat bran (FWB) on growth performance, nutrient digestibility, serum biochemistry, short-chain fatty acids, and intestinal microbiota of weaned piglets. One hundred twenty-eight weaned piglets were randomly assigned to 4 groups, each with 8 pens and 4 piglets per pen: basal diet group (BD), 5% wheat bran group (5% WB), 5% fermented wheat bran group (5% FWB), and 10% fermented wheat bran group (10% FWB) for a 28-day trial. Results showed that compared to the BD group, the diarrhea rate in the 5% WB group was significantly increased ($p < 0.05$) at d 15–28 and d 1–28. In contrast, at d 15–28 and d 1–28, the diarrhea rates in the 5% FWB and 10% FWB groups were significantly lower than those in the 5% WB group and showed no significant difference compared to the BD group. Moreover, the apparent total tract digestibility (ATTD) of DM, GE, CP, EE, CF and ADF at d 1–14, and EE and NDF at d 15–28 in the 5% FWB group were significantly improved compared to the 5% WB group ($p < 0.05$). However, only the ATTD of CP, EE and CF at d 1–14 in the 10% FWB group were significantly higher than those in the 5% WB group ($p < 0.01$). Compared to the BD group, the pH of cecum chyme and serum urea nitrogen content in the 5% FWB and 10% FWB groups were significantly reduced ($p < 0.05$), and those in the 10% FWB group were significantly lower than those in the 5% WB group ($p < 0.01$). The propionic acid content of cecum chyme in the 5% FWB and 10% FWB groups, and butyric acid content in the 10% FWB group were significantly higher than those in the BD group ($p < 0.05$). LEfSe analysis (LDA score > 3.0) identified 4 species, 6 species of Proteobacteria, 2 species, and 9 species that were enriched in the BD, 5% WB, 5%F WB and 10%F WB groups, respectively. Additionally, *Dialister*, *Prevotellaceae_NK3B31_group*, *Mitsuokella*, *Succinivibrio*, and *Prevotella* were significantly and positively correlated with the concentrations of valeric acid, propionic acid, and acetic acid ($p < 0.05$). In conclusion, 10% FWB supplementation in weaned piglet diets did not affect growth performance, it reduced the diarrhea rate compared to the 5% WB group, potentially due to enhanced nutrient digestibility, elevated SCFAs levels, and shifts in microbial composition.

KEYWORDS

fermented wheat bran, growth performance, intestinal health, nutrients digestibility, weaned piglet

1 Introduction

In recent years, the shortage of corn and soybean meal has severely restricted the rapid development of China's livestock and poultry breeding, necessitating urgent exploration of unconventional feed materials to address this situation. China possesses abundant unconventional feed resources, such as rapeseed meal, cottonseed meal, rice bran, wheat bran (WB), etc. WB is a by-product of wheat milling, remaining after the extraction of flour and germ. As the world's largest wheat producer and consumer, China generates 20–30 million tons of WB annually. This by-product is nutritionally dense, containing approximately 15% crude protein (CP), substantial levels of B vitamins (1), and 35–60% dietary fiber (DF) (2, 3). WB is widely used in pig diets and offers multiple benefits, such as improving intestinal health in piglets when supplemented at ~5%, increasing average daily feed intake (4, 5) (ADFI) and enhancing gut microbiota in sows when supplemented with 18% during gestation (6). However, the use of WB in weaned piglet's diet is limited due to its inferior amino acid profile compared to soybean meal (7), low energy value, high crude fiber (CF) content, antinutritional factors such as phytate, and poor palatability (8, 9). Additionally, WB contains up to 46% non-starch polysaccharides (NSP) (10) and 5% phytic acid, which may significantly impede digestion and absorption in piglets (11, 12).

Weaned piglets have underdeveloped digestive organs, limited organ volume, and insufficient digestive enzymes secretion capacity, making them highly susceptible to weaning stress due to physiological and environmental challenges (13). The digestive capacity of the gastrointestinal tract improves with age. Weaning leads to reduced protease activity in piglets, with typically recover within two weeks post-weaning (14, 15). The low water-holding capacity of WB increases intestinal chyme viscosity in piglets. Supplementation with 5% WB in a fiber-free diet has been shown to exacerbate piglet diarrhea (16, 17). Furthermore, diets containing 20% WB have been found to reduce nutrient digestibility in growing pigs (18).

Microbial fermentation is a widely used method for enhancing the nutritional value of fibrous materials. *Saccharomyces cerevisiae* contains a high bacterial protein content (40–80%) and generates free nucleotides and amino acids during the fermentation, thereby increasing the CP content of the substrate (19, 20). *Lactobacillus* fermentation produces lactic acid, which enhances substrate palatability while inhibiting harmful bacterial colonization (21). *Bacillus subtilis* secretes proteases and cellulases, and its metabolically active spores can reduce intestinal oxygen levels, thereby suppressing harmful bacteria growth (22). Fermented feed components have health-promoting properties as a source of probiotic microbes, digestive enzymes, and antioxidant compounds (23). Mixed fungi-fermented WB can increase the soluble dietary fiber (SDF) content from 5.6 to 13.4% (3), improve the intestinal flora of piglets and enhance their immune function (24). When enzymes are used in combination with microbial fermentation, the essential amino acid content and nutritional value of fermented wheat bran (FWB) become significantly higher than those of unfermented WB (25, 26). However, there is a lack of research on the application of FWB in weaned piglets. Therefore, the purpose of this study was to explore the effects of FWB on growth performance, nutrient digestibility, and intestinal microbiota of weaned piglets.

2 Materials and methods

2.1 Animal ethics

The experiment was conducted in accordance with the recommendations of "Laboratory Animal-Guideline of Welfare and Ethics of China (GB/ T 35892-2018)" and approved by Institutional Animal Care and Use Committee of Sichuan Agricultural University.

2.2 Materials and diet

Wheat bran was obtained from a commercial company (Chengdu Xiongjian Powder Industry Co., Ltd., Chengdu, Sichuan, China), and stored in dry conditions. The fermentation process of FWB was as follows: Based on the weight of WB, 0.5 times (w/v) the volume of water containing cellulase at 200 U/mL was added, and the mixture was thoroughly mixed and allowed to undergo enzymatic hydrolysis at room temperature for 24 h. Then, the bacterial solution equivalent to 0.5 times the weight of WB (w/v) was added, which contained amylase at 1000 U/mL, 8% $(NH_4)_2SO_4$, and a mixture of *Candida utilis* BNCC 336517, *Lactobacillus plantarum* CGMCC 1.12934, and *Bacillus subtilis* CICC 21095 in a ratio of 3:2:2, resulting in a total bacterial count of 3×10^9 CFU/kg WB. The mixture was stirred again and fermented at 34°C for 3 days, then stored at 4°C for later use. The nutrient composition of wheat bran before and after fermentation was shown in Table 1.

The diets were supplemented with minerals and vitamins to meet or exceed the requirements for piglets (body weight, 5~7 kg and 7~11 kg) according to the NRC (2012). The ingredients and nutrient compositions of the diets were reported in Table 2.

2.3 Animals, experiment design and management

A total of 128 crossbred (Duroc × Landrace × Yorkshire) weaned piglets, with an average body weight (BW) of 7.59 ± 0.99 kg, were randomly divided into 4 groups, each with 8 pens and 4 piglets per pen ($n = 8$). The 4 groups included the basal diet group (BD), 5% wheat bran group (5% WB), 5% fermented wheat bran group (5% FWB), and 10% fermented wheat bran group (10% FWB). The addition amount of FWB was calculated based on the weight of WB before fermentation, and the diets were provided in powder form. The piglets were housed in floor pens, the room temperature was maintained at 28 ± 1°C, and they were fed *ad libitum* with free access to water. The trial lasted for 28 days and was divided into two stages: d 1–14 and d 15–28. Compared to the BD group, the 5% FWB and 10% FWB groups contained 10.14 and 20.07% less corn

TABLE 1 The nutrient levels of wheat bran before and after fermentation (as air-dry matter basis, %).

Items	WB	FWB
Ether extract	3.75	4.51
Crude protein	19.55	24.62
True protein	15.82	16.84
Acid-soluble protein	2.47	10.75
Crude fiber	12.36	11.41
Neutral detergent fiber	45.29	39.80
Acid detergent fiber	13.50	12.49
Soluble dietary fiber	8.68	11.96
Insoluble dietary fiber	62.34	56.74

WB, wheat bran; FWB, fermented wheat bran.

and soybean meal at 1–14 d, and 8.43 and 16.56% less at 15–28 d, respectively. All diets contained 0.3% of Cr_2O_3 as an indigestible marker to calculate the apparent total tract digestibility (ATTD) of energy and nutrients.

2.4 Growth performance and diarrhea rate

All piglets were weighed on days 0, 14 and 28 after an overnight fast, and their feed intake was recorded. The ADG, ADFI and feed-to-gain ratio (F/G) for each pen were then calculated. The general health of all piglets was checked daily during the experimental period. The diarrhea score was based on previous descriptions: 0, normal; 1, pasty; 2, semi-liquid; and 3, liquid (27). Piglets were considered to have diarrhea when the fecal score was ≥2. The diarrhea rate was calculated as follows: Diarrhea rate (%) = (total number of diarrhea piglets × days of diarrhea) / (total number of piglets × days) × 100 (27).

2.5 Samples collection

Approximately 500 g of raw WB, FWB, and each group's diet at each stage were collected and stored at −20°C for analysis. Fresh fecal samples per pen were collected from days 12 to 14 and 26 to 28. Then, 10 mL of 5% H_2SO_4 solution was added to each 100 g of fresh fecal sample to fix excreta nitrogen. All samples were then dried at 65°C for 72 h and finely ground for ATTD analysis.

On day 28, after an overnight fast, 6 piglets from each group ($n = 6$) with the average BW from each pen were selected for sample collection. Blood samples (8 mL) were collected from the vena cava into anticoagulant-free tubes and kept at room temperature for 0.5 h. After centrifugation (3,500 × g for 15 min at 4°C), the supernatant (serum) was collected and stored at −20°C for later analysis. The same piglets were then anesthetized with a lethal injection of sodium pentobarbital (200 mg/kg BW) and slaughtered immediately. After opening the abdomen, the tissues of the jejunum, colon, cecum and gastric were quickly removed. The contents of colon, cecum and gastric were then transferred to sterile beakers, and the pH values were measured using a pH meter (FE-28, Mettler Toledo, Switzerland). The chyme from the middle cecum was collected into sterile tubes, placed in liquid nitrogen, and stored at −80°C for microbial analysis. A 10 cm section of the middle jejunum was removed, emptied, and washed with normal saline. A 2 cm section was then cut and fixed in 4% paraformaldehyde solution for histological analysis.

2.6 Physicochemical characteristics analyses

The samples of WB, FWB, feed, and feces were dried at 65°C for 72 h, regained moisture for 24 h at room temperature, and then ground and analyzed in duplicate. The WB and FWB samples were placed on double-sided adhesive tape, fixed onto the sample holder of a scanning electron microscope (SEM, Aztec X-Max80, UK), and scanned using ion sputtering to observe microstructural changes. Dry matter (DM) and ether extract (EE) were determined using AOAC method 930.15 (2019) and 920.39 (2019) (28), respectively. Gross energy (GE) was measured using an oxygen bomb calorimeter (Model 6,400, Parr Instrument Company, Moline, IL, United States). N content was determined using AOAC method 990.03 (2019) (28) on a Kjeldahl K-360 (Buchi Corp., Flawil, Switzerland), and CP was calculated as N × 6.25. True protein (TP) content of the WB and FWB was determined by the method of Saavedra-Jiménez (29). Acid-soluble protein (ASP) content in the WB and FWB was determined according to China National Standard (30). CF, acid detergent fiber (ADF), and neutral detergent fiber (NDF) contents were measured using the methods of Van Soest et al. (31). SDF and insoluble dietary fiber (IDF) contents were determined using AOAC method 991.43 and 2011.25 (2019), respectively. Cr content was determined using an atomic absorption spectrometer (contrAA700, Jena, Germany) as described by Kemme et al. (32). The ATTD of nutrient was calculated using our previously reported formula: $$\text{Digestibility}\ (\%) = \left\{1 - \frac{\left(\text{【Cr】}_{(\text{content in diet})} \times \text{nutrient content in fecal}\right)}{\left(\text{【Cr】}_{(\text{content in fecal})} \times \text{nutrient content in diet}\right)}\right\} \times 100$$ (33).

2.7 Serum biochemistry analysis

The concentrations of serum urea nitrogen (SUN), albumin (ALB), alkaline phosphatase (ALP), alanine aminotransferase (ALT), aspartate aminotransferase (AST), and total protein were measured using reagent kits (CH0101051, CH0101002, CH0101203, CH0101201, CH0101202, CH0101008; Maccura, Sichuan, China) with an automatic biochemical analyzer (3,100, HITACHI, Tokyo, Japan). All measurements were performed in duplicate.

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TABLE 2 Formulation and chemical compositions of diets (as air-dried fed basis, %)[a].

Ingredients	Treatments							
	1–14 d				15–28 d			
	BD	5% WB	5% FWB	10% FWB	BD	5% WB	5% FWB	10% FWB
Corn	51.58	46.75	47.24	43.02	57.54	52.80	53.16	49.09
Soybean meal	8.00	6.68	6.30	4.60	14.00	12.68	12.35	10.60
Low protein whey powder	10.00	10.00	10.00	10.00	6.00	6.00	6.00	6.00
Soy protein concentrate	6.00	6.00	6.00	6.00	4.00	4.00	4.00	4.00
Extruded soybean	8.00	8.00	8.00	8.00	6.00	6.00	6.00	6.00
WB	-	5.00	-	-	-	5.00	-	-
FWB	-	-	5.00	10.00	-	-	5.00	10.00
Whole milk powder	5.00	5.00	5.00	5.00	2.00	2.00	2.00	2.00
Fish meal	4.00	4.00	4.00	4.00	2.00	2.00	2.00	2.00
Soybean oil	1.00	2.10	2.00	2.90	2.00	3.00	3.00	3.80
Sucrose	2.00	2.00	2.00	2.00	2.00	2.00	2.00	2.00
L-lysine HCl (78%)	0.73	0.76	0.76	0.79	0.67	0.70	0.70	0.73
DL-Methionine (99%)	0.28	0.30	0.30	0.30	0.25	0.26	0.25	0.26
L-Threonine (99%)	0.28	0.29	0.28	0.29	0.23	0.25	0.23	0.24
L-Tryptophan (99%)	0.09	0.10	0.10	0.10	0.07	0.07	0.07	0.08
Choline chloride (50%)	0.16	0.16	0.16	0.16	0.16	0.16	0.16	0.16
Limestone	0.38	0.40	0.40	0.44	0.48	0.52	0.52	0.54
$CaHPO_3$	0.94	0.90	0.90	0.84	1.24	1.20	1.20	1.14
NaCl	0.51	0.51	0.51	0.51	0.51	0.51	0.51	0.51
Zinc oxide (75%)	0.20	0.20	0.20	0.20	-	-	--	
Acidifiers	0.50	0.50	0.50	0.50	0.50	0.50	0.50	0.50
Vitamin-mineral premix[b]	0.35	0.35	0.35	0.35	0.35	0.35	0.35	0.35
Total	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00
Nutrient levels (analyzed values, %)								
Crude protein	19.33	19.21	19.64	19.66	17.54	17.85	17.95	17.89
Dry matter	90.42	90.37	88.99	86.38	88.91	89.02	88.07	86.22
Ether extract	4.75	3.68	4.69	4.95	5.31	3.83	6.40	4.75
Crude fiber	2.62	2.80	4.36	5.16	3.71	4.05	4.72	5.02
Neutral detergent fiber	10.68	11.26	11.22	11.68	10.45	11.89	12.86	13.29
Acid detergent fiber	2.57	3.38	2.85	2.86	3.40	4.06	4.08	4.14
Nutrient levels (calculated values, %)								
Digestible energy (Mcal/kg)	3.59	3.59	3.59	3.59	3.55	3.55	3.55	3.55
Ca	0.85	0.85	0.85	0.85	0.80	0.80	0.80	0.80
AP	0.45	0.45	0.45	0.45	0.40	0.40	0.40	0.40
SID[c] Lysine	1.50	1.50	1.50	1.50	1.35	1.35	1.35	1.35
SID methionine	0.57	0.59	0.59	0.58	0.51	0.51	0.50	0.51
SID methionine + cysteine	0.82	0.82	0.83	0.82	0.74	0.74	0.74	0.74
SID threonine	0.88	0.88	0.88	0.88	0.79	0.79	0.79	0.79
SID tryptophan	0.28	0.28	0.28	0.28	0.24	0.24	0.24	0.24

[a]All nutritional requirements in the diets of 1–14 d and 15–28 d were met or exceeded NRC (2012) recommendations for 5–7 kg and 7–11 kg piglets, respectively.

[b]Provided the following per kg of complete diet: Vitamin A, 15000 IU; Vitamin D_3, 5,000 IU; Vitamin E, 40 IU; Vitamin K_3, 5.0 mg; Vitamin B_1, 5.0 mg; Vitamin B_2, 12.5 mg; Vitamin B_6, 6.0 mg; Vitamin B_{12}, 0.6 mg; Nicotinamide, 50 mg; D-pantothenic acid, 25.0 mg; Folic acid, 2.5 mg; D-biotin, 2.5 mg; Fe ($FeSO_4 \cdot 7H_2O$), 100 mg; Cu ($CuSO_4 \cdot 5H_2O$), 6.0 mg; Zn ($ZnSO_4 \cdot H_2O$), 100 mg; Mn ($MnSO_4 \cdot H_2O$), 4.0 mg; I (KI), 0.14 mg; Se (Na_2SeO_3) 0.3 mg.

[c]SID: Standardized ileal digestible.

BD, basal diet group without wheat bran; 5% WB, 5% wheat bran group; 5% FWB, 5% fermented wheat bran group; 10% FWB, 10% fermented wheat bran group.

2.8 Histomorphology measurements

The jejunum samples from 6 pigs per group were fixed in 4% paraformaldehyde solution, dehydrated, and infiltrated with paraffin wax. They were sectioned at 5 μm thickness, stained with hematoxylin and eosin (HE), and examined using a microscope (DM1000, Leica, Germany). Villus height (VH) and crypt depth (CD) were measured for at least 10 well-oriented villus and crypt columns at 10 × magnification with Image-Pro plus 6.0 (Media Cybernetics, Maryland, United States). The ratio of villus height to crypt depth (VH/CD) was then calculated.

2.9 Short-chain fatty acids analysis

The contents of acetic acid (AA), propionic acid (PA), butyric acid (BA), and valeric acid (VA) were analyzed using a gas chromatograph system (CP-3800, Varian, Palo Alto, USA) after the pretreatment of cecum chyme, as described by our group (33). Briefly, approximately 3 g of cecal chyme was mixed with ultra-pure water in a 1:1 (w/v) ratio and vortexed. After centrifugation at 10,000 × g for 15 min, 1 mL of supernatant was mixed with 0.2 mL of 25% metaphosphoric acid and 23 μL of 210 mmol/L crotonic acid, then incubated at 4°C for 30 min. Following centrifugation at 8,000 × g for 10 min, 0.3 mL of supernatant was mixed with 0.9 mL methanol (1:3, v/v), and centrifuged at 8,000 × g for 5 min. The final supernatant was filtered through a 0.22 μm membrane and analyzed by gas chromatograph.

2.10 Microbial analysis

Cecum chyme samples were analyzed using the 16S rRNA method (33). Briefly, after thawing, 0.25 g of chyme was uniformly sampled, then, the genomic DNA was extracted using the CTAB method. The DNA purity and concentration were assessed using 2% agarose gel electrophoresis and a Nanodrop 2000 (Thermo Fisher Scientific, Waltham, MA, United States). The V4 hypervariable regions of bacterial 16S rRNA genes were amplified using primers 515F (5′-GTGCCAGCMGCCGCGGTAA-3′) and 806R (5′-GGACTACHVGGGTWTCTAAT-3′). Pyrosequencing of bacterial 16S rDNA was performed on the Illumina NovaSeq platform to generate 250 bp paired-end reads. Initial Operational Taxonomic Units (OTUs) were obtained using the DADA2 or deblur module in QIIME2 for denoising. OTUs with an abundance of less than 5 were removed. Species annotations were performed using QIIME2 software. The sequences were submitted to NCBI's Sequence Read Archive for open access (PRJNA1247028).

2.11 Statistical analysis

All data were first tested for normal distribution using the Descriptive Statistics (explore) module in SPSS 27.0 software. For normally distributed data, one-way ANOVA was used to analyze group differences, and Duncan's multiple range test assessed variance homogeneity between groups. Data were expressed as the mean with pooled standard error (SE). Differences among the four groups were considered statistically significant at $p < 0.05$, whereas p-values between 0.05 and 0.10 were considered trends.

For the microbiota data, all indices were calculated using QIIME (Version 1.7.0) and displayed using R software (Version 2.15.3). One-way ANOVA was performed to identify significantly different species at each taxonomic level (Phylum and Genus). LEfSe analysis (LDA score threshold: 3) was performed using LEfSe software (Version 1.0). Spearman correlation analysis was conducted to evaluate the relationship between SCFAs and microorganisms, obtaining correlation and significance values.

3 Results

3.1 Physicochemical characteristics of FWB

Compared with WB, FWB increased EE, CP, TP, ASP and SDF by 20.27, 25.93, 6.45, 335.22 and 37.79%, respectively. Meanwhile CF, NDF, ADF, and IDF levels decreased by 7.69, 12.12, 7.48, and 8.98%, respectively (Table 1). The morphological characteristics of WB and FWB were observed under 1,000× SEM, with results presented in Figure 1. The apparent structure of WB was complete, dense, and regular (Figure 1A). After fermentation, the tearing area and crushing structure of FWB were significantly increased compared to before fermentation (Figure 1B).

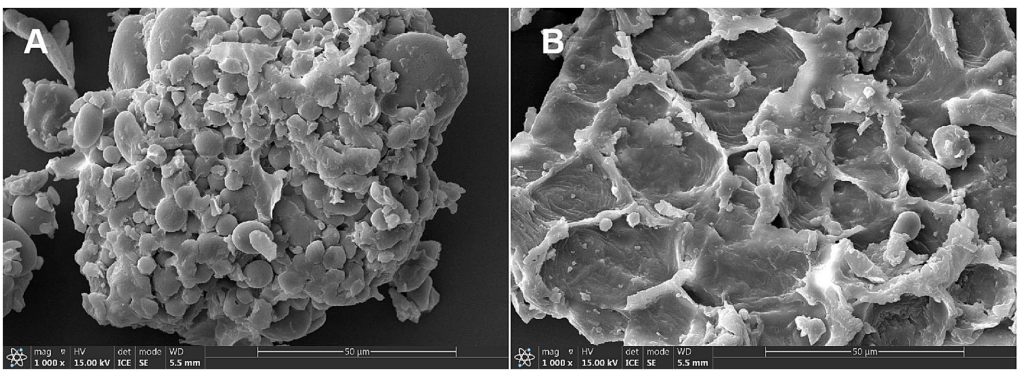

FIGURE 1
Surface structure of wheat bran **(A)** and fermented wheat bran **(B)**. Scanning electron microscope images at × 1,000-fold magnification.

3.2 Growth performance

As shown in Table 3, BW, ADFI, ADF, and F/G did not differ among the BD, 5% WB, 5% FWB, and 10% FWB groups ($p > 0.05$). Compared to the BD group, the diarrhea rate was significantly increased at 15–28 d and 1–28 d ($p < 0.05$) and tended to increase at 1–14 d ($p = 0.07$) in the 5% WB group. However, compared to 5% WB group, the diarrhea rate in the 5% FWB and 10% FWB groups was significantly reduced at 15–28 d and 1–28 d ($p < 0.05$). However, there was no significant difference in the diarrhea rate between the 5% FWB and 10% FWB groups.

3.3 Apparent total tract digestibility

In the first phase (1–14 d), the ATTD of DM, GE, EE, CF, and NDF in the 5% WB group was significantly lower than that in the BD group ($p < 0.05$). However, compared with the 5% WB group, the ATTD of DM, CP, GE, EE, CF, NDF, and ADF in the 5% FWB group was significantly increased ($p < 0.05$). Notably, the ATTD of CP, CF, and ADF in the 5% FWB group was significantly higher than that in the BD group. Furthermore, the ATTD of CF in the 10% FWB group was significantly higher than that in both the BD and 5% WB groups ($p < 0.01$) (Table 4).

In the second phase (15–28 d), the ATTD of DM, CP, GE and NDF in the 5% WB group was significantly higher than that in the BD group ($p < 0.05$). However, the ATTD of EE and NDF in the 5% FWB group was significantly increased ($p < 0.05$) compared to the 5% WB group. Moreover, the ATTD of CP, GE, EE, and NDF in the 5% FWB group was significantly higher than that in the BD group ($p < 0.05$). In the 10% FWB group, the ATTD of CP was significantly higher, while the ATTD of DM and CF were significantly lower than that in the BD group ($p < 0.01$) (Table 4).

3.4 Serum biochemical parameters

Compared to the BD group, the SUN levels in the 5% FWB and 10% FWB groups were significantly decreased, with no significant difference between the two groups. Additionally, the SUN level in the 10% FWB group was significantly lower than that in the 5% WB group ($p = 0.01$). However, neither WB nor FWB diets had no effect ($p > 0.05$) on serum levels of ALB, ALP, ALT, AST, or TP (Table 5).

3.5 The pH of intestinal and gastric chyme

In cecum chyme, compared to the BD group, the pH of the 5% WB group remained unchanged ($p > 0.05$), but the pH in the 5% FWB and 10% FWB groups was significantly decreased ($p < 0.01$) (Figure 2). However, there was no significant difference in the pH of colonic chyme and gastric contents among the four groups.

3.6 Intestinal morphology and short-chain fatty acids

According to the results of the histological analysis, there was no significant difference in jejunum morphology among the four groups of weaned piglets ($p > 0.05$) (Table 6). Compared to the BD group, the PA

TABLE 3 Effects of FWB on growth performance and diarrhea rate of weaned piglets ($n = 8$).

Item	BD	5% WB	5% FWB	10% FWB	SE	*P*-value
Body weight, kg						
0 d	7.57	7.59	7.61.	7.59	0.03	0.97
14 d	10.53	10.87	10.49	10.59	0.07	0.23
28 d	16.67	17.02	16.57	16.90	0.13	0.64
Average daily feed intake, g/d						
1–14 d	305.49	315.85	295.81	294.55	4.16	0.24
15–28 d	702.01	702.70	693.39	703.06	8.85	0.98
1–28 d	503.75	509.28	494.60	498.81	5.93	0.85
Average daily gain, g/d						
1–14 d	211.05	234.82	205.58	214.73	4.46	0.09
15–28 d	438.84	438.84	434.24	450.34	5.92	0.82
1–28 d	324.95	336.83	319.91	332.54	4.55	0.58
Feed/gain ratio, g/g						
1–14 d	1.46	1.35	1.44	1.38	0.02	0.15
15–28 d	1.60	1.60	1.60	1.57	0.01	0.78
1–28 d	1.55	1.51	1.55	1.51	0.01	0.30
Diarrhea rate, %						
1–14 d	7.14	10.49	4.24	5.58	0.89	0.07
15–28 d	2.46^{b}	5.58^{a}	3.35^{b}	3.13^{b}	0.40	0.03
1–28 d	4.80^{b}	8.04^{a}	3.80^{b}	4.35^{b}	0.56	0.02

BD, basal diet group without wheat bran; 5% WB, 5% wheat bran group; 5% FWB, 5% fermented wheat bran group; 10% FWB, 10% fermented wheat bran group. ab Mean values within a row with different letters differ significantly ($P < 0.05$).

TABLE 4 Effects of FWB on apparent total tract digestibility of weaned piglets (n = 8).

Nutrients	BD	5% WB	5% FWB	10% FWB	SE	*P*-value
1–14 d						
DM, %	85.07^{ab}	82.12^{c}	85.75^{a}	82.77^{bc}	0.49	0.01
CP, %	76.79^{bc}	74.12^{c}	81.41^{a}	79.29^{ab}	0.81	< 0.01
GE, %	84.54^{ab}	81.96^{c}	85.93^{a}	82.73^{bc}	0.49	0.01
EE, %	73.72^{a}	62.58^{b}	75.36^{a}	73.21^{a}	1.54	< 0.01
CF, %	62.21^{b}	53.70^{c}	75.22^{a}	69.56^{a}	1.80	< 0.01
NDF, %	63.25^{ab}	53.89^{c}	65.04^{a}	57.99^{bc}	1.27	< 0.01
ADF, %	37.29^{b}	37.18^{b}	49.11^{a}	34.15^{b}	1.97	0.03
15–28 d						
DM, %	75.37^{b}	78.74^{a}	76.38^{b}	72.69^{c}	0.53	< 0.01
CP, %	62.75^{b}	71.48^{a}	69.47^{a}	68.71^{a}	0.77	< 0.01
GE, %	75.43^{b}	79.00^{a}	77.75^{a}	73.62^{b}	0.51	< 0.01
EE, %	65.59^{b}	61.24^{b}	78.78^{a}	65.84^{b}	1.62	< 0.01
CF, %	46.67^{a}	44.60^{a}	49.28^{a}	38.56^{b}	1.21	0.01
NDF, %	30.68^{c}	39.09^{b}	46.26^{a}	27.25^{c}	1.74	< 0.01
ADF, %	22.94	30.36	34.88	26.41	1.63	0.05

^{abc} Mean values within a row with different letters differ significantly ($P < 0.05$). BD, basal diet group without wheat bran; 5% WB, 5% wheat bran group; 5% FWB, 5% fermented wheat bran group; 10% FWB, 10% fermented wheat bran group; DM, dry matter; CP, crude protein; GE, gross energy; EE, ether extract; CF, crude fiber; NDF, neutral detergent fiber; ADF, acid detergent fiber.

TABLE 5 Effects of FWB on serum biochemistry of weaned piglets (n = 6).

Items	BD	5% WB	5% FWB	10% FWB	SE	*P*-value
Alanine aminotransferase, U/L	70.84	94.61	74.66	76.12	4.19	0.19
Aspartate aminotransferase, U/L	41.03	41.14	44.12	40.29	2.34	0.95
Albumin, g/L	22.83	24.38	26.11	27.01	0.73	0.18
Alkaline phosphatase, U/L	278.00	260.83	302.00	269.17	14.61	0.80
Total protein, g/L	44.88	47.03	49.39	49.37	0.86	0.19
Serum urea nitrogen, mmol/L	2.01^{a}	1.82^{ab}	1.27^{bc}	0.88^{c}	0.14	0.01

^{abc} Mean values within a row with different letters differ significantly ($P < 0.05$). BD, basal diet group without wheat bran; 5% WB, 5% wheat bran group; 5% FWB, 5% fermented wheat bran group; 10% FWB, 10% fermented wheat bran group.

content in cecum chyme was significantly increased ($p < 0.05$) in the 5% FWB and 10% FWB groups, with no significant difference between these two groups. Additionally, the BA content in the 10% FWB group was significantly higher than in the BD group ($p < 0.05$), with no significant difference compared to the 5% WB and 5% FWB groups (Table 7).

3.7 Cecum microbiota composition

Dietary supplementation with 10% FWB tended to reduce the Chao 1 index ($p = 0.07$) in cecum chyme compared to the BD, 5% WB, 5% FWB groups (Table 8). There was no significant difference in the α-diversity indices of Shannon and Simpson among the four groups ($p > 0.05$).

The microbial characteristics of cecum chyme in piglets on d 28 post-weaning was presented in Figure 3. A total of 598,713, 615,078, 619,874, and 600,325 high-quality sequences were obtained from cecum chyme samples in the BD, 5% WB, 5% FWB, and 10% FWB groups, respectively. The Venn diagram shows 1,481, 1,575, 1,569 and 1,162 OTUs in the BD, 5% WB, 5% FWB, and 10% FWB groups, respectively (Figure 3A). The four groups shared 673 OTUs, with the BD, 5% WB, 5% FWB, and 10%FWB groups having 308, 276, 286, and 196 unique OTUs, respectively (Figure 4). Figures 3B,C showed species with significant differences among the 4 groups when the LDA

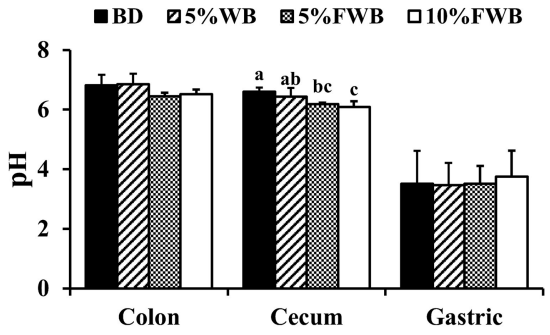

FIGURE 2
Effects of FWB on intestinal chyme and gastric contents pH of weaned piglets. Mean values with different letters on vertical bars differ significantly ($p < 0.05$). BD, Basal diet group; 5% WB, 5% Wheat bran group; 5% FWB, 5% Fermented wheat bran group; 10% FWB, 10% Fermented wheat bran group.

TABLE 6 Effects of FWB on jejunum morphology of weaned piglets ($n = 6$).

Items	BD	5% WB	5% FWB	10% FWB	SE	*P*-value
Villus height, μm	359.44	388.94	433.76	422.32	12.78	0.15
Crypt depth, μm	192.59	219.65	189.13	185.35	8.99	0.55
Villus height/crypt depth	2.04	1.95	2.38	2.38	0.09	0.22

BD, basal diet group without wheat bran; 5% WB, 5% wheat bran group; 5% FWB, 5% fermented wheat bran group; 10% FWB, 10% fermented wheat bran group.

TABLE 7 Effects of FWB on short-chain fatty acids in piglet cecum chyme ($n = 6$).

Item	BD	5% WB	5% FWB	10% FWB	SE	*P*-value
Acetic acid, mg/g	5.25	4.81	5.00	4.71	0.13	0.51
Propionic acid, mg/g	2.31^{b}	2.29^{b}	2.85^{ab}	3.33^{a}	0.15	0.03
Isobutyric acid, mg/g	0.04	0.05	0.02	0.02	0.01	0.64
Butyric acid, mg/g	0.76^{b}	0.95^{ab}	1.01^{ab}	1.13^{a}	0.05	0.03
Isovaleric acid, mg/g	0.14^{a}	0.09^{bc}	0.10^{b}	0.06^{c}	0.01	< 0.01
Valeric acid, mg/g	0.19	0.21	0.25	0.25	0.02	0.34

^{abc} Mean values within a row with different letters differ significantly ($P < 0.05$). BD, basal diet group; 5% WB, 5% wheat bran group; 5% FWB, 5% fermented wheat bran group; 10% FWB, 10% fermented wheat bran group.

TABLE 8 Effects of FWB on α-diversity of cecum microorganisms of weaned piglets ($n = 6$).

Items	BD	5% WB	5% FWB	10% FWB	SE	*P*-value
Shannon	6.59	6.54	6.59	5.84	0.14	0.13
Simpson	0.97	0.97	0.97	0.95	0.01	0.22
Chao 1	629.38	630.22	611.62	463.98	26.88	0.07

BD, basal diet group without wheat bran; 5% WB, 5% wheat bran group; 5% FWB, 5% fermented wheat bran group; 10% FWB, 10% fermented wheat bran group.

score was greater than 3.0. LEfSe analysis identified 4 species (s_*Clostridium_butyricum*, g_*Turicibacter*, s_*Selenomonas_sp_oral_clone_JI021*, g_*Fournierella*), 6 species of Proteobacteria (f_*Spirochaetaceae*, p_Spirochaetota, c_Spirochaetia, o_Spirochaetales, g_*Treponema*, s_*Treponema_porcinums*), 2 species (g_*NK4A214_group* and g_*Dialister*), and 9 species (g_*Mitsuokella*, g_*Holdemanella*, g_*Solobacterium*, p_Actinobacteriota, c_Coriobacteriia, o_Coriobacteriales, g_*Erysipelotrichaceae_UCG_002*, g_*Collinsella*, f_*Coriobacteriaceae*) enriched in the BD, 5% WB, 5% FWB, and 10% FWB groups, respectively.

Ten bacterial genera with relative abundance greater than 1% at the genus level were analyzed for Spearman correlation with SCFAs in cecum chyme. As shown in Figure 4, the concentration of VA was significantly positively correlated with *Dialister* ($r = 0.48$, $p < 0.05$) and significantly negatively correlated with *Prevotellaceae_NK3B31_group* ($r = -0.55$, $p < 0.01$) and *Phascolarctobacterium* ($r = -0.44$, $p < 0.05$). The concentration of isovaleric acid (IVA) was significantly negatively correlated with *Dialister* and *Mitsuokella* ($r = -0.41$, $p < 0.05$; $r = -0.46$, $p < 0.05$). *Succinivibrio* was significantly negatively correlated with BA concentration ($r = -0.46$, $p < 0.05$) and

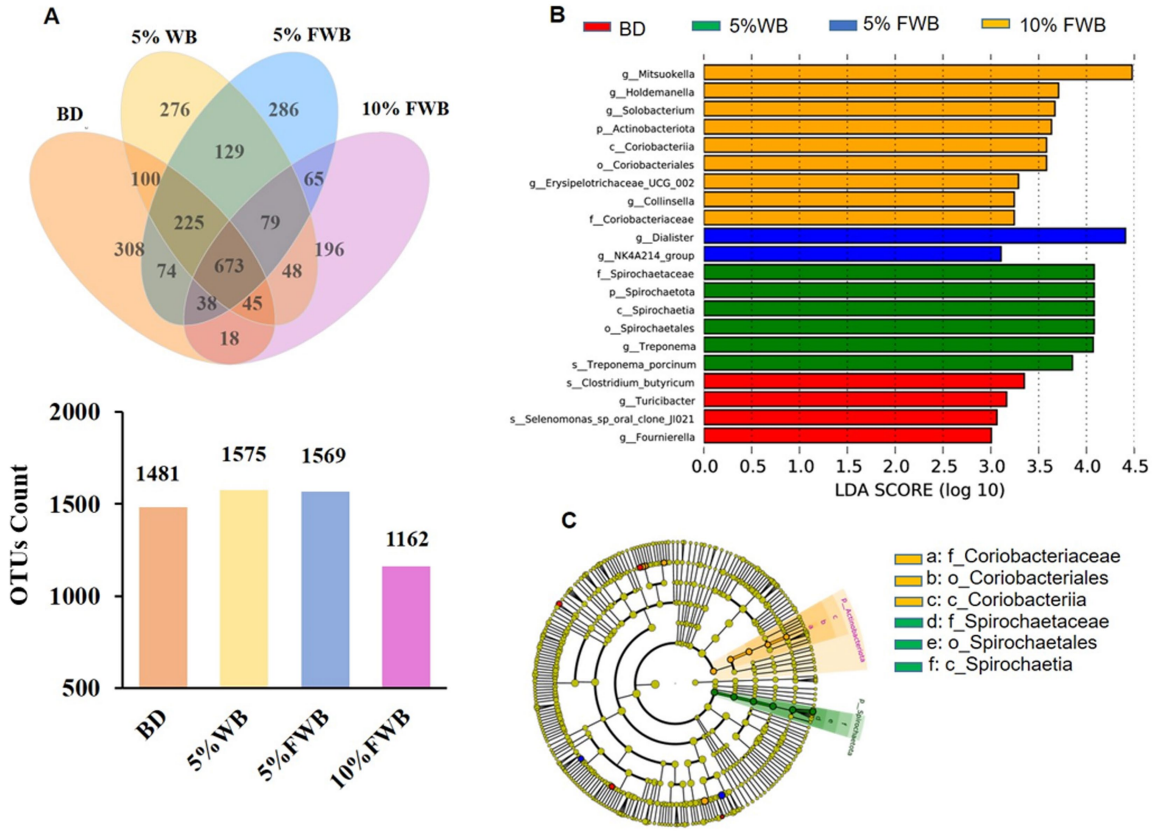

FIGURE 3
Effects of FWB on cecum chyme microbial characteristics of piglets on day 28 post-weaning (n = 6). **(A)** The unique and shared OTUs in the each group; **(B)** LDA scores show the significant bacterial differences among the groups ($p < 0.05$, LDA score > 3.0). **(C)** Cladogram using the LEfSe method shows the phylogenetic distribution of the cecum microbes among the groups. FWB, fermented wheat bran; LDA, Linear discriminant analysis; OTUs, Operational taxonomic units.

significantly positively correlated with PA concentration (r = 0.41, $p < 0.05$). The concentration of PA was significantly positively correlated with *Dialister* (r = 0.48, $p < 0.05$) and *Mitsuokella* (r = 0.52, $p < 0.01$) and significantly negatively correlated with *Clostridium_sensu_stricto_1* (r = −0.71, $p < 0.01$). *Prevotellaceae_NK3B31_group* (r = 0.62, $p < 0.01$) and *Prevotella* were significantly positively correlated with AA concentration (r = 0.41, $p < 0.05$).

4 Discussion

Wheat bran has not been widely used because it contains a high content of anti-nutrients (such as CF), which negatively affect growth performance and nutrient digestibility in weaned piglets (24). After solid-state fermentation (SSF), CF, NDF and ADF content of FWB were significantly reduced, while the SDF and protein quality were improved (3, 24). This is consistent with the findings of this study. The original dense structure of WB developed obvious tearing surface and pore structure after SSF (24, 34). This looser structure is more favorable for microorganisms to access and fully ferment the nutrients (34).

Piglets have limited tolerance to CF, and excessive levels can reduce their growth performance (35). Due to its large amount of fiber and strong resistance to natural intestinal degradation and digestion, WB is largely used in animal feed, but it is not used to feed young animals such as piglets (36). Thus, dietary supplementation of WB could not improve the growth performance of pigs (24). Similarly, replacing 7.2% of corn in the diet with 8% FWB did not significantly affect the growth performance of weaned piglets (24). In this study, dietary supplementation with 5% or 10% FWB did not significantly affect the growth performance of piglets, consistent with previous studies (25), indicating that FWB can feasibly replace some soybean meal and corn in piglet diets. However, dietary supplementation of 5% FWB significantly increased ADG and F/G in growing-finishing pigs (37), which should be related to the well-developed intestinal system of pigs. Additionally, the high content of NSP in WB limits its use in piglet feed, as piglets often suffer stress-induced diarrhea due to the inadequate development of their intestines and microbiota (38). The increase in NSP concentration increases the viscosity of the chyme and digestive fluid, resulting in undigested chyme entering the colon, where elevated osmotic pressure and water influx lead to diarrhea (16, 39). In this study, dietary supplementation with 5% WB significantly increased the diarrhea rate of piglets, which is consistent with previous study (18). However, the diarrhea rate in the 5% FWB and 10% FWB groups was significantly lower than in the 5% WB group. This is likely related to the fact that FWB has lower CF, NDF, ADF, but higher SDF than WB. SDF is well-known for being largely degraded by microbes in the hindgut of pigs, thus benefiting intestinal homeostasis (39).

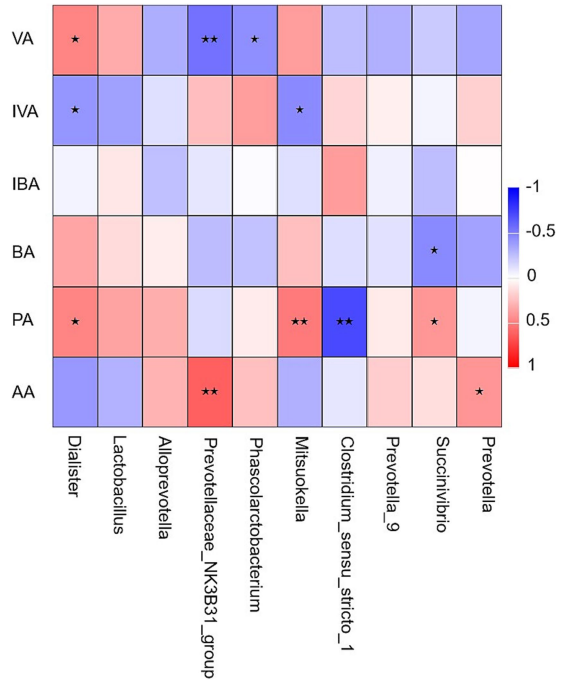

FIGURE 4
Heat map of the correlation analysis between short-chain fatty acids and microorganisms at genus level. VA, valeric acid; IVA, isovaleric acid; IBA, isobutyric acid; BA, butyric acid; PA, propionic acid; AA, acetic acid. * Indicates a significant correlation between microbes and short-chain fatty acids ($P < 0.05$). ** Indicates an extremely significant correlation between microbes and short-chain fatty acids ($P < 0.01$).

Additionally, the higher SDF concentration in FWB can slow chyme transit, improve fecal formation, and thereby reduce diarrhea incidence (16, 24).

The high IDF content of WB accelerates gastric emptying and shortens diet retention time in the gastrointestinal tract of piglets (39). However, the gastrointestinal tract of weaned piglets is not capable enough of secreting digestive enzymes, so WB is seldom added to their diets. The current results demonstrated that the ATTD of DM, GE, EE, CF and NDF in the 5% WB group was lower than that in the BD group on d 0–14. However, the ATTD of DM, CP, GE and NDF in the 5% WB group was higher than that in the BD group on d 15–28, but this had no significant effect on the growth performance, which may be related to factors such as energy loss associated with fiber fermentation in the hindgut and shorter experimental period and the treatment replicates, among other factors. Previous studies have shown that the digestibility of CP and EE in the 8% WB group significantly increased compared to the control group at 40 days post-weaning (24). This may be related to the maturation of the piglets' intestinal development in the later stage of nursery, allowing better secretion of digestive enzymes. Fermented feeds are more palatable (40). The acidic environment enhances intestinal secretion of proteases and other digestive enzymes (17). Additionally, degradation of large-molecule proteins in FWB improves nutrient digestibility in piglets. In this study, the ATTD of DM, CP, GE, EE, CF, NDF, and ADF in the 5% FWB group was higher than in the 5% WB group at 0–14 d. Notably, fiber digestibility in piglets was equal to or better in the 5% FWB and 10% FWB groups compared to the BD group. This is attributed to effective fiber degradation after SSF, indicating FWB did not negatively affect the fiber digestibility in piglets, consistent with a previous study (24), while has found no significant difference in nutrient digestibility between piglets in the 8% WB and 8% FWB groups.

The microbial fermentation process can produce beneficial substances, such as small-size peptides, exoenzymes, vitamins, and organic acids, which can enhance the physiological metabolism of animal (41). Serum biochemical indicators visualize physiological and metabolic functions in animals (42). SUN is an important index reflecting overall protein metabolism (43). The SUN content of growing pigs in the 10% *Aspergillus niger*-fermented canola meal group was found to be significantly reduced by 26.48% compared to the unfermented group (44). In this study, the SUN levels in the 5% FWB and 10% FWB groups were significantly decreased compared to the BD group. This indicates that piglets in the 5% FWB and 10% FWB groups showed greater protein utilization, suggesting that FWB is beneficial for regulating protein digestion and metabolism.

Diarrhea is closely related to small intestinal health, especially the jejunum, which is crucial for nutrient digestion and absorption (38). However, this study found no significant differences in jejunum morphology among the four groups. Additionally, a more acidic intestinal environment benefits piglets' digestion and absorption and has a positive effect on their intestinal health (45). In this study, the pH of colon and cecum chyme was decreased in the 5% FWB and 10% FWB groups compared to the BD group. The addition of wheat to the diet enhances SDF fermentation in the hindgut, significantly reducing cecal chyme pH in piglets (46), which aligns with the findings of this study. The pH of cecum and colonic chyme serves as an important indicator for evaluating intestinal health in weaned piglets. A lower colon pH would reduce the colonization of enterotoxin-producing bacteria like *Anaplasma* sp. and *Clostridium* sp., beneficial for maintaining intestinal homeostasis in piglets (46).

Probiotic-fermented food or feeds may effectively regulate gut microbiota and its metabolites, such as SCFAs (33). SCFAs are mainly produced in monogastric animals through the fermentation of SDF by beneficial flora like *Lactobacilli* and *Bifidobacteria* in the hindgut (47). They play a crucial role in glycolipid metabolism and intestinal homeostasis in piglets. The rate of microbial fermentation is related to the solubility and viscosity of DF (48), and the loose structure of WB after fermentation enables easier access for microorganisms, accelerating the fermentation rate (24). PA is a substrate for hepatic gluconeogenesis (49). BA is a major energy source for colonic epithelial cells (50). Butyrate can prevent pathogenic microorganisms from attaching to the intestinal mucosa, alleviating intestinal inflammation in *E. coli* infected piglets (51). In this study, PA content in the 5 and 10% FWB groups and BA content in the 10% FWB group were significantly higher than in the BD group. Moreover, SCFAs, as important intermediate products during anaerobic digestion, can inhibit harmful flora, promote the colonization of beneficial flora, and improve intestinal microorganism metabolism in piglets (13). This may explain the decreased diarrhea rate in piglets in this study. *In vitro* digestion of sugarcane polyphenols and fiber significantly decreased IVA, while increasing in BA and total SCFAs (52). These findings are consistent with the significant reduction in cecal chyme IVA content following WB or FWB supplementation observed in our study. As a minor SCFA, IVA production may be reduced by the combined dietary increase of fiber and polyphenols (53, 54), which appear to suppress IVA-producing bacterial metabolism while promoting BA and PA production.

Intestinal microflora plays a crucial role in regulating intestinal health in pigs (55). The Chao 1 index in the 10% FWB group tended to decrease compared to the other groups. This indicates fewer microbial species in the cecum chyme of piglets in the 10% FWB group, likely related to the introduction of *Candida utilis*, *Lactobacillus plantarum*, and *Bacillus subtilis* in FWB. *Lactobacillus* promotes intestinal health in piglets by producing organic acids and bacteriocins, lowering environmental pH and inhibiting harmful bacteria like *E. coli* and *Salmonella* (56). LEfSe analysis showed that the relative abundance of f_*Spirochaetaceae*, p_Spirochaetota, c_Spirochaetia, o_Spirochaetales, g_*Treponema*, s_*Treponema_porcinums* increased in the 5% WB group. Previous studies have found that *Spirochaetes* was significantly and positively correlated with the diarrhea rate in piglets (57). In this study, *Dialister* was enriched in the 5% FWB group, which is consistent with previous reports that enriched SCFAs in piglet feces by *Lactobacillus*-fermented feed, thereby increasing the beneficial effects on piglet intestinal health (58). Dietary yeast supplementations may also promote *Dialister* colonization in the porcine intestinal tract, leading to elevated BA levels (59). Previous studies have shown that *Dialisters* abundance increases significantly by 27 days post-weaning compared to 7 days (60), and the changes in its abundance may be related to the differences in diarrhea among piglets at different stages after weaning. Increased relative abundance of *Dialister* in growing pig's feces leads to higher total AA and SCFA in the cecum (59). The 10% FWB group showed increased relative abundance of beneficial bacteria like p_Actinobacteriota, g_*Mitsuokella* and g_*Holdemanella*. Higher relative abundance of Actinobacteriota in the cecum of fattening pigs significantly increased intramuscular fat content in the longest dorsal back muscle (61). Moreover, higher abundance of Actinobacteriota can promote BA production (62). *Mitsuokella* can produce fermentation acids and lower the pH to inhibit the growth of *Salmonella Typhimurium* (63), which helps maintain gut health. The increase in relative abundance of Actinobacteriota, *Mitsuokella*, *Holdemanella* and *Dialister* indicates that FWB benefits the colonization of beneficial bacteria in the intestines of weaned piglets, thereby improving their intestinal health, which may be related to its fiber structure (64).

The gut microbiota composition is associated with SCFAs (55). Spearman's correlation analysis revealed significant positive correlations between the relative abundance of *Dialister*, *Succinivibrio*, *Prevotellaceae_NK3B31_group*, and *Prevotella* and the concentrations of VA, PA, and AA. Higher SDF content in FWB indicates a close relationship between microbial fiber fermentation in the piglet hindgut and SCFAs production. *Prevotella*, prevalent in the pig cecum, produce SCFAs by degrading SDF (65). Similarly, *Succinivibrio* ferments carbohydrates into metabolites like AA and succinic acid (66). Spearman's correlation analysis indicated that FWB enhanced the colonization of the intestinal tract by genera involved in the metabolism of DF and polysaccharides, increasing the levels of PA and BA, potentially benefiting piglet intestinal health.

5 Conclusion

In this study, substituting some soybean meal and corn with FWB in the weaned piglet diet enhanced nutrient digestibility, increased intestinal SCFAs levels, and improved the structure of intestinal microflora, thereby reducing the diarrhea rate. Therefore, this study provides valuable insights into alleviating the shortage of feed resources and supports the application of FWB in weaned piglets.

Data availability statement

The original contributions presented in the study are publicly available. This data can be found at the NCBI with accession number: PRJNA1247028.

Ethics statement

The animal study was approved by Institutional Animal Care and Use Committee of Sichuan Agricultural University. The study was conducted in accordance with the local legislation and institutional requirements.

Author contributions

NJ: Data curation, Investigation, Validation, Writing – original draft. JJ: Investigation, Writing – original draft. XW: Data curation, Investigation, Writing – original draft. MT-M: Writing – review & editing. GJ: Data curation, Investigation, Writing – original draft. QZ: Data curation, Writing – original draft. RZ: Data curation, Writing – original draft. HuL: Data curation, Methodology, Writing – original draft. FW: Data curation, Writing – original draft. HZ: Investigation, Supervision, Writing – original draft. HeL: Investigation, Writing – original draft. LC: Supervision, Writing – review & editing. JT: Data

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Conflict of interest

HeL was employed by Dekon Food and Agriculture Group.

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Glossary

AA - Acetic acid

ADF - Acid detergent fiber

ADFI - Average daily feed intake

ADG - Average daily gain

ASP - Acid soluble protein

ATTD - Apparent total tract digestibility

BA - Butyric acid

CF - Crude fiber

CP - Crude protein

DF - Dietary fiber

DM - Dry matter

EE - Ether extract

F/G - Feed/Gain

FWB - Fermented wheat bran

IDF - Insoluble dietary fiber

NDF - Neutral detergent fiber

NSP - Non-starch polysaccharides

OTUs - Operational taxonomic units

PA - Propanoic acid

SCFAs - Short-chain fatty acids

SDF - Soluble dietary fiber

SEM - Scanning electron microscope

TP - True protein

VA - Valeric acid

WB - Wheat bran

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Effects of water and feed based RISCO-NUTRIFOUR probiotic supplementation on the technological and physicochemical quality of broiler breast meat

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Introduction: Consumer interest in poultry and high-quality meat products has increased. Probiotics are used in the diet to improve the quality of broiler meat. The aim of this study was to investigate the effects of multi-strain probiotics (RISCO-NUTRIFOUR®, RNF) on the quality and physicochemical properties of broiler meat.

Methods: A total of 288 broilers received six feed treatments for 1-14 days in water and 15-28 days in feed. T1-T3 received 0.4%, 0.2%, and 0.1% RNF, respectively; T4 received 0.1% *Bacillus subtilis* (BS; CLOSTAT®); T5 received 0.1% *Saccharomyces cerevisiae* (SC); and T6 received 0.0% probiotic (NC). The meat quality and physicochemical properties of the broiler breast were evaluated on day 28.

Results and discussion: RNF, especially at 0.1% RNF, significantly reduced cooking losses was more tender (required the least SF), and improved average body weight at day 28 and total numerical feed conversion ratio compared to controls. The RNF probiotic had a positive effect on the texture profile (especially 0.4% RNF), sensory properties, and body weight (especially at 0.2% RNF). In conclusion, 0.4% RNF is recommended to achieve the best texture profile, 0.2% RNF to achieve the best juiciness and overall sensory acceptability as well as the best target weight of the broilers, and 0.1% RNF to achieve the most tender texture and the lowest cooking losses at day 28 compared to the controls.

KEYWORDS

broilers meat quality, feed conversion ratio, meat texture, RISCO-NUTRIFOUR probiotic, physicochemical properties, sensory attributes

1 Introduction

Chicken is the most popular poultry species and accounts for one third of the world's meat production for human consumption, providing both meat and eggs (1). Chicken meat is an important source of high-quality protein and is in high demand worldwide due to its nutritional value and affordability (2, 3). As consumer preference shifts toward healthier diets, meat quality has become a critical factor in poultry production (4, 5).

The widespread use of antibiotics in poultry has raised concerns about antibiotic resistance, imbalance of the intestine microbiota, and the accumulation of residues in meat products. As

a result, probiotics have emerged as viable, safe, and effective alternatives to antibiotics that promote gut health, immunity, and meat quality without undesirable side effects (1, 6). Among the various dietary supplements (7), probiotics such as *Bacillus subtilis* and *Saccharomyces cerevisiae* have shown potential to improve meat quality (5, 8). Probiotics have gained considerable attention in poultry nutrition as natural growth promoters and alternatives to antibiotics that improve gut health, feed efficiency, and overall meat quality.

Meat quality is significantly influenced by the microbial and physicochemical properties of the meat. Physicochemical properties, such as pH and color, and technological meat quality, such as water retention, drip loss, cooking loss, and shear force, are the most important and noticeable indices (1, 2, 9, 10). However, the effects of probiotics on these parameters are inconsistent across studies, and conflicting results are reported regarding their effects on broiler meat characteristics (4, 11–14).

This well-designed study addresses the demand for high-quality poultry meat by testing RISCO–NUTRIFOUR, a multi-strain probiotic (*Bacillus substiles*, *Lactobacillus Parabunchneri*, *Saccharomyces cerevisiae* yeast, *Lactobacillus harbinensis*, *Rhodopseudomonas palustris*, *Rhodopseudomonas shaeroides*, and *Candida ethanolic*) and its effects on meat quality parameters such as pH, color, water-holding capacity, cooking loss, texture, and sensory properties. The effects of multi-strain probiotics, such as RISCO-NUTRIFOUR® (RNF) on the physicochemical and technological quality of broiler meat, especially when administered by different routes, continue to be the subject of active research. The aim of this trial was to evaluate the effects of RNF probiotic supplementation-administered in water from day 1 to 14 and in feed from day 15 to 28-on the physicochemical properties, technological meat quality parameters, texture profile analysis (TPA), and sensory attributes of broiler breast meat, carcass characteristics, and feed conversion ratio of broiler chickens.

2 Materials and methods

2.1 Housing birds and experimental design

This study was conducted in June 2022 at King Saud University (KSU) Experimental Poultry Research Unit using 288 day-old Ross 308 chicks. The experiment complied with all applicable methods and procedures approved by the KSU Scientific Research Ethics Committee under the institutional approval code KSU-SE-21-47. The chicks were separated according to feather sex, weighed individually, and randomly divided equally into 6 experimental groups. Each treated group had 8 replicates with 6 birds each (3♂ and 3♀) (48 chicks per group). Experimental groups 1–3: groups treated with RNF at the three RNF doses 1, 2, and 3 (0.4, 0.2, and 0.1% RNF, respectively). Group 4: group treated with *Bacillus substiles* (BS, Clostat®). Group 5: group treated with *Saccharomyces cerevisiae* yeast (SC). Group 6: non-treated group (negative control).

The study investigated the effects of RISCO–NUTRIFOUR probiotic supplementation using two different administration methods: Water supplementation from 1 to 14 days and feed supplementation from 15 to 28 days. Supplementation in drinking water ensures a steady intake, especially in the first two weeks when feed intake is still in progress and the digestive system is not yet mature, from the 15th onwards, as the digestive system matures and feed intake increases, nutrient absorption becomes more efficient through feed. The broilers were treated with 1 of six water–based (from 1 to 14 days) and feed–based (from 15 to 28 days) treatments: 4 L/ton (0.4%), 2 L/ton (0.2%), 1 L/ton (0.1%), 0.1% Clostat "1:128," 0.1% SC, and negative control (NC) 0%. Al Raya Specialties Industrial Factory in Riyadh, Saudi Arabia, manufactures the RISCO-NUTRIFOUR® solution, a probiotic mixture. RNF contains *Bacillus substiles* ($1 \times 10^{\wedge 9}$ colony-forming units (CFU)/ml), *Lactobacillus Parabunchneri* ($1 \times 10^{\wedge 9}$ CFU/mL), *Saccharomyces cerevisiae* yeast ($1 \times 10^{\wedge 5}$ CFU/mL), *Lactobacillus harbinensis* ($1 \times 10^{\wedge 9}$ CFU/mL), *Rhodopseudomonas palustris* ($1 \times 10^{\wedge 7}$ CFU/mL), *Rhodopseudomonas shaeroides* ($1 \times 10^{\wedge 7}$ CFU/mL), and Candida ethanolic ($1 \times 10^{\wedge 5}$ CFU/mL).

The experimental starter (0–14 days) and grower (15–28 days) diets were formulated as a mash according to the nutritional requirements of the Ross 308 Management Guide recommendations (Aviagen, 2019, New York, NY, USA), as shown in Supplementary Table S1. Feed and water were provided *ad libitum*. Chicks were housed in electrically controlled heated battery cages, with a room temperature of 35°C on arrival and a gradual decrease (2°C every 3 days) until day 24. The average outside temperature was approximately 26.4°C, and humidity ranged from light to moderate. The light bulb program was on continuously for 24 h during the first week of life and was on 23 h and off for 1 h during the rest of the experimental period. The broilers were housed in the same cage, which was 58 cm long, 50 cm wide, and 35 cm high. The stocking density was 6 birds per 0.30 m^2. All chicks were immunized against Gumboro disease, Newcastle disease, and infectious bronchitis (Fort Dodge Animal Health-USA).

2.2 Bioactive chemicals analysis of RISCO–NUTRIFOUR

The procedures for the separation of chemical mixtures by gas chromatography–mass spectrometry (GC–MS; Agilent Technologies, Palo Alto, CA, USA) was described in detail by Azzam et al. (15). The bioactive chemicals were expressed as a proportion of the extracted samples.

2.3 Carcass traits, target weight, feed conversion ratio, and meat quality evaluation

The weights of the chicks at arrival and at the end of the trial were converted to the average target weight and used to calculate the daily weight gain. Then, the feed conversion ratio (1–28 d) was calculated by dividing the feed intake by the gain throughout these periods. On day 28, the broilers were slaughtered according to standard practice in Saudi Arabia, and eight male broilers per group ($n = 8$) were randomly selected to evaluate meat quality and carcass traits. The slaughter of broilers according to Islamic law complies with halal standards, with an emphasis on humane and respectful treatment during the slaughter process without anesthetic. The slaughtering was done with a very sharp knife and by a qualified person to allow for a faster process while minimizing the suffering of the birds, which is critical under halal standards, with an emphasis on maintaining the cleanliness and consistency of the meat. Slaughter weight and carcass weight

(excluding head, neck, feathers, shanks, abdominal fat, and eviscerated organs) were measured. The carcass yield (%) was calculated as the ratio between carcass weight and slaughter weight. Breast, leg, wing, kidney, pancreas, lymphoid organs (liver, bursa, spleen, and thymus), and offal (gizzard, proventriculus, heart, and liver without gall bladder) were separated and weighed in the same manner. The percentage yield for each portion was estimated in relation to the live weight at slaughtering (16). After cutting, samples of the pectoral muscle were taken from each carcass to determine the physicochemical parameters (pH and color) and samples of the pectoralis major muscle to determine the textural characteristics and sensory properties. The samples were stored in the refrigerator at 4°C for 24 h after slaughter to measure the ultimate physicochemical parameters and then immediately stored at −20°C to determine the meat quality parameters. The frozen samples were thawed at 4°C before being tested for water-holding capacity (WHC), cooking loss (CL), myofibrillar fragmentation index (MFI), shear force (SF), texture profile analysis (TPA), and sensory evaluation.

2.4 Physico-chemical properties (pH value, core temperature, and color measurements)

The internal temperature and pH parameters of the pectoral muscles were determined 15 min and 24 h post-mortem using a thermocouple thermometer, taking the average of three pH measurements on the inner surface of the pectoral muscles at different locations for each sample. The pH was measured by inserting electrodes into the meat samples using a Hanna Instruments pH meter with microprocessor (model pH 211, Woonsocket, RI, USA).

The color parameters (L^*, a^*, and b^* values and their derivatives) of the breast samples were measured 24 h post-mortem, whereby the average of two color measurements on the inside of the breasts was determined for each sample. Breast muscle color measurements were taken using a Minolta Chroma-Meter (Konica Minolta, Tokyo, Japan) with a CR400 head at an illumination setting compatible with D_{65} illumination (17). The coordinates L^*, a^*, and b^* were evaluated according to the CIELAB system, where L^* corresponds to lightness, a^* to redness (between green and red), and b^* to yellowness (between blue and yellow). The measurements were made after calibrating the device with a white reference tile at $Y = 86.10$, $x = 0.3188$, and $y = 0.3362$.

The center of the plane is neutral, and the distance from the center axis represents the color saturation "chroma," while the angle on the chromaticity axes refers to the hue angle (18). In order to obtain a particularly realistic assessment of how consumers imagine the color of meat, chroma, delta color change (ΔE), browning index (BI), whiteness index (WI), and hue angle (h*) were derived from the color coordinates and calibration values and formulated as described by Valizadeh et al. (19) and Cázares-Gallegos et al. (20).

2.5 Meat quality indicators

To measure water-holding capacity (WHC), the compression method described by Wilhelm et al. (21) was used. Thawed samples with a wet weight of about 2 to 3 g were carefully clamped between two sheets of filter paper and pressed for 5 min with a pressure device over two acrylic plates with a force of 10 kg; the samples were weighed again. The samples were analyzed in duplicate. Finally, the percentage of WHC was determined using the following equation: WHC (%) = 100 − [((Initial weight of sample − Final weight of sample/Initial weight of sample)) × 100]. Cooking loss (CL) is a common method for evaluating the water-holding capacity of meat during cooking. It is calculated as the percentage weight loss during the cooking process as described by Hussein et al. (22). In brief, the breast meat samples were weighed raw (initial weight, W_1). Samples are placed on a standard tabletop grill preheated to a specific temperature (e.g., 170–180°C). The samples are cooked until they reach an internal temperature of 75°C, which is recommended for poultry meat. The internal temperature is measured with a thermometer inserted into the thickest part of the breast. After cooking, the samples are cooled to room temperature (~20–25°C). The cooked samples are weighed again (final weight, W_2). To calculate the cooking loss (%):

$$CL\,(\%) = \frac{(W1 - W2)}{W1} * 100$$

Where: W_1 = Initial raw weight (g), W_2 = Final cooked weight (g).

The MFI of muscle samples was measured as an indirect indicator of calpain activity using the method described by Suliman et al. (23). Thawed, scissor-cut samples (4 g) were homogenized in 40 mL MFI buffer (2°C) for 30 s using a blender. After washing several times with MFI buffer, the absorbance was measured at 540 nm using a spectrophotometer (HACH DR/3000, Loveland, CO, USA). The MFI was calculated by multiplying the absorbance of the resulting 0.5 mg/mL solution by 200. To calculate the shear force (SF) as an index of breast meat tenderness, five rectangular core samples of 2*1*1 cm^3 in size from each chilled, cooked sample were cut longitudinally parallel to the muscle fibers using a manual corer. The greatest force (N/cm^2) of the TA-HD Texture Analyzer (Stable Micro Systems Ltd., Godalming, UK) equipped with a Warner-Bratzler shear barb with a triangular opening blade, could be applied vertically to the fibers. The crosshead was configured to move at 200 mm/min. From a distance of 15 mm, the device was operated at speeds of 2, 2, and 10 mm/s during the pre-, during-, and post-tests. The SF values were calculated using the maximum point of the generated curve.

2.6 Texture profile analysis (TPA)

The TPA was performed with a TA-HD Texture Analyzer. To determine the TPA, the cooked breast muscle fibers were scored parallel to the longitudinal direction using a hand-held coring device. A cylindrical piston was used to compress the samples to 80% of their original height over two test cycles. The force-time curves of the deformation were determined using test-specific analyzes in the texturometer. The velocities used were 2, 5 and 5 mm/s in the pre-, intermediate and post-test. The hardness, springiness, cohesiveness, and chewiness of the samples were measured as described by Novaković and Tomašević (24).

2.7 Sensory evaluation

The frozen meat samples were thawed overnight at 4°C, then wrapped in aluminum foil and cooked in the oven at 200°C until a core temperature of 70°C was reached. After cooking, the samples were cut into small pieces of approximately 2 cm^3 and given a random code number for identification. Twenty-four trained KSU taste panelists were asked for a sensory evaluation of the meat. The mean of all panel ratings was calculated to determine the characteristics of the sample. The evaluation was carried out according to the method described by Grunert et al. (25) using a 9-point hedonic scale, whereby the meat samples used for the sensory evaluation were divided into the following groups based on the category scaling: 9, 8, 7, 6, 5, 4, 3, 2, 1 = extremely like, very like, moderately like, somewhat like, neither like nor dislike, somewhat dislike, moderately dislike, very like dislike, extremely dislike, respectively. Water and crackers were served to remove any residual taste in the mouth from the previous samples.

2.8 Statistical analysis

The Ryan-Einot-Gabriel-Welsch and Quiot (REGWQ) test, also known as the "Ryan's method," is used to determine statistically significant differences ($p < 0.05$) between independent treatment groups in a balanced 1-way ANOVA reporting means ± standard error of the mean (SEM) based on a completely randomized design using the general linear model (GLM) of SAS (26) software (Cary, NC, USA).

The equation of the model was:

$$\gamma_{ij} = \mu + T_i + e_{ij}$$

Where Y_{ij} is an individual observation, μ is the overall mean, T_i is the effect of the i^{th} treatment, and e_{ij} is the random residual error. Before starting the statistical analysis, the Kolmogorov–Smirnov test was performed to ensure that the data were normal.

For the statistical analysis, a typical experimental design was established for this study, which included multiple treatment groups with standardized protocols for humane slaughter and random sampling, and data collection with a sufficient number of birds (8 per group) for the evaluation of carcass traits and meat quality. Six chicks per cage (8 cages per group) were used to evaluate growth performance.

3 Results

3.1 Physico-chemical traits, meat quality, texture profile analysis, and sensory evaluation of the breast

The data on the physicochemical parameters the breast meat of 28-day-old broilers treated with RNF in water from 1 to 14 days and with feed from 15 to 28 days are presented in Table 1. The treatments had no significant effect ($p > 0.05$) on pH 15 min, core temperature 24 h post-mortem, ultimate color components and color derivatives. However, 0.4% RNF had a significantly higher ultimate breast pH (pH_{24} h) compared to the other treatment groups.

The meat quality characteristics for the breast samples at 28 days of age are shown in Table 2. CL% and SF of the breast samples differed ($p < 0.05$) between treatments. The 0.1% RNF treated group had the

TABLE 1 Initial and ultimate pH, core temperature, color components and color derivatives at day 28 in breast meat of broiler treated with RISCO–NUTRIFOUR probiotics in a water and feed bases.

Treatments[1]	RNF			0.1% *Bacillus subtilis*	0.1% *Saccharomyces cerevisiae*	Negative control	Standard error	*p* value
Item	0.4%	0.2%	0.1%					
Physicochemical properties								
Initial and ultimate pH								
Initial pH	6.19	6.11	6.20	5.97	6.08	6.07	0.062	0.109
Ultimate pH	6.08[a]	5.89[b]	5.92[b]	5.79[b]	5.86[b]	5.83[b]	0.039	0.0001
Core temperature	16.1	15.8	15.7	16.0	16.3	15.9	0.158	0.138
Ultimate color components and their derivatives								
Lightness (L^*)	48.0	46.0	50.4	47.2	47.7	45.4	1.14	0.055
Redness (a^*)	4.34	6.53	4.40	4.79	5.24	5.24	0.614	0.151
Yellowness (b^*)	18.2	17.5	18.3	16.7	16.6	16.9	0.665	0.293
ΔE	48.5	50.5	46.4	48.9	48.5	50.8	1.03	0.053
Chroma	18.8	18.9	18.9	17.4	17.5	17.7	0.634	0.261
Hue angle	76.3	69.6	76.0	74.1	72.5	72.7	2.00	0.195
Browning index	53.7	57.8	50.9	50.5	50.3	54.2	2.26	0.159
Whiteness index	44.7	42.8	46.8	44.4	44.8	42.5	1.01	0.053
a/b ratio	0.245	0.378	0.254	0.288	0.316	0.313	0.039	0.206

[1]Basal diet with 0.4, 0.2, and 0.1% RISCO–NUTRIFOUR (RNF), or with 0.1% *Bacillus subtilis*, or with 0.1% *Saccharomyces cerevisiae*, or without probiotics (Negative control). Each mean is based on measurements from 8 birds per treatment at 28 days of age. a-b Means in the same row with different superscripts differ significantly ($p < 0.05$). ΔE: total ultimate color change; Chroma: Saturation index; a^*/b^* ratio: redness to yellowness ratio.

TABLE 2 Meat quality characteristics on day 28 of age in breast meat of broilers treated with RISCO–NUTRIFOUR probiotics in a water and feed base.

Treatments[1]	RNF			0.1% *Bacillus subtilis*	0.1% *Saccharomyces cerevisiae*	Negative control	Standard error	*p* value
	0.4%	0.2%	0.1%					
WHC (%)	66.7	67.7	68.5	66.6	65.9	66.7	1.17	0.636
CL (%)	26.8^{a}	20.5^{ab}	8.42^{c}	12.3^{bc}	29.3^{a}	12.1^{bc}	2.51	<0.0001
MFI	103.4	105.4	112.7	102.2	105	108.5	5.47	0.779
SF (N)	4.21^{b}	4.25^{b}	3.42^{c}	4.40^{ab}	4.92^{a}	4.35^{ab}	0.193	0.0002

[1]Basal diet with 0.4, 0.2, and 0.1% RISCO–NUTRIFOUR (RNF), or with 0.1% *Bacillus subtilis*, or with 0.1% *Saccharomyces cerevisiae*, or without probiotics (Negative control). Each mean is based on measurements from 8 birds per treatment at 28 days of age. $^{a-c}$Means in the same column with different superscripts differ significantly ($p < 0.05$). WHC, Water holding capacity; CL, Cooking loss; MFI, myofibril fragmentation index; SF, shear force.

lowest CL% and SF values, indicating that the 0.1% RNF treated group had the most favorable CL% (8.42%) and tenderness (the lowest tenderness; 3.42). Although WHC% and MFI were similar between groups ($p > 0.05$), the 0.1% RNF treated group had the highest numerical ($p > 0.05$) from WHC and MFI, which decreased with increasing RNF dosage.

The TPA for the broiler samples on day 28 are shown in Table 3. Hardness (N), springiness, and cohesiveness of the breast meat samples differed ($p < 0.05$) between the treatment groups. Hardness was higher in groups treated with 0.1% BS and 0.1% SC probiotics, lower in groups treated with the RNF-probiotic mixture, and intermediate in the negative control group.

The RNF treatments, particularly at 0.4%, gave the most significant values for springiness and cohesiveness in the meat samples when compared to the negative control, with the 0.4% RNF treated group having the best values for springiness and cohesiveness. The chewiness values were similar between treatments ($p > 0.05$).

The sensory evaluation of the broiler samples at 28 days of age is shown in Table 4. Consumer ratings of tenderness and flavor were similar between treatments ($p > 0.05$), while juiciness and overall acceptability were highest in broilers treated with 0.2% RNF in water from 1 to 14 days of age and in feed from 15 to 28 days of age compared to the other groups.

3.2 Carcass traits, target weight, and feed conversion ratio

The carcass characteristics (live slaughter weight (g), hot carcass weight (g), carcass yield (%), and relative carcass composition weights (% to live weight)) of 28-day-old broilers treated with RNF in water from 1 to 14 days and with feed for 15 to 28 days are shown in Table 5. The live weight, carcass weight, carcass yield, and carcass composition of the broilers did not differ ($p > 0.05$) between the experimental groups.

Figure 1 shows the FCR of broilers treated with RNF in water for 1–14 days and with feed for 15–28 days. Water supplementation with RNF resulted in a lower ($p > 0.05$) FCR in broiler chicks compared to the negative control. However, FCR improved quantitatively between days 15 and 28, indicating that treatments with water supplementation may not be as effective in achieving optimal results. During the 1–14 days, 0.2% RNF treatment reduced FCR by 5.08% compared to the negative control (1.24 vs. 1.18). During 15–28 days, 0.2% RNF treatment improved ($p > 0.05$) FCR by 5.30% compared to negative control (1.51 *vs* 1.43). Thus, RNF was more effective when administered via feed rather than water compared to the control. The average body weight at day 28 and the overall FCR of broilers receiving RNF in water from 1 to 14 days and in feed from 15 to 28 days are shown in Figure 2. The 0.2% RNF treatment resulted in a significantly ($p < 0.05$) higher body weight (1.558 kg) on day 28 and a trend toward better feed conversion (1.40) throughout the period compared to the other treatments.

4 Discussion

Probiotics, live non-pathogenic microorganisms added to human and animal diets, colonize the intestinal environment to promote a balanced flora consisting of species commonly found in the poultry gut (27–30). Probiotics have the ability to reduce pathogens, and improve the quality of broiler meat (30–32). The effectiveness of probiotics in exerting beneficial effects depends on their ability to colonize the intestine, which is influenced by various factors. These include the feeding program, the type, dose, and frequency of probiotic administration, the presence of prebiotics, and host-related factors such as age, health status, genetic characteristics, the pH of the intestine. In addition, external environmental conditions play a crucial role in determining probiotic colonization and functionality (30, 33–35).

In this study, the effects of probiotic supplementation on the meat quality of broilers were investigated. Meat quality parameters such as pH, lightness, redness, yellowness, cooking loss, water holding capacity, shear force, texture profile, and sensory evaluation were assessed. Analysis of REGWQ showed that the physicochemical data of the breast meat at day 28 were similar in the RNF groups, except for the ultimate pH, which was higher in the highest RNF group compared to the other groups. The pH was considered a general signal for meat quality testing, reflecting the conversion of glycogen to lactic acid in the muscle pre and post mortem (9). At that time, there was a direct relationship between pH and meat quality, including tenderness, water-holding capacity, color, juiciness, and shelf-life. Meat generally had a pH between 5.0 and 7.0 (36). Some of the studies that examined meat quality found that the use of probiotics increased redness and yellowness of breast meat and decreased lightness (5, 37), while probiotics increased all parameters of meat color in thigh meat (13, 38). In contrast, some studies found that probiotic supplementation had no effect on yellowness, redness, or lightness (5, 13, 37, 39). The addition of probiotics has been shown to consistently improve the redness and yellowness of broiler meat, which could be an indication of improved meat quality as perceived

TABLE 3 Texture profile analysis (TPA) on day 28 of age in breast meat of broilers treated with RISCO–NUTRIFOUR probiotics in a water and feed base.

Treatments[1]	RNF			0.1% *Bacillus subtilis*	0.1% *Saccharomyces cerevisiae*	Negative control	Standard error	P value
	0.4%	0.2%	0.1%					
Hardness (N)	5.09^{b}	5.36^{b}	5.01^{b}	6.83^{a}	7.26^{a}	5.79^{ab}	0.309	<0.0001
Springiness	0.91^{a}	0.86^{ab}	0.90^{a}	0.78^{c}	0.79^{bc}	0.78^{c}	0.019	<0.0001
Cohesiveness	0.66^{a}	0.60^{ab}	0.64^{ab}	0.58^{b}	0.60^{ab}	0.58^{b}	0.018	0.014
Chewiness	3.07	2.94	2.91	3.15	3.46	2.66	0.221	0.218

[1]Basal diet with 0.4, 0.2, and 0.1% RISCO–NUTRIFOUR (RNF), or with 0.1% *Bacillus subtilis*, or with 0.1% *Saccharomyces cerevisiae*, or without probiotics (Negative control). $n = 8$ samples per treatment. $^{a–c}$Means in the same column with different superscripts differ considerably ($p < 0.05$).

TABLE 4 Sensory attributes on day 28 of age in breast meat of broilers treated with RISCO–NUTRIFOUR probiotics in a water and feed base.

Treatments[1]	RNF			0.1% *Bacillus subtilis*	0.1% *Saccharomyces cerevisiae*	Negative control	Standard error	P value
	0.4%	0.2%	0.1%					
Juiciness	6.08^{ab}	7.00^{a}	6.42 ^{ab}	6.33 ^{ab}	5.75^{b}	6.00 ^{ab}	0.26	0.03
Tenderness	6.42	6.75	6.83	6.25	5.75	6.33	0.27	0.086
Flavor	6.00	6.33	6.00	6.25	6.25	6.50	0.21	0.513
Acceptability	6.67^{ab}	7.25^{a}	6.84^{ab}	6.76^{ab}	6.17^{b}	7.00^{a}	0.195	0.01

[1]Basal diet with 0.4, 0.2, and 0.1% RISCO–NUTRIFOUR (RNF), or with 0.1% *Bacillus subtilis*, or with 0.1% *Saccharomyces cerevisiae*, or without probiotics (Negative control). $n = 8$ samples per treatment. $^{a–c}$Means in the same column with different superscripts differ considerably ($p < 0.05$).

TABLE 5 Carcass traits measured at day 28 of broilers supplemented with RISCO–NUTRIFOUR probiotics in a water and feed bases.

Treatments[1]	RNF			0.1% *Bacillus subtilis*	0.1% *Saccharomyces cerevisiae*	Negative control	Standard error	P value
Item	0.4%	0.2%	0.1%					
Live weight (Kg)	1.55	1.62	1.64	1.54	1.57	1.49	0.046	0.334
Carcass weight (g)	0.942	0.996	1.065	0.944	0.967	0.908	0.039	0.168
Carcass yield (%)	60.9	61.5	65.2	61.4	61.6	60.9	1.78	0.577
Percentage weights (g/100 g BW of the broilers)								
Breast	28.2	28.3	28.7	28.2	27.1	29.9	0.802	0.372
Leg	24.6	24.4	24.5	25.9	25.5	25.8	1.50	0.350
Wing	5.25	4.49	4.92	5.27	5.12	4.98	0.857	0.494
Thymus	0.367	0.404	0.333	0.314	0.371	0.476	0.046	0.088
Bursa	0.216	0.204	0.252	0.217	0.161	0.224	0.021	0.286
Spleen	0.081	0.068	0.084	0.081	0.074	0.088	0.137	0.558
Kidney	0.502	0.531	0.547	0.457	0.578	0.509	0.031	0.372
Pancreas	0.268	0.290	0.276	0.273	0.293	0.300	0.401	0.559
Giblets	5.75	5.86	5.72	5.57	6.01	5.49	0.094	0.741

[1]Basal diet with 0.4, 0.2, and 0.1% RISCO–NUTRIFOUR (RNF), or with 0.1% *Bacillus subtilis*, or with 0.1% *Saccharomyces cerevisiae*, or without probiotics (Negative control). $n = 8$ samples per treatment. $^{a–c}$Means in the same row with different superscripts differ significantly ($p < 0.05$). [2]Giblets (Gizzard, Proventriculus, heart, and liver without gallbladder).

by the consumer. These differences between trials were more significant for thigh meat than breast meat, indicating that probiotics had a different impact depending on the anatomical location of the muscle.

This study showed that the use of RNF probiotics in broilers significantly improves meat quality. The 0.4% RNF improved the breast texture profile of the breast through improved springiness by 16.67% indicating better elasticity and resilience of meat after compression, and improved cohesiveness by 13.79% compared to the negative control, indicating improved structural integrity and firmness of the meat, resulting in better meat texture.

The 0.2% RNF showed a 21.7% increase in juiciness, increasing meat palatability and consumer satisfaction, and a 17.5% increase in overall acceptability, indicating a higher consumer preference for RNF-treated meat, compared to the SC group, and a 7.45% increase in average body weight at day 28 compared to the negative control, indicating improved growth performance with the 0.2% RNF supplementation. Also, the lowest (best) FCR was observed at RNF 0.2%, indicating better feed efficiency compared to the other groups. The lower FCR value indicates that the broilers fed 0.2% RNF utilized the feed more efficiently, resulting in higher weight gain per unit of feed consumed.

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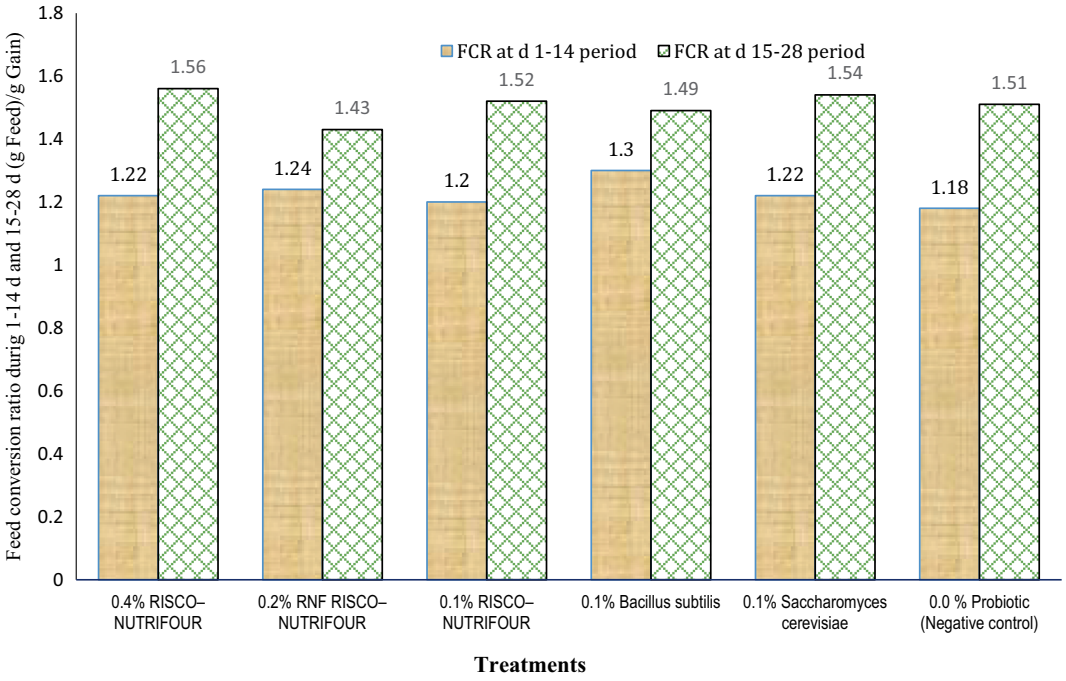

FIGURE 1
Feed conversion ratio (FCR) during 1–14 days' period (p = 0.225; SEM ± 0.394); FCR during 15–28 days' period (p = 0.394; SEM ± 0.042); n = 8 replicated cages.

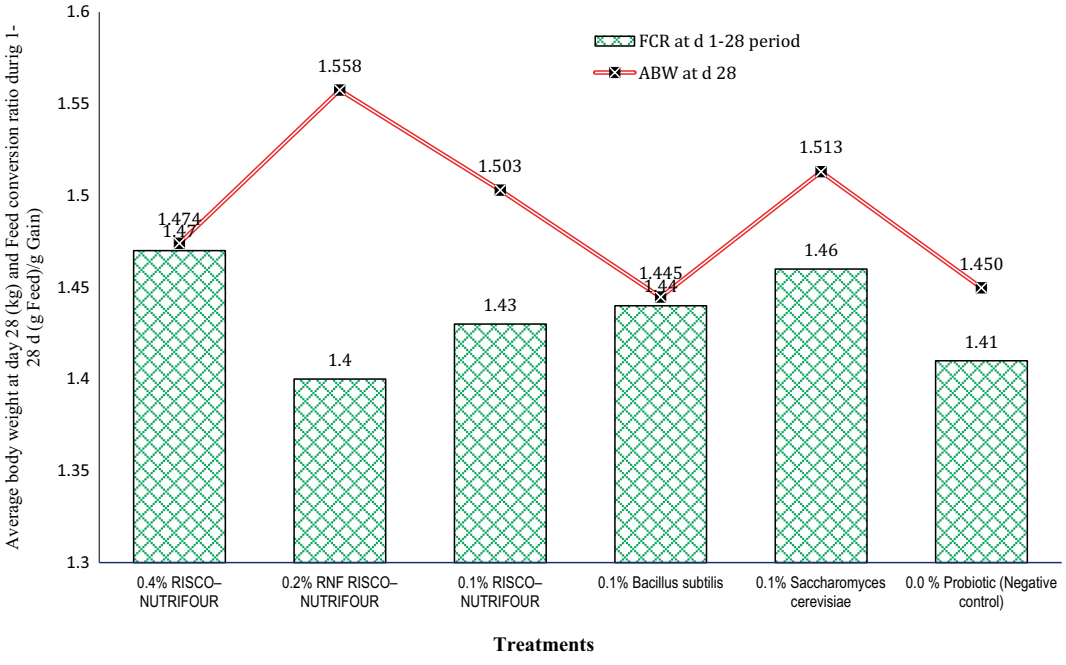

FIGURE 2
Average body weight at day 28 (kg; p = 0.041; SEM ± 0.027) and feed conversion ratio during 1–28 days' periods (p = 0.041; SEM ± 0.027; FCR = feed intake (g)/gain (g)); n = 8 replicated cages.

The 0.1% RNF showed a 71.3% reduction in cooking loss, which improved water-holding capacity, as well as a 30.5% reduction in shear force and 30.99% reduction in hardness, which improved meat tenderness compared to the SC group at day 28. Thus, the study shows that RNF supplementation significantly improves meat quality, especially in terms of water retention, tenderness, and sensory properties. Tang et al. (5) found that dietary supplementation with BS can improve meat quality and carcass characteristics of broilers, which

is beneficial to consumers due to the improved fatty acid profile and amino acid composition. Other parameters studied were not significantly altered by the treatments. Previous research on probiotics in broilers has primarily focused on growth rather than meat quality, leaving a gap in the literature (40, 41). As a result, this study presents information about meat quality.

Similarly, studies on other meat quality metrics show that probiotic supplementation has no effect on pH, cooking loss, shear force, or drip loss of broiler meat (5, 13, 39). In addition, some studies found that probiotic supplementation increased pH and WHC in breast meat (42, 43) while reducing cooking loss, shear force, and drip loss in breast and thigh meat (13, 37, 38). The increase in WHC% and decrease in CL% in breast meat show that RNF probiotics, especially at the 0.1% level, can alter protein structures in muscle, improving their ability to bind moisture during cooking. This improves both the sensory properties of the meat and its nutritional value. The various research studies on the effect of adding probiotics to broilers vary widely, including the breed of chicken, the type of probiotic, the level and dosage, and the location of measurement. Therefore, a study was needed to further investigate these effects on meat quality indicators.

Several factors contribute to the heterogeneity of the study results. In some studies, measurements were taken on both the leg and the breast, while in others only the breast or the leg was examined. These differences highlight the complex relationships between probiotic supplementation and meat quality in broilers. This study provides a detailed assessment of the effects of probiotics on numerous meat quality traits in broilers. The study found that the addition of RNF probiotics, particularly at 0.1%, had a significant effect on meat texture profile, sensory characteristics, cooking loss, and shear force, all of which were significantly improved in the broiler breast meat. In addition, numerical improvements in WHC and MFI were observed in the breast meat portions of broilers receiving RNF at a low concentration (0.1%). The results have important implications for the chicken industry, particularly with regard to improving meat quality by optimizing feed formulation.

5 Conclusion

Based on the finding obtained in this study, the 0.4% RNF is recommended to achieve a 16.67% improvement in springiness and a 13.79% improvement in cohesiveness meat texture compared to the negative control. With 0.2% RNF, a 21.7% increase in juiciness and a 17.5% increase in overall acceptability compared to the SC group, and a 7.45% increase in average body weight at day 28 compared to the negative control and overall feed conversion compared to other groups is recommended. It is recommended that 0.1% RNF achieves a 71.3% reduction in cooking loss, improving water-holding capacity, a 30.5% reduction in shear force and a 30.99% reduction in hardness, improving meat tenderness compared to the SC group on day 28. The study thus shows that supplementation with RNF significantly improves meat quality, particularly in term of water retention, tenderness, and sensory properties, and points to avenues for further research and standardization in poultry production. These results also contribute to a better understanding of the role of RISCONUTRIFOUR probiotics in improving meat quality and meeting consumer demands for nutritious and high-quality poultry products.

Data availability statement

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

Ethics statement

The animal study was approved by King Saud University (KSU-SE-21-47). The study was conducted in accordance with the local legislation and institutional requirements.

Author contributions

AA-a: Funding acquisition, Project administration, Resources, Software, Supervision, Validation, Visualization, Writing – review & editing. MA-G: Conceptualization, Investigation, Supervision, Validation, Writing – review & editing. MQ: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Writing – original draft, Writing – review & editing. AM: Data curation, Investigation, Methodology, Writing – review & editing. MA: Investigation, Writing – review & editing. MA-B: Data curation, Software, Writing – review & editing. EH: Data curation, Validation, Writing – review & editing. GS: Conceptualization, Writing – review & editing.

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Conflict of interest

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

Generative AI statement

The authors declare that no Gen AI was used in the creation of this manuscript.

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The Supplementary material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fvets.2025.1517078/full#supplementary-material

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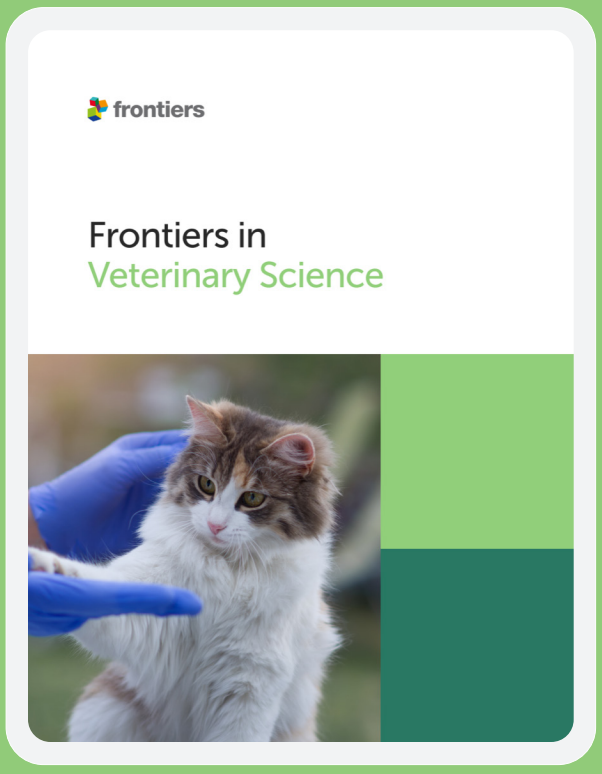

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