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Gut microbiota: Allied with livestock nutrition, health, and welfare

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Gut microbiota: Allied with livestock nutrition, health, and welfare

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Editorial: Gut microbiota: allied with livestock nutrition, health, and welfare

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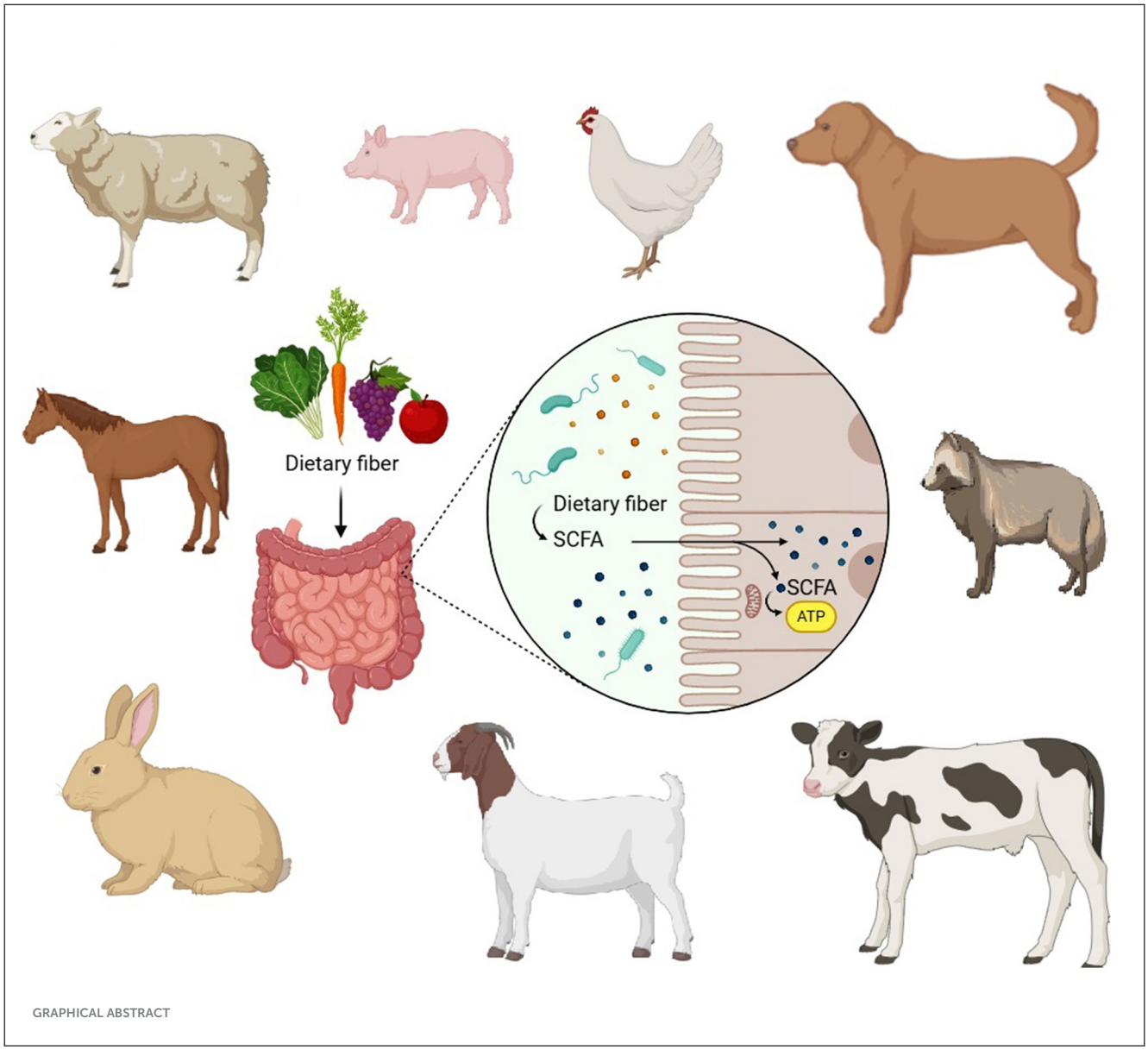
Editorial on the Research Topic
Gut microbiota: allied with livestock nutrition, health, and welfare

Introduction

The majority of digestion and assimilation occur in the livestock's gastrointestinal tract. The availability of essential nutrients in a balanced diet is the key factor for successful animal production. The gut is associated with many microbiota that act as an extensive barrier, play an active role in immune development, and accelerate dietary challenges. Furthermore, the gut microbiome aids in communication at the cellular/tissue level and mediates the animal's overall metabolism. In short, the proper functioning of the gut is necessary to perform multiple functions to enhance the health, productivity, and sustainability of livestock farming. Thus, intestinal disorders can be minimized by understanding the role of the gut in animals.

From another perspective, several antibiotics are used to counteract the diseases and infections associated with intestinal disorders. However, a comprehensive approach to the use of probiotics, involving nutritional genetics and animal behavior, increases the possibility of resilience and robustness in animals. This lowers the rate of gut-related diseases and reduces the consumption of commercial drugs. However, clinical drugs will be used to treat other infections and diseases. In other words, the intestinal microbiome performs an important barrier and digestive role in the gut, which has been well evidenced in *in vivo* livestock models. Gut functionality and microbiome colonization are the triggers and supports for the immune system. Moreover, early interventions to strengthen gut health provide cues for overall livestock development. A wealth of research has demonstrated the complementary association between the gut microbiome, the immune system, and the brain. The gut microbiome also influences behavioral traits, for stress and anxiety. Overall, gut health is influenced by the diet, composition, and function of the GIT barrier with efficient digestion and assimilation factors, which in turn modulate the overall immune state of the animal. The microbiota aids in fermentation absorption, enhances immunity and growth, and improves host development. In addition, it regulates the stabilization of the gut environment and maintains the ruminal pH. Thus, the gut microbiota accelerates feed efficacy, and high-performance animals are vital objectives in livestock agriculture to meet the growing demand for animal products.



GRAPHICAL ABSTRACT

In total, we received 16 articles that emphasize the critical role and significance of gut microbiota in growth, development, immune modulation, gut-brain barrier, digestion, feed utilization, and disease resistance (Graphical Abstract). This editorial aims to provide the essence of the research articles published in this domain to facilitate community stakeholders.

Characterization and role of fecal microbiota in livestock farming

The microbiota is a specific microbial community that inhabits a particular setting within the animal's gut. Therefore, they serve as essential identification tools for understanding the insights of genetic traits across the specific breed or lineage characterization of the microbial community and aid in understanding the co-evolutionary relationships within the specific breed characters. The study by Carrillo Heredero et al. characterized the fecal microbiota composition of the *Bardigiano* horse and compared the data with the athlete breeds. Samples were identified using 16S rRNA sequencing. The results indicated that the *Bardigiano* breed showed prominent differences at the lower taxonomic levels. In addition, factors such as weight, origin, and breeding sites accelerated the microbiota composition. Moreover, a comparison with the athlete breed confirmed that environmental factors contribute to the microbial population, which is critical for safeguarding animal diversity, preserving animal health, and developing livestock farming.

Interestingly, differences in the gut microbiome and metabolism have been noted as the physical and pathological conditions of the host change The mechanism of association between the microbiome likely aids in the development of dairy cow health, which provides cues for the advancement of probiotics in cattle production. A recent study by Hsieh et al. investigated the gut microbiota and metabolome biomarkers in healthy and mastitis-lactating dairy cows. *In vitro* fermentation

and cow-to-mouse fecal microbiota transplantation (FMT) were investigated. The results showed that *Ruminococcus flavefaciens* and *Bifidobacterium longum* subsp. *longum* rumen metabolites correlated with the healthy cow group. Inter-relationship similarity was verified by the upregulation of putrescine, xanthurenic acid, and pyridoxal in the mastitis ruminal fluid. These results evidenced the close alliance between the microbiome of dairy cows and their respective metabolites. The FMT confirmed the role of *R. flavefaciens* and *B. longum* subsp. *longum* in reducing mortality and improving the recovery of body weight loss. Adding organic and inorganic components to animal feeds has been shown to have significant growth-promoting properties. A recent study (Du et al.) reported that Copper (Cu) is an essential trace element for the growth of rabbits and has an influence on the intestinal microbiota and short-chain fatty acids (SCFAs) in growing rabbits. The cecal contents of the experimental rabbits (basal, inorganic Cu, and organic Cu fed) were investigated using 16S rDNA gene amplification sequencing and chromatography analysis. The relative abundance of *Rikenella Tissierella, Lachnospiraceae* NK3A20 group, *Enterococcus,* and *Paeniclostridium* was highest in the cupric citrate-fed group of rabbits, which was a factor in the lower incidence of diarrhea in these animals. The results revealed that the organic Cu-fed experimental groups had an abundance of *Rikenella* and *Enterococcus*.

The potential of dietary supplementation to enhance the gut microbiota

The inappropriate usage of antibiotics in livestock and poultry has become an alarming and severe health problem. The impact of antibiotics has been observed as residues present in the egg; however, this can be overcome by adopting probiotics as feed additives to improve the health of breeders and their egg quality. In this context, a recent study reported the effect of β-mannanase and probiotics on the growth performance, serum levels, gut morphology, and egg quality traits of hens (Carvalho et al.). The β-mannanase and probiotic-supplemented feed improved egg-laying capacity in the hens by 11% and 7%, respectively. The combination effect of the (β-mannanase + probiotics) improved several yolk traits and serum biochemical parameters such as total cholesterol, uric acid, and triglycerides. Notable morphological variations in villus height and crypts were also reported, improving nutrient absorption levels (Carvalho et al.).

Salmonella is one of the most common infectious bacteria, causing severe infections in humans and livestock animals. However, *Salmonella enterica serovar Typhimurium* (ST) causes non-typhoidal infections and inflammation in the gut, disrupting the microbiome and leading to enterocolitis and dehydration in pigs (Yi et al.). Strengthening gut health through feed additives such as resistant starch (RS) increases the production of SCFAs in the intestinal tract and reduces gut inflammation. Weaned experimental pigs were treated with feed supplemented with raw potato starch (RPS), which resulted in better gut health and increased production of SCFAs. Histopathological lesions were reduced in the treated groups compared to the control group animals. The expression of IL-18 was low in the RPS-treated groups. However, Reg 3γ expression varied significantly across the different gut sites of the cecum and colon in both groups. Thus, the study concluded that RPS augmentation reduced inflammation and infection severity, preventing fecal shedding in ST-infected pigs (Yi et al.). Similarly, another study investigated the differences in the growth, biochemical, antioxidant, and gut microbiome of *Cyberlindnera jadinii*-supplemented feeds in raccoon dogs (Zhao et al.). The results reported improved immunity, gut health, growth development, and behavioral performance at the supplement concentration of 1×10^9 CFU/g. Furthermore, the *C. jadinii*-treated groups exhibited improved average daily gain and a decreased feed-to-weight ratio (Zhao et al.).

Dang et al. investigated the dietary effect of tributyrin and anise mixture (TA) in weaned pigs. The experimental results showed that the TA mixture improved body weight, weight gain, average daily feed intake, and efficiency in pigs. Furthermore, noticeable improvements were observed in dry matter, crude protein, and energy digestibility, and jejunal villus height accelerated fecal microbial diversity. Populations of LAB strains such as *Lactobacillus reuteri*, *Lactobacillus amylovorus*, and *Clostridium butyricum* increased, improving digestibility and reducing ammonia emissions (Dang et al.). Feed augmentation of lambs with *Lemus chinensis* hay and *Alfalfa* hay was investigated, and observations on rumen microbiota populations and their metabolism were studied (Wang H. et al.). Increased carcass weight, loin-eye area, kidney weight, and abundance of the species *Fibrobacteres*, *Bacteroidetes*, and *Spirochaetes* were noted. Metabolomic approaches to several pathways were studied, while BF31, *Alistipes*, *Faecalibacterium*, *Eggerthella*, and *Anaeroplasm*a were associated with growth and metabolic indices. The effects of growth and weight gain indices were evaluated in the Boer crossbred goats fed with selected commercial diets supplemented with *Hemarthria altissima*, *Pennisetum sinese*, and forage maize. Differences in the carcass traits and semi-eviscerated and eviscerated slaughter percentages were reported (Wu et al.). However, there was no significant difference in growth performance. The maize-fed animals exhibited abundant amino-acid content in the semimembranosus muscles. Microbiome analysis using 16S rRNA gene sequencing revealed the abundance of *Firmicutes*, *Bacteroidetes*, and *Proteobacteria* phyla. In particular, the *Rikenellaceae*_RC9 and *Oscillospiraceae*_UCG-005 correlated with the lipid metabolism and fatty acid composition, respectively. Therefore, the relative abundance of these rumen microbiota impacted the fattening, meat quality, and nutritional indices of goats (Wu et al.).

The role of probiotics in accelerating the richness of the gut microbiome

The change in habitat from the inside of the uterus to the outside world requires additional nutrition and sustenance for calves. Developing immunity and digestive capacity plays a critical role in growth, survival, and gut health. In the study by Wang J. et al., it was demonstrated that the mode and composition of feed accelerated the impact on the development of the gut microbiome in newborn calves. According to the study, the Holstein bull

calves were fed three types of diets for an experimental period of 80 days. The results showed that the SH group showed marked differences in the development of intestinal epithelium with goblet cells, and higher daily gain was observed with no pathogenicity or inflammation. Furthermore, improved digestibility and absorption impacted the immune status of the calves (10). Thus, strengthening the feed during pre-weaning is considered for the overall growth and development of calves. The gut microbiome of young animals includes a variety of bacteria colonized in the gut by maternal exposure, habitat, and feeding patterns.

To explore the entity of the microbiome, full-length sequencing in the rumen samples of Pastured yaks (in different age) was investigated by using 16S and 18S rRNA sequencing platforms to study bacterial and fungal communities (Yang et al.). The results showed that the bacterial diversity increased exponentially from the first to the eighth week of birth. However, the *Prevotella* species was the most abundant among all the other groups. Interestingly, the development of the fungal communities was observed at 90 days, which facilitated their growth and reproduction. Therefore, the study reported the occurrence of the rumen bacterial and fungal microbiota of Zhongdian yaks at different ages, which illuminated the potential changes of the main microflora aligned with the age and growth of yaks (Yang et al.).

In a similar work (Song et al.), the efficacy of the dietary stimbiotic (STB) supplement on growth performance, intestinal morphology, microbiota, and immune response in piglets challenged with Shiga toxigenic *E. coli* (STEC) was studied. The STB-supplemented diets decreased WBC, neutrophil, lymphocyte, and tumor necrosis factor-alpha expression levels and interleukin-6 levels. However, iliac villus height and claudin1 expression levels were found to be increased. STB supplementation increased the abundance of *Desulfobacterota* and *Fibrobacterota* to counteract the inflammatory response induced by *E. coli* in the challenged piglets (Song et al.). The feeds of pigs contain non-digestible carbohydrates such as cellulose. A recent study revealed that the microbiome is involved in the fermentation of non-digestible carbohydrates (NDC) such as resistant starch, xylo-oligosaccharide, and fructo-oligosaccharide in the large intestine of swine (Pandey et al.). However, inadequate enzymes make it difficult for swine to digest this component. Therefore, they depend on the gut microbiota for their degradation and energy utilization. Thus, a study reported identifying such microbiome involved in the fermentation of the NDC in the intestine of swine through next-generation high-throughput sequencing to provide insights into their metabolism-related and nutritional roles (Pandey et al.).

Similarly, weaned pigs were treated with a combination of *Lacticaseibacillus casei* and *Saccharomyces cerevisiae,* and growth, hematology, and immunological parameters were evaluated (Kim et al.). Adequate weight gain and feed utilization efficiency were increased in the treated group, while there were no marked differences in hematological and immune response parameters. Microbiota, such as the *Treponema* genus, were comparatively higher than *Lactobacillus* and *Roseburia* genera in the treated animals than in the control. Thus, the study provided cues to understand the association between gut microbiota and growth performance (Kim et al.). The effects of ammonia toxicity on the on-rumen microbiota and fermentation were investigated (Shen et al.) using the *in vitro* rumen fermentation technique. Correlation analysis revealed a negative association between free ammonia nitrogen (FAN) and the microbiome, *in vitro* rumen fermentation profile, total volatile fatty acid, and total ammonia nitrogen (TAN). Fluctuations in microbiome structure were observed as a function of TAN levels. Elevated TAN levels induced an abundance of *Firmicutes* and *Actinobacteria* but reduced the populations of *Fibrobacteres* and *Spirochaetes*. Thus, the study established that *in vitro* rumen fermentation by high ammonia is dependent on the rumen microbial communities, accelerating the pH dependence *in vitro* (Shen et al.).

A similar study demonstrated the effects of *Pediococcus acidilactici*, prebiotic lactulose, and their symbiotic combination on gut microbiota using 16S rRNA gene sequencing in weaned piglets challenged with STEC. *Prevotella*, *Roseburia*, and *Succinivibrio* were increased in the probiotic-fed groups, while the abundance of *Phascolarcto bacteria* was reduced in the challenged group. Thus, the study demonstrated that the administration of symbiotics improved intestinal health by modulating the gut microbiota in piglets (Guevarra et al.). Therefore, the overall research findings show that improving dietary practices with probiotic-rich sources provides effective cues for healthy livestock production. It may also serve as a reinforcing objective to enhance antibiotic-free animal products and augment gut microbiota modulation.

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BB: Conceptualization, Investigation, Validation, Visualization, Writing – original draft, Writing – review & editing. W-CL: Conceptualization, Supervision, Validation, Visualization, Writing – original draft, Writing – review & editing.

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Gut microbial shifts by synbiotic combination of *Pediococcus acidilactici* and lactulose in weaned piglets challenged with Shiga toxin-producing *Escherichia coli*

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Development of alternatives to in-feed antibiotics in the swine industry have been the focused of many pig gut microbiota studies to improve animal health. In this study, we evaluated the effects of probiotic *Pediococcus acidilactici* (PRO), prebiotic lactulose (PRE), and their synbiotic combination (SYN) on gut microbiota using 16S rRNA gene sequencing in weaned piglets challenged with Shiga-toxin producing *Escherichia coli* (STEC). Our data showed that prebiotics, probiotics and synbiotics improved the intestinal health in weaned piglets. No significant differences were observed in species richness and species diversity in weaned piglets fed prebiotics, probiotics and their synbiotic combination. However, beta diversity analysis revealed distinct clustering of the microbiota of according to dietary treatment and by oral challenge of STEC. At the phylum level, Firmicutes to Bacteroidetes ratio was lower in the dietary treatment groups than the control group. Oral supplementation of prebiotics, probiotics and synbiotics enriched the abundance of *Prevotella* and *Roseburia*. *Succinivibrio* was elevated in PRO group; however, *Phascolarctobacterium* was depleted with STEC challenge regardless of dietary treatment. Overall, our data showed that administration of synbiotics in piglets improved intestinal health through gut microbiota modulation. Our data indicated that prebiotics, probiotics and their synbiotic combination could promote intestinal health through gut microbiota modulation in weaned piglets.

KEYWORDS

microbiota, piglet, Shiga-toxin producing *Escherichia coli*, 16S rRNA gene, synbiotics

Introduction

Mortality of piglets at weaning causes major economic losses and it is a serious concern for the global pork industry (1). Weaning is a critical and stressful stage in the life cycle of pigs and it is frequently associated with severe enteric infections such as post-weaning diarrhea (2). Biological stresses during the first week after weaning induce changes in the intestinal barrier function and structure leading to poor growth performance such as reduced feed intake, slow growth rate and reduced feed conversion of post-weaning piglets (3). The weaning transition generally causes gastrointestinal infections which are associated with piglet mortality of ∼15% in the swine industry (1). Antibiotic growth promoters, which are mainly used to treat and prevent disease and improve growth rate, have been banned in many developed countries including the United States and in the European Union due to increased conflicts about drug residues and antimicrobial resistance (4). Hence, there is a need for alternatives to in-feed antibiotics to reduce mortality and to improve gut health in pigs at the critical weaning period.

The intestinal microbiota has received a lot of attention in the recent years because of its role in immune system development and function, and for improvement of overall health, growth and performance of pigs (5). To mitigate the negative impact of the stress on early-weaned piglets, effective measures are required to promote gut health. The most widely researched antibiotics alternatives include probiotics (6), prebiotics (7) and their combination known as synbiotics. The effects of probiotics and prebiotics in the swine gut microbiota have been demonstrated in a number of studies. For example, probiotic *Lactobacillus plantarum* PFM 105, isolated from the rectum of a healthy sow, showed to improve the growth and promote intestinal development through modulation of gut microbiota in weaning piglets (8). Interestingly, the prebiotic lactulose has been shown to significantly increase the fecal diversity, decrease the abundance of pathogenic bacteria and increase the number of beneficial bacteria in weaned piglets (9). However, evaluation of synbiotics to improve gut health and improve nutrient utilization have received less much attention compared to other alternatives. Moreover, limited information is available regarding the protective effects of synbiotics on intestinal microbiota to control post-weaning diarrhea in piglets. Further research is still needed in this area since the perfect alternative to antibiotics does not yet exist and the effects of probiotics on swine gut microbiota are relatively limited and often contradictory.

Shiga toxin-producing *Escherichia coli* (STEC) is an important pathogen, which can cause pig diseases, including hemorrhagic colitis (10). The STEC bacterium is characterized by the ability to produce a cytotoxin, known as Shiga toxin (*Stx*), which is encoded by stx genes carried on bacteriophages (11). Pigs are important reservoir for human STEC infections, hence effective mitigation strategies including the use of prebiotics, probiotics, and synbiotics are required to improve animal health and address public health concerns.

In this study, we evaluated the effects of probiotic *Pediococcus acidilactici* (PRO), prebiotic lactulose (PRE), and their synbiotic combination (SYN) on weaned pig gut microbiota using 16S rRNA gene sequencing in weaned piglets challenged with Shiga-toxin producing *Escherichia coli* (STEC).

Materials and methods

Animals and housing

A total of 50 healthy weanling pigs [Duroc x (Landrace x Yorkshire)] with average body weight of 5.33 ± 0.60 kg weaned at the age of 28 days were used in this study at experimental research center of Dankook University, Cheonan, South Korea. All pigs in this study were selected from one delivery room and had similar husbandry practices. Each pen was equipped with a one-sided self-feeder and a nipple water-feeder for *ad libitum* access to feed and water throughout the experiment. The experimental procedures used in this study were approved by the Animal Care and Use Committee of Dankook University (No. DK-1-1645).

Diet and experimental design

On the day of weaning, piglets were divided into five groups consisting of ten pigs per treatment and housed in pens of five animals per pen using a randomized complete block design for the 7 weeks trial. Basal diet was provided in a mash form and formulated to meet or exceed the nutrient requirements (Table 1). The 5 dietary treatments were: (i) CONT, basal diet without any antibiotics or feed supplements, (ii) PRE, basal diet + 0.05% prebiotics, (iii) PRO, basal diet + 0.1% probiotics (*Pediococcus acidilactici*), (iv) SYN1, basal diet + 0.05% synbiotics, (v) SYN2, basal diet + 0.1% synbiotics. The synbiotics was formulated with a prebiotic lactulose at a concentration of 10 g/kg feed combined with *Pediococcus acidilactici* GB-U15 KCCM 11856P at a concentration of 5.0 x 10^9 colony forming units (CFU)/mL. The synbiotics used in the study were provided by Genebiotech Co., Ltd. (Seoul, Korea). We selected lactulose as the prebiotics, the low doses of lactulose can help to stimulate the growth of health-promoting bacteria in the gastrointestinal tract.

STEC challenge and clinical evaluation

At week 5 of the experiment (56-day-old), five pigs from each group were inoculated with the pathogenic STEC to

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TABLE 1 Composition of basal diet for weaned pigs (as-fed basis).

Item	Diet
Ingredient (%)	
Corn	56.09
Soybean meal, 44%	26.00
Soy protein concentrate	12.00
Soybean oil	3.00
Limestone	1.30
Monocalcium phosphate	1.20
Vit-Min premix[a]	0.04
L-lysine-HCl	0.24
DL-methionine	0.09
L-threonine	0.04
Total	100
Calculated energy and nutrient contents	
ME, Mcal/kg	3.48
CP, %	24.17
Calcium, %	0.84
Phosphorus, %	0.66
Lysine, %	1.54
Methionine, %	0.45
Cysteine, %	0.39
Threonine, %	0.96
Tryptophan, %	0.28
Arginine, %	1.60
Histidine, %	0.67
Isoleucine, %	1.03
Leucine, %	2.05
Phenylalanine, %	1.21
Valine, %	1.09

[a]Provided per kilogram of diet: vitamin A, 12,000 IU; vitamin D3, 2,500 IU; vitamin E, 30 IU; vitamin K3, 3 mg; D-pantothenic acid, 15 mg; nicotinic acid, 40 mg; choline, 400 mg; and vitamin B12, 12 μg; Fe, 90 mg from iron sulfate; Cu, 8.8 mg from copper sulfate; Zn, 100 mg from zinc oxide; Mn, 54 mg from manganese oxide; I, 0.35 mg from potassium iodide; Se, 0.30 mg from sodium selenite.

determine the impact of the dietary treatments on intestinal microbiota of piglets challenged with STEC. STEC strain used in this study was isolated from the sick pig and had the virulent genes, such as F18, F6, heat-labile enterotoxin (LT), Shiga toxin type 2 (*stx2)*, Shiga toxin type 2e (*stx2e*) genes. The STEC, which was isolated from piglet feces, was grown in fresh LB broth and incubated at 37°C with shaking for 24 h. The final concentration of *E. coli* used was ∼2 x 10^9 CFU/mL. Five piglets from each group orally inoculated with 5 mL of *E. coli* (2 x 10^9 CFU/mL) diluted in PBS. PBS was orally administered in the remaining five piglets from each group. The health status of piglets during the experiments was assessed by fecal consistency scoring using a five-grade system. The scoring system for stool consistency that indicate stool hardness or softness is as follows: 1 = hard, dry pellets in a small, hard mass; 2 = hard, formed stool that remains firm and soft; 3 = soft, formed and moist stool that retains its shape; 4 = soft, unformed stool that assumes the shape of the container; 5 = watery, liquid stool that can be poured.

Fecal collection and intestinal histology

Fresh fecal samples were collected individually from the rectum of each piglet at week 7 of the experiment. Two pigs from each group were euthanized at week 7 of the experiment (2 weeks after *E. coli* oral challenge) for intestinal morphological analysis. The proximal segments of ileum, colon and cecum were sampled for histological examination. For histology, 3-cm sections from the ileum, cecum and colon were removed, opened longitudinally, and fixed in a 10% neutral formalin solution. Tissue samples were dehydrated and embedded in paraffin wax, sectioned at 5 μm, and stained with hematoxylin and eosin. Morphological measurements were performed with a light microscope.

Genomic DNA extraction

Total DNA from the feces was extracted from 200 mg of feces per sample using QIAamp Fast DNA Stool Mini Kit (QIAGEN, Hilden, Germany) according to the manufacturer's instructions. Cell lysis was performed by bead-beating the samples twice for 2 min at 300 rpm, with an incubation period of 5 min in a water bath at 70°C between beatings. The concentrations of DNA were measured using a Colibri Microvolume Spectrometer (Titertek Berthold, Pforzheim, Germany) and samples with OD260/280 ratios of 1.80–2.15 were processed further.

16S rRNA gene library preparation and sequencing

The PCR primers 799F-mod6 (5′ CMGGATTAGATA CCCKGGT-3′) and 1114R (5′-GGGTTGC GCTCGTTGC-3′) were used to amplify the V5 to V6 hypervariable regions of the 16S rRNA genes. The amplification mix contained 5 X PrimeSTAR Buffer (Mg^{2+}) (Takara Bio, Inc., Shiga, Japan), 2.5 mM concentrations of each deoxynucleotide triphosphates, 2.5 U/μL of PrimeSTAR HS DNA Polymerase, 10 pmol of each primer, and 25 ng of DNA in a reaction volume of 50 μL. The thermal cycling parameters were as follows: initial denaturation was at 98°C for 3 min, followed by 30 cycles of 98°C for 10 s,

55°C for 15 s, and 72°C for 30 s, and a final 3-min extension at 72°C. PCR products were purified using PCR a Wizard® SV Gel and PCR Clean-Up System purification kit, (Promega, Wisconsin, USA).

After sample preparation and quality control, 16S rRNA gene amplicons were sequenced using the Illumina MiSeq platform at Macrogen Inc. (Seoul, Republic of Korea) according to the manufacturer's instructions. Briefly, the sequencing library was prepared by random fragmentation of the DNA samples followed by 5′ and 3′ adapter ligation. This step attaches dual indices and Illumina sequencing adapters using the Nextera XT Index Kit. The final products were normalized and pooled using the PicoGreen, and the size of libraries were verified using the TapeStation DNA ScreenTape D1000 (Agilent). The PCR conditions were as follows: initial denaturation (3 min at 95°C) 8 amplification cycles (95°C for 30s, 55°C for 30s, 72°C for 30s) and final elongation (72°C for 5 min).

16S rRNA gene analysis

All the raw sequence data from Illumina MiSeq platform were checked for quality using FastQC. Then, Mothur software was used to remove low-quality sequences (12). Briefly, sequences that did not match the PCR primers were eliminated from demultiplexed sequence reads. Sequences containing ambiguous base calls and sequences with a length of <200 bp were also removed to minimize the effects of random sequencing errors. Chimeric sequences were identified and excluded for downstream analysis using the UCHIME algorithm implemented in Mothur. Next, the QIIME (Quantitative Insights into Microbial Ecology) pipeline (version 1.9.1) was used to perform operational taxonomic unit (OTU) picking using the open-reference OTU picking workflow with the SortMeRNA and SUMACLUST methods for reference OTU and *de novo* OTU picking, respectively. Taxonomy was assigned using the naïve Bayesian Ribosomal Database Project (RDP) classifier based on GreenGenes taxonomy reference database version 13_8. Low-abundance OTUs and singletons were filtered from the OTU table for downstream analysis with minimum count of 4 and low-count filter based on 20% prevalence in samples. Then, data normalization was performed by rarefying the data to the minimum library size and by data scaling using the total sum scaling before any statistical comparison to address the variability in sampling depth and the sparsity of the data.

Statistical analysis

Statistical analysis was performed using the R package MicrobiomeAnalystR and GraphPad Prism v7.00 (La Jolla, CA, USA). Alpha diversity measures including observed OTUs, Chao1, Shannon and Simpson indices were computed using the MicrobiomeAnalystR. Significant differences in alpha diversity among the groups and pairwise comparisons were calculated based on analysis of variance tests. Significant difference level was set at $P < 0.05$. The principal coordinate analysis (PCoA) plots at the OTU level based on the weighted and unweighted UniFrac distances. Significant differences in beta-diversity were performed using the Analysis of Similarities (ANOSIM) based on the unweighted and weighted UniFrac distances. The heatmap of core microbiota was performed with the default parameters at 20% sample prevalence and 0.2% relative abundance.

Results

Growth performance and intestinal morphology

The effects of synbiotics administration on growth performance of weaned piglets is shown in Supplementary Table 1. Final body weight, average daily gain, and gain to feed ratio were significantly increased in SYN1 and SYN2 group compared to the CONT, PRO and PRE groups, suggesting that supplementation of synbiotics may improve growth performance of weaned piglets. The effects of prebiotic, probiotic and synbiotic supplementation on fecal index is shown in Supplementary Table 2. Piglets of the SYN1 and SYN2 group significantly decreased the fecal score ($P < 0.05$), while no differences were observed between PRE and PRO as compared to the CONT group suggesting that synbiotics administration decreased the diarrhea incidence in weaned piglets.

Histological examinations of ileal tissue revealed that STEC-challenged CONT group increased the inflammatory cells including neutrophils and macrophages in the lamina propria as compared to the healthy CONT group, which were given PBS only. Interestingly, oral administration of PRE, PRO, SYN1 and SYN2 group decreased these inflammatory cells (Supplementary Figure 1). In the cecum tissue, STEC-challenged CONT group expanded the mucosal crypt and increased the number of plasma cells as compared to the healthy CONT group. However, these observations were lower in STEC-challenged pig groups fed PRO, PRE, SYN1 and SYN2 (Supplementary Figure 2). In the colon tissue, neutrophils and plasma cells were increased in the STEC-challenged CONT group compared to the healthy CONT group. Similarly, PRO, PRE, SYN1 and SYN2 decreased these inflammatory cells with higher reduction in the SYN2 (Supplementary Figure 3).

DNA sequencing data

Total DNA was extracted from fecal samples of pigs and the extracted community DNA was PCR amplified and sequenced

using primers specific for the V5 to V6 hypervariable regions of the 16S rRNA genes. The 16S rRNA gene sequencing produced a total of 4,875,951 raw sequence reads from 48 fecal samples ranging from 66,397 to 165,469 reads. The average quality score (Phred scores) across all the samples ranged from 32 to 36. Phred scores >Q30 indicated that that there was <0.1% chance that a base was called incorrectly. Further data filtering was performed in the OTU table to remove low quality sequences and to improve downstream statistical analysis (Supplementary Figure 4). The total number of reads after quality filtering was 3,409,184 ranging from 46,296 to 118,099 with an average counts per sample of 71,024. For alpha and beta diversity analyses, all samples were rarefied to the minimum number of sequences to account for unequal sequencing depth. A total of 1,688 OTUs were obtained after data filtering.

Alpha diversity

Alpha diversity indices were compared between the treatment groups (PRE, PRO, SYN1, and SYN2) as compared to the CONT. Also, the alpha diversity between the STEC-challenged treatment groups and their non-challenged counterpart were compared to determine the effects of STEC on alpha diversity and the protective effects of PRE, PRO, SYN1, and SYN2. The rarefaction curves for 16S rRNA gene sequences of all the samples with an OTU definition at 97% identity cut-off were shown in Supplementary Figure 5, indicating that sampling depth was sufficient for downstream OTU-based analysis. The number of observed OTUs and Chao1 were used to measure species richness, whereas Shannon and Simpson diversity indices were used to measure species diversity.

Interestingly, Chao1 and observed OTUs were significantly lower in the CONT challenged with STEC compared to those of CONT without STEC challenge, indicating that STEC challenge significantly decreased the microbial species richness (Figures 1A, B). However, no significant differences in Chao1 and number of observed OTUs were observed between the PRE, PRO, SYN1, and SYN2 and their STEC-challenged counterparts, suggesting that probiotics, prebiotics or their combination may play a role in maintaining the balance of microbial communities in the gut of piglets against STEC infection (Figures 1A, B).

In addition, oral challenge of STEC significantly decreased the Shannon diversity in weaned piglets as compared to the non-challenged CONT group, indicating that STEC infection altered the species diversity of microbial communities (Figure 1C). Furthermore, no significant difference in Simpson diversity was observed between the treatment groups compared to the non-challenged healthy CONT group, and between those groups infected with STEC compared to the STEC-challenged CONT group ($P > 0.05$) (Figure 1D).

Beta diversity

Beta diversity, which is defined as the diversity among the treatment groups, was measured using the weighted and unweighted UniFrac distances with the former takes into account the relative abundance of species and the latter considers the presence or absence of OTUs in the community. Principal coordinate analysis (PCoA) was used to visualize the separation of microbial community among the treatment groups (CONT, PRE, PRO, SYN1, SYN) and effects of STEC infection. The PCoA plots based on both unweighted (Figure 2A) and weighted UniFrac (Figure 2B) distances showed significant differences in the separation of microbial communities in pigs in response to the different treatment groups (PRE, PRO, PRO, SYN1, and SYN2) and oral challenge of STEC as measured using ANOSIM ($P < 0.05$). PCoA results indicated that fecal microbial communities differ in pigs between treatment group (PRE, PRO, SYN1, and SYN2) and CONT groups. Moreover, separation of microbiota obtained were also significantly different between the unchallenged treatment groups and pigs treatment groups orally challenged with STEC based on ANOSIM ($P < 0.05$). These results suggest that prebiotics, probiotics and their synbiotic combination had individual effects on the intestinal microbial community structure in pigs.

Microbial compositions associated with the administration of prebiotics, probiotics, and synbiotics in weaned piglets

We examined the bacterial compositions associated with oral administration of probiotics, prebiotics and synbiotics and STEC oral challenge in weaned piglets. At the phylum level, a total of 11 phyla were identified and the top 5 most abundant phyla were Bacteroidetes (42.63–51.10%), Firmicutes (32.94–46.78%), Proteobacteria (3.11–13.82%), Spirochaetes (0.19–4.48%), and Tenericutes (0.05–2.61%). Phylum Bacteroidetes and Firmicutes collectively ranged from 83.70 to 94.58% of the total sequences among the groups (Figure 3A).

At the genus level, 50 unique genera were identified from at least one sample in each group. Regardless of the treatment group, the top 5 most abundant genera were *Prevotella* (25.73–37.52%), *Lactobacillus* (1.59–9.97%), *Oscillospira* (1.60–6.24%), *Succinivibrio* (0.27–5.63%), and *Roseburia* (0.26–3.72) (Figure 3B).

Without STEC challenge, differential abundance analysis revealed that each additive resulted in different alterations of the pig intestinal microbiota at the genus level (Figure 4). Relative bundance of *Lactobacillus* was not different among PRO, SYN1 and SYN2 groups (Figure 4A). One of the most interesting observations of the present study is the significant increase in

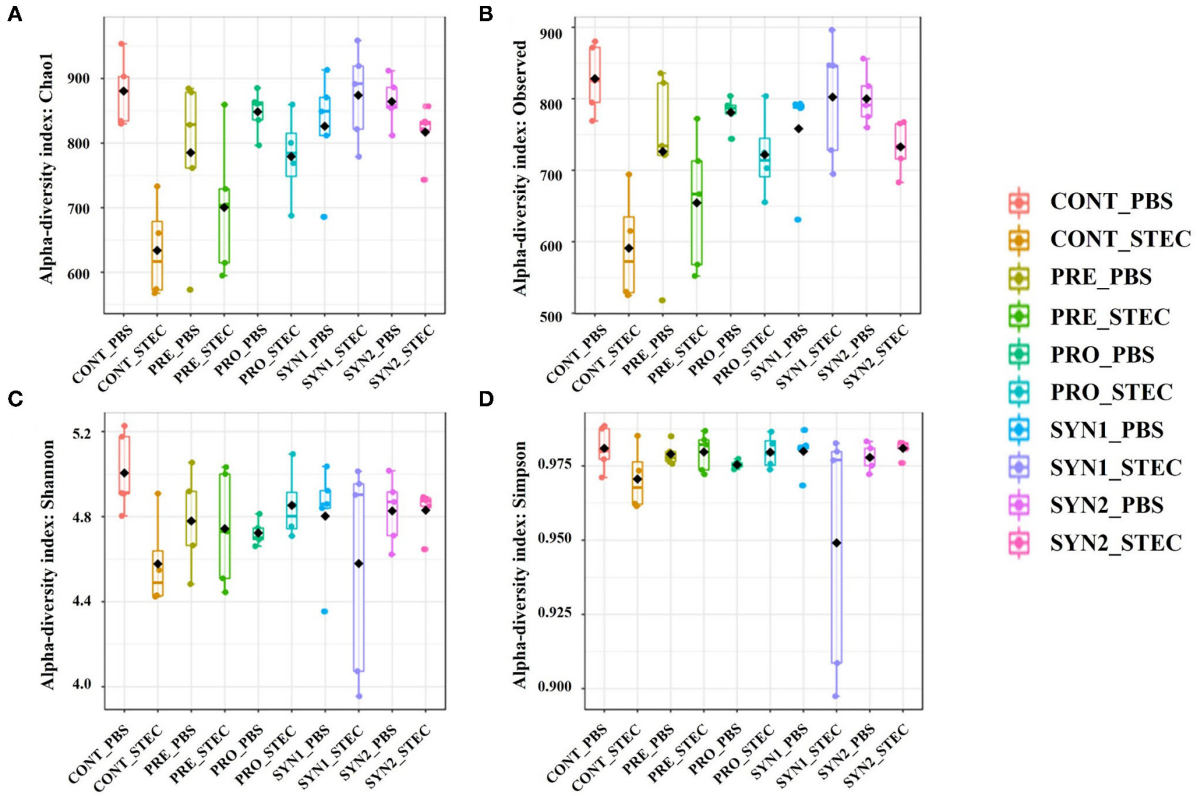

FIGURE 1
Box plots showing the alpha diversity indices of the pig groups PRE, PRO, SYN1 and SYN2 with or without STEC challenge. Species richness was measured using **(A)** Chao1 index and **(B)** number of observed OTUs, while species diversity was measured using **(C)** Shannon and **(D)** Simpson diversity indices. Boxes represent the interquartile range (IQR) between the 25th and 75th percentile, and the black dot inside the box denotes the median value. Whiskers represent the lowest and highest values within 1.5 time from the 25th and 75th quartiles, respectively. Boxes were colored according to treatment group (CONT, PRE, PRO, SYN1, SYN2) challenged with STEC or PBS.

the abundance of *Prevotella* in all the dietary treatments (PRE, PRO, SYN1 and SYN2) as compared to the CONT ($P < 0.05$) (Figure 4B). Moreover, abundance of *Roseburia* was significantly elevated by oral administration of PRE, PRO and SYN2 as compared to the negative CONT group ($P < 0.05$) (Figure 4C). Another interesting finding of the study is the significant increase of *Succinivibrio* in the PRO group as compared to the CONT group ($P < 0.05$) (Figure 4D). These findings indicate that prebiotics, probiotics and synbiotics have unique effects on gut microbial composition in weaned piglets.

Differential abundance in microbial composition associated with STEC infection in weaned piglets receiving prebiotics, probiotics, and synbiotics

Differences in microbial composition at the genus level between non-challenged and STEC-challenged CONT groups were compared to determine the effects of STEC infection in weaned piglets (Figure 5). STEC-challenge resulted to significant increase in the population of *Phascorlarctobacterium* and *Prevotella* while there was a significant decrease in abundance of *Lactobacillus* in comparison to the CONT pigs fed PBS ($P < 0.05$). No significant differences were observed between STEC-challenged CONT and PRE group challenged with STEC ($P > 0.05$). On the other hand, a significant increase in the abundance of *Prevotella* and *Lactobacillus* and significant reduction of *Phascolarctobacterium* were observed in PRO group challenged with STEC ($P < 0.05$). However, in SYN1 group challenged with STEC, significant depletion in the abundance of *Prevotella* and *Phascolarctobacterium* were observed ($P < 0.05$). In the SYN2 group challenged with STEC, we observed a significant increase in the relative abundance of *Prevotella* and a significant depletion of *Phascolarctobacterium* similar to those observations in the PRO group with STEC ($P < 0.05$). These findings suggest that STEC infection significantly altered the composition of the pig gut microbiota, however, prebiotics, probiotics or their synbiotic combination have inhibitory effects to fight against STEC.

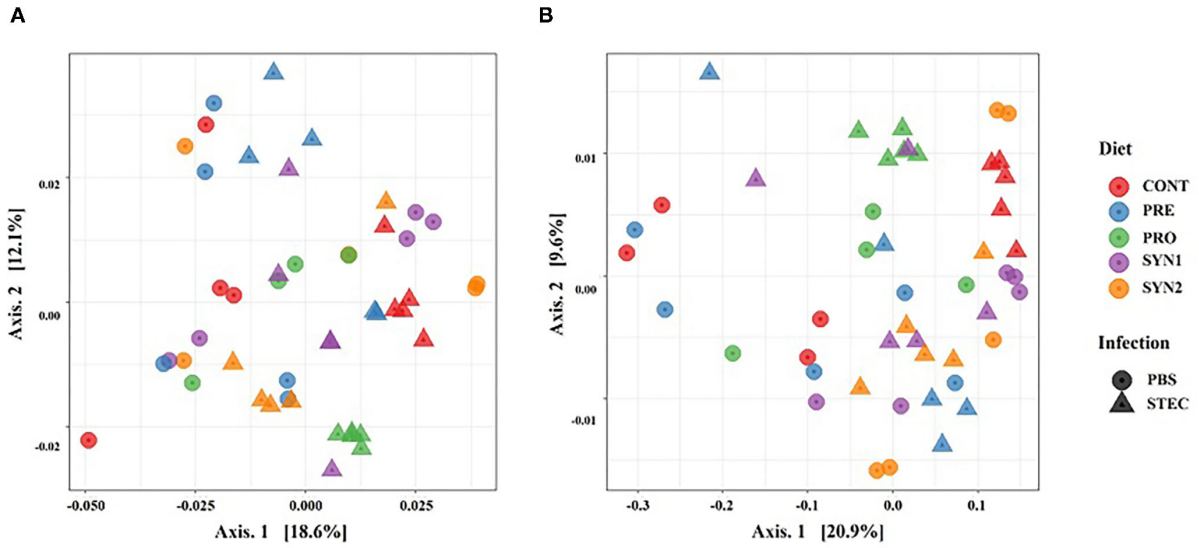

FIGURE 2
Beta diversity analysis of the pig gut microbiota of the pig groups PRE, PRO, SYN1 and SYN2 with or without STEC challenge. Principal coordinates analysis (PCoA) plots based on the weighted **(A)** and unweighted **(B)** UniFrac distances of gut microbial communities. Each symbol represents the microbiota from individual pig sample and were color coded according to treatment group (CONT, PRE, PRO, SYN1 and SYN2) while shape represents the oral challenge with STEC **(circle)** and PBS **(triangle)**. The axes show the percent variation.

Core microbiota in weaned piglets orally administered with prebiotics, probiotics, and synbiotics

The core microbiome analysis was performed at the genus level based on the sample prevalence and relative abundance cut-off value at 20 and 0.02%, respectively. Regardless of the treatment group, the six core bacterial genera were identified as *Prevotella*, *Lactobacillus*, *Oscillospira*, *Succinivibrio*, *Roseburia*, and *Parabacteroides*, which were shown in descending order according to prevalence (Figure 6). Our results were similar to a previous study on meta-analysis of the swine gut microbiota using published data sets from 16S rRNA gene sequences to define a core microbiota in the pig gut (13). These findings suggest that pig gut microbiota may be used for future gut microbiota manipulation studies for potential health benefits.

Discussion

In this study, we investigated the influence of prebiotics, probiotics and their synbiotic combination on the intestinal microbial composition in weaned pigs. Probiotics and prebiotics are two commonly used feed additives in swine nutrition and they have been extensively studied due to their perceived health benefits (14). Probiotics are defined as "live microorganisms which, when consumed in adequate amounts as part of food, confer a health benefit on the host" (5, 15). On the other hand, prebiotics are defined as "a non-digestible food ingredient that beneficially affects the host by selectively stimulating the growth and/or activity of one or a limited number of bacteria in the colon and thus improves health" (16). To date, the focus has been primarily around the use of prebiotics and probiotics for reducing post-weaning diarrhea, with little research evaluating synbiotics as possible interventions on pig gut microbiota modulation. Post-weaning diarrhea has been a particular concern to the swine industry, hence many studies including the use of prebiotics and probiotics as alternatives to in-feed antibiotics have been reported to improve swine gut health and reduce mortality in weaning piglets (17, 18). However, only a few of them have been performed with STEC challenge in weaned piglets. The present study revealed that mortality was reduced by synbiotic administration and improved gut health by reduction of inflammatory cells in the small intestine in weaned piglets.

In this study, prebiotics, probiotics and their synbiotic combination had no significant effects on species richness and species diversity in weaned piglets. There were no significant differences in the number of observed OTUs and Chao1 were detected in pigs receiving probiotics, prebiotics or their synbiotic combination, indicating that these feed additives had no effects on species richness in the fecal microbiota of pigs. These observations were similar with that of Wang et al. (8) who found that alpha diversity indices were not affected by

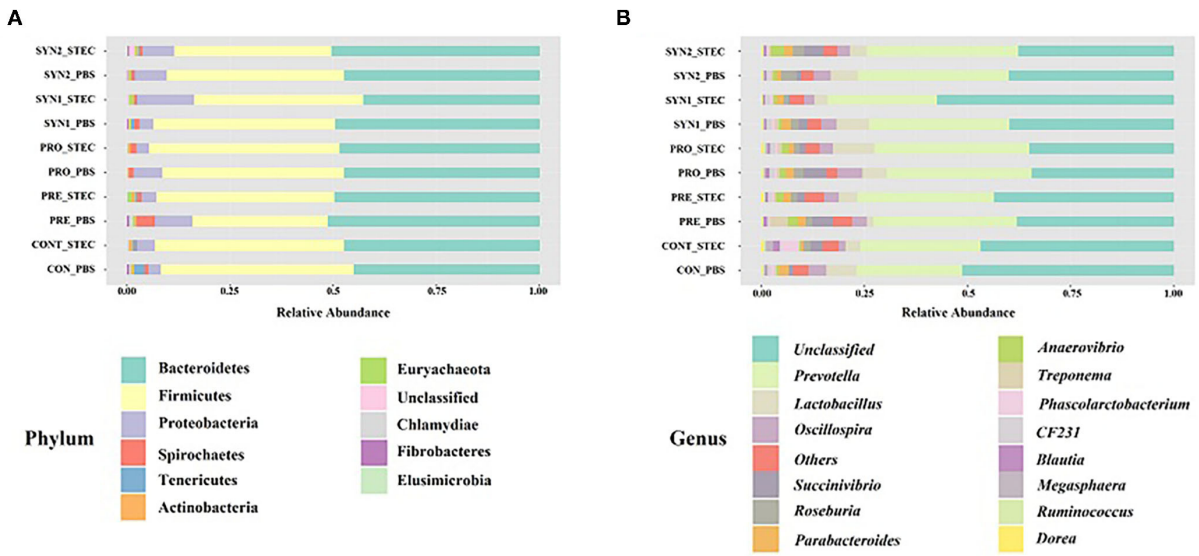

FIGURE 3
Gut microbiota composition of the pig groups PRE, PRO, SYN1 and SYN2 with or without STEC challenge. Bar plots showing, the relative abundance of taxa at the phylum **(A)** and genus **(B)** levels in the different treatment groups (CONT, PRE, PRO, SYN1 and SYN2) orally challenged with STEC or PBS.

treatment with probiotics in weaned piglets. Umu et al. (19) also revealed that prebiotic alginate and resistant starch diet decreased the species richness in the swine microbiome. Our results were also in agreement with a previous study in infant microbiome which indicated that probiotic supplementation did not alter the overall bacterial community richness and evenness (20). In contrast, recent metagenome studies revealed that microbial diversity and richness were higher in weaned piglets fed probiotics (21), and synbiotic combination of lactulose and probiotic enterococci (9). Our results support the evidence that effects of probiotics, prebiotics and synbiotics in microbial diversity varies widely in weaned pigs.

In addition, STEC challenge significantly decreased the alpha diversity in the non-treated CONT group, indicating low bacterial community diversity in weaned piglets without synbiotic treatment. In beef cattle operations, presence of pathogenic *E. coli* is correlated with lower bacterial community diversity and composition (22), since cattle are the most important STEC reservoir (23). However, no significant differences in alpha diversity were observed between piglets fed dietary treatments and STEC-challenged CONT group. This could explain that probiotics have protective effects against pathogenic bacteria by producing antimicrobial compounds, decreasing the intestinal pH, and competing with pathogens for adhesion and colonization in the gut (24, 25). Our results indicate that use of probiotics, prebiotics and their synbiotic combination is effective in reducing the negative effects of STEC and may balance and restore the gut microbial diversity in a piglet challenge model.

Beta-diversity analyses based on the PCoA plots using both unweighted and weighted UniFrac distances showed that microbial community structure was perturbed by oral administration of synbiotic additives and by challenge with STEC in weaned piglets. Both PCoA plots showed that microbiota of the non-challenged pigs fed additives were clustered separately, suggesting that administration of prebiotics, probiotics and synbiotics have unique effects on the microbial community structure in weaned piglets. However, oral challenge of STEC in treatment groups lead to segregated clustering of the microbiota, suggesting that STEC caused perturbation of the microbiota in weaned piglets receiving prebiotics, probiotics and synbiotics. To verify this result, microbiota of the non-challenged CONT group were compared to the STEC-challenged CONT, resulting to significant clustering of the two groups. This suggest that STEC significantly altered the microbial community structure and composition regardless of oral administration of prebiotics, probiotics or their synbiotic combination in weaned piglets. Our results were similar to those obtained by Chae et al. (9) who showed significant clustering of pig gut microbiota according to dietary treatment of prebiotics, probiotics and their synbiotic combination. Furthermore, findings of this study suggest that prebiotics, probiotics and synbiotics in addition to oral challenge of STEC in pigs had individual effects on intestinal microbial community structure in pigs. The results of the present study also indicate that STEC is associated with gut microbiota dysbiosis, while prebiotics, probiotics and synbiotics have inhibitory effects against STEC.

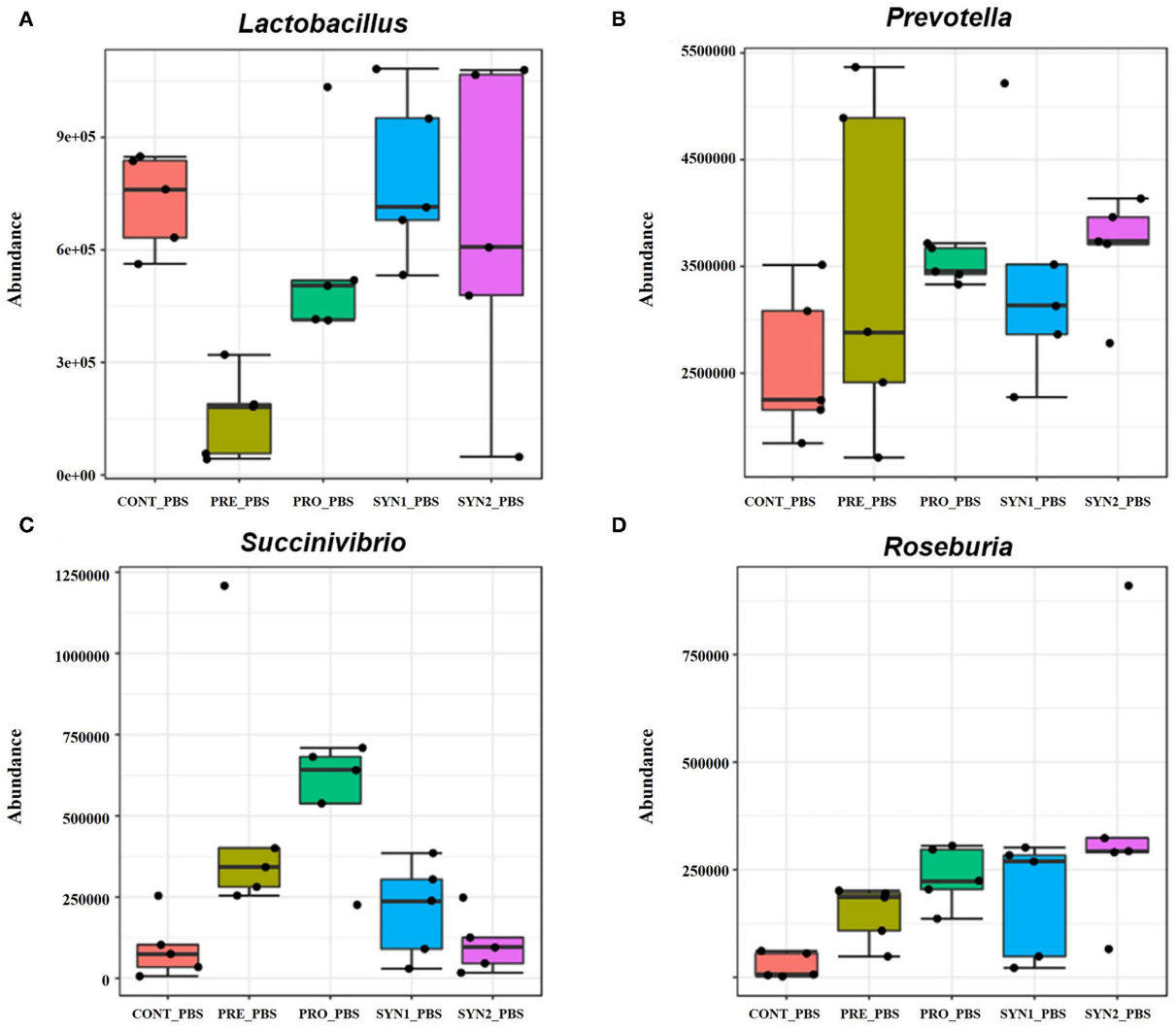

FIGURE 4
Box plots showing significantly different genera in weaned pigs supplemented with prebiotics, probiotics or synbiotics without STEC challenge. Abundance of bacterial genera including **(A)** *Lactobacillus*, **(B)** *Prevotella*, **(C)** *Succinivibrio,* and **(D)** *Roseburia* were compared between PRE, PRO, SYN1 and SYN2 in comparison to the negative CONT group.

Consistent with a previous study on pig gut microbiota (26), Firmicutes and Bacteroidetes were the two most abundant taxa at the phylum level. Previously, an increased in Firmicutes to Bacteroidetes ratio was detected in piglets after oral administration of prebiotics, probiotics and synbiotics (9). While intestinal microbial communities play important roles in modulating host physiology (27), Firmicutes to Bacteroidetes ratio in pig gut microbiota is a major contributor to adiposity (28, 29). Firmicutes has key roles in starch and fiber degradation (30, 31) and Bacteroidetes contribute significantly in degradation of proteins and polysaccharides in the plant cell wall, producing short-chain fatty acids (SCFAs) that can be absorbed by the host (32) and they can modify the host lipid metabolism, increasing fat retention and adipogenesis (33). Conversely, the present study observed higher Firmicutes to Bacteroidetes ratio in CONT group than in the treatment groups, suggesting that supplementation of prebiotics, probiotics and synbiotics have varying effects on pig microbiota and may improve gut health and performance of piglets. In addition, we detected in this study that the phylum Tenericutes was significantly depleted by oral administration of prebiotics, probiotics and synbiotics. It has been reported that Tenericutes are correlated with apparent crude fiber digestibility in pigs (34) and they are associated with healthy human gut microbiota along with decreased abundance of Proteobacteria, which are known pathogenic phyla (35). These findings imply

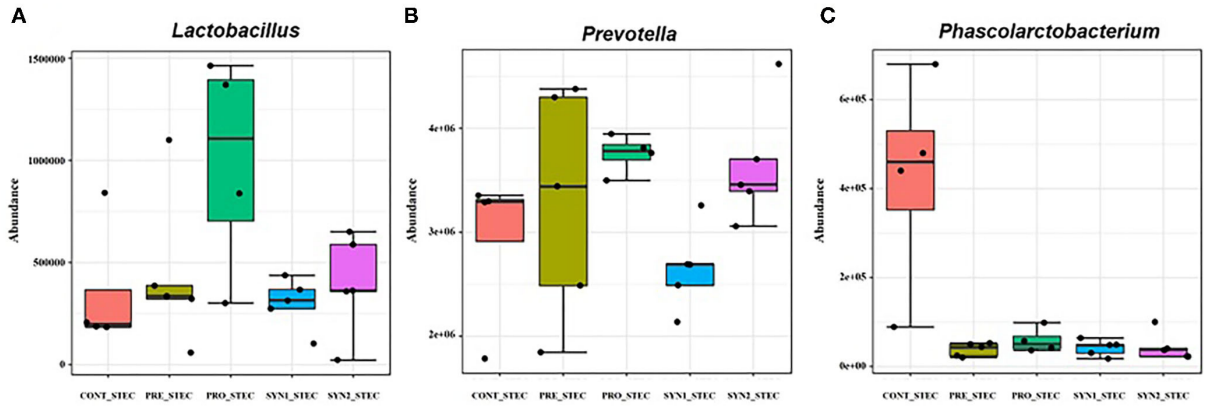

FIGURE 5
Box plots showing significantly different genera in weaned pigs supplemented with prebiotics, probiotics or synbiotics with STEC challenge. Abundance of bacterial genera including **(A)** *Lactobacillus*, **(B)** *Prevotella*, **(C)** *Phascolarctobacterium* were compared between PRE, PRO, SYN1 and SYN2 in comparison to the STEC-challenged CONT group.

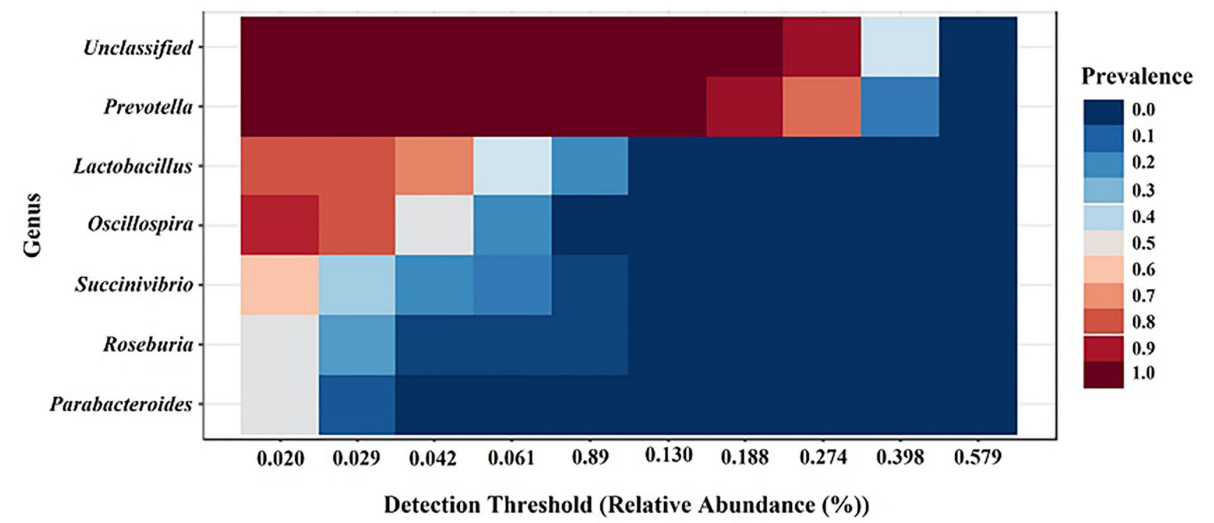

FIGURE 6
Core gut microbiota among the groups regardless of treatments. Heatmap depicting the core OTUs and their prevalence at different detection thresholds.

that synbiotic administration significantly improved the gut health status of weaned piglets by decreasing the populations of pathogenic bacteria. Interestingly, STEC challenge had no significant effects on microbial composition at the phylum level in pigs fed the dietary treatment. In a previous report, it has been suggested that synbiotics are as effective as antibiotics on growth performance, nutrient digestibility and enhancement of gut microbiota in weaned piglets (36). Our study suggests that prebiotics, probiotics, and synbiotics have protective effects against pathogenic STEC and induce beneficial effects in improving gut health in weaned piglets. The discrepancies between the present study and previous studies may have resulted from the use of pigs in different ages, environmental conditions, probiotic strains and types of prebiotics.

To further illuminate whether the changes in the composition of the microbiota were associated with dietary treatment and STEC infection, the distribution of microbiota at the genus level were investigated. At the genus level, *Prevotella* and *Lactobacillus* were detected as the two most abundant genera in the swine gut microbiota regardless of synbiotic supplementation and STEC challenge. Interestingly, PRO and SYN1 significantly elevated the relative abundance of

Lactobacillus, while it decreased in PRE in comparison to the CONT group. It has been reported that *Lactobacillus* is the most common probiotic agent in swine production due to their ability to improve growth performance and prevent gastrointestinal infection (37). Moreover, *Lactobacillus* has been known to improve feed conversion efficiency, nutrient utilization, intestinal microbiota, gut health and regulation of immune function in pigs (38). Interestingly, STEC challenge significantly depleted the abundance of *Lactobacillus* in the negative CONT group, however PRO reversed this result, suggesting that probiotics may play a role in proliferation of beneficial organisms during infection and fight against pathogenic bacteria. In a similar study, synbiotic combination of *L. plantarum* with maltodextrins and fructooligosaccharides showed significant reductions in the population of *E. coli* K-88 in the intestinal mucosa in pigs (39). Moreover, in the present study, *Prevotella* was significantly elevated in PRE, PRO, SYN1 and SYN2 as compared to the non-challenged CONT. *Prevotella* strains are associated with plant-rich diets but are also linked with chronic inflammatory conditions (40). Previously, the abundance of *Prevotella* was significantly increased with supplementation of fermentable non-starch polysaccharides (41) and low-molecular-weight chitosan which are potential prebiotics in weaned piglets (42). One of the most striking observation in this study was the significant depletion of *Phascolarctobacterium* in all dietary treatment groups challenged with STEC. *Phascolarctobacterium* was found to be a beneficial microbe that play a significant role in SCFA production including acetate and propionate and can be associated with the metabolic state and mood of the host (43). Conclusively, it could be speculated that synbiotics improve the survival and activity of beneficial microorganisms mainly due to synergistic effects of prebiotic and probiotics in the regulation of intestinal microbiome.

Conclusion

It is evident that the pig intestinal microbiome plays an important role in modulating gut health and disease. In this study, lactulose and *Pediococcus acidilactici* showed unique effects and their synbiotic combination resulted in different alterations of the gut microbial communities in weaned piglets. Oral supplementation of prebiotics, probiotics and their synbiotic combination modulated the pig gut microbiota by increasing the abundance of beneficial microbes including *Lactobacillus* and *Prevotella*. Conclusively, it could be speculated that synbiotics 1 (SYN1) was most effective on improving the activity of beneficial microorganisms and growth performance mainly due to synergistic effects of prebiotic and probiotics. Overall, findings of this study may be used for development feeding strategies such as alternatives to in-feed antibiotics in the swine production for intestinal development and modulation of the gut microbiota.

Data availability statement

All raw 16S rRNA gene data used in this study were deposited in the National Center for Biotechnology Information (NCBI) under BioProject accession number PRJNA576307.

Ethics statement

The animal study was reviewed and approved by Institutional Animal Care and Use Committee of the Dankook University, South Korea (Approval number: DK-1-1645).

Author contributions

JiC, MS, SW, CL, WC, and HBK designed the research. RG, JaC, JL, HK, SK, EK, GK, and CL participated in animal trial and data analysis. RG, MS, and SK validated and interpreted the data. RG, JaC, MS, SW, and HBK wrote the manuscript. HBK were the principal investigator. All authors read and approved the final manuscript.

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Conflict of interest

Authors CL and WC were employed by Gene Bio Tech Co., Ltd. This study received funding from Gene Bio Tech Co., Ltd. The funder had the following involvement with the study design.

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.

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fvets.2022.1101869/full#supplementary-material

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Diversity of the fecal microbiota in Chinese ponies

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Introduction: The gut microbiomes of equine are plentiful and intricate, which plays an important part in the growth. However, there is a relative lack of information on the microbial diversity in the pony's gut.

Methods: In this article, 118 fecal samples from DeBa pony, NiQi pony and GuZh horse were studied by 16S rRNA amplicon sequencing.

Results: Diversity analysis was used to determine the difference of gut microbiota composition among different breeds. Alpha diversity analysis showed that the gut microbiota of NiQi ponies were abundant and various. Beta diversity analysis showed that the microorganisms constitution of DeBa ponies was more similar to that of NiQi ponies. LDA Effect Size (LEfSe) analysis result that the microorganism biomarkers for NiQi pony at the genus level were Phascolarctobacterium, Paludibacter, and Fibrobacter; the bacterial biomarker for DeBa pony was Streptococcus and Prevotella; and the bacterial biomarkers for GuZh horses was Treponema, Treponema Mogibacterium, Adlercreutzia, and Blautia. The correlation analysis between genera with >1% abundance and horse height found that Streptococcus ($P < 0.01$), Treponema ($P < 0.01$), Coprococcus ($P < 0.01$), Prevotella ($P < 0.01$), Phascolarctobacterium ($P < 0.01$), and Mogibacterium ($P < 0.01$) were significantly associated with horses' height. The functional prediction results indicated that DeBa pony have a microbiota functional more similar to NiQi pony.

Discussion: For the first time, our results announce the species composition and structure of the gut microbiota in Chinese ponies. At the same time, our results can provide theoretical reference for further understanding the healthy breeding, feeding management and disease prevention of horses.

KEYWORDS

equine, 16S rRNA sequencing, fecal microbiota transplantation, intestinal microorganisms, microbial community diversity

Introduction

Chinese ponies are mainly distributed in the mountainous areas of southwest China, which is one of the most famous ponies producing areas in the world. On the basis of providing important means of production for agricultural production, ponies have also promoted the development of rural tourism industry mainly for children's entertainment and sightseeing. With the development of society and the improvement of people's quality of life, people began to pursue a richer spiritual and cultural life. Because ponies are gentle, intelligent, and have the attributes of riding, racing and ornamentation, they have higher value, so the health of ponies is greatly concerned, especially their intestinal health. In fact, it is widely known that domestic horses were vulnerable to diseases originating in the gut, while the microbiota in the gut were prone to disturbances and malfunctions (1).

Research in recent decades has highlighted the importance of the microbiota in the normal development and physiological development and maintenance of the gut, including digestion and nutrient absorption, metabolism, tissue development, and immunity (2). The animal gastrointestinal tract is home to a large and diverse microflora, and constitutes a large and complex system (3). As an ecosystem where organisms coexist, microorganisms are considered the "second largest gene pool" of genetic information for animals (4), forms a complete organism with the host (5). Compared with the widely studied human microflora, animal microflora has received less attention (6). For herbivores, they are unable to synthesize in their own bodies the hydrolytic enzymes needed to degrade plant lignocellulose (7). In general, it is often the microorganisms in the organism that convert indigestible feed into easily absorbed compounds, thus providing nutrition to the host and effectively promoting the physiological health of the host. Thus, by better understanding the equine microbiome, we can inform interventions that will improve health and welfare, performance, value and longevity of the horse.

One study found that host genetics, diet and geography affect the structure of the gut microbial community (8). It has since been sufficient established that gut microbiota plays an importance role in nutritional absorption, vitamin synthesis, food digestion, energy harvest, and metabolism in humans and other animals (9). The horse gut microbiota is a complex ecosystem comprised of thousands of microorganisms (10, 11). Through the microbiological analysis of the feces of Mongolian and Thoroughbred horses, it was found that there were significant differences in 5 phyla and 30 genera (12). Another research found that Przewalski horse fecal microbiomes have a distinct and more diverse community of bacteria compared with the domestic equine (13). There is also study analyzed the gut microbiota of Quarter horse, Azteca, warmblood, Thoroughbreds, and Andalusian breeds and found that the family Christensenellaceae has been found in animals belonging exclusively to the Thoroughbreds breed (14). A recent investigation has revealed diversity in the microbiome of equine from different geographical situation (15). However, those researches are limited to tall horses, and there is lack of understanding of the intestinal flora of ponies.

Animal fecal samples are typically used as a substitute for intestinal microflora and have been widely used in the study of intestinal microorganisms in animals (16–19). Here, we used 16S rRNA amplicon sequencing to research the microorganism in the feces of equine from three different region, we collecte118 fecal samples from Guangxi and Shannxi provinces in China. First, we investigate the diverse in the community structure and species composition of the gut microbiota of pony and tall horses; secondly, to explore the relationship between gut microbes and body height in horses.

Materials and methods

Animals and sample collection

All animal work was conducted according to the guidelines for the care and use of experimental animals established by the Ministry of Agriculture of China. And all methods are reported in accordance with ARRIVE guidelines (https://arriveguidelines.org) for the reporting of animal experiments. The project was also approved by Animal Care and Use Committee (ACUC) in Institute of Animal Sciences, Chinese Academy of Agricultural Sciences (ethical permit: IAS2019-24). Our study included 118 healthy horses. There were including Debao (DeBa) ponies (n = 31), Ningqiang (NiQi) ponies (n = 47) and Guanzhong (GuZh) horses (n = 40) (Supplementary Table S1). All horses were house fed forage and concentrate supplement. The adult animals were selected based on the following criteria: no drugs affecting gastrointestinal microbes were used within 6 months, no reported illness within the past 6 months of the study and, no gut-related disorders recorded until the beginning of the study. All the samples are in the same growing stage. Furthermore, the animals were clinically healthy based on their parasite profiles. One hundred and eighteen fecal samples were collected from the rectum of horses using long arm gloves. Samples were aliquoted into 2 ml cryovials and immediately snap frozen in liquid nitrogen. Then take it to the laboratory and store it in −80°C refrigerator until DNA extraction.

DNA extraction, library construction, and sequencing

Microbiome DNA was extracted with the Omega Stool DNA kit (Omega, Norcross, GA, USA) follow the appropriate instructions. The DNA concentration and purity were quantified with a Nanodrop 2000® (ThermoFisher Scientific, USA) and Qubit3.0 (Life Invitrogen, USA), respectively. 1% agarose gel electrophoresis was used to examined DNA quality. According to the literature describe (20, 21), We amplified the v3-v4 (338F-806R) region of the 16S rRNA gene. Brief, the PCR components contained 5 μl of buffer (5×), 0.25 μl of Fast pfu DNA, Polymerase (5 U/μl), 2 μl (2.5 mM) of dNTPs, 1 μl (10 uM) of each Forward and Reverse primer, 1 μl of DNA Template, and 14.75 μl of ddH2O. The PCR amplification program is carried out according to the Hai-Bin Yang program (20), as follows: 98°C for 5 min, followed by 25 cycles consisting of denaturation at 98°C for 30 s, annealing at 53°C for 30 s, and extension at 72°C for 45 s, with a final extension of 5 min at 72°C (20, 21). PCR amplicons were purified with Vazyme VAHTSTM DNA Clean Beads (Vazyme, Nanjing, China) and quantified using the Quant-iT Pico Green dsDNA Assay Kit (Invitrogen, Carlsbad, CA, USA) (20, 21). After the individual quantification step, amplicons were pooled in equal amounts, and pair-end 2×250 bp sequencing was performed using the Illlumina MiSeq platform with MiSeq Reagent Kit v3 at Shanghai Personal Biotechnology Co., Ltd (Shanghai, China).

Sequence analysis

QIIME2 (Quantitative Insights into Microbial Ecology), version 2019.04 (22) were used to process sequences. Analyze according to the guidance of the official website (https://docs.qiime2.org/2019.4/tutorials/). Removal of primers, quality control, denoising, splicing of double-terminal sequences, removal of chimera and identification of amplicon sequence variants (ASVs) were performed using DADA (23). For taxonomic classification, we selected the Greengenes database (version 13.8) using the Naïve Bayes classifier in QIIME2 (24, 25). ASVs that were identified in only a single sample or classified as non-bacterial were discarded. The sequence of each horse was

randomly selected to achieve a uniform sequencing depth for fair comparison (26).

Statistical analysis of data

Rarefaction curves were plotted for each sample to determine the abundance of communities and sequencing data of each sample (27, 28). The QIIME2 software has been used to calculate alpha diversity index and evaluated by Observed_species index and Shannon index (29). The Observed_species index is used to evaluate species of abundance, and the higher the value of the index, the more species are included in the sample. The Shannon index measure species diversity, affect the species richness and evenness in the sample microbial community. Differences in alpha diversity indices between groups were analyzed by Kruskal-Wallis Rank sum test, and $P < 0.05$ was considered significant (30). Beta diversity measurements, including gut microbiota trees, were calculated as described to compare species diversity between different samples (31). Bacterial taxonomic distributions of sample communities were visualized using the R package software. Bacterial biomarkers with markedly different abundance between horses and ponies were analyzed using the LEfSe (linear discriminant analysis effect size) method. LDA (Linear discriminant analysis) was performed on the identified divergent species to estimate the effect size of each divergent species abundance on the difference between groups, with LDA scores >3.5 (32). The relationship between genera and body height was statistically analyzed using Pearson correlation. $P < 0.05$ was considered statistically significant (32).

Results

Bacterial composition of horses fecal

Microbial genomic DNA was obtained from 118 samples, and the V3–V4 region of the 16S rRNA was sequenced. The reads of 118 samples were denoised, and a total of 6,657,888 clean reads were obtained. In total, 211, 683 ASVs were obtained by DADA2 software and were annotated 24 phylum and 368 genera. The Shannon-Wiener and grade abundance curves generated by R software are shown in Supplementary Figures S1A–F. This result indicates that the number of ASVs per sample is relatively homogeneous.

Analysis of microbial diversity in the equine gut

The Good's coverage of DeBa ponies, NiQi ponies, and GuZh horses was 0.952 7, 0.942 6, and 0.958 6, respectively, indicating that the proportion of undetected species in the sample is relatively small (Supplementary Figure S2). The Observed species index of DeBa ponies and NiQi ponies was significantly higher than those in GuZh horses (Figure 1A). The Shannon index of DeBa ponies was 9.15, which was significantly lower than that in NiQi ponies (10.16, $P < 0.01$) and GuZh horses (10.07, $P < 0.01$) (Figure 1B). The statistical analysis showed that NiQi ponies had a richer and more various gut microbiota. Interestingly, DeBa ponies had more observed species than GuZh ponies, the shannon index value of DeBa ponies was smaller than that of GuZh. The reason for this phenomenon may have something to do with the distribution of horses. DeBa ponies are distributed in the south and GuZh horses in the north (8).

We investigated the relationship between the 118 feces samples from three different regions using Bray–Curtis distances. The UPGMA cluster tree of intestinal microbial structure of three horse breeds was drawn. Each subfield on the tree represented one regions of gut microbiota. Even more interesting is that the gut microbiota of the DeBa pony and NiQi pony clustered together, but those of the GuZh horse located on different subfields (Figure 2A).

We used PCoA (principal coordinate analysis) to examine the gut microbiotas community structures of the three breeds equines. On the PCoA plot (Figure 2B), the bacterial communities from the DeBa pony and NiQi pony clustered tightly and were separated from those from the GuZh horse along principal coordinate axis 1 (PC1), and cluster analysis was similar, which explained the largest amount of variation (16.5%). This result indicating that the composition of intestinal microorganisms in animals of the same body size tends to be similar, which is consistent with the findings of previous studies (33).

Horse gut microbial composition

We annotate a total of 24 phyla and 368 genera (Supplementary Figure S3). Analysis of the intestinal microbial composition of three species found that the abundance of Firmicutes was the highest, accounting for 28.87–84.98%. The second most abundant phylum was Bacteroidetes with 7.49–66.71% (Figure 3A). At the genus level, Treponema, Oscillospira, BF311 and Ruminococcaceae_Ruminococcus were rich in all samples (Figure 3B). However, bacterial taxa were different between the pony and horse fecal samples. At the phylum level, Firmicutes and Bacteroidetes showed a noteworthy difference in the three groups (Figures 4A–F). In addition, the ratio of F and B (Firmicutes and Bacteroidetes) in the GuZh horses (6.66 ± 1.28) was significantly higher than that in the DeBa (1.04 ± 0.55) and NiQi (1.36 ± 0.41) ponies ($P < 0.01$) (Table 1). The abundance of Streptococcus was significant higher in the DeBa (12.21%) and NiQi (4.56%) ponies than in the GuZh horses (0.06%). In addition, the abundance of Coprococcus in the GuZh horses (2.16%) than in the DeBa (0.63%) and NiQi (0.53%) ponies (Figures 5A–J). LEfSe analysis found that Phascolarctobacterium, Paludibacter, and Fibrobacter were markedly enriched in NiQi ponies. The relative abundances of Streptococcus and Prevotella were dramatically higher in DeBa ponies than in NiQi ponies and GuZh horses. The relative abundances of Treponema, Treponema Mogibacterium, Adlercreutzia and Blautia were dramatically higher in GuZh horses than in the DeBa ponies and NiQi ponies (Supplementary Figure S4).

Microflora function prediction and correlation with horse height

The intestinal microbial function of the three horses breeds was predicted using PICRUSt2. PCoA based on the KEGG module revealed differences in microbial function among the DeBa ponies, NiQi ponies, and GuZh horses (Figure 6A). Analogous to the results

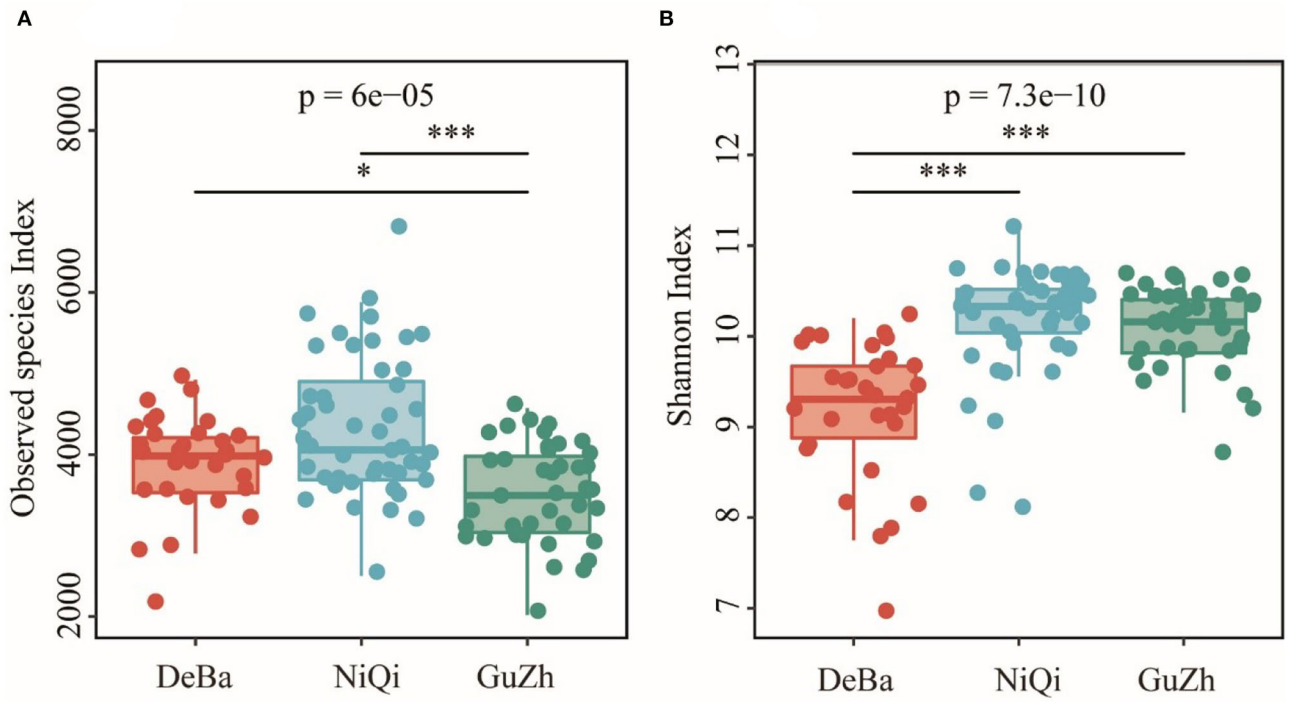

FIGURE 1
Differential analysis of the alpha diversity index. **(A)** Observed_species index. **(B)** Shannon index. Statistical method: one-way ANOVA with Tukey's *post-hoc* test. ***$P < 0.001$, *$P < 0.05$.

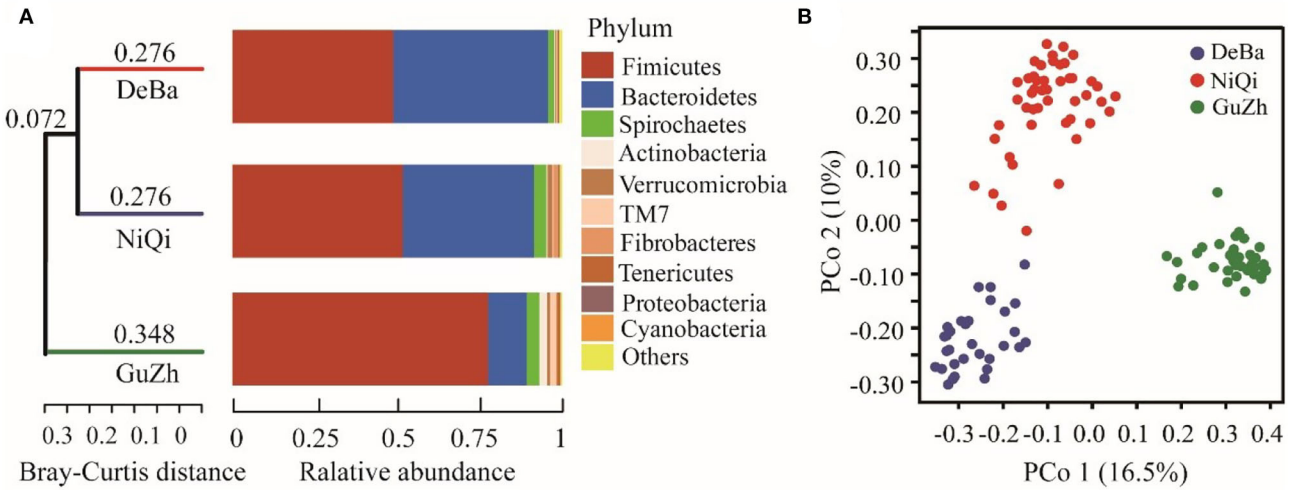

FIGURE 2
Relationship of the gut microbiota of the equines from three populations. **(A)** UPGMA cluster tree based on Bray-Curtis analysis of the structure of intestinal microorganisms in the three horses breeds. The UPGMA (unweighted pair group method with arithmetic mean) cluster tree structure is shown on the left, and the relative abundance distribution map of species at the gate level of each sample is shown on the right. **(B)** Maps representing the beta diversity based on Bray-Curtis analysis. Plots are generated base on the Bray-Curtis distance. Blue dots represent the Debao (DeBa) group, red dots represent the Ninqiang (NiQi) group and green dots represent the Guanzhong (GuZh) group.

of PCoA used for assessing beta diversity, the DeBa and NiQi ponies had a similar microbial composition and parallel functions, which were quite different from those of the GuZh horse.

We selected the bacteria with relative abundance more than 1% to analyses their correlation with body height and found that six genera were significantly correlated with body height, including Streptococcus ($r = -0.48$, $P < 0.01$), Treponema ($r = 0.35$, $P < 0.01$), Coprococcus ($r = 0.71$, $P < 0.01$), Phascolarctobacterium ($r = 0.31$, $P < 0.01$), Prevotella ($r = -0.45$, $P < 0.01$) and Mogibacterium ($r = 0.53$, $P < 0.01$). Among the six genera, Coprococcus, Streptococcus, Phascolarctobacterium and Mogibacterium were classified as Firmicutes; Prevotella was classified as Bacteroidetes and Treponema was classified as Spirochaetes. We speculated that Streptococcus, Treponema, Coprococcus,

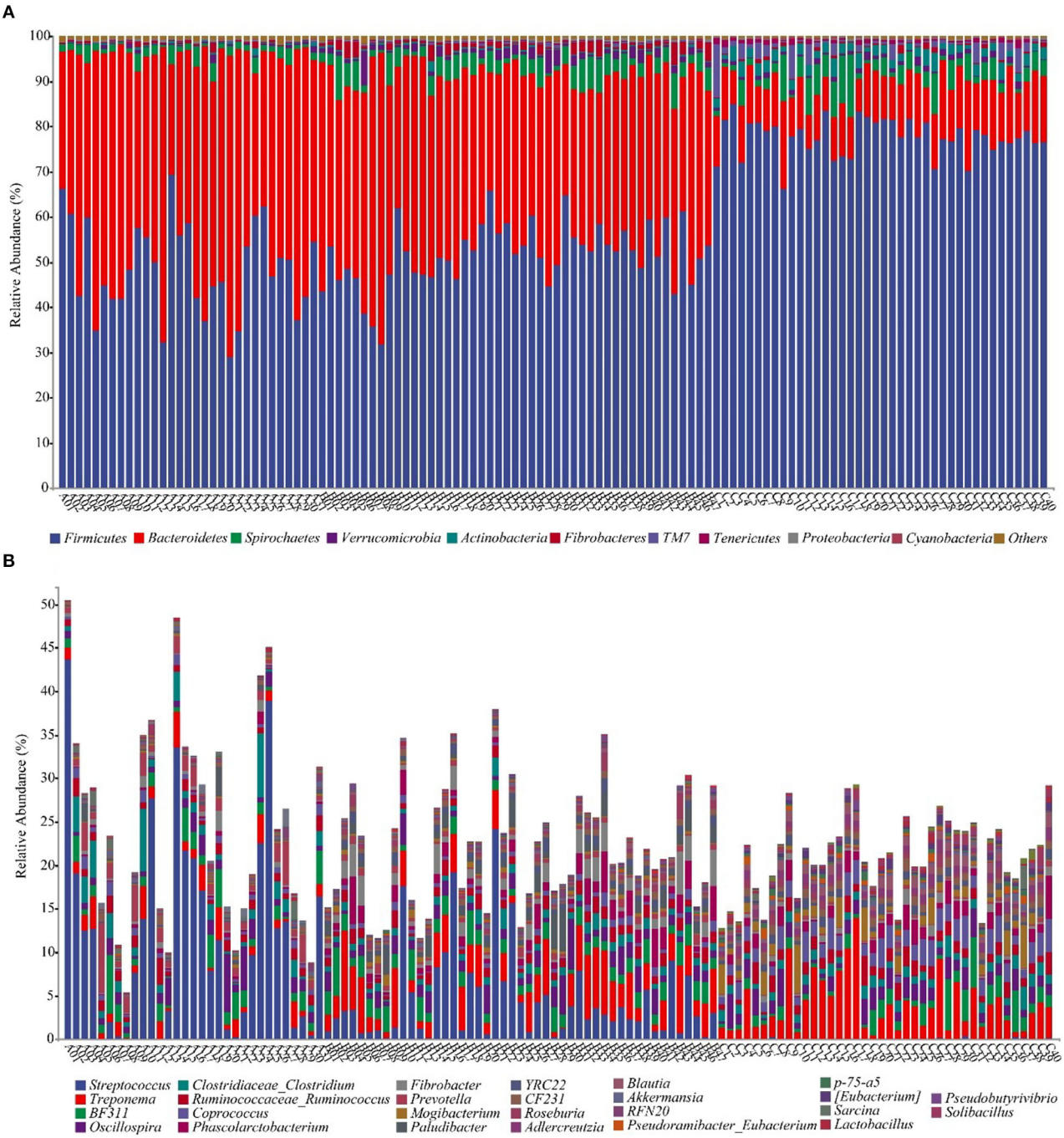

FIGURE 3
Fecal bacterial community at the phylum **(A)** and genus **(B)** levels. **(A, B)** Relative abundance of bacterial groups in the feces of 118 equines. Less than 1% abundance of the phyla was merged into others. Thirty-one samples from DeBa pony (A1–A31), 47 samples from NiQi pony (B1–B47), and 40 samples from GuZh horse (C1–C40).

Phascolarctobacterium, Prevotella, and Mogibacterium were the potential microbiota that may affected the body height (Figure 6B).

Discussion

The association of gut microbiota diversity and function with horse health and phenotypes is currently an active area of research. In the present study, 118 equine gut microbiotas were explored through 16SrRNA high-throughput sequencing, and announced the species composition of microbes existed in the gut tract of ponies. Meanwhile we assess microbiota correlation with the height of equines. We concluded that the DeBa ponies, NiQi ponies and GuZh horses had highly diverse microbial communities. We found that the Firmicutes, Bacteroidetes, and Spirochaetes were the major bacteria phylum, which is consistent with the results of previous studies on herbivorous animals (34, 35).

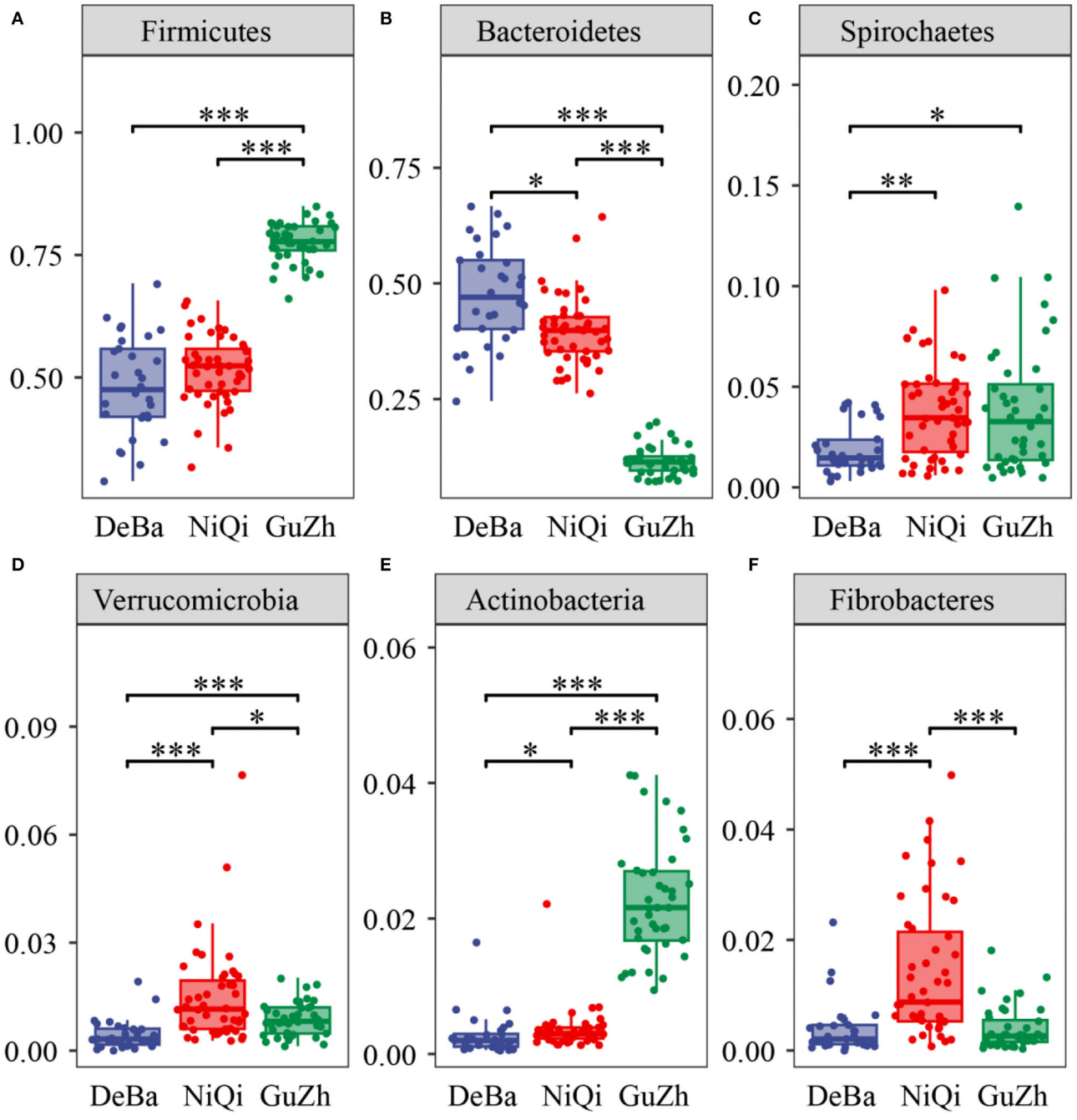

FIGURE 4
Statistical comparison of the relative abundance. **(A–F)** Comparison of dominant phyla in the DeBa, NiQi, and GuZh groups (***$P < 0.001$, **$P < 0.01$, *$P < 0.05$).

However, we observed that the composition of intestinal microorganisms in the DeBa ponies and NiQi ponies was different. At the phylum level, Firmicutes is the most abundant phylum in GuZh horses, while Bacteroidetes was the most abundant phylum in DeBa and NiQi ponies. At the genus level, Streptococcus was the most abundant genus in DeBa and NiQi ponies, and Coprococcus was the most abundant genus in GuZh horses. There were many reasons for the difference among the three varieties, including differences in body size (tall vs. short), geographical distribution differences (north vs. south), and daily management (house feeding vs. grazing).

Notably, the ratio of Firmicutes and Bacteroidetes (F/B) and the relative abundance of Firmicutes in the GuZh horses were significantly higher than those in the of DeBa and NiQi ponies. Prevenient studies have shown that the higher the F/B ratio in the intestine, the stronger the ability of the host to absorb energy from food (36). Firmicutes can promote the decomposition of fibred into short-chain fatty acids (37). SCFAs (Short-chain fatty acids) can promote the absorption of calcium and induce the production of hormone-like insulin growth factor (IGF-1), which can promote bone development and affect bone health (38). A high F/B ratio and superior abundance of Firmicutes promote enhanced nutrient

TABLE 1 Proportions of Firmicutes and Bacteroidetes and the F/B ratios of three breeds horse.

Group	Relative abundance of Firmicutes (Mean values ± SD)	Relative abundance of Bacteroidetes (Mean values ± SD)	Firmicutes/Bacteroidetes (F/B) ratios (Mean values ± SD)
DeBa	48.74 ± 10.21%	46.93 ± 10.95%	1.038 ± 0.55
NiQi	51.60 ± 6.92%	39.83 ± 7.29%	1.364 ± 0.41
GuZh	77.59 ± 4.06%	11.65 ± 3.17%	6.658 ± 1.28**

** $P < 0.01$.

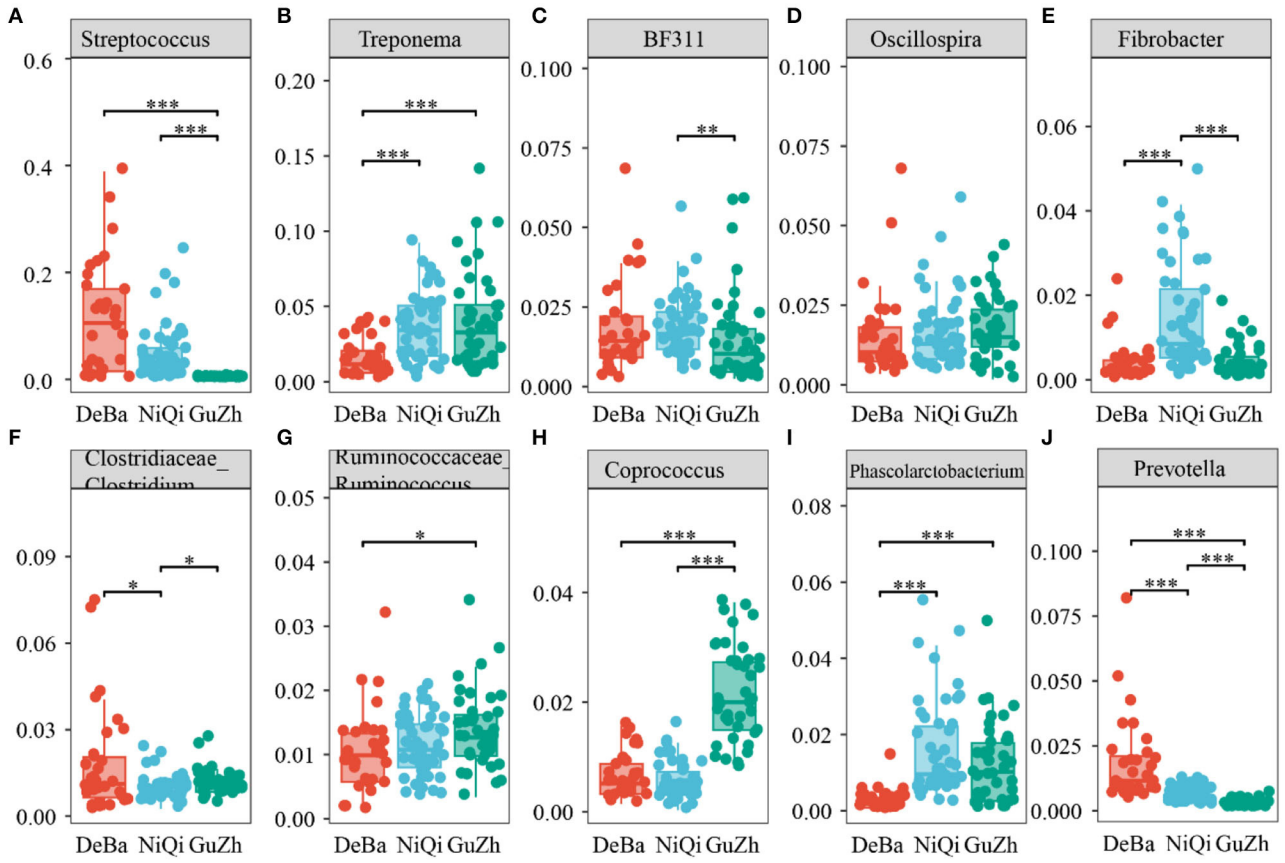

FIGURE 5
Statistical comparison of the relative abundance. **(A–J)** Comparison of dominant genera in the DeBa, NiQi, and GuZh groups (***$P < 0.001$, **$P < 0.01$, *$P < 0.05$).

absorption in the GuZh horses, thus may be contributing to their big size.

Further correlation analysis suggested that Coprococcus, Mogibacterium, Treponema and Phascolarctobacterium were positively correlated with body height, whereas Streptococcus and Prevotella were negatively correlated with body height. Coprococcus is a short-chain fatty acid-producing bacterium that produces butyric acid through the phosphate transferase, the butyric acid kinase pathway and the butyryl-CoA transferase pathway. Butyric acid can promote the proliferation and development of intestinal epithelial cells. In addition, Coprococcus can use lactic acid as a substrate to produce propionic acid through the acrylic acid pathway, which is mainly involved in glycogen synthesis (39, 40). Furthermore, Treponema and Coprococcus are closely associated with pectin degradation in roughage, promote protein synthesis, and improve animal production performance (41). Phascolarctobacterium can use other bacteria to degrade succinate produced by crude fibers, and succinate can be used as a carbon source to produce SCFAs to provide nutrition for the body (42). Mogibacteriumin is associated with ammonia assimilation (43). Streptococcus mutans can stimulate the release of pro-inflammatory cytokines and promote immune regulation (44). Prevotella can degrade and utilize plant non-fibers polysaccharides such as pectin, starch and xylan (45). This means that GuZh horse may make more full use of forage, which is beneficial to its own development.

Conclusions

The microflora analysis of equine showed that there was a significant difference in microbial composition between pony and horse. For the first time, our study characterized the Chinese

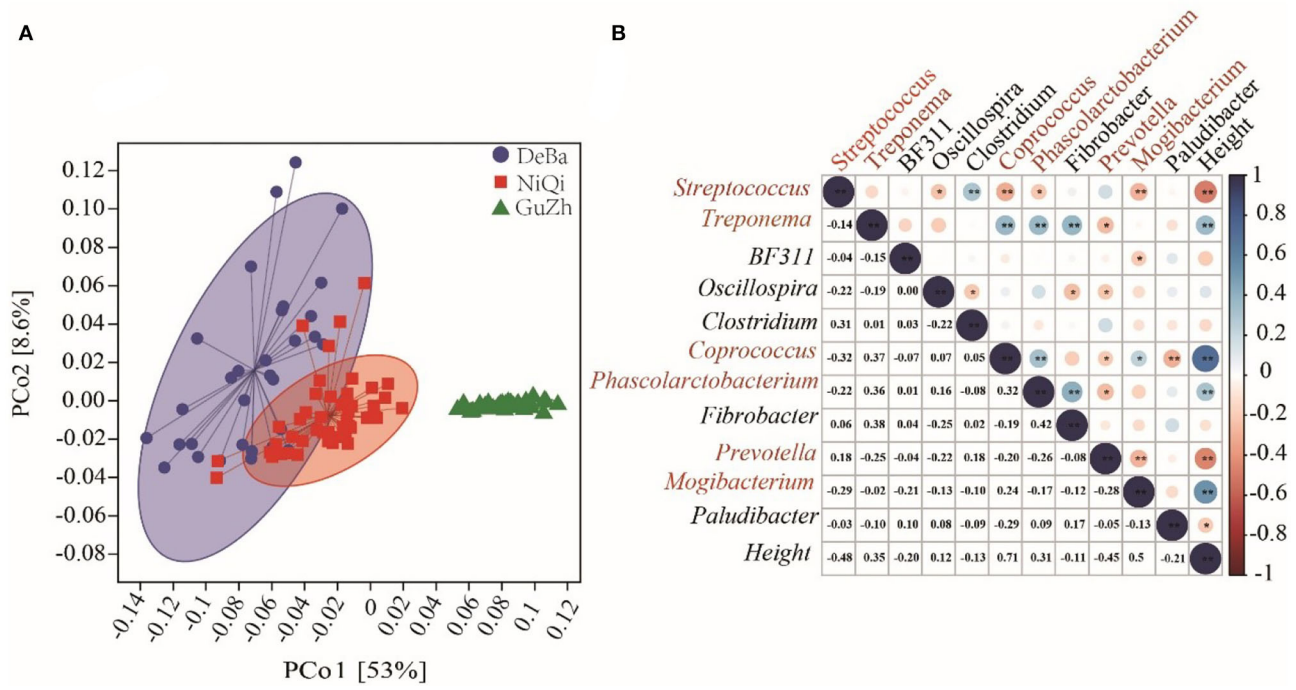

FIGURE 6
Microflora function prediction and correlation with horse height. **(A)** PCoA based on the Bray–Curtis distance of the KEGG modules in DeBa ponies, NiQi ponies, and GuZh horses. **(B)** Correlation between different bacterial genera and horse height. The size of the circle represents the correlation; the larger the circle, the stronger the correlation. The blue color denotes a positive correlation, and the red color indicates a negative correlation (**$P < 0.01$, *$P < 0.05$).

ponies gut microbiota by 16S rRNA amplicon sequencing. The comparison of intestinal microbial diversity of different breeds showed that the microbial diversity of NiQi ponies was higher than that of GuZh horses. Based on clustering and PCoA analysis found that the gut microbiota of DeBa and NiQi ponies were clustered closer than those of GuZh horse. LEfSe analysis found that the content of fiber decomposing bacteria was more abundant in the gut of GuZh horses. Meanwhile, correlation analysis found that six genera were significantly correlated with equine's body height. These bacteria can degrade polysaccharides to produce SCFAs, which may affect body height. In conclusion, there may be an association between horse body height and gut microbiota. Our results can provide theoretical reference for improve health and welfare, performance, value and longevity of the horse.

Data availability statement

The datasets generated and/or analyzed during the current study are available in the NCBI repository (https://www.ncbi.nlm.nih.gov/bioproject/, project number: PRJNA761841) with accession numbers SRR18750362 to SRR18750479.

Ethics statement

All animal work was conducted according to the guidelines for the care and use of experimental animals established by the Ministry of Agriculture of China. And all methods are reported in accordance with ARRIVE guidelines (https://arriveguidelines.org) for the reporting of animal experiments. The project was also approved by Animal Care and Use Committee (ACUC) in Institute of Animal Sciences, Chinese Academy of Agricultural Sciences (ethical permit: IAS2019-24).

Author contributions

SL and YZ performed data analysis and wrote the manuscript. SL, YZ, ZZ, YP, and SM conducted an animal trial and laboratory studies. YM, WL, LJ, XL, and LL conceived animal trial and revised the manuscript. All authors have read and agreed to the publication of this manuscript.

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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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Tributyrin and anise mixture supplementation improves growth performance, nutrient digestibility, jejunal villus height, and fecal microbiota in weaned pigs

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Introduction: The objective of this study was to investigate the effects of dietary supplementation of tributyrin and anise mixture (TA) on growth performance, apparent nutrient digestibility, fecal noxious gas emission, fecal score, jejunal villus height, hematology parameters, and fecal microbiota of weaned pigs.

Methods: A total of 150 21-day-old crossbred weaned pigs [(Landrace × Yorkshire) × Duroc] were used in a randomized complete block design experiment. All pigs were randomly assigned to 3 groups based on the initial body weight (6.19 ± 0.29 kg). Each group had 10 replicate pens with 5 pigs (three barrows and two gilts) per pen. The experimental period was 42 days and consisted of 3 phases (phase 1, days 1–7; phase 2, days 8–21; phase 3, days 22–42). Dietary treatments were based on a corn-soybean meal-basal diet and supplemented with 0.000, 0.075, or 0.150% TA.

Results and discussion: We found that dietary supplementation of graded levels of TA linearly improved body weight, body weight gain, average daily feed intake, and feed efficiency ($P < 0.05$). TA supplementation also had positive effects on apparent dry matter, crude protein, and energy digestibility ($P < 0.05$) and jejunal villus height ($P < 0.05$). The emission of ammonia from feces decreased linearly with the dose of TA increased ($P < 0.05$). Moreover, TA supplementation was capable to regulate the fecal microbiota diversity, manifesting in a linearly increased Chao1 index and observed species and a linearly decreased Pielou's index ($P < 0.05$). The abundance of *Lactobacillus reuteri*, *Lactobacillus amylovorus*, *Clostridium butyricum* were increased, while the abundance of *Prevotella copri* was decreased, by treatment ($P < 0.05$). Therefore, we speculated that TA supplementation would improve growth performance and reduce fecal ammonia emission through improving nutrient digestibility, which was attributed to the increase of jejunal villus height and the regulation of fecal microbiota.

KEYWORDS

tributyrin, anise, weaned pig, villus height, growth performance, nutrient digestibility

Introduction

Post-weaning is a critical phase in swine husbandry. During this period, weaned pigs will encounter challenges in intestinal microflora disorder and/or gastrointestinal tract dysfunction, which will limit their digestion and absorption capacity, resulting in growth retardation and/or diarrhea (1, 2). For decades, antibiotics have been used to cope with the post-weaning challenges

in swine husbandry (3). However, due to antibiotic resistance, the use of antibiotics in livestock has been considered as a threat to the safety in animal production and human health (4). For this reason, seeking suitable alternatives to antibiotics has always been the direction in animal husbandry research.

Tributyrin, also known as tributyrate glyceride, is a triglyceride containing 3 butyrate molecules, which is regarded as a precursor of butyrate (5, 6). When taken orally, it can directly release into the hindgut (7), thus exerting several biological effects such as regulating intestinal microflora (8, 9), improving intestinal health (2, 6), enhancing muscle development (10, 11), alleviating antioxidant stress (12, 13), promoting nutrients absorption (14), and ameliorating growth retardation (13, 15).

When feeding animals with a mixture of tributyrin and herb-derived extract, an increase in growth performance and a decrease in intestinal harmful bacteria were observed (16). Chen et al. (17) found that tributyrin and herb-derived extract mixture supplementation was capable to improve growth performance, maintain intestinal mucosal integrity, and regulate intestinal microbiota in weaned pigs.

Anise has long been used as an aromatic for mammals in order to induce imprinting effects (18, 19). Anise is an important traditional Chinese medicine. Bioactive compounds presented in anise, such as anethole, estragole, limonene, pinene, β-phellandrene, and α-terpineol allows it anti-inflammatory (20, 21), bacteriostatic (22, 23), antioxidant (22, 23), resist pathogenicity bacterial infection (21), and growth promotion (24, 25) characteristics. Therefore, it is reasonable to believe that the combination of tributyrin and anise has positive effects on the performance of weaned pigs.

However, no study has investigated the effects of tributyrin and anise mixture (TA) supplementation on the productive performance of pigs.

The technique of 16S rRNA high-throughput sequencing provides a reliable method for bacterial identification. The gene of 16S rRNA is a conserved sequence region that exists in all bacteria and can be targeted by broad-range polymerase chain reaction (PCR) primers (26). The 16S rRNA sequencing has been used to describe the species composition of various communities in the study of bacterial diversity (27). Therefore, we use the technique of 16S rRNA high-throughput sequencing to investigate the effects of TA supplementation on intestinal microbiota of weaned pigs. We hypothesized that feeding weaned pigs with TA containing diet had positive effects on jejunal villus height and the abundance of beneficial bacteria in intestinal microbiota, so as to improve growth performance by increasing nutrient digestibility, limiting fecal noxious gas emission, reducing fecal score.

Materials and methods

Experimental design

A total of 150 21-day-old crossbred weaned pigs [(Landrace × Yorkshire) × Duroc] with an average initial body weight of 6.19 ± 0.29 kg were used in a completely randomized block design experiment. The protocol (DK-1-2034) of this study was approved by the Animal Care and Use Committee of Dankook University (Cheonan, South Korea).

Based on the initial body weight, all pigs were randomly assigned to three groups. Each group had 10 replicate pens with five pigs (three barrows and two gilts) per pen. The experimental period was 42 days and divided into 3 phases (phase 1, days 1–7; phase 2, days 8–21; phase 3, days 22–42). Dietary treatments were based on a corn-soybean meal-basal diet, which were formulated to meet the nutrient requirements of the NRC (28), and supplemented with 0.000, 0.075, or 0.150% TA to form control, TRT1, and TRT2 groups. The chosen dose was determined in a preliminary study. Feed ingredients and analyzed nutrient composition of the basal diet were shown in Table 1. The commercial TA (ElanPlus® TB50) used in this study was obtained from Olus Plus BV (8 Randweg, Hasselt, The Netherlands). The additive was composed of 50% tributyrin, 5% anise coated by potato starch (10% w/v), and 45% *Vehicle q.s.* (Silica).

All pigs were housed in an environmentally controlled room. The temperature during week 1 was maintained at 30°C and then gradually reduced by 1°C every week to maintain in 24°C. The relative humidity within the room was 60%. The room was equipped with a mechanical ventilation system and the floor was slatted plastic. A nipple drinker was installed in each pen to ensure that pigs could drink freely. In addition, stainless steel self-feeders were installed on one-side of the pens to ensure that pigs had free access to feed. All pigs were allowed *ad libitum* access to feed and water.

Feed composition analysis

On days 7, 21, and 24, representative feed samples were taken to analyze feed composition. Feed samples were dried in an oven with 70°C for 72 h, and later they were ground to pass through a 1-mm sieve and collected. Powder feed samples were analyzed for dry matter (DM; method 930.15), crude protein (CP; nitrogen × 6.25; method 968.06), calcium (method 984.01), phosphorus (method 965.17), and crude fat (method 954.02) following the procedures established by AOAC (29). The lysine and methionine contents in the diet were measured using an AA analyzer (Beckman 6300; Beckman Coulter, Inc., Fillerton, CA). The combustion heat was measured by a bomb calorimeter (Parr 6100; Parr Instrument Co., Moline, IL, USA) to determine the energy content in the feed sample.

Experimental parameters measurement

Growth performance

Individual body weight of pigs was measured on days 1, 7, 21, and 42. Data of body weight was pooled on a pen basis to determine average daily gain (ADG) during days 1–7, 8–21, 22–42, and 1–42. Pen-based feed intake was measured daily to calculate the average daily feed intake (ADFI) during days 1–7, 8–21, 22–42, and 1–42. The feed efficiency (gain to feed ratio) during days 1–7, 8–21, 22–42, and 1–42 was calculated using ADG and ADFI values.

Apparent nutrient digestibility

During days 1–7, 14–21, and 35–42, 0.20% chromium oxide as an indigestible marker was added to the diet of each group for measuring apparent nutrient digestibility. On day 7, 21, and 42, two pigs (one barrow and one gilt) were randomly selected from each replicate pen for fecal taking (about 250 g) by the rectal massage method. Fecal

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TABLE 1 Formula and composition of experimental diet (as fed-basis).

	Phase 1 (days 1–7)	Phase 2 (days 8–21)	Phase 3 (days 22–42)
Ingredients, %			
Corn	37.92	48.39	58.40
Soybean meal (crude protein 47.5%)	16.44	19.40	22.27
Fermented soybean meal (crude protein 53.2%)	5.00	4.00	3.00
Spray-dried porcine plasma (crude protein 77.3%)	6.00	3.00	–
Tallow	3.32	3.08	2.83
Lactose	12.88	7.78	3.18
Sugar	3.00	3.00	3.00
Whey protein	11.00	7.00	3.00
Monocalcium phosphate	1.60	1.54	1.40
Limestone	1.12	1.06	1.06
NaCl	0.20	0.10	0.10
$_{DL}$-Methionine (50%)	0.22	0.22	0.20
$_{L}$-Lysine- H_2SO_4 (51%)	0.49	0.62	0.75
Mineral mixture[a]	0.20	0.20	0.20
Vitamin mixture[b]	0.20	0.20	0.20
Choline chloride (50%)	0.03	0.03	0.03
Zinc oxide	0.38	0.38	0.38
Total	100.00	100.00	100.00
Analyzed composition, %			
Metabolizable energy, MJ/kg	14.45	14.24	14.03
Crude protein	20.00	19.00	18.00
Calcium	0.90	0.85	0.80
Phosphorus	0.75	0.70	0.65
Lysine	1.60	1.55	1.50
Methionine	0.50	0.48	0.46
Crude fat	4.96	5.09	5.19

[a]Provided per kg diet: Fe, 100 mg as ferrous sulfate; Cu, 17 mg as copper sulfate; Mn, 17 mg as manganese oxide; I, 0.5 mg as potassium iodide; and Se, 0.3 mg as sodium selenite.
[b]Provided per kilograms of diet: vitamin A, 10,800 IU; vitamin D_3, 4,000 IU; vitamin E, 40 IU; vitamin K_3, 4 mg; vitamin B_1, 6 mg; vitamin B_2, 12 mg; vitamin B_6, 6 mg; vitamin B_{12}, 0.05 mg; biotin, 0.2 mg; folic acid, 2 mg; niacin, 50 mg; D-calcium pantothenate, 25 mg.

samples were dried in an oven with 70°C for 72 h. After that, samples were ground into powder, which can pass through a 1-mm sieve, and be collected in duplicate. Fecal samples were analyzed for DM (method 930.15) and CP (nitrogen × 6.25; method 968.06) following the procedures established by AOAC (29). The combustion heat was measured by a bomb calorimeter (Parr 6100; Parr Instrument Co., Moline, IL, USA) to determine the energy in feces. The chromium levels were analyzed *via* UV absorption spectrophotometry (UV-1201, Shimadzu, Kyoto, Japan). The apparent total tract digestibility was calculated according to the equation provided by Liu and Kim (30).

Jejunal villus height

Two pigs per pen (one barrow and one gilt) were selected randomly and euthanized with an intravenous injection of 3 mg/kg body weight of chlorpromazine hydrochloride injection on day 42. The entire intestine of euthanized pigs was then removed and dissected free of mesenteric attachments and placed on a smooth and cold surface. The jejunum was separated. The isolated intestinal segments were immediately opened lengthwise following the mesentery line and flushed with ice-cold saline. Approximately 2 cm segments of the jejunum at consistent locations were collected immediately, fixed in 10% formalin, then subsequently embedded, sectioned and stained with hematoxylin and eosin by routine methods. Villus height of the jejunum was measured in ∼10 microscopic fields using an image analysis system by a blinded investigator.

Fecal score

Fecal scores of pigs were recorded daily using the scoring system proposed by Hashem and Shehata (31) in the first week post-weaning.

Hematology parameters

On the last day of the experiment, before euthanizing the pigs used above, they were bled for collecting blood samples *via* anterior vena cava puncture into non-heparinized vacuum tubes (Becton Dickinson Vacutainer Systems, Franklin Lakes, NJ, USA). The blood samples were collected during 11:00 to 12:00 h in order to exclude the circadiurnal fluctuations in hormone concentrations. Pigs did not receive any feed before sampling. Blood samples were centrifuged (3,000 × *g*) for 15 min at 4°C to obtain serum samples and then stored at −20°C until analysis. The concentrations of total protein and albumin were assayed using colorimetric methods. Additionally, globulin levels were evaluated through the difference between total protein and albumin. Serum total cholesterol, triglyceride, and high-density lipoprotein cholesterol (HDL-C) concentrations were determined enzymatically using reagent kits (Wako Pure Chemical Industries Ltd., Tokyo, Japan).

Fecal noxious gas emission

On day 7, 21, and 42, the method of rectal massage was used to take fecal samples (about 300 g) from two randomly selected pigs (one barrow and one gilt) in each pen for measuring fecal ammonia (NH_3), hydrogen sulfide (H_2S), acetic acid, carbon dioxide (CO_2), and total mercaptans (R-SH) emission by the method provided by Dang et al. (32). The fecal samples from the same pen were pooled, mixed, and transported to lab for further analysis of fecal noxious gas emission.

Metagenomic DNA extraction & 16S rRNA gene V3–V4 amplicon sequencing

Fresh stool samples were taken from 15 pigs (five pigs/group) after feeding phytogenic TA additives for 6 weeks. The specimens were kept in liquid nitrogen until they arrived at the laboratory. Metagenomic DNA (mDNA) from 15 fecal samples was extracted using QIAamp Power Fecal Kit (Qiagen, Germany). The mDNA extraction experimental steps were performed according to the manufacturer's instructions with the inclusion of a homogenization

step in which 100 mg fecal samples were pooled in 1.4 ml lysis buffer. When the samples were thoroughly homogenized, tissue lysis stages were implemented for 6 min at 30 Hz, followed by the ending process at 95°C for 5 min. The extracted mDNA was eluted in 100 µl with buffer provided in the kit. Thereafter, the quality check of all mDNA samples was conducted using NanoDrop One (ThermoFisher Scientific, USA), and all samples were stored at 4°C until the next process. All PCR steps were performed with 2X KAPA HiFi Hot Start Ready Mix (Roche, Germany). A primer pair suitable for the 16S V3–V4 amplification was used, and the sequences were as follows: 341F forward primer is 5′-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG-3′. 806R reverse primer is 5′-GTCTCGTGGG CTCGGAGATGTG TATAAGAGACAG-3′. After PCR amplification, all amplicons were purified with the AMPure XP beads (Beckman Coulter, USA). The second PCR amplification was then conducted at lower cycles to add the Illumina adapter and multiplexing indices included in Nextera XD Index (Illumina, USA). All second PCR products were purified with AMPure XP beads once again. The final amplicon products were pooled with normalized concentration, and the library size was checked using the TapeStation system (Agilent, CA, USA). Finally, high-throughput amplicon sequencing was carried out using the Illumina Miseq™ paired-end (2 × 300) platform (Illumina, USA).

Microbial 16S V3-V4 sequencing data pre-processing

Sequencing reads created from microbial 16S V3–V4 regions were demultiplexed by the split_libraries_fastq.py function of QIIME 2, a next-generation microbiome bioinformatics platform (33). The sequences were then trimmed using the Divisive Amplicon Denoising Algorithm 2 (DADA2) plugin which detects and corrects amplicon errors. Also, sequence quality was controlled with DADA2 by filtering out PhiX chimeric sequences. Sequences including ambiguous base calls and <100 bp were trimmed to minimize the random errors. After the denoising step, the data was specified with pre-trained Naïve Bayes classifier artifact using the machine learning Python library scikit-learn in the QIIME2 pipeline. The classifier artifact was trained on SILVA database v138 which is trimmed to include only V3–V4 regions, pre-clustered with 99% sequence identity, and classified bacterial taxonomy using a 70% confidence threshold (default).

Alpha and beta diversity analyses

For all samples, alpha and beta diversity analyses were performed with the "diversity" QIIME2 algorithm to measure changes or differences in microorganisms. The alpha-diversity was estimated by observed ASVs, Chao1, Shannon's abundance, and Pielou's evenness indices, indicating microbial richness and evenness measures within a single sample. The Kruskal–Wallis non-parametric test was then calculated to identify statistical differences among the three groups. The beta-diversity measures were estimated using principal coordinate analysis (PCoA) of both Unweighted UniFrac and Bray-Curtis abundance dissimilarity metrics with the non-parametric permutational multivariate analysis of variance (PERMANOVA) test which is used to compare the differences between the groups. All non-parametric statistical analyses were conducted by GraphPad PRISM v8 (GraphPad Software. Inc., CA, USA), and the P-value < 0.05 was considered statistically significant. All visualization data was created using R bioinformatics packages.

Relative abundance analysis

A relative abundance analysis was carried out to determine the relative frequency of bacteria from phylum to species level of each group. The Kruskal–Wallis non-parametric test was also calculated to confirm statistical differences among three groups. Furthermore, we visualized the statistically significant species of the three groups with the Venn diagram ($P < 0.05$). The heatmap visualization was performed on 15 species among the shared species shown in the Venn diagram except for the unclassification and uncultured bacteria using R studio version 4.1.2. Finally, we compared the relative frequency of the representative four species to analyze the linear effects of the phytogenic additive on the weaning pigs.

Statistical analysis

Before the analysis, all the percentage data were transformed by arcsine transformations. Data were then subjected to statistical analysis in a randomized complete block design using the General Linear Models procedures (SAS Institute, Cary, NC, USA). The normality of data was examined by the Shapiro–Wilk test and QQ plots. The replicate pen was used as the experimental unit. Orthogonal polynomials were used to assess the linear and quadratic effects of storage duration. Differences among groups were evaluated by the one-way ANOVA for multiple comparisons. Variability in the data was expressed as the pooled standard error of means (SEM). $P < 0.05$ was considered statistically significant.

Results

Growth performance and fecal score

In comparison to control group, feeding weaned pigs with 0.15% TA containing diet led to higher body weight on day 42 ($P = 0.038$), ADG during days 8–21 ($P = 0.012$), 22–42 ($P = 0.009$), and 1–42 ($P = 0.006$), ADFI during days 22–42 ($P = 0.040$) and 1–42 ($P = 0.028$), and feed efficiency during days 1–42 ($P = 0.034$). Above growth performance parameters were increased linearly with the dose of TA increased. Moreover, no significant difference in fecal score has been observed among groups (Table 2).

Apparent nutrient digestibility and jejunal villus height

Apparent DM digestibility on day 7 ($P = 0.008$), day 21 ($P = 0.043$), and 42 ($P = 0.029$), apparent CP digestibility on day 42 ($P = 0.033$), and apparent energy digestibility on day 7 ($P = 0.002$), day 21 ($P = 0.028$), and 42 ($P = 0.010$) were the highest in the group of TRT2 in comparison to other groups and they were increased linearly with the dose of TA increased in the diet. Additionally, feeding weaned pigs with graded levels of TA containing diet increased jejunal villus height (Figure 1) in a dose-dependent manner (linearly; $P = 0.043$), of which the highest value was presented in TRT2 group (Table 3).

TABLE 2 Effects of tributyrin and anise mixture (TA) supplementation on growth performance and fecal score of weaned pigs[1].

Items	TA, %			SEM	*P*-value		
	0.000	0.075	0.150		ANOVA	Linear	Quadratic
Body weight, kg							
Day 1	6.18	6.19	6.19	0.294	1.000	0.985	0.998
Day 7	8.01	8.08	8.06	0.293	0.979	0.911	0.896
Day 21	13.96	14.23	14.30	0.299	0.611	0.426	0.783
Day 42	24.53^{b}	25.24^{ab}	25.58^{a}	0.338	0.041	0.038	0.660
ADG, g							
Days 1–7	261.23	271.03	266.80	4.302	0.164	0.368	0.194
Days 8–21	425.01^{b}	439.51^{ab}	446.09^{a}	5.508	0.008	0.012	0.562
Days 22–42	503.37^{b}	520.39^{ab}	533.23^{a}	7.479	0.007	0.009	0.821
Days 1–42	436.89^{b}	453.88^{ab}	461.26^{a}	5.828	0.004	0.006	0.507
ADFI, g							
Days 1–7	292.86	301.43	297.14	4.511	0.280	0.508	0.255
Days 8–21	526.57	541.29	545.14	7.769	0.118	0.052	0.484
Days 22–42	706.48^{b}	719.52^{ab}	734.00^{a}	11.013	0.097	0.040	0.950
Days 1–42	577.57^{b}	590.43^{ab}	598.24^{a}	7.637	0.081	0.028	0.741
Feed efficiency[2]							
Days 1–7	0.89	0.90	0.90	0.007	0.692	0.588	0.646
Days 8–21	0.81	0.81	0.82	0.007	0.368	0.255	0.930
Days 22–42	0.71	0.72	0.73	0.007	0.237	0.186	0.624
Days 1–42	0.76^{b}	0.77^{ab}	0.77^{a}	0.006	0.070	0.034	0.349
Fecal score	1.17	1.15	1.14	0.058	0.924	0.694	0.968

ADG, average daily gain; ADFI, average daily feed intake; SEM, standard error of the mean.
[1]Values represent the means of 10 pens with 5 pigs per replicate pen (n = 10) per treatment.
[2]Gain to feed ratio.
[a,b]Means in the same row with different superscript differ significantly ($P < 0.05$).

Hematology parameters

The concentrations of serum total protein, albumin, globulin, cholesterol, triglyceride, and HDL-C did not differ among groups (Table 4).

Fecal noxious gas emission

Weaned pigs fed the diet supplemented with TA linearly decreased fecal NH_3 emission on day 42 ($P = 0.040$), while did not affect the emissions of H_2S, R-SH, acetic acid, and CO_2. Moreover, the emission of NH_3 in control group was lower than that in TRT2 group (Table 5).

Alpha and beta diversity in fecal microbiota

The average number of total raw reads for 16S metagenome sequencing was 189,215 and the average number of filtered reads, denoised reads, merged reads, and non-chimeric reads were 39,551, 35,450, 23,394, and 14,794, respectively. We confirmed that non-chimeric read data was sufficient enough to analyze the microbiome and the number of ASVs taxonomy classified by SILVA v138 reference database with a >70% confidence threshold was 1,045.

We performed the alpha-diversity analysis to analyze in abundance and evenness differences of each group. The alpha-diversity was measured using Chao1 index, Observed species, Shannon index, and Pielou's index (Table 6). As a result of diversity estimation, observed Chao1 index ($P = 0.042$) and observed species ($P = 0.044$) measures linearly increased when feeding weaned pigs with graded levels of TA containing diet. In contrast, Pielou's evenness estimated scores linearly decreased with the dose of TA increased, showing a significant statistical value ($P = 0.020$). Additionally, the highest value of Chao1 index and Observed species were presented in TRT2 group, whereas the lowest value of Pielou's index was presented in TRT1 and TRT2 groups, in comparison to other groups.

We analyzed beta-diversity to affirm the change of species in each group by coordinates analysis (PCoA) using phylogenetic qualitative Unweighted UniFrac (A) and Bray-Curtis (B) abundance dissimilarity distance (Figure 2). We found that both indices showed statistical importance ($P = 0.001$) based on the PERMANOVA test. In

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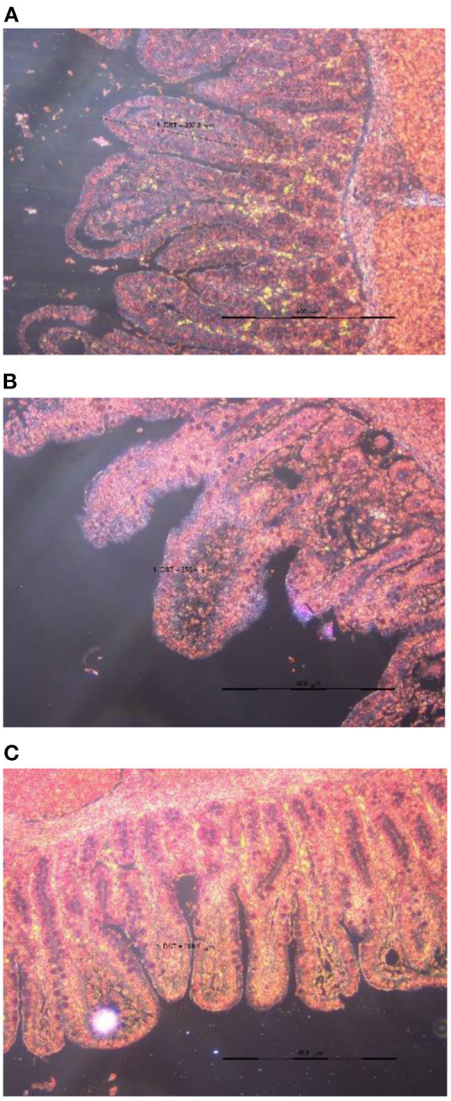


FIGURE 1
Slice of the jejunum in weaned piglets fed the diet supplemented 0.000 **(A)**, 0.075 **(B)**, or 0.150 **(C)** % tributyrin and anise mixture.

addition, pseudo-F, which indicates the ratio of the between-cluster variations to the within-cluster variation, showed higher scores in Bray-Curtis than Unweighted UniFrac (Table 7).

Relative abundance in fecal microbiota

We confirmed a relative bacterial frequency at the genus level based on the taxonomy classification results using SILVA v138 16S rRNA gene database (Figure 3). The 10 most abundant bacteria among groups were mainly involved in *Subdoligranulum*, *Muribaculaceae*, *Megasphaera*, *Faecalibacterium*, *Prevotellaceae_NK3B31_group*, *Blautia*, *Agathobacter*, *Clostridium_sensu_stricto_1*, *Lactobacillus*, and *Prevotella*.

In order to accurately determine the effects of the TA diets on the gut microbiome, we first selected the species showing statistical significance, and 106 species were identified in the three groups (Supplementary Table 1). Then, we identified the species of each group using the Venn diagram and classified three species, five species, and 11 unique species in the control, TRT1, and TRT2 groups, respectively. There were also three species shared between the control and TRT1 groups, common two species between the control and the TRT2, and five species between the TRT1 and TRT2 groups. In line with the purpose of the study, we focused on 64 common species in the three groups (Figure 4). Finally, we compared the relative abundance of the 15 species, which was converted into a *z*-score using a heatmap, excluding ambiguous species (e.g., unclassification or uncultured_bacterium; Figure 5A). Among the 15 species, we picked representative four species to investigate the effects of the dietary additive. The abundance of *Lactobacillus reuteri* in TRT2 group was higher than that in control and TRT1 groups ($P < 0.05$). The abundance of *Lactobacillus amylovorus* and *Clostridium butyricum* in TRT1 and TRT2 groups were higher than that in control group ($P < 0.05$). Moreover, the abundance of *Prevotella copri* was significantly reduced by treatment, of which higher value was observed in TRT2 group in compared to that in TRT1 group (Figure 5B).

Discussion

In the present study, feeding weaned pigs with graded levels of TA containing diet had positive effects on growth performance. Body weight and ADG as important economic parameters are closely related to ADFI and feed efficiency. A high ADFI ensures a good nutrition supply. A high feed efficiency allows good absorption of nutrients from the feed. It is reported that tributyrin supplementation was capable to regulate appetite (8, 13, 34). Gu et al. (8) reported that feeding lipopolysaccharide-challenged weaned pigs with 0.6 g/kg tributyrin containing diet prevented growth retardation by stimulating appetite. Wang et al. (13) noted that dietary supplementation of 0.75 g/kg tributyrin increased ADFI and ADG in diquat-challenged weaned pigs. However, the mechanism of tributyrin promoting feed intake is still unclear, which is probably related to butyrate systemic circulation mediated by tributyrin (7, 35, 36). In addition, dietary supplementation of aromatic substances has been reported to increase the appetite, thus promoting voluntary feed intake and further improving growth performance (25, 37). The flavor of trans-anethole is described as sweet (38), and its chemosensory features could improve palatability and feed preferences of weaned pigs by exerting pleasant sensations (37). Charal (23) reported that feeding weaned pigs with 50 mg/kg anise oil containing diet had positive effects on feed intake and growth performance. Dang et al. (19) noted that the ADG of weaned pigs increased by anise-containing mixture supplementation, which was attributed to the promotion of feed intake. Therefore, as the components of TA, the tributyrin and anise were capable to regulate appetite, which partially contributed to the improvement of growth performance. Additionally, Hou et al. (14) reported that feeding 5 g/kg tributyrin containing diet to weaned pigs was capable to increase feed efficiency and then improve their body weight. Sotira et al. (39) noted that weaned pigs fed the diet supplemented with 2 g/kg tributyrin increased feed efficiency, thus improving ADG. In addition, the supplementation of biochemical substances from anise also has been reported to improve the feed efficiency of weaned

TABLE 3 Effects of tributyrin and anise mixture (TA) supplementation on apparent nutrient digestibility and jejunal villus height of weaned pigs[1].

Items	TA, %			SEM	*P*-value		
	0.000	0.075	0.150		ANOVA	Linear	Quadratic
DM, %							
Day 7	79.91[b]	81.01[ab]	81.75[a]	0.639	0.013	0.008	0.749
Day 21	82.15[b]	82.37[b]	83.73[a]	0.674	0.087	0.043	0.398
Day 42	84.55[b]	86.30[ab]	86.51[a]	0.848	0.033	0.029	0.309
CP, %							
Day 7	78.09	78.67	80.60	1.331	0.116	0.071	0.567
Day 21	81.31	81.49	82.51	1.251	0.119	0.079	0.466
Day 42	82.02[b]	84.16[ab]	85.22[a]	1.423	0.059	0.033	0.665
Energy, %							
Day 7	79.12[b]	79.77[b]	81.23[a]	0.626	0.003	0.002	0.465
Day 21	81.38[b]	82.28[ab]	83.56[a]	0.651	0.004	0.028	0.813
Day 42	83.44[b]	85.19[ab]	85.65[a]	0.794	0.012	0.010	0.358
Villus height, μm	301.97[b]	344.23[ab]	353.21[a]	18.556	0.095	0.043	0.438

DM, dry matter; CP, crude protein; SEM, standard error of the mean.
[1]Values represent the means of 10 pens with 2 pigs per replicate pen (n = 10) per treatment.
[a,b]Means in the same row with different superscript differ significantly ($P < 0.05$).

TABLE 4 Effects of tributyrin and anise mixture (TA) supplementation on hematology parameters of weaned pigs[a].

Items	TA, %			SEM	*P*-value		
	0.000	0.075	0.150		ANOVA	Linear	Quadratic
Total protein, g/dl	5.30	5.48	5.18	0.313	0.796	0.791	0.543
Albumin, g/dl	2.92	2.68	2.78	0.097	0.253	0.327	0.178
Globulin, g/dl	2.38	2.80	2.40	0.305	0.563	0.964	0.294
Cholesterol, mg/ml	83.20	87.40	80.40	4.073	0.494	0.636	0.284
Triglyceride, mg/ml	53.40	53.40	55.60	6.112	0.958	0.803	0.886
HDL-C, mg/ml	30.20	30.60	26.80	2.101	0.401	0.275	0.430

HDL-C, high-density lipoprotein cholesterol; SEM, standard error of the mean.
[a]Values represent the means of 10 pens with two pigs per replicate pen (n = 10) per treatment.

pigs (21, 23). Charal (23) reported that the supplementation of anise oil increased the growth of weaned pigs in nursery stages, and had a positive effect on feed efficiency. Yi et al. (21) noted that supplementing 300 mg/kg anethole to Enterotoxigenic *Escherichia coli*-infected weaned pigs increased feed efficiency. The improvement of digestibility, utilization rate, and retention rate of energy and nutrients partly explain the improvement of feed efficiency in pigs (40, 41). In this study, weaned pigs fed the diet supplemented with TA had positive effects on apparent nutrient digestibility. Similarly, some studies have reported that the supplementation of tributyrin was beneficial to improve nutrient digestibility in weaned pigs (42–44). However, no study has investigated the effects of anise supplementation on nutrient digestibility in weaned pigs. Therefore, we considered that dietary supplementation of TA had positive effects on nutrient digestibility, feed efficiency, and ADFI, and therefore contributed to the improvement in body weight and ADG.

It is reported that manipulating intestinal microbiota communities is a strategy to improve nutrient digestibility and/or feed efficiency (45, 46). Our diversity (alpha and beta) results demonstrated that the TA treatment explicitly influenced the composition of the weaning pigs' gut microbiome, especially in the aspects of evenness and abundance. The classification results at the genus level showed that the microbiome transition was related to additive treatments to some extent. However, species-level classification was further necessary because the genera have functions in both beneficial and pathogenic ways depending on the specific species. Therefore, we first selected 64 species shared by the three groups and selected 15 species showing statistical significance except for unclassified and uncultured bacteria. Finally, four representative species were analyzed to measure the effects of the additive. We found that the abundance of *L. reuteri*, *L. amylovorus*, and *C. butyricum* were positively, whereas *P. copri* was negatively, affected by treatment. A substantial number of studies have shown that *L. reuteri* and *L. amylovorus* in the intestine of pigs had positive effects on growth performance and intestinal health (e.g., gut integrity, nutrient digestion) (47–49). In particular, *L. reuteri* is crucial commensal bacteria in pigs that produce exopolysaccharides to increase intestinal adhesion and

TABLE 5 Effects of tributyrin and anise mixture (TA) supplementation on fecal noxious gas emission of weaned pigs[1].

Items	TA, %			SEM	*P*-value		
	0.000	0.075	0.150		ANOVA	Linear	Quadratic
NH_3, ppm							
Day 7	1.13	1.38	1.13	0.267	0.516	1.000	0.256
Day 21	1.38	1.25	1.13	0.317	0.701	0.404	1.000
Day 42	1.75[a]	1.38[ab]	1.25[b]	0.247	0.098	0.040	0.534
H_2S, ppm							
Day 7	1.00	1.05	0.98	0.187	0.908	0.887	0.681
Day 21	1.05	1.15	1.05	0.206	0.834	1.000	0.552
Day 42	1.30	1.35	1.38	0.225	0.935	0.722	0.945
R-SH, ppm							
Day 7	2.38	2.25	1.88	0.377	0.348	0.167	0.684
Day 21	1.75	1.88	1.63	0.377	0.776	0.724	0.542
Day 42	2.13	1.75	1.88	0.429	0.636	0.536	0.475
Acetic acid, ppm							
Day 7	6.63	7.25	6.75	0.545	0.438	0.807	0.212
Day 21	6.88	6.75	6.50	0.498	0.714	0.425	0.877
Day 42	6.75	7.13	7.13	0.429	0.561	0.356	0.591
CO_2, ppm							
Day 7	9,775.00	9,600.00	9,275.00	262.202	0.137	0.052	0.725
Day 21	10,050.00	10,100.00	10,050.00	435.890	0.990	1.000	0.888
Day 42	10,225.00	10,175.00	10,575.00	257.256	0.211	0.157	0.288

NH_3, ammonia; H_2S, hydrogen sulfide; R-SH, total mercaptans; CO_2, carbon dioxide; SEM, standard error of the mean.
[1]Values represent the means of 10 pens with 2 pigs per replicate pen (n = 10) per treatment.
[a,b]Means in the same row with different superscript differ significantly ($P < 0.05$).

TABLE 6 Effects of tributyrin and anise mixture (TA) supplementation on diversity and abundance indexes in fecal microbiota of weaned pigs[1].

Items	TA %			SEM	*P*-value		
	0.000	0.075	0.150		ANOVA	Linear	Quadratic
Chao1 index	173.25[b]	232.38[ab]	254.09[a]	25.102	0.036	0.042	0.554
Observed species	171.60[b]	228.80[ab]	247.40[a]	23.828	0.037	0.044	0.521
Shannon index	6.60	6.68	6.80	0.149	0.519	0.360	0.939
Pielou's index	0.89[a]	0.86[b]	0.86[b]	0.009	0.004	0.020	0.068

SEM, standard error of the mean.
[1]Values represent the means of 5 pigs per treatment (n = 5).
[a,b]Means in the same row with different superscript differ significantly ($P < 0.05$).

inhibit the proliferation of *E. coli*, thereby promoting growth performance (50, 51). Further, reutericyclin, produced by *L. reuteri*, is capable to prevent *Clostridium difficile* infection (52). Shen et al. (49) also reported that *L. amylovorus* showed the characteristics of probiotics in livestock production. Based on these previous studies, we speculated that the additive had a positive effect on weaned pigs by increasing *Lactobacillus* species. In addition to the two species, we also found a more than 20-fold increase in *C. butyricum* and an approximately half decrease in *P. copri* in the treatment groups. Studies have examined the positive effects of *C. butyricum* on intestinal morphology, intestinal microflora balance, and growth performance (53, 54). Moreover, *Clostridium butyiricum*, a butyric acid producer, is capable to provide energy to intestinal epithelium (55) and beneficial to improve nutrient digestibility (56). Regarding *P. copri*, it is capable to induce dysbiosis, reduce short-chain fatty acids, and increase the risk of colitis (57–61). Additionally, research indicated that the *P. copri* is a potential pathogen that causes a series of metabolic diseases (62). Therefore, the increase of beneficial bacteria such as *L. reuteri*, *L. amylovorus*, and *C. butyricum* as well as the decrease of harmful bacteria such as *P. copri* caused by TA supplementation was beneficial to the improvement of nutrient digestibility and/or feed efficiency, and therefore contributed to the improvement of growth performance.

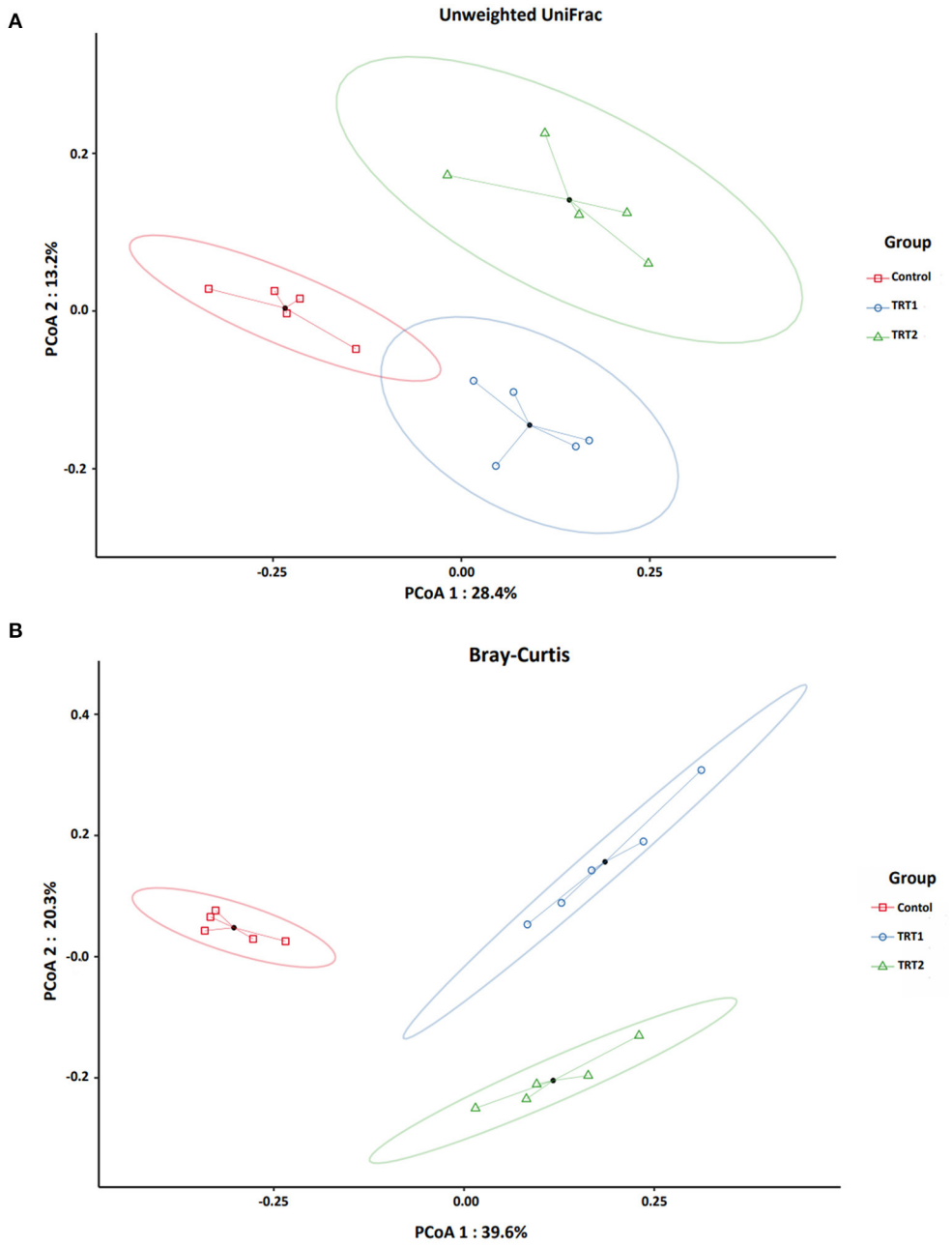


FIGURE 2
Beta-diversity analysis of the three groups between each weaning pigs ($n = 5$). Microbial beta-diversity analysis of the three groups is measured by phylogenetic qualitative Unweighted UniFrac **(A)** and Bray-Curtis **(B)** abundance dissimilarity distance matrix for all 15 samples. The colored circular clusters denote the control (red), TRT1 (blue), and TRT2 (green). Control was defined as the weaned pigs fed the basal diet. TRT1 was defined as the weaned pigs fed the diet supplemented with 0.075% tributyrin and anise mixture. TRT2 was defined as the weaned pigs fed the diet supplemented with 0.150% tributyrin and anise mixture.

On the other hand, the physiology of the intestinal tract is the cornerstone for ensuring high nutrient digestibility and/or feed efficiency (63, 64). The villus height condition is related to the ability of nutrient absorption (42, 65). The supplementation of tributyrin to the diet has been reported to increase the villus height in the intestine of weaned pigs (2, 8, 13, 66, 67). Tributyrin is a strong mitosis promoter and a differentiation agent in the growth of villi in gastrointestinal tract (7, 68), which is considered to be the mechanism by which the height of intestinal villus increases (65). In addition, feeding *E. coli* K88-challenged weaned pigs with 300 mg/kg anethole containing diet has been reported to increase the villus height in the duodenum, thus increasing feed efficiency (21). Taken together, these data indicated that both tributyrin and anise bioactive components have the ability to enhance the intestinal absorption of nutrients by increasing villus height.

Feeding manipulation to alleviate post-weaning diarrhea is also a strategy to improve growth performance (69). It has been reported that dietary supplementation of tributyrin decreased fecal score

during the post-weaning (67). However, Zhang et al. (70) noted that feeding weaned pigs with 1 g/kg tributyrin containing diet did not affect the diarrhea rate. In this study, pigs were healthy and showed no signs of diarrhea. In addition, results of 16S rRNA analysis indicated that pig feces were rich in probiotics, such as *Prevotella* (71), *Lactobacillus* (72), *Agathobacter* (73), *Blautia* (71), Prevotellaceae_NK3B31_group (74), *Faecalibacterium* (75), *Megasphaera* (76), and *Muribaculaceae* (77). Indeed, the efficacy of tributyrin varies according to the health status of animals (15). In addition, Hamer et al. (78) reported the dual effect of butyrate on intestinal epithelial permeability, that is, low dose of butyrate enhanced tight junction and decreased intestinal permeability *in vitro* and *in vivo* in pigs, while high concentration of butyrate increased permeability both in some intestinal cell lines and in murine models. Therefore, we considered that the effect of tributyrin on fecal score was variable. Its supplementation may not be useful for pigs with no diarrhea symptoms. Studies that evaluated the effects of anise supplementation on fecal score in weaned pigs are still limited. In this study, we considered that the supplementation of TA did not affect the fecal score in weaned pigs, which was probably due to the lack of diarrhea symptoms in pigs.

TABLE 7 Statistical significance of beta-diversity using PERMANOVA-test.

Metric name	pseudo-*F*	R^2	*P*-value
Unweighted UniFrac	3.70845919	0.381982261	0.001
Bray-Curtis	7.641977174	0.560181056	0.001

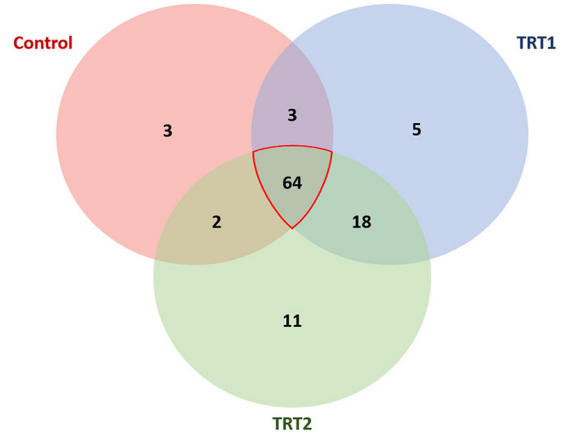
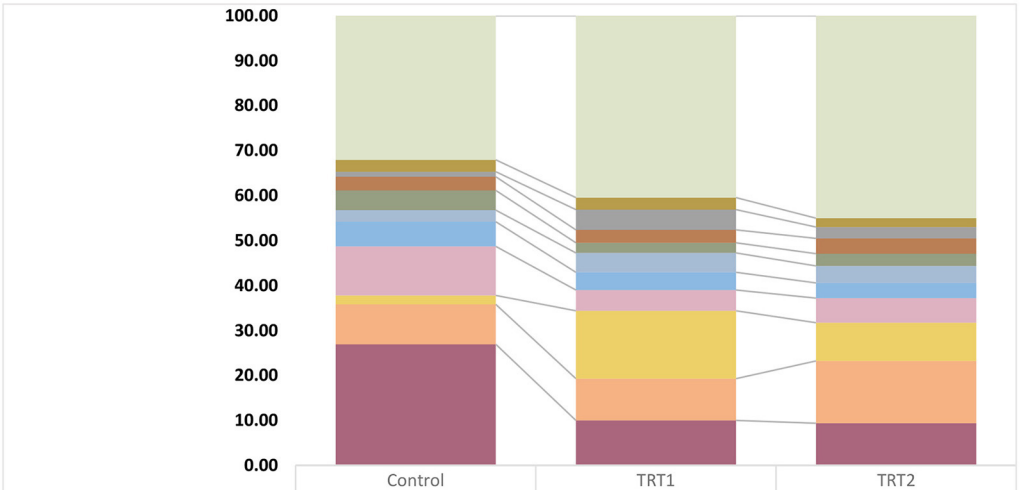


FIGURE 4
Venn diagram visualization at the species level. The Venn diagram represents the species with statistical significance in the three groups. Among the 106 species, we select 64 species shared by all three groups to analyze the linear effect of the additive. Control was defined as the weaned pigs fed the basal diet. TRT1 was defined as the weaned pigs fed the diet supplemented with 0.075% tributyrin and anise mixture. TRT2 was defined as the weaned pigs fed the diet supplemented with 0.150% tributyrin and anise mixture.



	Control	TRT1	TRT2
Others	32.05	40.46	45.04
Subdoligranulum	2.65	2.65	1.97
Muribaculaceae	1.12	4.52	2.52
Megasphaera	3.04	2.88	3.40
Faecalibacterium	4.39	2.23	2.72
Prevotellaceae_NK3B31_group	2.58	4.32	3.78
Blautia	5.49	3.93	3.38
Agathobacter	10.90	4.62	5.47
Clostridium_sensu_stricto_1	1.98	15.10	8.50
Lactobacillus	8.88	9.30	13.85
Prevotella	26.93	9.98	9.37

FIGURE 3
Relative bacterial abundance of the groups at the genus level ($n = 5$). The relative abundance box plots indicate the composition of the three groups at the genus levels which are classified based on the 16S V3–V4 metagenome profiling. Each graph bar is ordered from the highest proportion. Control was defined as the weaned pigs fed the basal diet. TRT1 was defined as the weaned pigs fed the diet supplemented with 0.075% tributyrin and anise mixture. TRT2 was defined as the weaned pigs fed the diet supplemented with 0.150% tributyrin and anise mixture.

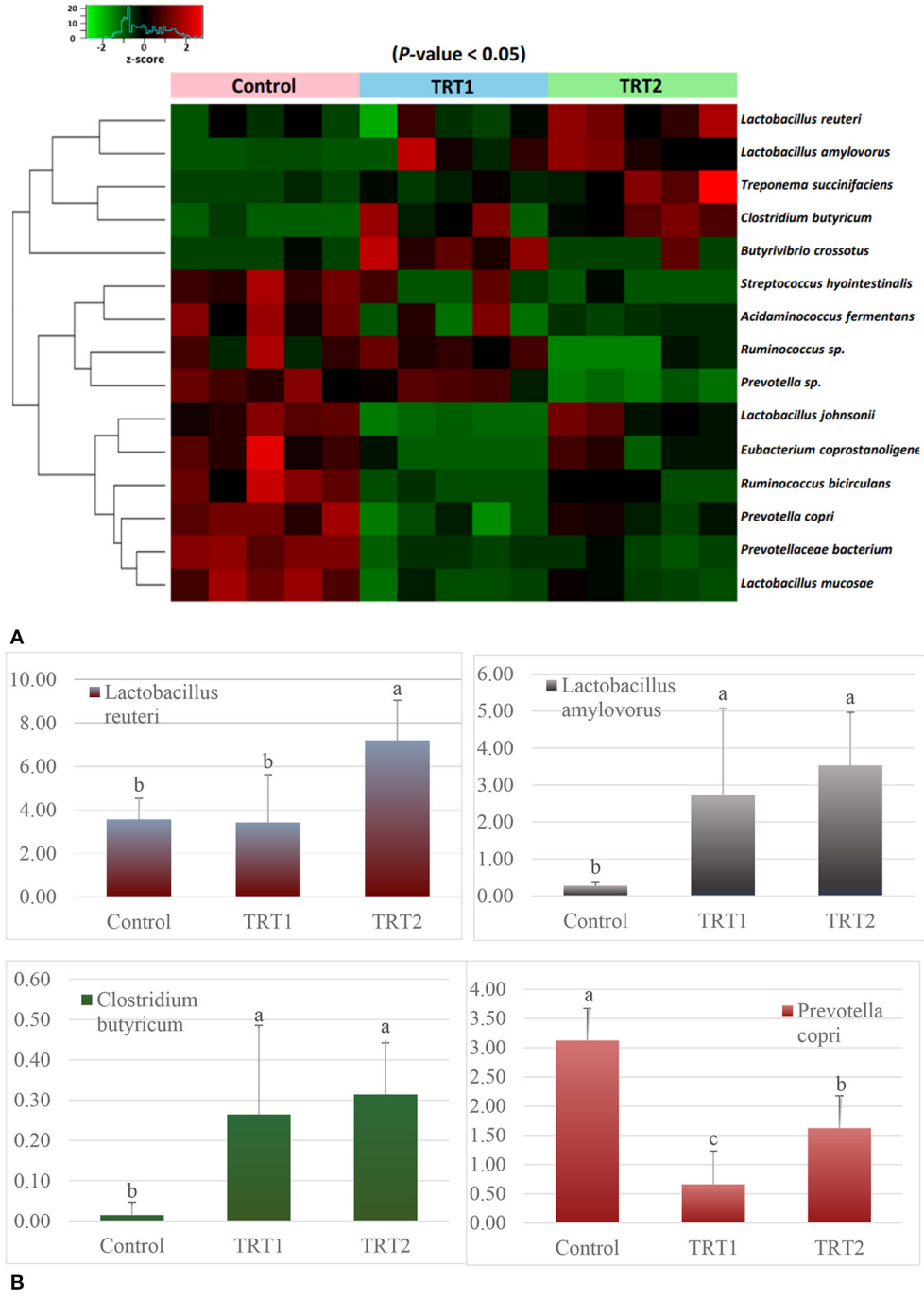


FIGURE 5
Analysis of bacterial relative abundance at the species level ($n = 5$). **(A)** Heatmap plot shows the changes in the relative abundance of the shared 15 species of each weaning pig except for ambiguous species. The relative frequency of the shared bacteria is converted to z-score value. **(B)** A bar chart presents the four species' relative frequency. Control was defined as the weaned pigs fed the basal diet. TRT1 was defined as the weaned pigs fed the diet supplemented with 0.075% tributyrin and anise mixture. TRT2 was defined as the weaned pigs fed the diet supplemented with 0.150% tributyrin and anise mixture. $^{a-c}$Means in the same figure with different superscript differ significantly ($P < 0.05$).

The noxious gas from feces is produced by the unabsorbed nutrients fermented by intestinal microbiota (79). Decrease the fermentation substrate by improving nutrient digestibility and regulate the fermentation process by regulating intestinal microbiota are strategies to reduce fecal noxious gas emission (32, 80). In this study, we observed that feeding weaned pigs with TA containing diet improved apparent nutrient digestibility and upregulated the abundance of beneficial microbiota in feces. We speculated that the reduction of fecal NH_3 emission can be explained by the improvement in nutrient digestibility and the increase in beneficial microbiota abundance.

The total protein, albumin, and globulin concentrations *in vivo* are always linked to nutritional status (81, 82). In this study, dietary supplementation of TA had no significant effects on serum total protein, albumin, and globulin concentrations. Similarly, several studies reported that feeding weaned pigs with tributyrin containing diet did not affect the concentrations of total protein, globulin, and albumin in serum (2, 39, 70, 83). The effect of anise on serum total protein, albumin, and globulin concentrations are not yet known, but at least, it does not generate any damaging effect.

Tributyrin supplementation has been reported to regulate blood lipids (39, 84). Butyrate could promote the secretion of the main proteins that make up HDL-C, thus promoting the synthesis of HDL-C (85, 86). Xiong et al. (87) noted that feeding lipopolysaccharide-challenged broiler chicks with tributyrin containing diet increased serum HDL-C concentrations. In addition, feeding weaned pigs with anise containing herbal mixture also has been reported to increase the serum HDL-C concentrations, whereas decrease the serum total cholesterol concentrations. However, in this study, weaned pigs fed the diet supplemented with TA had no effects on serum total cholesterol, triglyceride, and HDL-C concentrations, which was affirmed by the studies of Dell'Anno et al. (83) and Weber et al. (88). Therefore, as far as the results obtained in this study were concerned, the contribution of TA to the regulation of serum lipid metabolism parameters was limited.

Conclusion

Our findings confirmed that TA supplementation would improve growth performance and reduce fecal ammonia emission through improving nutrient digestibility, which was attributed to the increase of jejunal villus height and the regulation of fecal microbiota. Dose of TA at 0.15% seems suitable to be used in the diet of weaned pigs.

Data availability statement

The data presented in this study are deposited in the figshare repository, accession number https://doi.org/10.6084/m9.figshare.21626027.v3.

Ethics statement

The protocol (DK-1-2034) of this study was approved by the Animal Care and Use Committee of Dankook University (Cheonan, South Korea).

Author contributions

IK, KL, and JS: conceptualization and methodology. DD and SL: formal analysis. DD, HL, SM, and KH: writing—original draft preparation. DD and JS: investigation. IK: supervision. DD, HL, SM, KH, and IK: writing—reviewing and editing. All authors reviewed the manuscript.

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Conflict of interest

KL was employed by Semi Feed Tech 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.2023.1107149/full#supplementary-material

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Saccharomyces cerevisiae fermentation product improves robustness of equine gut microbiome upon stress

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Introduction: Nutritional and environmental stressors can disturb the gut microbiome of horses which may ultimately decrease their health and performance. We hypothesized that supplementation with a yeast-derived postbiotic (*Saccharomyces cerevisiae* fermentation product-SCFP) would benefit horses undergoing an established model of stress due to prolonged transportation.

Methods: Quarter horses ($n = 20$) were blocked based on sex, age (22 ± 3 mo) and body weight (439 ± 3 kg) and randomized to receive either a basal diet of 60% hay and 40% concentrate (CON) or the basal diet supplemented with 21 g/d Diamond V TruEquine C (SCFP; Diamond V, Cedar Rapids, IA) for 60 days. On day 57, horses were tethered with their heads elevated 35cm above wither height for 12 h to induce mild upper respiratory tract inflammation. Fecal samples were collected at days 0, 28, and 56 before induction of stress, and at 0, 12, 24, and 72 h post-stress and subjected to DNA extraction and Nanopore shotgun metagenomics. Within sample (alpha) diversity was evaluated by fitting a linear model and between sample (beta) diversity was tested with permutational ANOVA.

Results: The SCFP stabilized alpha diversity across all time points, whereas CON horses had more fluctuation ($P < 0.05$) at 12, 24, and 72 h post-challenge compared to d 56. A significant difference between CON and SCFP was observed at 0 and 12 h. There was no difference in beta-diversity between SCFP and CON on d 56.

Discussion: Taken together, these observations led us to conclude that treatment with SCFP resulted in more robust and stable microbial profiles in horses after stress challenge.

KEYWORDS

equine, horse, *Saccharomyces cerevisiae* fermentation product (SCFP), postbiotic, stress, microbiome

Introduction

The role of the microbiome and its importance has been well-established in several systems over the past two decades, including in environmental (1, 2), biomedical (3–5), and agricultural (6–8) contexts. Herbivores are particularly impacted by the gastrointestinal microbiome, given their interdependency with metabolic pathways only present in microbes that are necessary for digestion of complex carbohydrates present in forages that typify the equine daily diet.

In horses relatively fewer research studies investigating the microbiome are available, however the number of such studies is increasing rapidly (8, 9). Among the many factors that can impact the equine microbiome, stress is one of the most preeminent ones. Both diet- (10) and exercise- (11, 12) induced stress have been associated with microbiome changes in horses. Unstable microbiomes represent an open niche for opportunistic pathogen establishment and are associated with worse health outcomes. In fact, colonization resistance is one of the biggest roles played by the microbiome in maintaining host health (13–15). Thus, maintaining a robust microbiome upon stressful events would be beneficial for horse health.

Several techniques can be applied to intentionally manipulate the diversity and composition of the gut microbiome in the quest to maintain an optimal microbial community. Diet modification, pre-, pro-, and postbiotic administration, and more drastic therapeutics such as antibiotic therapy and fecal microbiota transplantation are also used for microbiome modulation (16). Postbiotics are defined as a "preparation of inanimate microorganisms and/or their components that confers a health benefit on the host" (17, 18). These preparations do not necessarily originate from probiotic microorganisms and must contain an unpurified mixture of inanimate organisms and their metabolites. Because the mode of action of postbiotics does not rely on presence of live organisms in the final product, they represent an attractive alternative for feed supplementation given their better stability during feed processing (19). Several studies evaluating the efficacy of postbiotic supplementation of *Saccharomyces cerevisiae* fermentation products (SCFP) in bovine (20, 21), avian (22), and equine (23) species have been performed. While the mechanisms by which postbiotics confer benefits to the host have not yet been completely elucidated, much of the literature indicates that postbiotic supplementation is associated with microbiome optimization (24) and improvement of immune function (20, 22, 25). However, less is known about the effects of SCFP on horses. A few recent studies have indicated improvement in immune parameters in a vaccine challenge model (23, 26) while no difference was observed in the microbiota of racehorses fed a yeast supplement (27). Taking the wealth of evidence of the beneficial effects of postbiotic administration in many species, it is reasonable to hypothesize that postbiotic administration would benefit horses under stress.

Horses are exposed to stressful situations daily, including transportation, exercise, and diet changes. Although several studies have demonstrated the impact of stressful events on the equine fecal microbiome (10, 28) little evidence is available on how postbiotic administration can impact the robustness of microbiome in horses under stress. Thus, the objective of this study was to determine if supplementation with SCFP would result in more robust microbiome in an established equine model to simulate stress due to prolonged transportation. We hypothesized that SCFP supplementation would result in a more robust microbiome that would be less impacted by experimental stress.

Abbreviations: CAZy, Carbohydrate Active enZyme; CLR, Centralized Log-Ratio; FDR, False Discovery Rate; SCFP, *Saccharomyces cerevisiae* fermentation product.

Materials and methods

Experimental design, animals, and sample collection

The animal experiment for present microbiome study was described by Tench et al. (29). The protocol for the use of experimental animals was approved by the Institutional Animal Care and Use Committee at the University of Florida in Gainesville, FL (#201810324) under the Guide for the Care and Use of Agricultural Animals in Research and Teaching (30).

Briefly, 20 young and clinically healthy horses in training (mean ± SEM; initial age 22 ± 0.3 mo and BW 439 ± 3 kg) were paired by age and sex and randomly assigned to one of the two experimental treatments for 60 days. Treatments included supplementation with 0 g/d (Control; no treatment Control) or 21 g/d Diamond V TruEquine C (SCFP; Diamond V, Cedar Rapids, IA). A basal diet of 60% Coastal bermudagrass hay and 40% concentrate formulated to meet the nutrient requirements of horses at a moderate rate of growth (31) was offered to all horses. Treatment administration was done by top dressing SCFP on the concentrate ration. Horses were exercised 4 days per week for 30–45 min/d at light to moderate intensity. On day 57, horses were placed in individual stalls and tethered with their heads elevated 35 cm above wither height for 12 h to induce mild upper respiratory tract inflammation according to a previously established protocol to mimic long-distance transport stress (32, 33). Induction of inflammation was confirmed by significantly elevated serum cortisol and blood leukocyte measurements performed after stress induction compared to pre-stress (34, 35). The stress period was relieved after the 12 h timepoint by untethering of the horse heads. Fecal samples were collected into sterile containers at seven time points: days 0, 28, and 56 before induction of stress, and at 0, 12, 24, and 72 h post-stress, where 0 h is the time at which the horses were untethered. Samples were immediately placed on ice and transported to the laboratory where they were kept in a −80°C freezer until DNA extraction. A schematic of the experimental design and sample collection is given in Figure 1.

DNA extraction and shotgun metagenomic sequencing

DNA extraction

Fecal samples were removed from the −80°C freezer 1 day prior to DNA extraction and thawed in a 4°C refrigerator overnight. The ZymoBIOMICS 96 MagBead DNA kit (Zymo Research Corporation, Irvine, CA) was used in a Biomek i7 (Beckman Coulter, Indianapolis, IN) workstation for DNA extraction according to manufacturer's instructions. Four extraction blanks were included in each 96 well plate to confirm that cross contamination did not occur.

Nanopore sequencing

Libraries were constructed using the SQK-RPB004 Rapid PCR Barcoding kit (ONT, Oxford, UK). Library preparation included

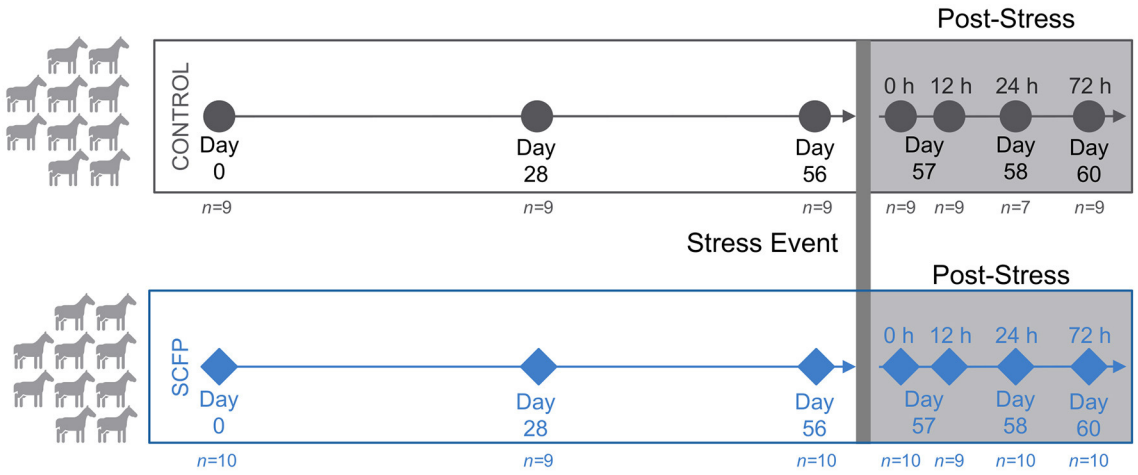

FIGURE 1
Study overview. Young and clinically healthy horses were paired by age and sex and randomly assigned into Control ($n = 10$) or *Saccharomyces cerevisiae* fermentation product (SCFP; $n = 10$). Horses received diets for 60 days. On day 57, horses were subjected to a previously established stress protocol to induce mild upper respiratory tract inflammation that mimicked long-distance transportation. Samples were collected on days 0, 28, and 56 before stress and at 0, 12, 24, and 72 h post-stress.

DNA extraction blanks for quality control. Shotgun metagenomic sequencing was performed using R9.4.1 FLO-MIN 106 flow cells on the GridION platform (ONT, Oxford, UK), multiplexing 12 samples in each flow cell. Each sequencing run lasted 70 h. The MinKNOW ONT software (v 3.6.5) with Guppy basecaller was used for sequencing using the high-accuracy basecalling setting, followed by de-multiplexing, adapter trimming, and quality control using default settings.

Bioinformatics and statistical analyses

Taxonomic assignment and microbial diversity

Fastq files obtained from the MinKNOW ONT workflow were used for microbial taxonomic classification. First, host DNA was removed by mapping fastq files to the horse genome (assembly EquCab3.0) using Minimap2 (36) followed by the removal of any reads matching the horse genome using SAMtools (37). The remaining reads were assumed to be from microbial origin and used for taxonomic assignment. To improve microbial classification, a custom database was made, which contained high quality genomes from the RefSeq database (38) and published metagenome-assembled genomes (38–40). The Kraken2 pipeline (41) was used for species identification and Bracken was used to estimate species abundances (42). Diversity metrics were calculated in R (43) using the Phyloseq package (44, 45) with the rarefied species count table from Bracken as input. Species tables were center-log transformed using the microbiome package (46) after imputation of zeros using a Bayesian multiplicative replacement method from the zCompositions package (47). Species with non-zero presence in at least 75% of samples and relative abundance >0.001% were identified separately in the pre-stress and post-stress periods and the superset containing all species was used for differential abundance analysis.

Functional potential

To have a better understanding of the microbiome functional potential, the Carbohydrate-Active enZymes (CAZy) (48) present in the microbiome communities for each sample were identified. First, genomes from microbial species identified with Kraken2 were annotated using PROKKA (45), followed by additional assessment of gene function using EggNOG-mapper v2 (49). After the annotation process was completed, a custom python script was used to compile the CAZy for each genome, generating a table with the accumulated CAZy potential for all the microbes identified for each sample. Results were compiled into a final table containing numbers of annotated features for each sample.

Statistical analyses

Diversity metrics

Within sample (alpha) diversity was evaluated by fitting a linear model with the lmer function of the lme4 package (50) in R. The model included Shannon diversity index as the dependent variable, horse as a random effect, treatment, timepoint, and their interactions as independent variables. Because stress is nested within timepoint, the effect of stress only is evaluated in a separate model. Between sample (beta) diversity was tested with permutational ANOVA using the adonis function in the vegan package (51) in R. The model included Aitchison distances (52) calculated based on CLR transformed values as the dependent variable, and treatment, timepoint, and their interactions as independent variables. Data was visualized with PCA using the Phyloseq package (44, 45).

Differential abundance

A modified version of the linear discriminant analysis from the LinDA package (53) was used to fit linear models that included relative abundances as the dependent variable, treatment,

timepoint, and their interaction as independent variables, and horse as a random effect. The output from each model was then analyzed with the emmeans package (54) to calculate fold changes of centralized log ratio (CLR) transformed data of each measurement (species) for each animal with respect to their initial sample collected at day 0. False discovery rate (FDR) correction (55, 56) was used to identify species within each timepoint that significantly differed between treatment and Control.

Correlation networks

An adaptation of the CoNet framework (57), which includes generation of a combination of diverse measures of correlation (including Pearson's, Spearman's, and Kendall's correlation coefficients) using CLR transformed data was used for correlation network analyses. Distributions of all pair-wise scores between the nodes were computed for each timepoint. Only edges (correlations) with p-values < 0.05 after FDR correction (55, 56) were taken into further consideration, and edges not supported by at least two measures were discarded.

Clustering

Identification of the optimal number of clusters and clustering was calculated and performed using gap statistics (58) in MATLAB R2019b (59) using the spearman correlation for species and CAZy identifiers, and Aitchison distance for samples. The difference of the CLR transformed values at any time point and its corresponding value at day 0 were used as the input. The data was sorted based on experimental variables or clusters and visualized.

Results

Sequencing parameters

A total of 140 samples were sequenced. On average, 389,680 reads were obtained per sample (mean 389,680, median 377,834, SD 118,596). Read N50 lengths averaged 4,043 bp (mean 4,043 median 4,052, SD 318). Reads had an average quality score of 12 (mean 12, median 12, SD 0.6). On average, 1,429,212,272 total bases were obtained per sample, with a standard deviation of 413,891,226 bases per sample. Four samples had low sequencing throughput and were removed from further analysis.

Taxonomic assignment

On average, 67% of reads were assigned at the species level (mean 66.9%, median 67.4%, SD 4.4%). A total of 119 taxa were identified (Supplementary Table 5). Of those, 27 taxa fit the criteria of being present in at least 75% of samples and relative abundance $>0.001\%$ in the pre-stress period and 18 taxa fit the criteria in the post-stress period. The final superset that was used for differential abundance analysis contained 27 taxa.

Stress significantly impacts microbial diversity and SCFP treatment leads to a more robust microbiome after stress

Alpha diversity was similar between Control and SCFP groups in the pre-stress period (Figure 2), indicating treatment with SCFP did not significantly alter Shannon microbial diversity index values. Stress impacted ($P < 0.0001$) diversity levels both in the Control and SCFP groups. However, stress had a lower impact in changing the SCFP group's diversity levels when compared to Controls. Overall, horses treated with SCFP exhibited robust microbial diversity after stress, with less variation and overall lower stress-induced drop in diversity when compared to the Control group (Supplementary Table 1). When within-group comparisons were made, statistical differences were observed in the Control group between several timepoints (Figure 2, gray dotted lines; Supplementary Table 2). On the other hand, fewer timepoints were significantly different from one another when within-group comparisons were made in the SCFP group (Figure 2, blue dotted lines; Supplementary Table 3), indicating that SCFP treatment might have contributed to more stable diversity levels post-stress.

Beta diversity was variable in the pre-stress period (Figure 3). At time 0 h (time at which the horses were untethered), horses assigned to the SCFP treatment formed two subclusters, whereas horses assigned to the Control treatment clustered in the same overall region (Figure 3, panel 1). On day 28, treated and untreated horses clustered in two overlapping groups (Figure 3, panel 2), and became homogeneous over time, with no clear difference between Control and SCFP-treated horses on day 56 (Figure 3, panel 3). However, Control and SCFP-treated horses had two completely different clustering trajectories after stress, with SCFP and Control horses clustering separately at 0 and 12 h post-stress (Figure 3, panels 4 and 5) and culminating again in a homogenous group at 72 h post-stress (Figure 3, panel 7).

The stress impact was greater for Control horses

Stress challenge and SCFP treatment significantly influenced microbial composition at the species level (PERMANOVA of Aitchison distances: Treatment, $P = 0.01$; Timepoint, $P = 0.01$; Treatment $\times$ Timepoint, $P = 0.01$). Two species clusters were identified (Figure 4A, vertical clusters A and B). The larger cluster (cluster A—18 species) comprised mainly species that increased in abundance after stress challenge. The smaller cluster (cluster B—nine species) comprised species that decreased in abundance after stress challenge (Figure 4A, vertical clusters A and B, Supplementary Table 5). Notably, Control horses had a much more marked reduction in species belonging to cluster B after stress when compared to those treated with SCFP. When total microbial composition was used as a basis for clustering analysis of the samples, five major sample clusters were identified (Figure 4A, clusters I, II, III, IV, and V). Very different trajectories were observed between the SCFP and Control treatments after stress (Figure 4B), with microbial composition of Control horses

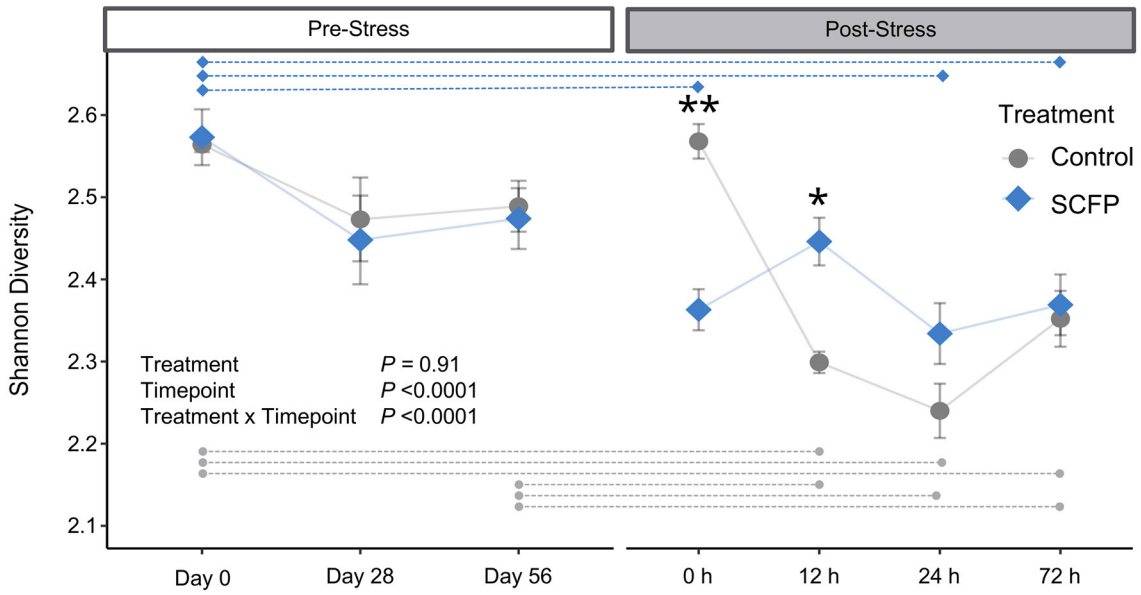

FIGURE 2
Alpha diversity comparisons. Shannon diversity metrics were analyzed with a linear model which included horse as a random effect, treatment, timepoint, and their interactions. Raw means and standard deviations are shown in the plot. Blue diamonds represent SCFP, and gray circles represent Control. Transparent lines represent the hypothetical trajectory of diversity. Dashed horizontal lines represent within-group pairwise significant differences at the 0.05 level after Bonferroni multiple comparison adjustment. Asterisks indicate timepoints in which SCFP significantly differs from Control $^{**}P < 0.01$, $^{*}P < 0.05$.

mostly belonging to cluster V, while SCFP treated horses exhibited microbiome compositions representatives of all clusters.

Stress challenge resulted in significant differential abundances in a time-dependent manner

Treatment with SCFP significantly increased the abundances of *Erysipelotrichaceae* before stress challenge. In fact, this was the only significantly different taxa between Control and SCFP in the pre-challenge period (Figure 5A, panels 1 and 2), and SCFP treated animals had an overall positive log ratios throughout the entire study (Supplementary Figures).

Many more species were significantly differentially abundant after stress challenge particularly at times 0 and 12 h post-stress (Figure 5B). At time 0 h, eight species were significantly increased in the SCFP group compared to Control, and three species were significantly decreased (Figure 5B, panel 1). Statistically different species were observed between groups up to 24 h after stress (Figure 5B, panels 2 and 3), with no significantly different species observed at 72 h after stress (Figure 5B, panel 4).

SCFP treated horses demonstrated more robust microbial functionality post-stress as compared to Control horses

Clustering analysis of the functional potential of the samples, measured by CAZy families, identified two major functional sample clusters (Figure 6A, clusters I and II). The CAZy families identified were Auxiliary Activity Family (AA), Carbohydrate-Binding Module Family (CBM), Carbohydrate Esterase Family (CE), Glycoside Hydrolase Family (GH), Glycosyl Transferase Family (GT), and Polysaccharide Lyase Family (PL). A more pronounced increase in CAZy families was observed after stress in Control horses compared to SCFP horses (Figure 6A). Similar to compositional clustering outcomes, Control and SCFP groups exhibited markedly different functional profiles following imposition of the stressor (Figure 6B), with Control horses demonstrating a switch to cluster I immediately after stress challenge, and again completely switching to cluster II from 12 to 72 h post-stress. Conversely SCFP treated horses displayed microbiome functional potential representatives of both clusters throughout the entire study period.

Correlation networks reveal that post-stress microbial communities are more stable in SCFP treated horses

Overall, a smaller number of significant interactions were observed in the SCFP group compared to Control, particularly following stress (SCFP = 198 positive interactions and 30 negative interactions: Control = 304 positive interactions and 210 negative interactions; Supplementary Table 4). Treatment with SCFP resulted in a smaller number of significant species interactions overall (maximum of 310 interactions before challenge) while the Control group had a total of 520 interactions before challenge. Horses that received SCFP had fewer interactions in total compared

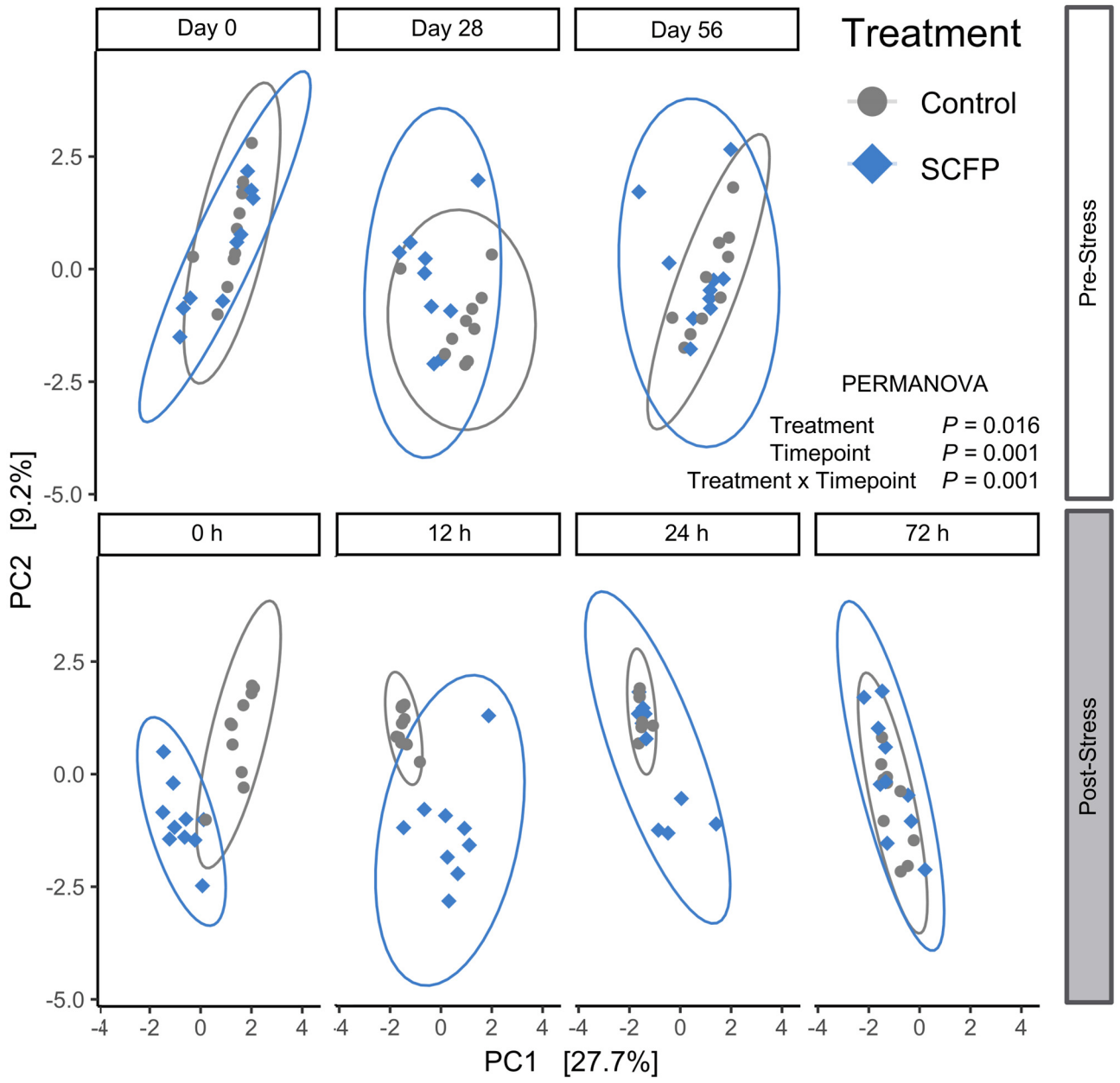

FIGURE 3
Beta diversity comparisons. Aitchison distances were analyzed with permutational ANOVA (PERMANOVA) model which included treatment, timepoint, and their interactions. Plots depict principal component analysis of Aitchison distances with CLR transformed data.

to Control, both pre- and post-stress. While no difference was observed in the percentage of positive interactions before stress, SCFP treated horses had a substantially higher number of positive interactions after stress when compared to untreated Control horses (87 vs. 59%).

Discussion

To evaluate the potential effect of supplementing horses under stressful conditions with a postbiotic, we sequenced the fecal metagenomes of 20 horses undergoing a previously established stress model that mimics prolonged transportation. The rationale that SCFP supplementation could lead to improved microbiome stability is based on recent reports of SCFP having a positive impact in other species undergoing stressful conditions (22, 24, 60–62). Here, we observed that untreated Control horses and treated (SCFP) horses presented very different microbiome trajectories upon stress, both in within- and between-sample diversity measurements. Moreover, a lower magnitude of changes was observed in the functional potential and microbial profile of SCFP horses vs. Control. Taken together, these observations led us to conclude that treatment with SCFP resulted in more robust and stable microbial profiles in horses after stress challenge.

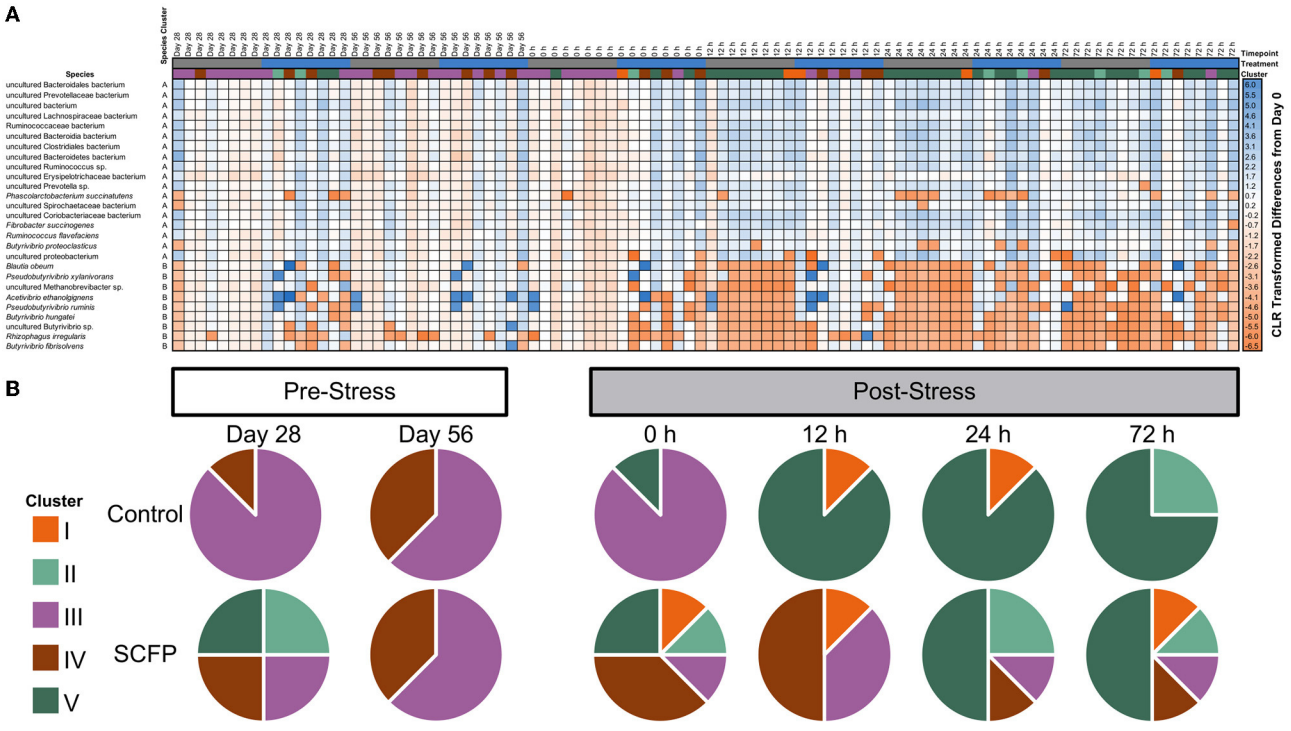

FIGURE 4
Stress challenge results in different microbial profiles in Control and SCFP treated horses. **(A)** Heatmap of centered log-ratio relative abundance of bacterial species detected in fecal samples of horses. **(B)** Pie charts represent the presence of samples across different clusters for Control and SCFP groups across time. Species cluster A contains species that drastically increase in relative abundance upon stress challenge, whereas species cluster B is comprised of species which decrease after stress challenge.

Less variation in microbial and functional profiles were observed for SCFP compared to Control horses. This was noted in several of our analyses including Shannon diversity index, total number of microbial network interactions, percentage of positive network interactions, and microbial and functional clustering profiles, which was illustrated in heatmaps with Control horses having a higher degree of change than SCFP treated horses. These data led us to conclude that dietary SCFP supplementation results in a more stable and robust community that is less impacted by stress. Our findings are in agreement with Tun et al. (24) who observed that postbiotic treatment tends to stabilize the microbiota of cows in a subclinical acidosis challenge. In that study authors concluded that SCFP supplementation attenuated the impacts of subacute ruminal acidosis on the composition and functionality of the rumen microbiome. Taken together, our results and those from others leads us to hypothesize that SCFP treatment results in a microbial community that is more robust (defined as resistance against change) in responding to stress. This hypothesis is corroborated by observations in our correlation network analyses, where the number of connections generally decreased with SCFP, but the percentage of positive interactions increased, indicating that a leaner (defined as a community with fewer connections among members but with a larger number of positive connections) and more connected microbial community in SCFP horses.

Our results are also in agreement with other studies that demonstrated that postbiotic supplementation is associated with improved microbiome balance which has been translated into host health in many species (23, 60, 62, 63). In horses, postbiotic supplementation resulted in increased relative abundances of fibrolytic bacteria (64) and attenuated exercise-induced stress markers (28). Additionally, Lucassen et al. showed that postbiotic-treated horses have more efficient response to vaccination (23). In contrast, a recent study by the same group evaluating SCFP supplementation did not identify significant alterations in the fecal microbiota of thoroughbred racehorses (27). Possible explanations for this disparity are the use of a lower resolution technique (16S rRNA gene sequencing) and the smaller dataset (11 horses) in that study when compared to our study, which used shotgun metagenomics to analyze the fecal microbiome of 20 horses. Additionally, those authors reported a high degree of horse-dependent effects of treatment, which can be attributed to high horse-to-horse variability.

The potential effects of stress and SCFP treatment on CAZy families was evaluated due to the importance and dependence of the horse on the degradation of structural carbohydrates of forages by gut microbiome for health and wellbeing. We observed that SCFP stabilized the composition and functionality of the hindgut microbial community. This was observed particularly immediately after stress relief (0 h) where lower CAZy abundance was observed in Control horses (light green in most cases) while abundances remained relatively unchanged or increased in SCFP (with the exception of one horse). At 12 h post-stress, Control horses displayed a dramatic switch in functional profile, with most

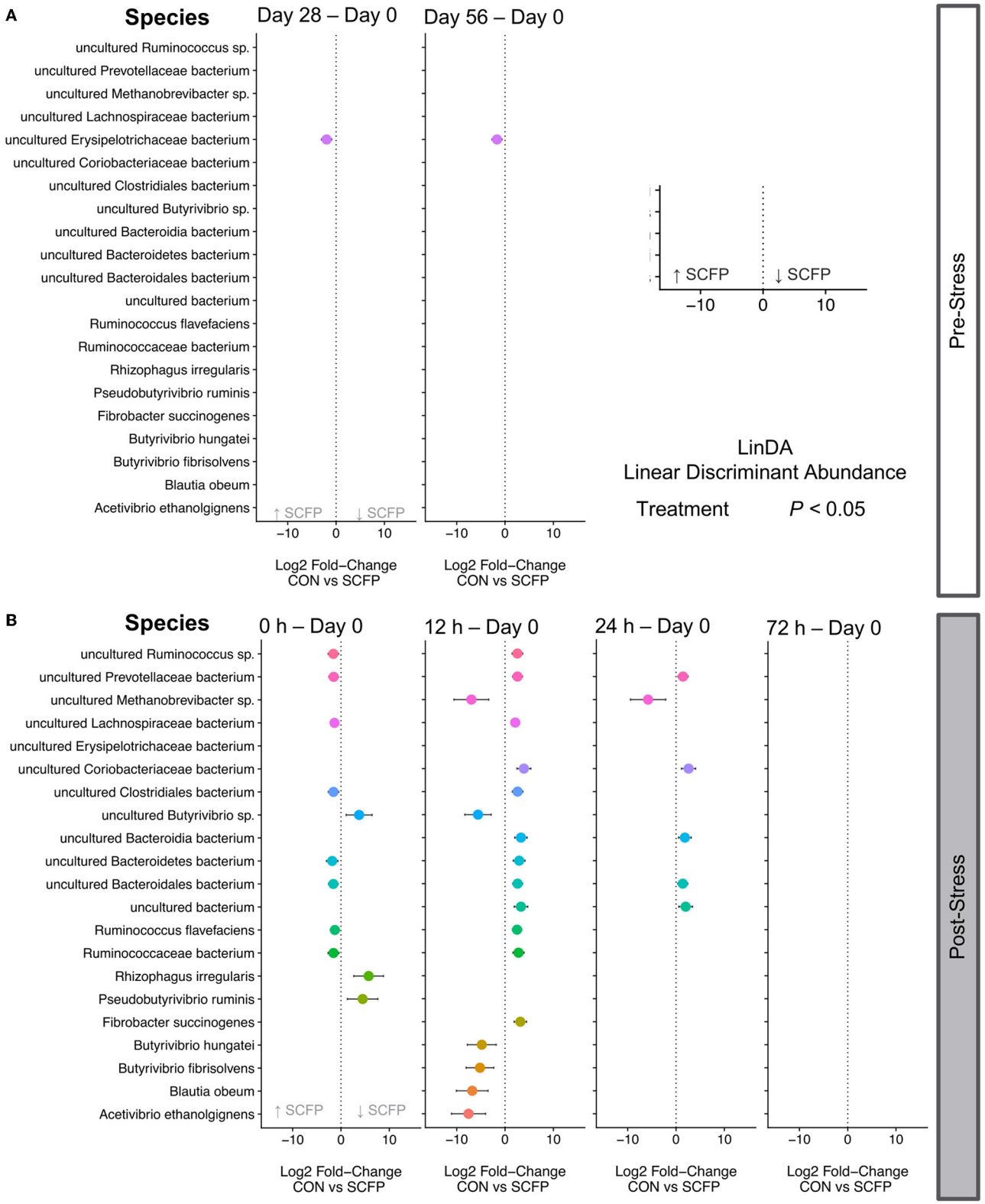

FIGURE 5
Differential abundances. The effect size (log2-fold change) is shown for each species, and only significantly different species are shown in each plot with their correspondent confidence interval. Statistical models included relative abundance as the dependent variable, horse as a random effect, treatment, timepoint, and their interactions as independent variables. Multiple hypothesis testing correction was performed with Benjamini Hochberg False Discovery Rate method. A negative fold change indicates an increase in relative abundance in SCFP compared to Control, and a positive fold indicates a decrease in relative abundance in SCFP compared to Control. **(A)** Differentially abundant species before stress. **(B)** Differentially abundant species after stress.

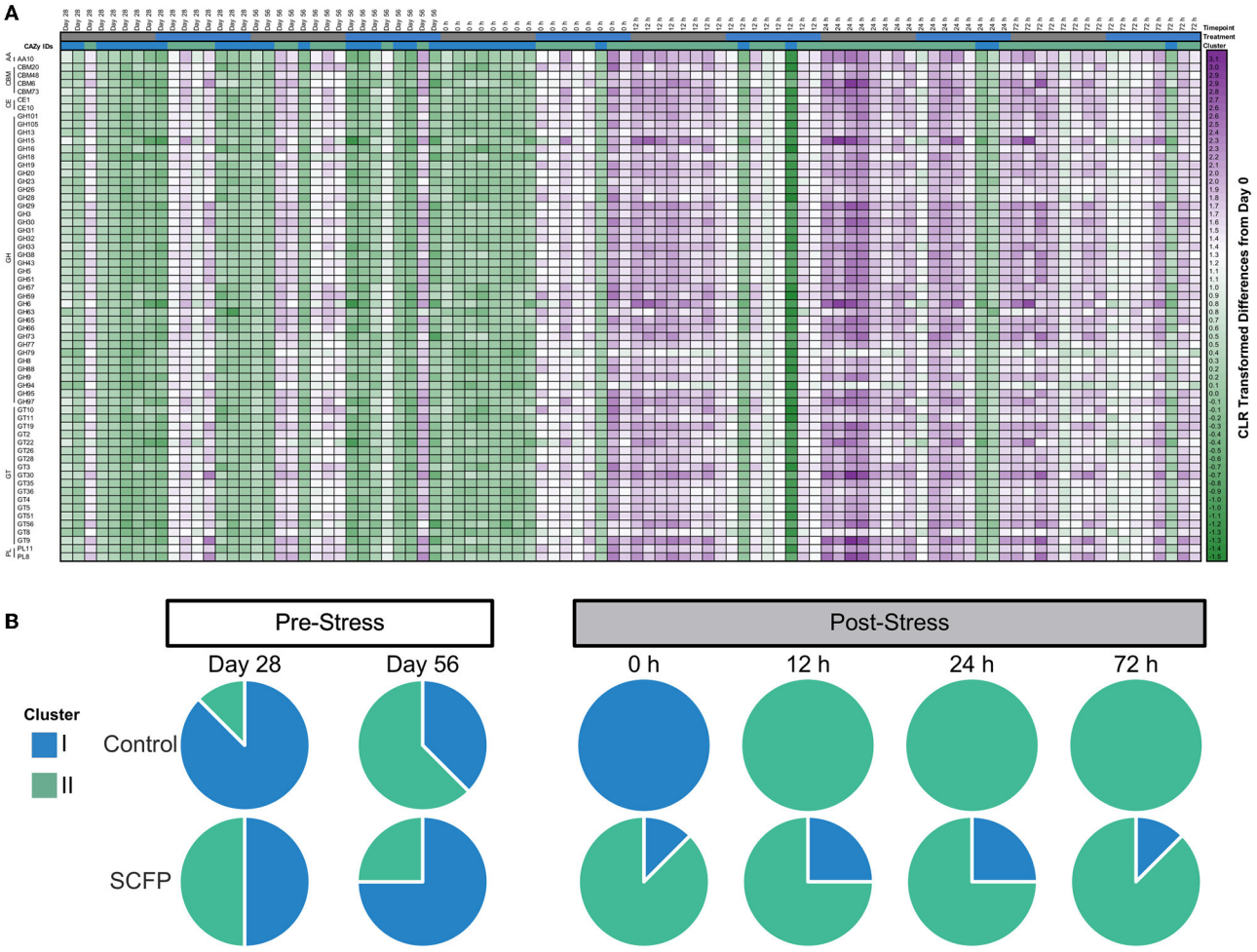

FIGURE 6
Functional potential is differentially impacted by treatment and stress in SCFP and Control horses. **(A)** Heatmap of centered log-ratio relative abundance of carbohydrate active enzymes (CAZy) families detected in fecal samples of horses. **(B)** Pie charts represent the presence of samples across different clusters for Control and SCFP groups across time. AA, Auxiliary Activity Family; CBM, Carbohydrate-Binding Module Family; CE, Carbohydrate Esterase Family; GH, Glycoside Hydrolase Family; GT, Glycosyl Transferase Family; PL, Polysaccharide Lyase Family.

showing increased relative abundances as illustrated in the heat maps in Figure 6. The small sample size of this study precludes us from making further statements regarding the functional potential, but what is evident from this study is that larger swings in relative abundances of CAZy families were associated with Control horses when compared to treated horses throughout the entire post-stress period. Additionally, a strong horse-to-horse effect was observed, indicating that treatment effect is highly dependent on the animal. These findings are similar to those of Lucassen et al. (27) who observed a high degree of horse-to-horse variability in their study of the equine microbiome of horses fed a postbiotic.

The bacteria identified in our study are in agreement with previous reports of healthy equine gut microbiomes, with a composition that is mainly dominated by fibrolytic bacteria (8, 9, 65). It is important to highlight that we chose a very strict threshold for taxa selection for statistical comparisons between treatment groups. Specifically, to be included in the statistical analysis, a microbial species had to be present in at least 75% of all samples. This was a deliberate choice to decrease the chances for spurious findings due to multiple hypothesis testing.

Here, we identified that SCFP treatment significantly impacted the relative abundance of *Erysipelotrichaceae* before stress, with a small, but significant increase in SCFP treated horses compared to Control horses. Biddle and colleagues also observed significant temporal changes in *Erysipelotrichaceae* in obese horses (66), and this family had previously been identified as part of the core microbial community of horse feces (67). However, little is known about the role of this species in the horse gut and diverging evidence has been presented about the role of *Erysipelotrichaceae* in other organisms, with varying levels of *Erysipelotrichaceae* reported in murine and human studies of disease (12, 68).

Immediately after stress 11 bacteria were identified to be significantly different albeit with very small effect sizes. From those, eight were increased in the SCFP group and three were increased in the Control group, with relatively higher effect sizes when compared to species increased in SCFP horses. Microorganisms significantly increased in Control horses included one uncultured *Butyrivibrio* species, *Pseudobutirivibrio ruminis*, and *Ryzophagus irregularis*. We identified *Ryzophagus irregularis*, an arbuscular mycorrhizal fungus that is common in plants, and which (69) has

not been previously reported in the horse gastrointestinal tract. Given the presence of this organism in many plant species, and the plant-based diet of horses, this finding is not completely surprising.

A larger number of significantly different species were observed at 12 h post stress. Out of 18 significantly different species, six had relatively high effect sizes and were increased in SCFP horses. These included three *Butyrivibrio* species in addition to *Blautia*, *Acetivibrio*, and *Methanobrevibacter*, which were found to have significantly higher relative abundances in SCFP treated horses 12 h after stress. *Butyrivibrio* are very versatile bacteria and encode a variety of enzymes to hydrolyze complex carbohydrates (70, 71). They have been reported to carry many genes encoding glycoside hydrolases (GH) that are involved in carbohydrate fermentation and butyrate production. Likewise, *Blautia* and *Acetivibrio* are also fiber fermenters (8). In agreement with increases with *Butyrivibrio* species, in our functional annotation analyses, 36 out of 62 enzymes found to be significantly different in the present study encode for glycoside hydrolases. Lastly, *Methanobrevibacter* was also identified to be increased in the SCFP group at 12 and 24 h post stress. The presence of methanogenic archaea in the horse gut has been previously reported, and the diversity of methane producers in the horse gut is believed to be high (8, 72).

Despite the wealth of data collected as part of this project we acknowledge that a sample size of 20 horses is relatively small. It was further substantiated that horse intrinsic factors impacted the response to stress or treatment, as it could be observed by single animals behaving differently than the remainder of the group at a given timepoint. In fact, horse-to-horse variability has been well-documented in immune parameters in horses subjected to this model of stress (33). Raidal et al. observed varying degrees of change in white cell count, neutrophil count, and total bacterial numbers in six horses subjected to prolonged head elevation (33). This added variability might have confounded our analyses and precluded us from identifying strong signals. Nevertheless, animals are different and thus further research should account for animal-to-animal changes and perhaps quantify the effect in terms of microbiome changes within an animal. Even with the relatively high heterogeneity of this dataset we were able to identify a clear overall signal that treatment with SCFP tends to promote microbiome robustness and stability after stress, as we observed in measures of alpha and beta diversity, as well as bacterial and functional profiles, and bacteria interaction dynamics.

This study adds to the body of knowledge regarding the beneficial impacts of postbiotic administration to horses undergoing stressful situations. While the specific mechanisms by which this robustness and stability are imparted in an equine's gut microbiome by postbiotic administration are not fully elucidated, studies in other species suggested that potential underlying mechanisms by which postbiotic supplementation led to improved health include effects in immunomodulatory pathways (73) and improved microbiome composition and functionality (24). From an immune perspective, animals receiving SCFP seem to be primed to respond with elevated (magnitude of response) and accelerated (speed of response) cytokine production when a threat is detected (61, 62). Additionally, at the site of challenge, increased phagocytic activity and killing ability of white blood cells and reduced activation of inflammatory system leads to a reduction in localized inflammation, and potentially immunopathology in SCFP supplemented animals (62, 74). From the microbiome perspective, ruminant studies have shown that SCFP supplementation boosts the abundances of influential members of the microbiome which promote richness and diversity, and hence, functionality of the microbiome resulting in increased VFA production and improved energetic efficiency of rumen fermentation (24, 75). Therefore, it can be speculated that the dual action of SCFP postbiotic *via* immunomodulatory pathways and optimized microbiome functionality increases robustness of animals against a wide range of infectious and metabolic stressors.

Our results indicate that prophylactic supplementation with a yeast-derived postbiotic might be a beneficial strategy for horses prior to exposure to stress. This exploratory study is limited in the ability to draw mechanistic conclusions on the effects of SCFP in horses subjected to a stress model. We observed a lower degree of change both in microbial diversity and functional profile of horses fed SCFP when compared to Control. Mechanistically, having a more robust and stable microbiome plausibly results in less opportunity for pathogen colonization and better health maintenance. Postbiotics have been demonstrated to have positive impacts in several species, and further research into the mechanisms by which these beneficial effects occur is warranted.

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 at: https://www.ncbi.nlm.nih.gov/, bioproject PRJNA788958.

Ethics statement

The animal study was reviewed and approved by Institutional Animal Care and Use Committee at the University of Florida in Gainesville, FL (#201810324).

Author contributions

LW designed the animal experiment. EK and BK designed the microbiome study. MT performed the animal experiment and collected samples. MS performed sequencing and bioinformatics. AC performed statistical analyses. EG, EK, and SN interpreted the data. EG and EK prepared the first draft. All authors read and approved the final manuscript.

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Conflict of interest

AC, MS, BK, SN, and EK were employed by the company Cargill Inc.

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.

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fvets.2023.1134092/full#supplementary-material

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The inhibition of high ammonia to *in vitro* rumen fermentation is pH dependent

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Ammonia is an important rumen internal environment indicator. In livestock production, feeding a large amount of non-protein nitrogen to ruminants will create high ammonia stress to the animals, which increases the risk of ammonia toxicity. However, the effects of ammonia toxicity on rumen microbiota and fermentation are still unknown. In this study, an *in vitro* rumen fermentation technique was used to investigate the effects of different concentrations of ammonia on rumen microbiota and fermentation. To achieve the four final total ammonia nitrogen (TAN) concentrations of 0, 8, 32, and 128 mmol/L, ammonium chloride (NH_4Cl) was added at 0, 42.8, 171.2, and 686.8 mg/100 mL, and urea was added at 0, 24, 96, and 384 mg/100 mL. Urea hydrolysis increased, while NH_4Cl dissociation slightly reduced the pH. At similar concentrations of TAN, the increased pH of the rumen culture by urea addition resulted in a much higher free ammonia nitrogen (FAN) concentration compared to NH_4Cl addition. Pearson correlation analysis revealed a strong negative correlation between FAN and microbial populations (total bacteria, protozoa, fungi, and methanogens) and *in vitro* rumen fermentation profiles (gas production, dry matter digestibility, total volatile fatty acid, acetate, propionate, etc.), and a much weaker correlation between TAN and the above indicators. Additionally, bacterial community structure changed differently in response to TAN concentrations. High TAN increased Gram-positive Firmicutes and Actinobacteria but reduced Gram-negative Fibrobacteres and Spirochaetes. The current study demonstrated that the inhibition of *in vitro* rumen fermentation by high ammonia was pH-dependent and was associated with variations of rumen microbial populations and communities.

KEYWORDS

ammonium chloride, free ammonia, microbiota, pH, rumen fermentation, urea

Introduction

Ruminants, which provide almost all of the milk and much of the meat consumed by humans globally, are of great importance in agricultural production (1, 2). The rumen is a unique digestive and metabolic organ of ruminants, and it contains a diverse microbiota consisting of bacteria, protozoa, fungi, archaea, and viruses (3). The rumen microbes can produce enzymes to digest crude fibers that cannot be digested by the host itself to short-chain fatty acids (primarily acetate, propionate, and butyrate), providing the main energy source and fat synthesis precursors for the host (4). Besides, these microbes also synthesize microbial proteins from ammonia, which is derived from deamination of amino acids and hydrolysis of non-protein nitrogen (NPN) such as urea, providing the primary protein

synthesis precursors for the host (5, 6). A proper rumen internal environment (pH, ammonia concentration, etc.) is critical to ensure efficient degradation of crude fibers and microbial protein synthesis in the rumen (3).

Ammonia can be utilized by many rumen microbes to synthesize microbial protein. In order to lower production costs, NPN (primarily urea) is used to replace part of high-quality protein sources (such as soybean meal) fed to ruminants (6–8). However, if a high dose of NPN is added, rumen ammonia concentration can increase rapidly, leading to ammonia toxicity (8). Total ammonia nitrogen (TAN) in aqueous phase exists in two different molecular forms, NH_3 as free ammonia nitrogen (FAN) or NH_4^+ as ammonium ions. The equilibrium concentration between NH_3 and NH_4^+ follows the Henderson-Hasselbalch equation and depends on pH and temperature (9). One previous study has found that ammonia toxicity is positively correlated with rumen pH and blood ammonia, but not with rumen TAN (10). Generally, the rumen temperature is relatively stable. Therefore, rapid increase of FAN concentration in the rumen at high pH is probably the primary cause for the rapid increase of ammonia absorption through the rumen epithelium leading to ammonia toxicity (7, 8, 11).

High ammonia stress is a major problem frequently encountered in anaerobic digestion for biogas production, a technology commonly used to treat organic wastes (9). The performance of an anaerobic reactor is directly associated with the structure of the microbial community present therein (12). Free ammonia levels are considered the foremost cause of inhibition of methanogens due to its high permeability to cell membrane (9, 13). For anaerobic digestion in biogas production, the goal is to maximize methane yield while reducing volatile fatty acid (VFA) accumulation (14). In contrast, for rumen fermentation, the goal is to maximize feed digestion and VFA production while reducing methane emissions (15). Besides, the operation parameters of anaerobic digesters (microbial composition, pH, temperature, ammonia concentration, etc.) are considerably different from those of rumen fermentation. Therefore, the specific response mechanism of the microbiota to ammonia toxicity may be different between the two different anaerobic digestion systems. Previous studies in ruminants have focused on the effects of ammonia toxicity on animal health (10, 16, 17). To our knowledge, however, no studies had reported the effects of ammonia toxicity on rumen microbiota even though ammonia toxicity adversely affects the animals. It is hypothesized that high FAN may affect the structure of rumen microbiota, leading to the inhibition of rumen fermentation.

Different NPN has varied acidity or alkalinity. Free NH_3 produced from urea hydrolysis mediated by microbial urease in the rumen is a weak base, and it can neutralize the acidity produced by rumen fermentation and buffer the rumen pH to some extent (7). In contrast, NH_4Cl, which is also an NPN additive commonly used in ruminants, is a weak acid after it is dissolved in water (17, 18). In this study, a rumen pH and ammonia level model was implemented by changing the amount and type of NPN (NH_4Cl vs. urea) in an *in vitro* rumen fermentation system, and this model was used to investigate the effects of NH_4Cl and urea addition on rumen microbial composition and fermentation profiles. The results helped reveal the microbial mechanism by which high ammonia inhibit rumen fermentation and could inform the improvement of ammonia utilization by the rumen microbiota.

Materials and methods

Experimental design

The experiment was designed in a 2 × 4 factorial arrangement: two nitrogen sources (NH_4Cl and urea) and four TAN levels (0, 8, 32, and 128 mmol/L). The rumen temperature is maintained quite stable varying within the narrow range of 38–41°C, and rumen TAN concentration and pH fluctuated between 1–40 mM and 5.5–7.2, respectively (19). In the present study, the tested TAN levels covered the TAN concentrations found in the rumen. To achieve the four final TAN concentrations of 0, 8, 32, and 128 mmol/L, NH_4Cl was added at 0, 42.8, 171.2, and 686.8 mg/100 mL (A-0, A-8, A-32, and A-128), and urea was added at 0, 24, 96, and 384 mg/100 mL (U-0, U-8, U-32, and U-128). Each treatment had four replicates.

Ruminal inoculum and *in vitro* incubation

All animal protocols were approved by the Animal Care and Use Committee of Nanjing Agricultural University (protocol number: SYXK2017-0007).

Three rumen cannulated male sheep (Body weight = 32 ± 2 kg) served as ruminal fluid donors for this *in vitro* study. The diet fed to these sheep contained (% DM basis) 45% forage (25% corn silage and 20% peanut vine) and 55% concentrate (42% ground corn, 4% soybean meal, 4% wheat bran, and 5% premix). The dietary nutrient composition (DM basis) of crude protein (CP), neutral detergent fiber (NDF), acid detergent fiber (ADF), and ether extract (EE) were 15.6, 32.7, 20.4, and 3.1% respectively, and digestible energy (DE) was 13.8 MJ/kg, which met the feeding standards of meat-producing sheep and goats (20). The sheep were fed twice daily at 08:00 and 18:00, and they had free access to feed and water. Ruminal contents were collected through rumen cannula from the three donor sheep before morning feeding, mixed with an equal volume, and then poured into a sterilized bottle (1,000 mL) leaving no headspace in the bottle, which was taken to the laboratory within 30 min. The mixed rumen sample was then squeezed through four layers of cheesecloth into a flask under a continuous flux of CO_2 in a water bath kept at 39°C until use.

The *in vitro* batch fermentation was performed in 180 mL serum bottles. The fermentation substrate was the same feed fed to the three sheep that donated the rumen sample. The buffered medium for the *in vitro* fermentation was prepared anaerobically as described by Theodorou et al. (21) with minor modification: Ammonium bicarbonate was replaced with equivalent amounts of sodium bicarbonate to eliminate the background nitrogen content in the buffered medium. The anaerobic buffer medium and strained rumen fluid inoculum were combined in each bottle in a 9:1 (v/v) ratio under anaerobic conditions. A 100-mL mixture was immediately dispensed into each incubation bottle containing 1 g of ground feed substrate and respective additions of NH_4Cl and

urea. To prevent exposure to air, the headspace of the bottles was continuously flushed with CO_2 before they were each sealed with a butyl rubber stopper and secured with an aluminum crimp seal. The *in vitro* cultures were incubated at 39°C for 24 h in a water bath with intermittent shaking by hand after gas measurement at each designed time point.

Sampling and chemical analysis

Gas production was measured at 0.5, 2, 4, 6, 8, 12, and 24 h using a pressure transducer (21). After gas measurement at each time point, 1 mL of each culture was collected for subsequent analysis for ammonia-N using a colorimetric method (22). At the end of the 24 h of incubation, the pH value of each *in vitro* culture was measured immediately using a portable pH meter. Then, 1 mL of culture each was preserved by adding 0.2 mL of 25% HPO_3 for VFA analysis using gas chromatography (7890A, Agilent, UK) according to the method described by Mao et al. (23). Also, 1 mL of culture each was collected for DNA extraction and subsequent microbial analysis. All the samples were stored at −20°C until analyses. The remaining content of each culture was filtered through a filter bag (ANKOM Technology, USA) to analyze apparent dry matter (DM) digestibility gravimetrically (24).

The free ammonia concentrations at 24 h after incubation were calculated based on the following equation as described by Rajagopal et al. (9):

$$\mathrm{FAN} = \mathrm{TAN} \times \left(1 + \frac{10^{-\mathrm{pH}}}{10^{-(0.09018 + \frac{2729.92}{\mathrm{T(K)}})}}\right)^{-1}$$

where FAN is the concentration of free ammonia (mmol/L); TAN is the concentration of total ammonia (mmol/L); T(K) is the rumen temperature (Klevin), which was fixed at 312.15 K (39°C) in the current study; and pH is the pH value of each culture measured after 24 h incubation.

DNA extraction and real-time quantitative PCR analysis

Microbial genomic DNA of the rumen culture samples was extracted using the bead-beating and phenol-chloroform extraction method (25). The DNA integrity was examined using agarose (1.2%) gel electrophoresis, and the DNA quantity of each sample was determined using a Nanodrop 2000 (Thermo Fisher Scientific, Inc., Madison, USA).

The PCR primers used for real-time quantitative PCR (qPCR) of total bacteria (25), fungi (26), protozoa (27), and methanogens (28) are listed in Supplementary Table 1. Real-time qPCR was performed on a StepOnePlus system (Applied Biosystems, California, USA) using the SYBR Premix Ex Taq dye (Takara Bio Inc.). Copies of 16S rRNA gene (total bacteria), methyl coenzyme-M reductase alpha-subunit gene (*mcr*A, methanogens), and 18S rRNA gene (fungi and protozoa) in each sample were performed in triplicate. Standard curves were generated using 10-fold serial dilutions of purified plasmid DNA containing the target gene sequences of each microbial group. The absolute abundance of each microbial population was expressed as copies of the target gene/mL of each sample.

Illumina sequencing of 16S rRNA gene amplicons and data analysis

The V3-V4 region of the 16S rRNA gene was amplified using primers 341F (5′-CCTACGGGAGGCAGCAG-3′) and 806R (5′-GGACTACHVGGGTWTCTAAT-3′). Unique barcodes were added to the 5'end of both primers for multiplexing. PCR products were verified on agarose gel (2%, w/v), and the expected bands were each extracted and purified using the QIAquick PCR Purification Kit (Qiagen, CA, USA). The concentrations of the purified DNA amplicons were each quantified using a QuantiFluor® dsDNA kit (Promega, Madison, WI, USA). Amplicons from all the samples were mixed in equal ratio and sequenced on an Illumina MiSeq platform to produce 250-bp paired-end reads. The raw sequence reads were deposited into the NCBI Sequence Read Archive (SRA) database under the accession number PRJNA940661.

Raw FASTQ files were de-multiplexed, quality-filtered (minimum Q score = 25), and analyzed using QIIME 1.9.1 (29). Operational taxonomic units (OTUs) were *de novo* clustered using UPARSE with a 97% sequence similarity (30), and possible chimeras were identified and removed using UCHIME (31). The most abundant sequence within each OTU was selected as the representative sequence and was taxonomically classified based on the SILVA database (version 138) (32). Sequences identified as of chloroplasts or mitochondria were removed before further analysis. The representative sequence of each OTU for each sample was aligned using MUSCLE (33), and the alignment was used to create a phylogenetic tree using FASTTREE (34). Principal coordinates analysis (PCoA) was performed based on Bray-Curtis dissimilarity to reveal overall differences in the bacterial communities among the different treatments. Analysis of similarities (ANOSIM) was performed to determine group similarity, where 0 = indistinguishable and 1 = dissimilar (35).

Statistical analysis

The real-time qPCR data were log-transformed to improve normality. Residual analysis was used to determine if transformation of variables was needed. If needed, cubic root transformations were performed. All data (*in vitro* rumen fermentation parameters, microbial populations quantified by qPCR, relative abundances of bacteria at the phylum and genus levels) were analyzed using the MIXED procedure of SAS version 9.4 (SAS Institute Inc., Cary, NC) in a 2 (nitrogen source) × 4 (ammonia level) factorial design. The model included nitrogen source, ammonia level, and interaction of nitrogen source × ammonia level as fixed effects. Degrees of freedom were calculated using the Kenward-Roger option. Mean separation was performed using the Tukey multiple range test. Differences were considered statistically significant at $P \leq 0.05$. Pearson correlation coefficients were calculated using SAS version 9.4 to examine the correlation

between TAN or FAN and *in vitro* rumen fermentation parameters, microbial populations, relative abundance of rumen bacteria at the genus level (data of A0 and U0 were not included for correlation analysis because of the insufficient ammonia concentration). A significant correlation was declared at $P \leq 0.05$.

Results

Rumen total ammonia, pH, and free ammonia concentration

The addition of the pre-set amounts of urea and NH_4Cl resulted in final TAN levels similar to that of the design, but at different rates (Figure 1A). NH_4Cl dissociation can reach the target TAN level instantly after inoculation, while urea hydrolysis, which is catalyzed by urease, needs time to release ammonia. In the U-8 group, urea was hydrolyzed completely within 0.5 h, but it took about 4 and 12 h to complete hydrolysis of urea in the U-32 and U-128 groups, respectively.

There was significant interaction ($P < 0.01$) between nitrogen source and ammonia level for pH value and FAN concentration. The addition of urea and NH_4Cl had different effects on *in vitro* rumen pH (Figure 1B). With the increase of urea addition, the rumen pH increased from 6.56 to 7.35. In contrast, NH_4Cl addition slightly reduced the pH of the *in vitro* rumen culture. But the reduction magnitude is not very small, <0.07 pH units (from 6.57 to 6.50).

In the present study, the increased *in vitro* rumen pH in response to the urea addition resulted in a much higher FAN concentration ($P < 0.05$) compared to NH_4Cl at a similar TAN level of 32 or 128 mmol/L (Figure 1C). For instance, the pH of the U-32 group (6.84) was 0.30 unit higher than that of the A-32 group (6.54), while the FAN of the U-32 group (0.31 mmol/L) was more than twice of that of the A-32 group (0.15 mmol/L). In contrast, the pH of the U-128 group (7.35) was 0.85 unit higher than that of the A-128 group (6.50), but the FAN of the U-128 group (3.96 mmol/L) was 7 times higher than that of the A-128 group (0.57 mmol/L). Therefore, pH value is the key factor to determine the concentration of FAN in the rumen.

Gas production, dry matter digestibility, and volatile fatty acids profile

There was significant interaction ($P < 0.01$) between nitrogen source and ammonia level to gas production, DM digestibility, and concentrations of volatile fatty acids. Total gas production, DM digestibility, and concentration of total VFA, acetate, propionate, and butyrate increased when the TAN level was raised from 0 to 8 mmol/L (A-8 and U-8), but these characteristics showed different responses to the urea and NH_4Cl additions higher than 8 mmol/L (Figure 2). When TAN reached 32 mmol/L by adding NH_4Cl (A-32), gas production, DM digestibility, total VFA, and propionate concentration were similar to those observed in A-8 and U-8 ($P > 0.05$), but the above parameters was decreased significantly in A-128 group ($P < 0.05$). In contrast, the *in vitro* rumen fermentation was inhibited by U-32 ($P < 0.05$) and further inhibited by U-128, and the inhibition was much stronger than that observed in the A-128 group ($P < 0.05$). These results indicate that urea is more inhibitory to rumen feed digestibility and fermentation than NH_4Cl at high concentrations within the current pH range (6.54–7.35).

Microbial population

Significant interaction ($P < 0.01$) between nitrogen source and ammonia level was detected with respect to the absolute abundance of total bacteria, methanogens, protozoa, and fungi (Figure 3). In the present study, the abundance of total bacteria, fungi, and protozoa remained similar when TAN varied between 0 and 32 mmol/L ($P > 0.05$), but when TAN reached 128 mmol/L, the abundance of the above microbial groups decreased significantly ($P < 0.05$) irrespective of the ammonia-N source. However, U-128 had a greater inhibition than A-128 ($P < 0.05$). These results indicate that high TAN reduced the abundance of microbial populations, and increased FAN from urea hydrolysis might have aggravated the inhibitory effect.

Bacterial community structure

Principal coordinates analysis based on Bray-Curtis dissimilarity showed clear separations of the microbiota between the highest TAN level of 128 mmol/L (A-128 and U-128) and other TAN levels (Figure 4A). Besides, the difference between A-128 and U-128, U-32 and A-0 or U-0, and A-32 and U-0 was also significant as analyzed using ANOSIM ($P < 0.05$, Figure 4B). These results indicate that the rumen bacterial community structure changed in response to the two different nitrogen sources and ammonia levels.

A total of 19 bacterial phyla were identified across all the treatments, with Bacteroidetes (32.12–47.82%), followed by Firmicutes (16.13–40.84%), Proteobacteria (11.17–26.53%), Fibrobacteres (0.03–9.00%), Tenericutes (1.77–7.10%), Spirochaetes (0.70–7.00%), Actinobacteria (0.91–8.68%), and Fusobacteria (0.04–2.42%) being the eight most predominant phyla, each of which was represented by more than 1.0% of the total sequences in at least one treatment (Table 1).

No interaction ($P \geq 0.18$) of ammonia-N source with ammonia level was detected with respect to any of the bacterial phyla except for Actinobacteria ($P < 0.01$) and Fusobacteria ($P = 0.01$). There were significant differences in the relative abundance of Firmicutes, Proteobacteria, Fibrobacteres, Tenericutes, and Spirochaetes among different ammonia levels ($P < 0.01$). Compared with other ammonia treatment levels, the high TAN treatments (128 mmol/L) significantly increased Gram-positive Firmicutes and Actinobacteria ($P < 0.05$), while decreasing Gram-negative Fibrobacteres and Spirochaetes significantly ($P < 0.05$) and Gram-negative Bacteroidetes numerically ($P > 0.05$). These results indicate that some Gram-negative bacteria were sensitive, while Gram-positive bacteria were tolerant to high TAN treatment.

Different responses of rumen bacterial genera to the incremental additions of urea and NH_4Cl are shown in Table 2. There was significant interaction ($P \leq 0.01$) between nitrogen source and ammonia level with respect to the relative

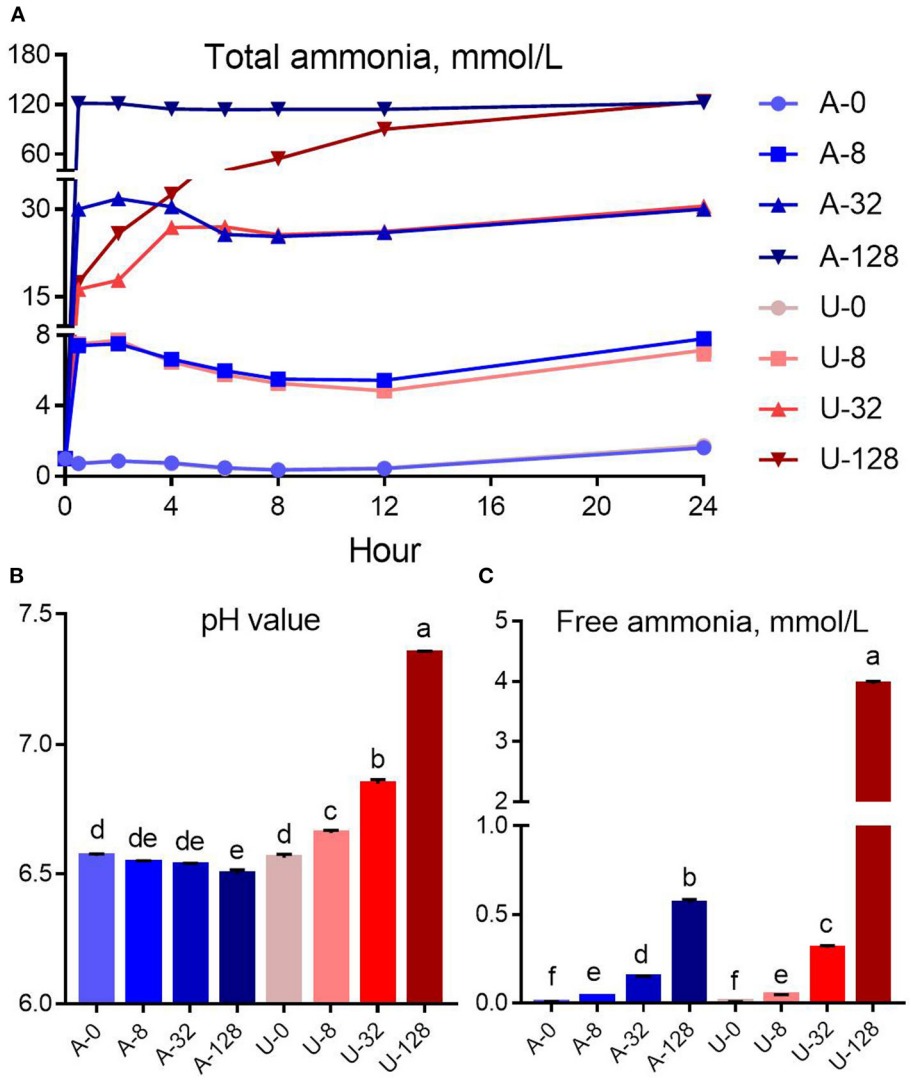

FIGURE 1
Effects of NH_4Cl and urea addition on the dynamic change of total ammonia **(A)**, pH value **(B)**, and free ammonia concentration **(C)** at 24 h of *in vitro* incubation. There was significant interaction ($P < 0.01$) between nitrogen source and ammonia level with respect to pH value and free ammonia concentration. Different letters on the top of the bars in each figure panel denote significant differences ($P < 0.05$).

abundance of *Prevotellaceae* YAB2003 group, *Prevotella* 7, *Selenomonas*, *Eubacterium* eligens group, *Anaerovibrio*, *Sharpea*, *Escherichia-Shigella*, *Bifidobacterium*, and *Fusobacterium*, but not for others.

Correlation analysis

To explore the correlations between TAN or FAN and *in vitro* rumen fermentation parameters, absolute abundance of microbial populations, the relative abundance of rumen bacteria at the genus level, Pearson's correlation analysis was performed. Pearson correlation analysis revealed a strong negative correlation between FAN and microbial populations quantified (i.e., total bacteria, protozoa, fungi, and methanogens) and *in vitro* rumen fermentation profiles (gas production, DM digestibility, total VFA, acetate, propionate, etc.) and a much weaker negative correlation between TAN and the above indicators (Figure 5). The correlation analysis between the relative abundance of bacterial genera and ammonia concentration (FAN and TAN, Figure 5) revealed the difference in tolerance to high ammonia between Gram-positive and Gram-negative bacteria. Most of the Gram-positive bacterial genera including *Agathobacter*, *Pseudobutyrivibrio*, *Butyrivibrio* 2, *Selenomonas*, *Ruminococcus* 1, *Ruminococcus* 2, *Eubacterium* eligens group, *Anaerobibrio*, *Sharpea*, and *Bifidobacterium* were positively correlated with FAN or TAN concentration ($P < 0.05$). In contrast, most of the Gram-negative bacterial genera including *Rikenellaceae* RC9 gut group, *Prevotellaceae* Ga6A1 group, *Bacteroidales* F082_norank, *Prevotellaceae* UCG-001, *Ruminobacter*, *Fibrobacter*, and *Treponema* were negatively correlated with the concentration of FAN or TAN concentration ($P < 0.05$). However, there are also a few exceptions. For example, Gram-negative bacteria of *Muribaculaceae*_norank, *Escherichia-Shigella*, and

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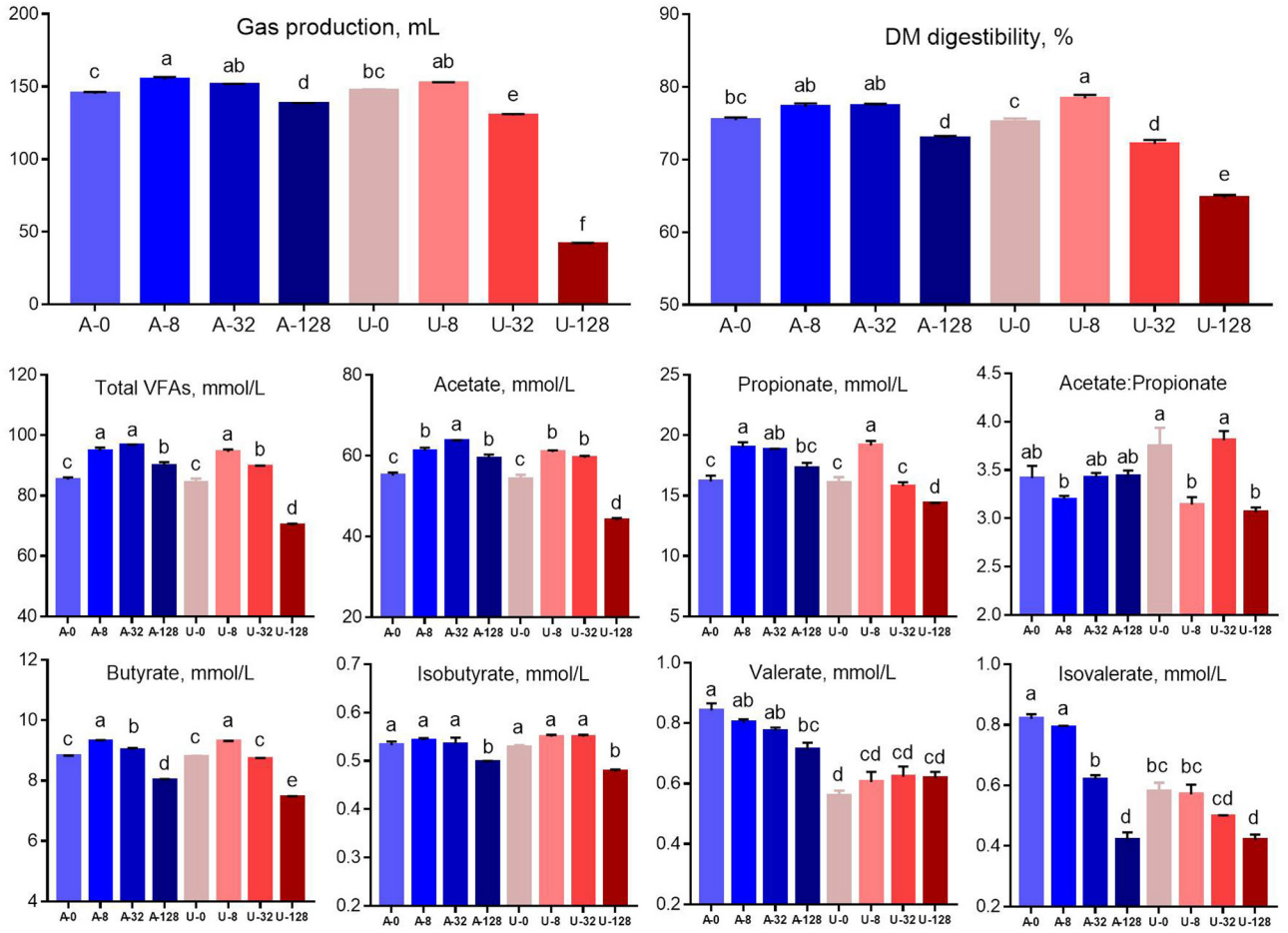

FIGURE 2
Effects of NH_4Cl and urea addition on gas production, dry matter (DM) digestibility, and concentrations of volatile fatty acids. There was significant interaction ($P < 0.01$) between nitrogen source and ammonia level with respect to gas production, DM digestibility, and concentrations of volatile fatty acids. Different letters on the top of the bars in each figure panel denote significant differences ($P < 0.05$).

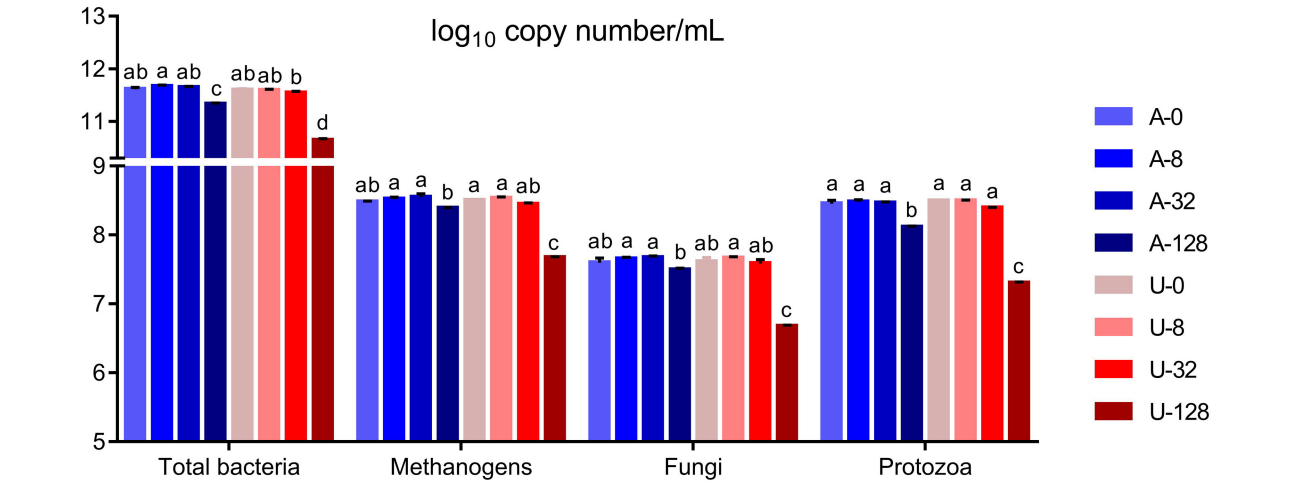

FIGURE 3
Effects of NH_4Cl and urea addition on the absolute abundance of total bacteria, methanogens, protozoa, and fungi (log10 copy number of the target genes/mL) in the *in vitro* rumen mixed cultures. There was significant interaction ($P < 0.01$) between nitrogen source and ammonia level with respect to the abundance of total bacteria, methanogens, protozoa, and fungi. Different letters on the top of the bars in each figure panel denote significant differences ($P < 0.05$).

Fusobacterium also showed a positive correlation with FAN or TAN concentration ($P < 0.05$).

Discussion

Ammonia and VFA are continuously produced by the rumen microbiota and then absorbed through the rumen wall. On the other hand, urea is constantly diffused back to the rumen. Therefore, an *in vitro* system was used to avoid the interference of absorption and diffusion across the rumen epithelia. The use of an *in vitro* system also allowed us to precisely control and test different levels of NH_4Cl and urea. Previous studies have demonstrated that high FAN induced rapid absorption of ammonia through the rumen epithelium leading to ammonia toxicity (10, 16), but no studies had reported the effects of high FAN on rumen microbiota and fermentation. Combining high-throughput sequencing and real-time qPCR using an *in vitro* fermentation system, this study for the first time evaluated the effects of high FAN on the rumen microbiota and fermentation. Moreover, this study also provided a practical guidance for the utilization of NPN to improve rumen fermentation.

In this study, it was found that the time required for complete hydrolysis of different doses of urea varied. Helmer and Bartley (36) reviewed previous literature and reported that 100 mL or g of rumen liquid/content could convert 80–100 mg of urea to ammonia per h, which is a rate much higher than that observed in the present study with a similar addition dose (U-32, 96 mg/100 mL). This could be explained by the small amount of rumen fluid inoculated [buffer medium: rumen fluid inoculum ratio = 9:1 (v/v)]. The initial microbial population was much lower than that of the rumen content, thus taking longer to hydrolyze the high doses of urea. In addition, high ammonia may inhibit urease activity (37), thus reducing the rate of urea hydrolysis. In order to increase the *in vitro* urea hydrolysis rate, large inoculum can be used in future research.

In the present study, the increase of rumen pH after urea hydrolysis is consistent with our recent *in vitro* study (38). Previous *in vivo* studies also found that infusion of urea into the rumen of Jersey cows caused an elevated rumen pH (39). In contrast, in line with our *in vitro* study with NH_4Cl addition, a previous ruminal NH_4Cl infusion study in Holstein cows also found reduced rumen pH (17). Moreover, in a study on anaerobic digestion for biogas production, similar changes of pH after urea and NH_4Cl addition were also found (18). The above results indicate that a pH and ammonia level model was successfully implemented by adding different doses of urea and NH_4Cl. This model can help examine

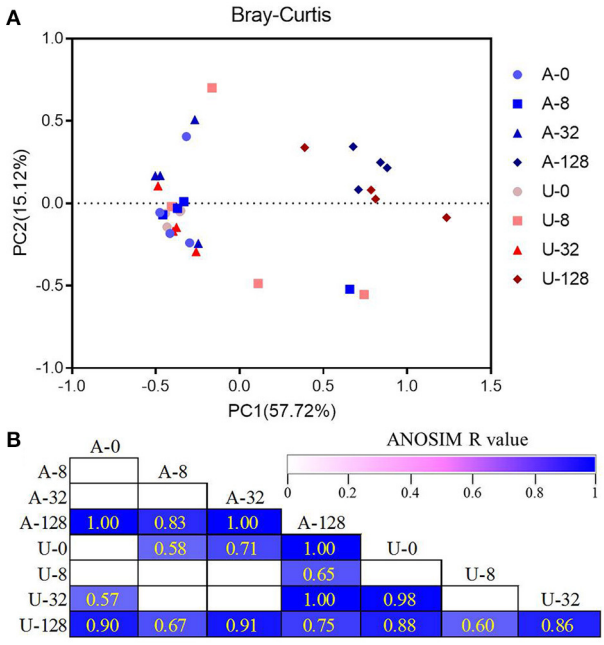

FIGURE 4
Principal coordinates analysis (PCoA) plots showing the overall differences in bacterial communities among the different treatments based on Bray-Curtis dissimilarity **(A)**. The statistical significance of the PCoA analysis of the overall bacterial communities among the treatments was analyzed using analysis of similarity (ANOSIM) **(B)**. ANOSIM *R*-values range from 0 (indistinguishable) to 1 (dissimilar), and ANOSIM *R*-values are showed only if $P < 0.05$, and cells are colored based on ANOSIM *R*-values.

TABLE 1 Effects of NH_4Cl and urea addition on the relative abundance of major ruminal bacterial phyla (each with a relative abundance ≥1.0% in at least one treatment).

Item	NH_4Cl				Urea				SEM	*P*-value		
	A-0	A-8	A-32	A-128	U-0	U-8	U-32	U-128		NS	AL	NS∗AL
Bacteroidetes	41.96	36.88	47.82	28.75	40.32	38.99	39.05	32.12	5.207	0.74	0.10	0.65
Firmicutes	20.33	20.69	16.13	40.84	21.87	20.18	21.47	39.06	3.402	0.64	<0.01	0.74
Proteobacteria	11.92	26.53	15.94	13.99	11.17	22.21	18.26	14.57	3.286	0.81	<0.01	0.78
Fibrobacteres	8.97	4.72	7.09	3.43	9.00	8.03	6.86	0.03	1.466	0.94	<0.01	0.18
Tenericutes	6.91	3.35	1.83	1.77	7.10	4.41	4.06	5.31	0.841	<0.01	<0.01	0.24
Spirochaetes	7.00	4.80	8.39	0.70	6.92	4.36	6.34	0.66	1.163	0.43	<0.01	0.80
Actinobacteria[1]	0.98^{c} (0.97)	1.06^{c} (1.21)	1.01^{c} (1.09)	2.04^{a} (8.68)	1.14^{c} (1.48)	0.95^{c} (0.91)	1.14^{c} (1.50)	1.65^{b} (4.63)	0.071	0.28	<0.01	<0.01
Fusobacteria[1]	0.33^{d} (0.04)	0.46^{cd} (0.10)	0.60^{c} (0.23)	0.99^{b} (1.01)	0.35^{d} (0.04)	0.47^{cd} (0.12)	0.62^{c} (0.25)	1.32^{a} (2.42)	0.051	0.01	<0.01	0.01

NS, Nitrogen source; AL, Ammonia level.
[a–d]Means with different superscripts within a row differ ($P < 0.05$).
[1]Data were cubic root transformed to ensure normality of residuals. Values in parentheses are the means of untransformed data in individual treatments.

TABLE 2 Effects of NH_4Cl and urea addition on the relative abundance of major ruminal bacterial genera (each with a relative abundance ≥1.0% in at least one treatment).

Phylum	Genus/other	NH_4Cl				Urea				SEM	*P*-value		
		A-0	A-8	A-32	A-128	U-0	U-8	U-32	U-128		NS	AL	NS∗AL
Bac	*Prevotella* 1	25.13	20.89	29.31	16.36	22.54	21.58	23.35	23.07	4.667	0.93	0.52	0.58
	Rikenellaceae RC9 gut group	4.10	4.45	4.05	2.25	5.25	5.26	4.52	2.85	0.685	0.13	<0.01	0.96
	Prevotellaceae Ga6A1 group	3.95	2.93	4.72	1.27	3.65	3.23	3.04	0.81	0.716	0.30	<0.01	0.58
	Prevotellaceae YAB2003 group	1.94^{b}	1.77^{b}	2.82^{b}	4.13^{a}	1.81^{b}	1.75^{b}	1.91^{b}	1.31^{b}	0.438	<0.01	0.13	0.01
	Bacteroidales F082_norank	1.42	1.33	0.75	0.18	1.72	1.82	1.05	0.25	0.242	0.10	<0.01	0.85
	*Muribaculaceae*_norank	1.23	1.00	0.80	1.85	1.14	1.02	0.77	1.83	0.219	0.85	<0.01	0.99
	Prevotellaceae UCG-001	1.16	1.20	1.44	0.34	1.14	1.18	1.34	0.27	0.192	0.68	<0.01	0.99
	Prevotella 7	0.51^{b}	0.71^{ab}	1.05^{ab}	1.17^{a}	0.45^{b}	0.56^{b}	0.37^{b}	0.17^{b}	0.129	<0.01	0.32	<0.01
Fir	*Agathobacter*	2.23	3.44	2.23	12.34	1.87	3.34	4.24	14.93	1.082	0.19	<0.01	0.44
	Anaerosporobacter	2.13	1.28	0.46	1.89	2.57	1.89	0.87	1.95	0.329	0.11	<0.01	0.86
	*Lachnospiraceae*_uncultured	1.98	1.94	0.90	0.88	1.67	1.81	1.99	2.15	0.434	0.13	0.69	0.18
	Pseudobutyrivibrio	1.92	1.75	1.80	3.58	1.99	2.06	1.72	2.25	0.361	0.33	0.01	0.13
	Butyrivibrio 2	1.04	0.73	0.72	0.97	1.00	0.83	0.68	1.45	0.188	0.34	0.045	0.49
	Selenomonas	0.80^{b}	0.77^{b}	1.06^{b}	4.24^{a}	0.70^{b}	0.29^{b}	1.11^{b}	1.39^{b}	0.258	<0.01	<0.01	<0.01
	Ruminococcus 1	0.57	0.68	0.60	1.36	0.69	0.73	0.85	1.03	0.146	0.84	<0.01	0.25
	Ruminococcus 2	0.52	0.80	0.65	0.92	0.91	1.09	0.88	1.42	0.213	0.03	0.16	0.93
	Eubacterium eligens group	0.49^{c}	0.54^{c}	0.46^{c}	2.67^{a}	0.41^{c}	0.30^{c}	0.49^{c}	1.50^{b}	0.154	<0.01	<0.01	<0.01
	Anaerovibrio	0.47^{b}	0.35^{b}	0.33^{b}	0.34^{b}	0.51^{b}	0.26^{b}	0.38^{b}	1.08^{a}	0.076	<0.01	<0.01	<0.01
	Sharpea	0.15^{c}	0.25^{c}	0.22^{c}	2.81^{a}	0.29^{c}	0.31^{c}	0.24^{c}	1.31^{b}	0.142	<0.01	<0.01	<0.01
Pro	*Ruminobacter*	9.75	22.12	13.82	11.60	8.73	20.29	15.80	10.32	3.121	0.81	<0.01	0.93
	Succinivibrio	1.77	2.14	1.78	1.99	1.96	1.53	1.95	2.27	0.279	0.99	0.69	0.37
	Escherichia-Shigella[1]	0.26^{b} (0.02)	0.27^{b} (0.02)	0.28^{b} (0.02)	0.38^{b} (0.06)	0.24^{b} (0.01)	0.25^{b} (0.02)	0.38^{b} (0.06)	1.06^{a} (1.25)	0.035	<0.01	<0.01	<0.01
Fib	*Fibrobacter*	8.97	4.73	7.09	3.43	9.00	8.03	6.86	0.03	1.466	0.94	<0.01	0.18
Spi	*Treponema* 2	6.94	4.77	7.33	0.67	6.84	4.30	6.30	0.66	1.169	0.43	<0.01	0.80
Ten	*Anaeroplasma*	6.64	3.19	1.70	1.66	6.87	4.25	3.90	5.04	0.820	<0.01	<0.01	0.27
Act	*Bifidobacterium*[1]	0.92^{c} (0.82)	0.98^{c} (0.94)	0.94^{c} (0.87)	2.00^{a} (8.11)	1.04^{c} (1.14)	0.90^{c} (0.79)	1.04^{c} (1.12)	1.60^{b} (4.29)	0.070	0.23	<0.01	<0.01
Fus	*Fusobacterium*[1]	0.33^{d} (0.04)	0.46^{cd} (0.10)	0.60^{c} (0.23)	0.99^{b} (1.01)	0.35^{d} (0.04)	0.47^{cd} (0.12)	0.62^{c} (0.25)	1.32^{a} (2.42)	0.051	0.01	<0.01	0.01

NS, Nitrogen source; AL, Ammonia level; Bac, Bacteroidetes; Fir, Firmicutes; Pro, Proteobacteria; Fib, Fibrobacteres; Spi, Spirochaetes; Ten, Tenericutes; Act, Actinobacteria; Fus, Fusobacteria.

[a–d]Means with different superscripts within a row differ ($P < 0.05$).

[1]Data were cubic root transformed to ensure normality of residuals. Values in parentheses are the means of untransformed data reatments.

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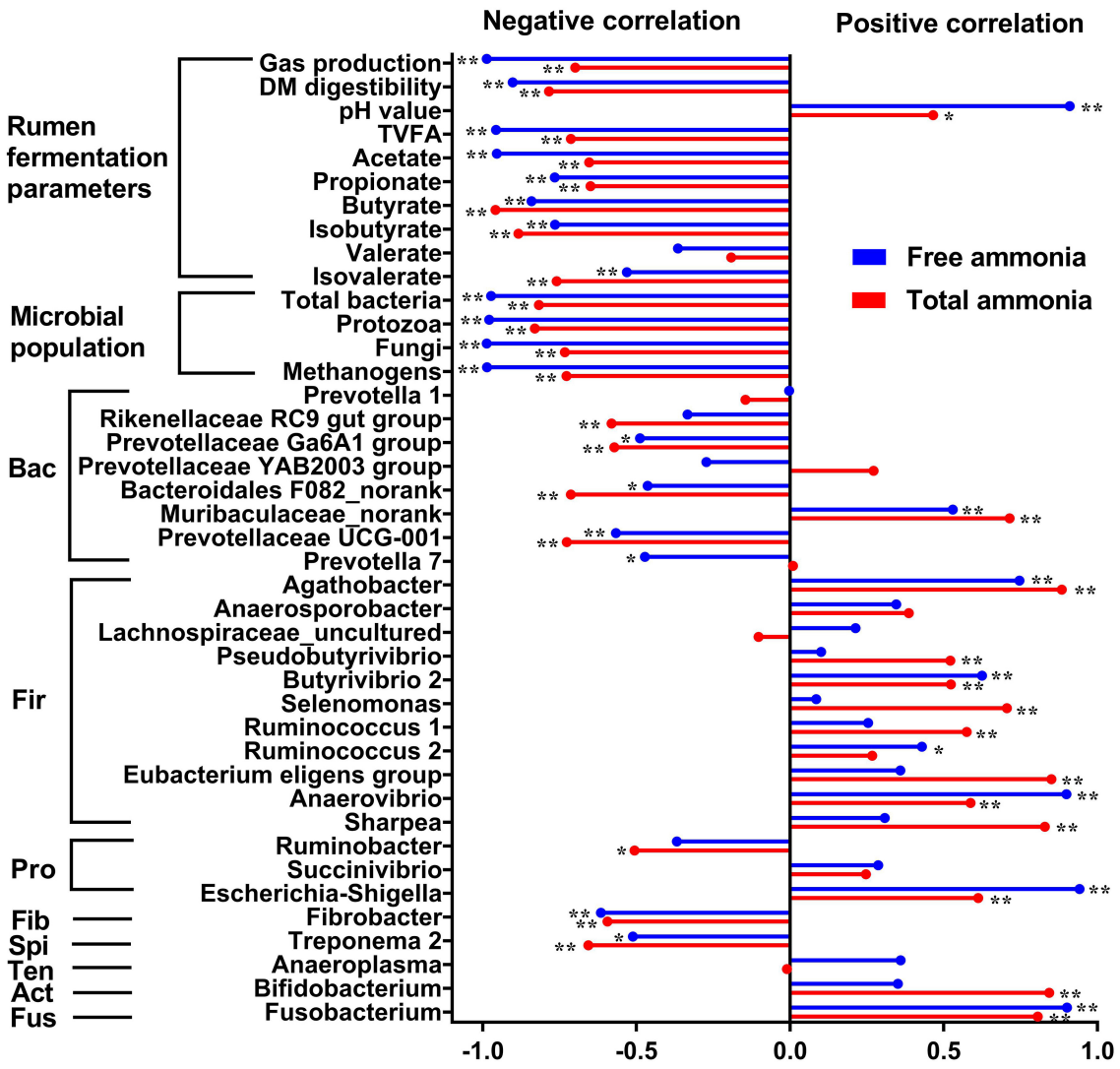

FIGURE 5
Pearson correlation coefficients (*r*) between total ammonia (red bars) or free ammonia (blue bars) and *in vitro* rumen fermentation parameters, absolute abundance of microbial populations (total bacteria, protozoa, fungi, and methanogens), the relative abundance of rumen bacteria at the genus level. *$P < 0.05$; **$P < 0.01$. Bac, Bacteroidetes; Fir, Firmicutes; Pro, Proteobacteria; Fib, Fibrobacteres; Spi, Spirochaetes; Ten, Tenericutes; Act, Actinobacteria; Fus, Fusobacteria.

the effect of varying FAN levels on the rumen microbiome (both individual taxa and functional guilds such as fibrolytic bacteria) and on *in vitro* rumen fermentation.

Under normal rumen environmental condition, the rumen pH is typically below the pKa (9.21) of ammonia, and thus virtually all ammonia is present in the rumen as NH_4^+ (40). However, as calculated according to Henderson-Hasselbalch equation (9), the amount of TAN present as NH_3 varies almost exponentially as a function of pH (41). Thus, in the present study, the increased *in vitro* rumen pH in response to the urea addition resulted in a much higher FAN concentration compared to NH_4Cl at a similar TAN level. Therefore, pH value is the key factor to determine the concentration of FAN in the rumen. For ruminants, the rumen pH is greatly influenced by dietary forage to concentrate ratio and buffers such as bicarbonate, calcium carbonate, and magnesium oxide (3). Thus, the rumen FAN level can be controlled to some extent by controlling the pH through modifying the buffer and diet composition.

Ammonia-N is an essential nutrient for microbial growth. The rumen microbiota needs 5–11 mmol/L ammonia to maximize microbial protein (42). Apparently, the reduced *in vitro* rumen fermentation in the groups of A-0 and U-0 was due to ammonia-N deficiency. High ammonia stress is a main issue in anaerobic digestion for biogas production (9). In a previous review, Jiang et al. (13) reported that the inhibitory concentrations of TAN for anaerobic digestion varied greatly, but if converted to FAN the inhibitory concentrations were more consistent. In livestock production, feeding a large amount of NPN to ruminants will create high ammonia stress to the animals, which increases the risk of ammonia toxicity (8). However, the effects of high FAN on *in vitro* rumen fermentation are unknown. In the present study, the FAN concentration of 0.31 mmol/L in the U-32 group resulted in

some inhibition of *in vitro* rumen fermentation (gas production, DM digestibility, total VFA concentration, etc.), and the increased FAN concentration of group A-128 (0.57 mmol/L) and U-128 (3.96 mmol/L) increased the inhibition magnitude significantly. Thus, to ensure efficient rumen fermentation, the rumen FAN level should be controlled. Moreover, based on the above results, it is speculated that rumen FAN level could serve as a potential biological marker to monitor rumen fermentation, but more studies are needed to confirm the inhibition threshold.

Rumen microorganisms are solely responsible for feed degradation and VFA production (43), and rumen bacteria are the predominant contributors (44). In addition, rumen protozoa, fungi, and methanogens also play important but different roles in rumen digestion and metabolism (45). In the present study, the stronger negative correlation between FAN and microbial populations quantified (i.e., total bacteria, protozoa, fungi, and methanogens) and *in vitro* rumen fermentation profiles (gas production, DM digestibility, total VFA, acetate, propionate, etc.) and a much weaker correlation between TAN and the above indicators indicate that high FAN inhibited *in vitro* rumen fermentation by reducing microbial populations. However, the mechanism(s) by which high FAN inhibited rumen microbes remain to be elucidated. Reviews of anaerobic digestion for biogas production reported that at least two possible mechanisms were underpinning the inhibition of anaerobic digestion by high ammonia (13): (1) direct inhibition of some enzymes in the cytoplasm of microorganisms, and (2) alteration of the intracellular environment upon absorption of free ammonia, resulting in ammonia toxicity to microorganisms. The operation environment of anaerobic digesters for biogas production (pH, temperature, microbial composition, etc.) is quite different from that of the rumen. Therefore, further research efforts are needed to elucidate the mechanism(s) of ammonia inhibition to rumen microorganisms.

The performance of rumen fermentation is not only related to the total bacteria population but also closely related to the rumen bacterial community structure. Generally, *Ruminococcus albus*, *R. flavefaciens*, *Fibrobacter Succinogenes*, *Butyrivibrio fibrisolvens*, and *Eubacterium cellulosolvens* are considered the major cellulolytic bacterial species cultured (46). Besides, a forage incubation study indicated that some unclassified bacteria assigned to the families *Lachnospiraceae*, *Christensenellaceae*, *Ruminococcaceae*, *Rikenellaceae*, *Prevotellaceae*, and *Bacteroidales* mightly also play an important role in fiber degradation in the rumen (47). In the present study, the reduced abundance of potential fibrolytic bacterial genera (e.g., *Rikenellaceae* RC9 gut group, *Prevotellaceae* Ga6A1 group, *Bacteroidales* F082_norank, *Prevotellaceae* UCG-001, and *Fibrobacter*) might have partially explained the decreased DM digestibility in the high ammonia treatments. However, some potential fibrolytic bacterial genera such as *Pseudobutyrivibrio*, *Butyrivibrio* 2, and *Ruminococcus* 1 were increased by the high ammonia treatments. This may be attributed to the different tolerance of fibrolytic bacteria to high ammonia stress. However, it should be noted that an increase or decrease of the relative abundance of a bacterial genus does not necessarily mean an increase or decrease of its absolute abundance. Quantitative analysis of absolute abundance using qPCR or other quantitative methods can determine how these genera respond to high ammonia stress.

In the present study, the correlation results between the relative abundance of bacterial genera and ammonia concentration indicate that some Gram-negative bacteria were sensitive, while Gram-positive bacteria were tolerant to high TAN treatment. However, to the best of the authors' knowledge, no literature to date has reported the tolerance response of Gram-positive vs. Gram-negative bacteria to high ammonia stress. Therefore, the difference in tolerance to high ammonia stress between Gram-positive and Gram-negative bacteria warrants future research.

Conclusion

In the present study, an *in vitro* rumen pH and ammonia difference model was implemented by adding urea and NH_4Cl. Urea hydrolysis increased, while NH_4Cl dissociation slightly reduced *in vitro* rumen pH. At the same TAN level, the increased rumen pH by urea addition resulted in much higher FAN concentrations compared to NH_4Cl addition. High FAN inhibited *in vitro* rumen fermentation by reducing the absolute abundance of total bacteria, fungi, protozoa, and methanogens. Additionally, bacterial community structure changed differently in response to nitrogen source and ammonia level. This study demonstrated that the inhibition of high ammonia to *in vitro* rumen fermentation is pH dependent.

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 at: https://www.ncbi.nlm.nih.gov/, PRJNA940661.

Ethics statement

The animal study was reviewed and approved by Animal Care and Use Committee of Nanjing Agricultural University (protocol number: SYXK2017-0007). Written informed consent was obtained from the owners for the participation of their animals in this study.

Author contributions

JS conceived and designed the experiments and wrote the paper. WZ and YX performed the experiments. JS, WZ, and YX analyzed the data. ZY revised the paper. All authors contributed to the article and approved the submitted version.

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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

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fvets.2023.1163021/full#supplementary-material

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Effects of forage type on the rumen microbiota, growth performance, carcass traits, and meat quality in fattening goats

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Forages fed to goats influence ruminal microbiota, and further contribute to affect growth performance, meat quality and its nutritional composition. Our objective for current study was to investigate the effects of different forages on growth performance, carcass traits, meat nutritional composition, rumen microflora, and the relationships between key bacteria and amino acids and fatty acids in the *longissimus dorsi* and *semimembranosus* muscles of goats. Boer crossbred goats were separately fed commercial concentrate diet supplemented with Hemarthria altissima (HA), Pennisetum sinese (PS), or forage maize (FG), and then slaughtered 90days after the beginning of the experiment. Growth performances did not vary but carcass traits of dressing percentage, semi-eviscerated slaughter percentage, and eviscerated slaughter percentage displayed significant difference with the treatment studied. Meats from goats fed forage maize, especially *semimembranosus* muscles are rich in essential amino acids, as well as an increase in the amount of beneficial fatty acids. Our 16S rRNA gene sequencing results showed that the Firmicutes, Bacteroidetes, and Proteobacteria were the most dominant phyla in all groups but different in relative abundance. Further, the taxonomic analysis and linear discriminant analysis effect size (LEfSe) identified the specific taxa that were differentially represented among three forage treatments. The spearman's correlation analysis showed that rumen microbiota was significantly associated with the goat meat nutritional composition, and more significant positive correlations were identified in *semimembranosus* muscles when compared with *longissimus dorsi* muscles. More specifically, the lipid metabolism-related bacteria Rikenellaceae_RC9_gut_group showed positively correlated with meat amino acid profile, while genera Oscillospiraceae_UCG-005 were positively correlated with fatty acid composition. These bacteria genera might have the potential to improve nutritional value and meat quality. Collectively, our results showed that different forages alter the carcass traits, meat nutritional composition, and rumen microflora in fattening goats, and forage maize induced an improvement in its nutritional value.

KEYWORDS

fattening goat, forage treatment, meat quality, rumen microbiota, 16S rRNA

1. Introduction

Mutton has conventionally been regarded as healthy food and is beneficial to the elderly, children, and pregnant persons (1). In general, goat meat has desirable fatty acids with moderately higher proportion of polyunsaturated fatty acids, lower cholesterol and saturated fat contents compared to beef and pork (2, 3). Meat quality and nutrients are determined by a considerable number of factors whereas genetics, diet, and management are highly ranked factors (4). Diet has a large economic impact on the raising processes as the feeding cost reaches 80% of total cost of production (5). It is important to note that adequate diet is critical for improving the quality and acceptability of animal-derived foods (6).

Forages are regarded as the cheapest and major source of nutrition for ruminant livestock. Ruminants are endowed with the ability to degrade and utilize forages with the help of rumen microbes, while providing adequate energy and protein for the body (7, 8). The rumen of ruminants is an extremely complex microbial ecosystem and hosts 100 trillion (10^{14}) microorganisms including bacteria, protozoa, fungi, archaea, and a small proportion of phages. Because these microorganisms are directly involved in the degradation and metabolization of plant materials in the rumen, any changes in nutrient availability will affect rumen microbiota community structure and microbial fermentation patterns (9). Normally, various groups of bacteria have been shown to be associated with the utilization of specific feedstuffs, such as starch or cellulose, which are digested by saccharolytic and cellulolytic bacteria, respectively (10). A forage-based diet is dominated by cellulolytic and fibrolytic bacteria, which degrades the cellulose and hemicellulose, while a concentrate-based diet is dominated by starch-degrading amylolytic bacteria, which ferment the starch and sugars (11). Meanwhile, the composition of the rumen bacterial community has been proven to be associated with feed efficiency (12) and fatty acid composition of meat (3), and fatty acids are closely linked to human health.

Finishing beef are often fed a forage-based diet in order to improve omega-3 polyunsaturated fatty acids, conjugated linoleic acid, and superior nutritional value (13). Similarly, in goats, mixed orchard hays can increase beneficial fatty acids and amino acids of meat, suggesting that feeding suitable type of forage is an important strategy for producing high-quality meat (14). In support of this, effects of forage-based diet types on growth performance, production quality, and rumen microbiome were widely studied (6, 10, 14, 15). However, it is still remains largely unknown about the link between different type of forages and rumen bacterial community composition. In addition, the relationship between rumen microbiota and fatty acids and amino acids in the *longissimus dorsi* and *semimembranosus* muscles of goats is limited. Therefore, in this study, growth performance, carcass traits, meat nutritional composition, rumen microflora of Boer crossbred goats under different forage treatments were determined. Subsequently, the sequential dynamic changes in rumen bacterial community composition and their relationships with the fatty acids and amino acids were analyzed comprehensively using high-throughput sequencing approach. Our work aimed to compare rumen bacterial community of diets differing in forage type, and reveal the dominant bacteria related to contributing to a good nutritional quality meat of goats.

2. Materials and methods

2.1. Ethics statement

All experimental procedures involved in this study were conducted in accordance with the guidelines duly approved by Institutional Animal Care and Use Committee of Chengdu University (SSXY-600008).

2.2. Animal treatments and sample collection

In this study, a total of 15 healthy 6-month-old male Boer crossbred goats with an average body weights (BW) of 19.61 ± 3.25 kg were enrolled and housed in individual wooden pens (1.20 m length × 0.80 m width × 1.50 m height) on a raised slatted floor. Goats were randomly divided into three forage treatment groups of concentrate + forage, within each group one of the forages including Hemarthria compressa, Pennisetum sinese, or forage maize was used, and then designated as HA, PS, and FG group, respectively. For each group, the concentrate was offered 3.5 kg per day for each, whereas forage and water were both offered *ad libitum*. The chemical compositions of the concentrate and forages are shown in Supplementary Table S1.

The study commenced with a 7-day adaptation period and lasted for 90 days. Of these enrolled goats, two animals in the HA group were sick and treated with drug during the experiment, these two goats were removed from the herd. Finally, a subset of 13 goats were obtained and then divided into three groups of HA ($N = 3$), PS ($N = 5$), and FG ($N = 5$), and all the animals were weighed and slaughtered according to standard protocol at the end of the experiment. Animals were subjected to electrical stunning at 220 voltages followed by exsanguination, skinning, evisceration, and washing procedures. Immediately after slaughter, the rumen fluid samples were collected from each goat, and the rumen contents were removed by using three layers of cheesecloth. Subsequently, the liquid fractions were transferred into plastic bottles, and stored at −80°C until further evaluation. On the other hand, the carcasses were cooled to 4°C for 24 h followed by further analyses.

2.3. Meat quality measurements

The *longissimus dorsi* and *semimembranosus* muscles were cut to measure the meat quality traits according to previous reports (3, 16). Briefly, the pH values were measured at 24 h (pH_{24h}) after slaughter. Meat color measurements cover indicators L* (lightness), a* (redness), and b* (yellowness) after slaughter 24 h (L_{24h}, a_{24h}, and b_{24h}). Drip loss, cooking loss, crude protein, and ash content were determined as described previously (3, 17). Amino acid profile and fatty acid composition were determined using gas chromatography–mass spectrometry (GC–MS 7890B-5977A, Agilent, Palo Alto, CA, United States) and liquid chromatography–mass spectrometry (Liquid phase was performed on Thermo Ultimate 3,000 system, Thermo Fisher Scientific Inc., Waltham, MA, United States; Mass Spectrometry was performed on Thermo Q Exactive Focus mass

spectrometer, Thermo Fisher Scientific Inc., Waltham, MA, United States), respectively.

2.4. DNA extraction and sequencing

Frozen rumen liquid (approximately 15 g) from each animal was subjected to microbial genomic DNA isolation using 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, United States) and gel electrophoresis, respectively. Amplicon libraries targeting the V3–V4 hypervariable region of the bacterial 16S rRNA gene were amplified by PCR using primers with barcoded tags (338F, 5′-ACTCCTACGGGAGGCAGCA-3′, 806R: 5′-GGACTACHVGGGTWTCTAAT-3′). The reaction was performed with the following cycle parameters: initial denaturation for 3 min at 95°C, 30 cycles of 95°C for 30 s, 55°C for 30s, and 72°C for 30s, and a final extension at 72°C for 10 min. The PCR products were separated on 1.5% agarose gel electrophoresis. Qualified amplicons were used to produce sequencing libraries using Illumina TruSeq (Illumina, San Diego, CA, United States) following manufacturer's specifications. Finally, the libraries were diluted and mixed in proportion and sequenced on Illumina HiSeq 2,500 platform for generating 250 bp paired-end reads.

2.5. Bioinformatics and data analysis

Paired-end reads were assigned to samples based on their unique barcode, and the high-quality clean reads were obtained by using the Cutadapt software (1.9.1) (18). Sequences analysis was performed by Qiime2 software (19). By which, representative sequences were established as operational taxonomic units (OTUs) and aligned through DEBLUR program (20) integrated within QIIME2. Then, OTUs were taxonomically classified and grouped by comparison with those in the Silva reference sequences (138 clustered at 99% similarity). Successive analyses of alpha diversity and beta diversity were conducted. The alpha diversity metrics including community richness parameters (Chao1 and Observed features) and diversity parameters (Shannon and Simpson indices) were calculated and significant differences between groups were assessed with the Kruskal-Wallis test (19, 21). The beta diversity metrics including Bray Curtis, Jaccard, Weighted UniFrac, and Unweighted UniFrac metric were calculated and significant differences were assessed using a PERMANOVA analysis (19, 21). Moreover, the rarefaction and rank curves were generated to assess the sequencing depth, richness, and evenness. To identify the bacterial taxa that were differentially represented at the genus or higher taxonomic levels, linear discriminant analysis coupled with effect size (LEfSe) was performed (22), where linear discriminant analysis (LDA) method was used to rank the features differing between the groups, and a LDA score > 2 was considered significant. Statistical analyses were performed using R (v4.1.3) software.[1] The criterion of significance was conducted at $p < 0.05$ and the values were presented as the means. Spearman's correlation between the identified ruminal genera and the contents of amino acids and fatty acids of all the enrolled goats was performed using corrplot package the R language (v4.1.3), and p-values <0.05 were selected as statistically significant.

2.6. Statistical analysis

Data were analyzed based on a general linear model of analysis of variance (ANOVA) using SPSS 21.0 (IBM Corp., New York, United States). Differences between mean values of different forage treatments were obtained by Fisher's LSD multiple range test, and a statistically significant difference was defined at $p < 0.05$.

3. Results

3.1. Effects of forage treatment on the growth performances and carcass characteristics

There was no significant difference in the initial body weight, the final body weight, and average daily weight gain of the goats among the forage treatment groups ($p > 0.05$; Table 1). Further, we observed no effect of forage treatment on the carcass parameters of semi-eviscerated weight and eviscerated weight. However, animals from HA group had significantly ($p < 0.05$) highest semi-eviscerated slaughter percentage and eviscerated slaughter percentage as well as the higher dressing percentage as compared to PS and/or FG group (Table 1).

Most of the carcass traits between *longissimus dorsi* and *semimembranosus* muscles were similar, except for cooking loss, moisture content, pH_{24h}, and a_{24h}. For these characteristics, the *longissimus dorsi* muscles presented significant higher cooking loss ($p < 0.05$), pH_{24h} ($p < 0.01$) and a_{24h} ($p < 0.05$), and lower moisture content ($p < 0.01$) when compared with *semimembranosus* muscle (Table 1). As for forage treatments, there was significant difference in the drip loss ($p < 0.05$), ash ($p < 0.05$), and L_{24h} ($p < 0.01$) of *longissimus dorsi* muscles among the three groups. In addition, animals from HA group presented significant higher crude protein, ash, and pH_{24h} of *semimembranosus* muscle, compared with PS and/or FG group (Table 1).

3.2. Effects of forage treatments on the amino acid and fatty acid composition

A total of 17 amino acids and 37 fatty acids were tested to examine whose relative content, and most of them did not differ among the forage treatment groups (Supplementary Table S2). The contents of serine (Ser) and proline (Pro) were significantly changed in both types of muscles. However, no fatty acids in *longissimus dorsi* muscles were found to be affected by the forage treatments. In *semimembranosus* muscles, a total of nine amino acids and three fatty acids were affected by forage treatments. Interestingly, the content of all these three fatty acids of linoleic acid (C18: 2n6C), arachidonic acid (C20: 4n6), and docosahexaenoic acid (C22: 6n3) were significant higher for FG than PS groups ($p < 0.05$; Table 2).

1 https://www.r-project.org/

TABLE 1 Growth and carcass traits in goats under different forage treatments.

Traits[1]		Group			*p* value	
		HA	PS	FG	Group	Tissue
Initial body weight (kg)		21.42 ± 3.75	19.49 ± 4.23	19.55 ± 2.65	0.478	/
Final body weight (kg)		33.47 ± 5.71	30.97 ± 5.68	32.84 ± 2.26	0.477	/
ADG[1] (kg/d)		0.13 ± 0.02	0.12 ± 0.09	0.14 ± 0.03	0.635	/
Dressing percentage (%)		52.41 ± 1.14^{a}	50.48 ± 2.04^{ab}	49.17 ± 0.32^{b}	0.011	/
Semi-eviscerated weight (kg)		24.74 ± 4.12	21.93 ± 4.19	22.49 ± 1.75	0.286	/
Semi-eviscerated slaughter percentage (%)		73.94 ± 0.53^{Aa}	70.72 ± 2.02^{ABb}	68.84 ± 1.97^{Bb}	0.002	/
Eviscerated weight (kg)		18.30 ± 3.49	16.34 ± 3.34	16.89 ± 1.17	0.345	/
Eviscerated slaughter percentage (%)		54.55 ± 1.18^{A}	52.60 ± 1.89^{AB}	51.42 ± 0.29^{B}	0.009	/
Longissimus dorsi	Drip loss (%)	14.47 ± 2.07^{a}	9.92 ± 2.29^{b}	11.03 ± 2.03^{ab}	0.016	0.113
	Cooking loss (%)	45.34 ± 2.79	45.67 ± 2.65	45.70 ± 0.92	0.825	0.022
	Moisture content (%)	75.49 ± 0.77	75.90 ± 1.07	75.30 ± 1.16	0.396	0.003
	Crude protein (%)	82.82 ± 3.35	79.16 ± 7.24	74.21 ± 8.39	0.131	0.354
	Fatty acid (%)	6.12 ± 1.26	10.00 ± 6.48	14.60 ± 7.15	0.087	0.133
	Ash (%)	7.91 ± 0.32^{a}	4.70 ± 1.69^{b}	6.26 ± 2.29^{ab}	0.035	0.468
	H_{24h}	5.44 ± 0.08	5.50 ± 0.12	5.52 ± 0.17	0.409	<0.001
	L_{24h}	43.77 ± 1.29^{Bb}	45.25 ± 2.35^{ABb}	48.05 ± 0.97^{Aa}	0.006	0.257
	a_{24h}	16.77 ± 1.43	17.28 ± 2.50	16.66 ± 2.31	0.671	0.048
	b_{24h}	7.73 ± 1.65	7.03 ± 0.73	8.27 ± 1.02	0.100	0.529
Semimembranosus	Drip loss (%)	12.30 ± 1.99	13.51 ± 1.79	12.84 ± 2.89	0.493	0.113
	Cooking loss (%)	42.47 ± 1.41	44.13 ± 2.58	43.06 ± 3.57	0.445	0.022
	Moisture content (%)	76.32 ± 0.73	76.83 ± 0.96	77.05 ± 0.99	0.309	0.003
	Crude protein (%)	83.09 ± 1.61^{a}	80.54 ± 2.41^{ab}	78.22 ± 3.88^{b}	0.049	0.354
	Fatty acid (%)	7.21 ± 1.67	7.68 ± 0.79	8.77 ± 1.29	0.109	0.133
	Ash (%)	7.85 ± 0.41^{A}	4.11 ± 0.33^{B}	8.49 ± 0.61^{A}	<0.001	0.468
	pH_{24h}	5.84 ± 0.15^{a}	5.61 ± 0.10^{b}	5.72 ± 0.07^{ab}	0.012	<0.001
	L_{24h}	42.82 ± 2.87	45.26 ± 2.07	45.71 ± 2.51	0.134	0.257
	a_{24h}	20.72 ± 1.97	19.59 ± 3.21	17.58 ± 3.38	0.193	0.048
	b_{24h}	8.67 ± 3.65	8.48 ± 1.17	7.35 ± 2.00	0.428	0.529

Values within a row with different lowerscripts and/or superscripts differ significantly at $p < 0.05$ and $p < 0.01$, respectively. [1]dage, average daily weight gain, pH_{24h} = pH of muscles after slaughter 24 h, L_{24h}, a_{24h}, b_{24h} = Lightness, redness and yellowness of muscles after slaughter 24 h.

3.3. Rumen bacterial community structure

A total of 1,039,145 high-quality reads remained after quality control processing and eliminating the unqualified data, with an average of 79,934 paired-end reads per sample. All sequences were subjected to OUT picking according to DEBLUR program, and herein produced a total of 1,489 OTUs, and these OTUs were assigned into 10 phyla, 13 classes, 37 orders, 66 families, and 119 genera. Alpha diversity consists of community diversity (Shannon and Simpson indices) and richness (Chao1 and Observed features) were assessed and compared for each treatment. Although variation in the inter-animal dynamics of the alpha diversity was observed, no significant differences in the overall alpha diversity indices of the rumen microbiome were found ($p > 0.05$) (Figures 1A–D). These results indicated that the forage treatments did not significantly change the rumen microbial abundance and diversity of goats. We next assessed the dissimilarities in community structure and membership of rumen microbiome between groups, and there were no significant difference in beta diversity indices (Supplementary Figure S1), the PCoA plots based on beta diversity metrics (Bray Curtis, Weighted UniFrac, Jaccard, and Unweighted UniFrac metric) were shown in Figures 1E–H. Beside HA group that has only three samples, individuals in the PS and FG group were clustered separately, indicating bacterial communities were positively correlated with the treatment of forage types.

TABLE 2 Significantly changed amino acids (g/100g dry matter) and fatty acids (g/100g dry matter) of goat meat under different forage treatments.

Traits		Group		
		HA	PS	FG
Longissimus dorsi	Ser	2.32 ± 0.11^{ABa}	2.56 ± 0.40^{Aa}	1.83 ± 0.14^{Bb}
	Pro	1.73 ± 0.74^{ab}	3.12 ± 2.13^{a}	0.91 ± 0.76^{b}
Semimembranosus	Asn	6.20 ± 0.24^{A}	3.86 ± 0.11^{B}	4.47 ± 0.82^{B}
	Glu	11.77 ± 0.30^{a}	10.62 ± 0.20^{b}	11.33 ± 0.87^{b}
	Ser	2.25 ± 0.09^{ABa}	2.64 ± 0.06^{Aa}	1.72 ± 0.37^{Bb}
	Thr	2.90 ± 0.10^{A}	2.97 ± 0.03^{A}	2.52 ± 0.34^{B}
	Ala	4.29 ± 0.16^{a}	3.84 ± 0.14^{b}	4.11 ± 0.26^{ab}
	Cys	0.44 ± 0.04^{B}	0.50 ± 0.02^{A}	0.40 ± 0.02^{B}
	Val	4.92 ± 0.17^{A}	4.22 ± 0.03^{B}	4.78 ± 0.37^{A}
	Phe	6.33 ± 0.26^{A}	5.36 ± 0.06^{B}	6.14 ± 0.51^{A}
	Pro	1.83 ± 0.62^{b}	4.02 ± 1.15^{ab}	4.72 ± 2.05^{a}
	C18:2n6C (linoleic acid)	0.76 ± 0.35^{ab}	0.51 ± 0.20^{b}	0.97 ± 0.38^{a}
	C20:4n6 (arachidonic acid)	0.30 ± 0.19^{ab}	0.22 ± 0.12^{b}	0.47 ± 0.12^{a}
	C22:6n3 (docosahexaenoic acid)	0.06 ± 0.03^{ab}	0.04 ± 0.03^{b}	0.08 ± 0.02^{a}

The results are presented as means and standard errors. Values within a row with different lowerscripts and superscripts differ significantly at $p < 0.05$ and $p < 0.01$, respectively.

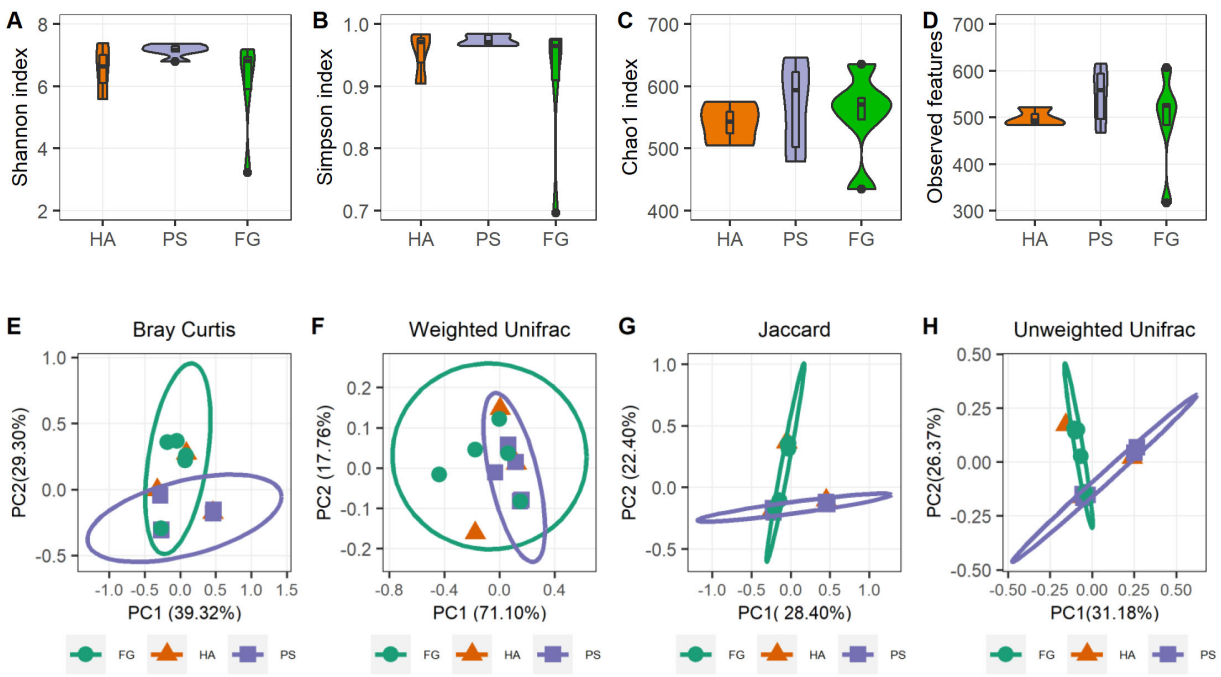

FIGURE 1
Rumen microbiota diversities of goats grouped by different forage treatments. Comparison of the diversity (Shannon and Simpson indices) **(A,B)** and richness (Chao1 and Observed features) **(C,D)** of the rumen microbiota community. The overall rumen microbiota structures showed by principal coordinate analysis (PCoA) of Bray Curtis distances **(E)**, Weighted UniFrac distances **(F)**, Jaccard distances **(G)**, and Unweighted UniFrac distances **(H)**.

3.4. Composition analysis of the rumen microbiota

Among these taxonomically OTUs, Bacteroidota and Firmicutes were absolutely predominated phyla in all of the three groups of goats, followed by Proteobacteria (Figure 2A). These three phyla accounted for 95.72, 93.28, and 96.70% of the sequences for HA, PS, and FG group, respectively. At the genus level, the relative abundance of the top 20 genera together made up 87–90% of the total composition, and Rikenellaceae_RC9_gut_group were the dominant genera in HA and PS group, reaching a proportion of 16.42 and 17.07%, respectively. Whereas Rikenellaceae_RC9_gut_group in the FG group were found

to be the subdominant genera with an abundance of 13.37%, following *Escherichia-Shigella* (17.20%). In addition, Prevotellaceae_UCG-004 and Prevotellaceae_UCG-003 were subdominant genera in HA and PS group, accounting for 9.15 and 11.81%, respectively (Figure 2B).

We further detected the specific bacteria associated with dietary treatment using Linear discriminant analysis Effect Size (LEfSe) analysis. As shown in Figure 3, a total of three, five, and five bacterial taxa that were abundant in HA, PS, and FG group, respectively. At the genus level, *Bifidobacterium* was significantly enriched in HA group, while *Monoglobus*, *Selenomonas*, and NK4A214_group were mostly associated with PS group based on LEfSe (Figure 3A). Furthermore, a cladogram representing the taxonomic hierarchical structure of rumen microbiota indicated significant difference phylogenetic distributions among different groups (Figure 3B). These results showed a remarkable difference in rumen microbiota composition due to different dietary treatment.

3.5. Correlation analysis of rumen microflora and meat quality composition

Robust correlations between amino acids and fatty acids and major rumen bacterial composition at the genus level were conducted, only the spearman correlation coefficients $|r| > 0.6$ and p-values <0.05 are drawn in the heat map (Figure 4). For *longissimus dorsi* muscles, bacteria genera of Oscillospiraceae_UCG-005, *Escherichia-Shigella*, *Bacillus* and *Psychrobacillus* was negatively correlated with amino acids of tyrosine (Tyr), serine (Ser), and proline (Pro). Saturated fatty

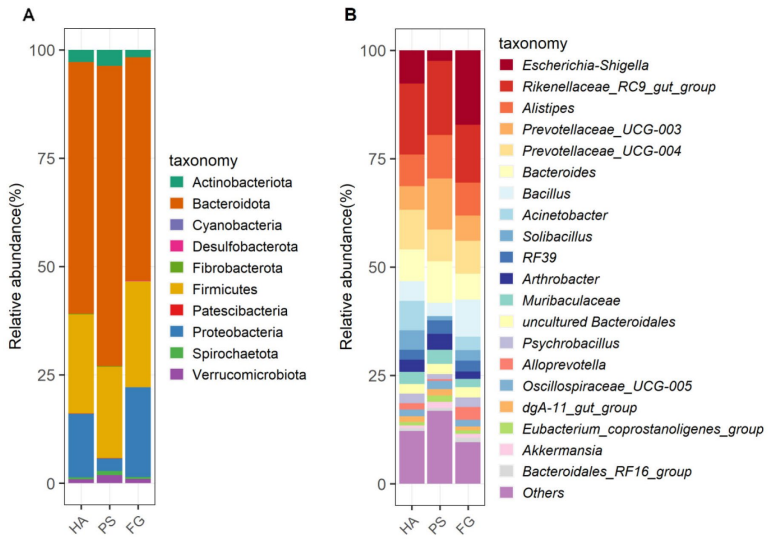

FIGURE 2
The composition and relative abundance of rumen microbial community of goats. Microbial community bar plot of phyla in rumen **(A)**. Microbial community bar plot of top 20 genera in rumen **(B)**.

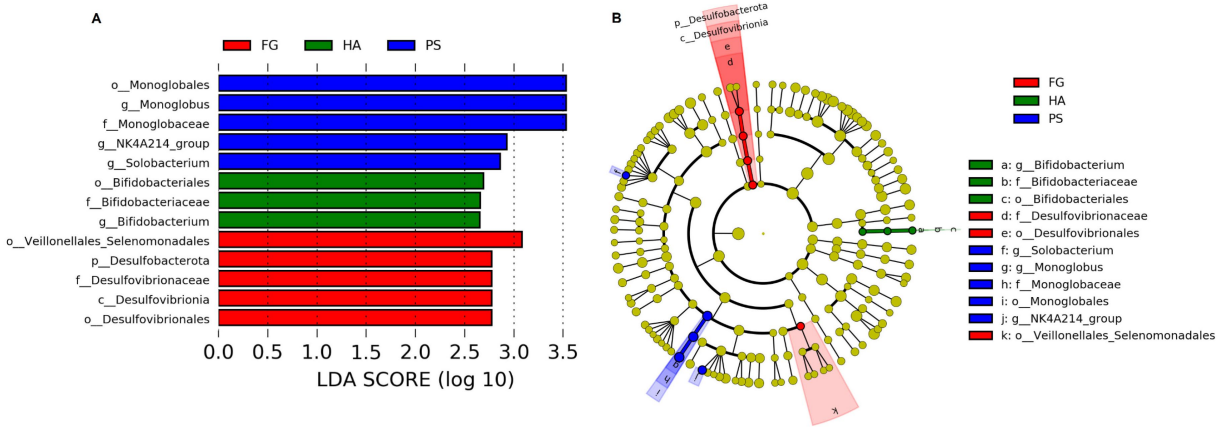

FIGURE 3
Linear discriminant analysis effect size (LEfSe) analysis integrated with linear discriminant analysis (LDA) revealed differentially abundant phylotypes in different groups. LDA scores indicated differences in abundance among forage treatments (LDA scores >2.0) **(A)**. Cladogram obtained from LEfSe analysis revealed the different taxa in microbiota of different groups of goats **(B)**.

acids such as capric acid (C10: 0), lauric acid (C12: 0), myristic acid (C14: 0), heptadecanoic acid (C17: 0), stearic acid (C18: 0), and heneicosanoic acid (C21: 0) were strongly correlated with several bacteria. Among which, myristic acid (C14: 0) was positively correlated with *Arthrobacter*, *Bacillus*, and *Psychrobacillus* abundances, but negatively correlated with Eubacterium_coprostanoligenes_group, *Alistipes*, *RF39*, *Akkermansia*, and *Bacteroides* abundances (Figure 4A). The effects of rumen bacteria abundances on amino acid and fatty acid content of the *semimembranosus* muscles are given in Figure 4B, Rikenellaceae_RC9_gut_group abundance was found to be positively correlated with amino acid content of tyrosine (Tyr), methionine (Met), leucine (Leu), isoleucine (Ile), and histidine (His). In addition, genera *Alloprevotella* and *Muribaculaceae* were positively correlated with several amino acids. Interestingly, Oscillospiraceae_UCG-005 abundance was positively correlated with fatty acid composition except amino acid composition. Together, we found more significant positive correlations in *semimembranosus* muscles were identified when compared with *longissimus dorsi* muscles.

4. Discussion

Roughage is an important part of feed for ruminants. Although a large number of forage species can utilize to provide roughage for domestic animals, there are diverse agronomic requirements for effective production forages (23). Meanwhile, supplementation with local and traditional forage is a strategy for reducing feeding cost and improve profitability for smallholders, and concentrate feeds on growth performance is often unsatisfactory in goat rearing (24). It is after these realizations that forage species namely Hemarthria altissima, Pennisetum sinese, and forage maize were chosen to fed local Boer crossbred goats in this study. Although goat growth rate was not affected by the forage type, animals from the HA group had the highest semi-eviscerated slaughter percentage and eviscerated slaughter percentage with the higher dressing percentage among the three groups (Table 1). This would imply that Hemarthria altissima is more suitable to improve the production efficiency.

Water-holding capacity is defined as the ability of meat to bind water and, therefore, always linked to the sensory properties of meat such as juiciness, texture, and flavor (25). The water released can be described as drip loss, and which is inversely related to water-holding capacity. In this study, the moisture content and cooking loss of goats were similar with no differences among different forage treatments, whereas animals fed with Hemarthria altissima forage have higher drip loss of *longissimus dorsi* muscles as compared to other two types of forages. This may be due to the different organizational structure of the meat between these three forage treatments. Meat color is one of the most important factor that can affect consumers' initial selection and purchase decision, and which may be contribute to combined effects of breed, aging, diet, intramuscular fat, and meat pH (3, 26). According Realini et al. (27), pasture-feed steers had darker color of *longissimus dorsi* muscles as compared to concentrate-fed steers. In the current study, the

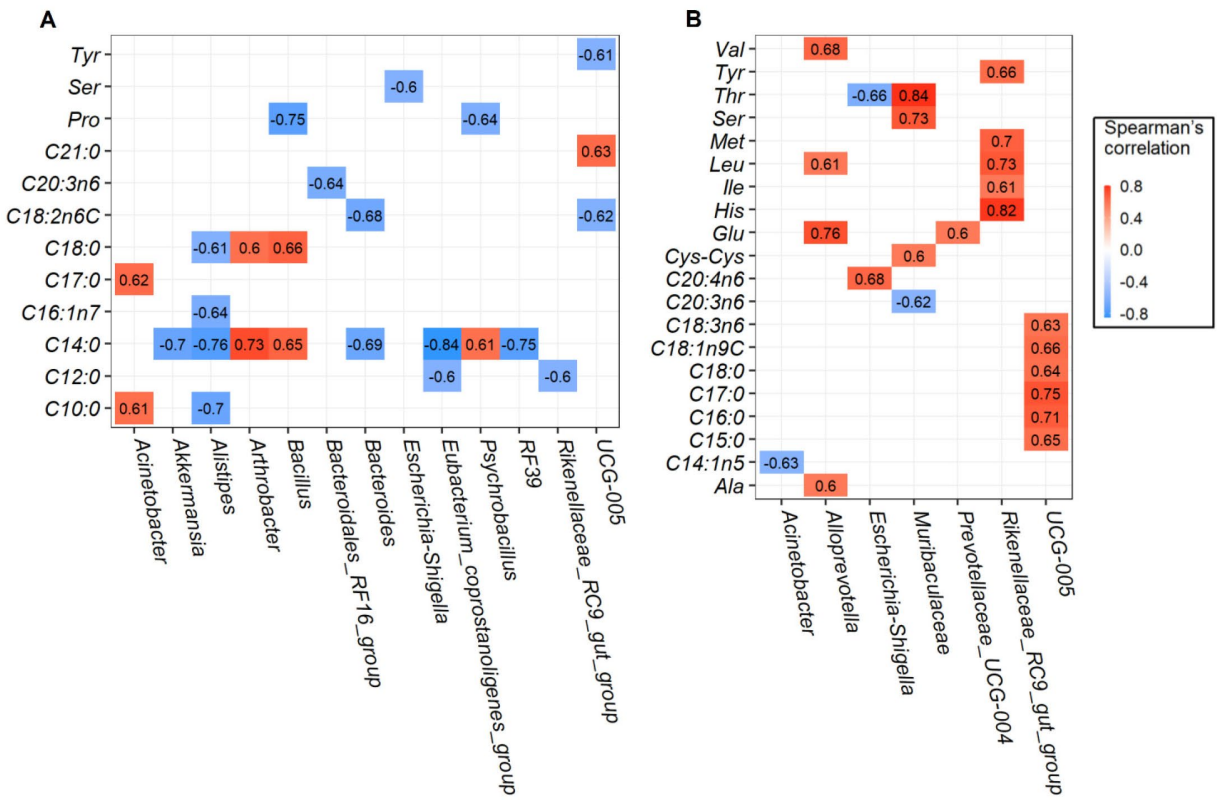

FIGURE 4
Correlation analysis between the rumen bacteria and amino acids and fatty acids. Significant correlations between the content of top 20 genera and amino acids and fatty acids in *longissimus dorsi* **(A)**, and *semimembranosus* **(B)**.

longissimus dorsi muscles from HA group had significantly lower L_{24h} values. The possible explanation was that it was related to factors such as forage composition and physical activities, which requires further investigation.

Amino acids are the basic components of animal protein, and changes in types and concentrations directly affect the nutritional value and flavor of meat. Forage treatments affected the amino acid profile of goat meat, especially whose profiles in *Semimembranosus* muscles. Goats fed forage maize had significant lower content of five of the non-essential amino acids measured (Asn, Glu, Ser, Ala, and Cys) but higher content of Pro than those of the other two groups in *Semimembranosus* muscles. Meanwhile, essential amino acids are critical for the body, and which is usually used to evaluate the biological value of protein (28). In this study, the contents of essential amino acid of threonine (Thr) was lower and other two essential amino acids of valine (Val) and phenylalanine (Phe) were higher in *Semimembranosus* muscles of goats fed forage maize. This result was consistent with the observation that the amino acids profile of goat meat protein was significantly affected by diet (29). Overall, meats from goats fed forage maize, especially *Semimembranosus* muscles are rich in essential amino acids, can be good sources of proteins for humans.

In addition to amino acid profile, a previous publication has showed that diet had effects on other physicochemical properties of goat meat, such as fatty acid profile and sensory qualities (30). In this study, the predominate fatty acids detected in *longissimus dorsi* and *semimembranosus* muscles were palmitic acid (C16: 0), oleic acid (C18: 1n9c), linoleic acid (C18: 2n6c), stearic acid (C18: 00), and arachidonic acid (C20: 4n6) (Supplementary Table S2). Similar results have been reported in a previous study conducted on Korean native black goats (6). The composition and concentration of fatty acids of *longissimus dorsi* muscles were not influenced by the forage treatments. However, goats fed forage maize had a significant higher contents of linoleic acid (C18: 2n6c), arachidonic acid (C20: 4n6), and docosahexaenoic acid (C22: 6n3) in the *semimembranosus* muscles compared with other two treatments (Table 2). Especially, docosahexaenoic acid has long been proposed to bestow health benefits by improving blood pressure control, attenuating the progression of Alzheimer's disease (31). Therefore, the docosahexaenoic acid is recognized as a beneficial dietary constituent, and goat meat is a desirable candidate for dietary docosahexaenoic acid enrichment (32). This suggests that, goats fed forage maize induced an increase in the amount of beneficial fatty acids and, therefore, improvement of its nutritional value. The fatty acids of meat mainly affected by diet composition and rumen microbiota because which absorbed by the duodenum are mainly from dietary origin as well as the result of rumen microbial biohydrogenation of dietary lipids (33). Moreover, several bacterial species are fond to be associated with beneficial fatty acids, such as *Butyrivibrio_2* was positively correlated to the α-linolenic acid (C18: 3 n-3, ALA) and conjugated linoleic acid (CLA) contents in sheep (34). In addition, several studies highlighted that the fat content of meat could be enhanced by providing a high energy diet (35). Here we analyzed the fatty acids of *semimembranosus* muscles among different forage treatments, it should be noted that the fatty acids in each diet of HA, PS, and FG as well as which in rumen remains unexplored. Future research would have to address these questions.

In the present study, neither the alpha diversity nor the relative abundances of main phyla were affected significantly by these three forage treatments. The relative abundances of Bacteroidota, Firmicutes, and Proteobacteria showed to be predominated phyla in the three groups (Figure 2A), which is consistent with the previous results conducted in goats (14) and other ruminants (36). At the genus level, Rikenellaceae_RC9_gut_group remained the dominant species in HA and PS group, whereas which were sub-dominated in FG group. Rikenellaceae_RC9_gut_group belong to the Rikenellaceae family, which play a key role in the digestion of crude fiber, and whose abundance decreased along with the content reduction of neutral detergent fiber in the diet (37). However, the highest neutral detergent fiber content in forage maize was found to be associated with the decrease of Rikenellaceae_RC9_gut_group in the rumen of FG goats. Future research would have to address this question. *Escherichia-Shigella* is a well-known member of the normal intestinal microflora of animals, and which is a potential pathogen known to delay the establishment of the anaerobic rumen environment (38). The high abundance of this genus in FG animals might be related to the ruminal fermentation parameters, ruminal enzymic activities, and ruminal epithelium development (39). However, the relationship between the *Escherichia-Shigella* and rumen fermentation parameters remains poorly understood and deserves further investigation.

Rumen bacteria are closely related to animal production and meat quality traits (40). Accordingly, we assessed whether the correlation existed between the bacterial genera and amino acids and/or fatty acids. The Rikenellaceae_RC9_gut_group genera played vital roles in carbohydrates degradation (41, 42), and was reported to be positively correlated with fatty acids production (14). However, no published research to date has yet to explore the correlation between genera Rikenellaceae_RC9_gut_group and amino acids. Results of the present study showed that strong positive correlations between bacterial genera and amino acids were found in the *semimembranosus* muscles, especially the lipid metabolism-related bacteria Rikenellaceae_RC9_gut_group were found to be positively correlated with amino acid content of tyrosine (Tyr), methionine (Met), leucine (Leu), isoleucine (Ile), and histidine (His), indicating this genus has played important role in modulating meat amino acid in ruminants. In addition, genera Oscillospiraceae_UCG-005 were positively correlated with several fatty acids such as pentadecanoic acid (C15: 0), palmitic acid (C16: 0), heptadecanoic acid (C17: 0), stearic acid (C18: 0), oleic acid (C18: 1n9C), and γ-linolenic acid (C18: 3n6), therefore, it is tempting to explore whether Oscillospiraceae_UCG-005 could improve goat meat nutritional value by increasing the amount of beneficial fatty acids.

5. Conclusion

Feeding suitable type of local and traditional forage is an important strategy for producing high-quality goat meat as well as reducing feeding cost for smallholders. In this study, we found that forage maize is more suitable to improve the production efficiency, and *longissimus dorsi* muscles of goats fed forage maize had significantly lower L_{24h} values. In addition, goats fed forage maize can increase beneficial fatty acids and amino acids and, thereby induce an

improvement in their meat nutritional value. Further, our 16S rRNA gene sequencing results showed that rumen microbiota was significantly associated with the goat meat nutritional compositions, and bacteria Rikenellaceae_RC9_gut_group and Oscillospiraceae_UCG-005 showed significantly positive correlated with the beneficial fatty acids contents.

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.

Author contributions

Zl-W, WW, JZ, and YX: conceived and designed the experiments. Zl-W, XY, HL, BH, TB, DL, YZ, and RZ: performed the experiments. Zl-W, XY, and HH: analyzed the data. Zl-W: wrote the paper. Zl-W, XY, and YX: reviewed and edited the manuscript. All authors contributed to the article and approved the submitted version.

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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

The Supplementary material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fvets.2023.1147685/full#supplementary-material

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Effect of *Cyberlindnera jadinii* supplementation on growth performance, serum immunity, antioxidant status, and intestinal health in winter fur-growing raccoon dogs (*Nyctereutes procyonoides*)

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Introduction: This study aimed to investigate the effects of *Cyberlindnera jadinii* supplementation on the growth performance, nutrient utilization, serum biochemistry, immunity, antioxidant status, and intestinal microbiota of raccoon dogs during the winter fur-growing period.

Methods: Forty-five 135 (±5) day-old male raccoon dogs were randomly assigned to three dietary groups supplemented with 0 (group N), 1×10^9 (group L) and 5×10^9 CFU/g (group H) *Cyberlindnera jadinii*, with 15 raccoon dogs per group.

Results: The results showed that *Cyberlindnera jadinii* in groups L and H improved average daily gain (ADG) and decreased feed-to-weight ratio (F/G) ($P < 0.05$). No significant difference was found in nutrient digestibility and nitrogen metabolism among the three groups ($P > 0.05$). Compared with group N, serum glucose levels were lower in groups L and H ($P < 0.05$). The levels of serum immunoglobulins A and G in group L were higher than those in the other two groups ($P < 0.05$), and the levels of serum immunoglobulins A and M in group H were higher than those in group N ($P < 0.05$). Supplementation with *Cyberlindnera jadinii* in groups L and H increased serum superoxide dismutase activity, and the total antioxidant capacity in group H increased compared with group N ($P < 0.05$). The phyla Bacteroidetes and Firmicutes were dominant in raccoon dogs. The results of principal coordinate analysis (PCoA) showed that the composition of microbiota in the three groups changed significantly ($P < 0.05$). The relative abundance of Campylobacterota was increased in the H group compared to the N and L groups ($P < 0.05$). The relative abundance of *Sarcina* was increased in group L compared with the other two groups ($P < 0.05$), while the relative abundance of *Subdoligranulum* and *Blautia* were decreased in group H compared with the other two groups ($P < 0.05$). Also, the relative abundance of *Prevotella*, *Sutterella* and *Catenibacterium* was higher in group L ($P < 0.05$) compared with group H.

Discussion: In conclusion, dietary supplementation with *Cyberlindnera jadinii* improved growth performance, antioxidant activity, immune status, and improved intestinal microbiota in winter fur-growing raccoon dogs. Among the concentrations tested, 1×10^9 CFU/g was the most effective level of supplementation.

KEYWORDS

***Cyberlindnera jadinii*, growth performance, immunity, antioxidant, microbiota, raccoon dogs**

Introduction

Cyberlindnera jadinii, an anamorphic form of *Candida utilis*, is used in the food and fodder industries (1, 2). *Cyberlindnera jadinii* is capable of producing valuable bioproducts and is an attractive source of biomass rich in protein and vitamins (3). It has been reported that *Cyberlindnera jadinii* has potential as an unconventional protein feed ingredients (4) and can replace 10% of soybean and meal crude protein in broiler chicken diets while maintaining growth performance and digestive function (5). More importantly, as a feed additive, *Cyberlindnera jadinii* can also improve animal health, promote growth and development, and improve feed efficiency. For example, the dietary supplementation with *Cyberlindnera jadinii* could not only improve the ruminal ammonia nitrogen contents of total volatile fatty acids and propionic acid of dairy cows and reduce the ruminal acetic acid concentration and acetic acid to propionic acid ratio, but also has a tendency to increase feed efficiency (6). Dietary supplementation with *Cyberlindnera jadinii* also improved feed conversion ratio and protein digestibility in Hu sheep (7), which was found to improve gut homeostasis and increase cecal microbial diversity in weaned piglets (8, 9). Recent studies demonstrated that dietary supplementation with *Cyberlindnera jadinii* increases feed intake of fodder in total mixed ratio and carcass weight in beef cattle aged 15 months, and also decrease dry matter intake and feed to weight ratio (10). However, no research data on *Cyberlindnera jadinii* in the diet of canine animals was available.

The Ussuri raccoon dog is a very ancient canid with a short, fat body somewhere between a raccoon and a dog. Besides, the Ussuri raccoon dog is the only hibernating animal among the canids, and its utilization of nutrients has its own unique characteristics, such as the demand for higher animal protein ingredients than that of red foxes, and it has certain requirements for fruits and grains in the diets. The main function of the gut is to digest food and absorb nutrients (11), and the gut microbiota is involved in the digestion and absorption of nutrients, maturation and regulation of the immune system (12, 13). In our previous study, we have found that supplementation with 1×10^9 CFU/g *Cyberlindnera jadinii* has a positive impact on growth performance and intestinal microbiota in growing raccoon dogs (14). However, the winter fur-growing period is a key stage for the growth and development of raccoon dogs, and a key stage for producing high economic value. Yet, the effect of *Cyberlindnera jadinii* on the winter fur-growing period of raccoon dog has not been studied to date. We hypothesized that *Cyberlindnera jadinii* might affect the growth and gut microbiota of winter fur-growing raccoon dogs. Therefore, the objectives of the present research were to study the effects of *Cyberlindnera jadinii* on the growth performance, nutrient digestibility, nitrogen metabolism, serum biochemical parameters, antioxidant capacity and gut microbiota of winter fur-growing raccoon dog. Our results may help to better apply *Cyberlindnera jadinii* to the production of raccoon dog and the development of canine products.

Materials and methods

All procedures involving animals were carried out in accordance with the guidelines for animal studies issued by Chifeng University.

Fungal strain

Cyberlindnera jadinii (Number: YTCJ91011) was preserved by the microbiology laboratory of the Chifeng University. Liquid potato dextrose culture medium (PDB) (Potato extract powder 20 g/L, glucose 20 g/L, distilled water 1 L) was used for resuscitating and subcultures (aerobic cultivation) at 28°C for 24 h.

Animal husbandry and experimental design

Forty-five healthy male raccoon dogs aged 135 ± 5 days and with similar body weight (7.78 ± 0.62 kg) were randomly assigned to three treatment groups, with 15 replicates per group. All animals were obtained from Shenyang Boyang Feed Co., Ltd. The *Cyberlindnera jadinii* was either not supplemented in the diet (group N) or supplemented with 1×10^9 CFU/g (group L) or 5×10^9 CFU/g (group H). The dosing basis for *Cyberlindnera jadinii* was based on the study in weaned piglets (15). The basal diet was formulated according to the management guidelines of the National Research Council (NRC, 1982) (16), and the composition and nutrient levels of the basal diet are shown in Table 1. All animals were housed individually in conventional cages (1.0 × 0.8 × 0.8 m). Raccoon dogs were fed twice daily at 7:00 AM and 15:00 PM with free access to water. After 7 days of adaptation, the experiment was lasted for 30 days.

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

Items Ingredients	Content (%)	Items Nutrient levels[b]	Content (%)
Extruded corn	42.81	Metabolizable energy (MJ/kg)[b]	14.78
Wheat bran	8.25	Crude protein	23.02
Corn germ meal	7.74	Ether extract	10.08
Rice bran meal	1.55	Ash	7.65
Soybean oil	7.22	Calcium	1.03
Soybean meal	10.83	Phosphorus	0.78
Distillers dried grains with solubles	6.71	Lysine	1.38
Fish meal	6.19	Methionine	0.95
Meat and bone meal	6.19	Methionine + Cysteine	1.22
Pork plasma protein powder	0.52		
NaCl	0.15		
Lysine	0.52		
Methionine	0.62		
Choline	0.08		
Premix[a]	0.62		
Total	100.00		

[a]The premix contains the following ingredients per kg of diet: VA 15 902 IU, VB_1 44 mg, VB_2 19 mg, VB_6 19 mg, VB_{12} 0.06 mg, VD_3 2 224 IU, VE 126 mg, VK_3 2 mg, biotin 0.55 mg, folic acid 1.0 mg, D-pantothenic acid 17 mg, nicotinamide 38 mg, antioxidant 0.5 mg, Cu (as copper sulfate) 32 mg, Fe (as ferrous sulfate) 118 mg, Mn (as manganese sulfate) 62 mg, Zn (as zinc sulfate) 100 mg, I (as calcium iodate) 0.31 mg, Se (as sodium selenite) 0.23 mg.
[b]Metabolizable energy was calculated based on the concentrations of crude protein, fat and carbohydrates according to from NRC (16), while others are measured values.

Experimental procedures and sample collection

The raccoon dogs were weighed before morning feeding at the beginning and end of the experiment, and the animals' initial body weight (IBW) and final body weight (FBW) were used to determine average daily gain (ADG). On the 30th day of the experiment, eight raccoon dogs with similar weight and good health were randomly selected in each group for digestion and metabolism experiments. Urine and feces were collected for three consecutive days. At the end of the experiment, the urine was preserved with 10% sulfuric acid. The total volume amount of urine from each animal was recorded and filtered through filter paper. 10 mL of each filtrate were stored at −20°C. 10% sulfuric acid was added to feces in accordance with 5% of the fresh weight. All feces from one animal were mixed and weighted. 10% of the total weight of feces was dried at 65°C to constant weight, and then ground through a 40-mesh screen. The daily amount of feed provided and the amount of remaining feed during the experimental period were recorded. Meanwhile, 5 mL of blood samples from the veins of lower limbs of three groups of raccoon dogs were collected, placed in a serum separation tubes (BD-Pharmingen, USA), and centrifuged at 3,000× g for 10 min to obtain serum. All serum samples were stored at −20°C for later analysis.

Chemical analysis

Diets and feces were analyzed for dry matter (DM), ether extract (EE), crude protein (CP), ash (17). Dietary calcium and phosphorus were estimated by the AOAC method (17). Nitrogen in urine was analyzed according to the procedures of AOAC (17). All chemical analyses were conducted in triplicate.

Serum sample analysis

Concentrations of serum total protein (TP), albumin (ALB), glucose (GLU), triglyceride (TG), highly density lipoprotein (HDL), low-density lipoprotein (LDL), cholesterol (CHO) and enzymatic activities of alkaline phosphatase (ALP), alanine aminotransferase (ALT) and aspartate aminotransferase (AST) (kits supplied by Zhongsheng Beikong Biotechnology Co., Ltd., Beijing, China) were analyzed by an automatic biochemical analyzer (Selectra-E, Witu). Serum immunoglobulins IgA, IgM, and IgG were measured using ELISA kits (Shanghai Shuangying Biotechnology Co., Ltd., Shanghai, China). The contents of glutathione peroxidase (GSH-Px), superoxide dismutase (SOD), total antioxidant capacity (T-AOC) and maleic dialdehyde (MDA) were determined using respective diagnostic kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, China).

DNA extraction, amplification, sequencing and bioinformatics analysis

Total bacterial DNA was extracted from the samples using the Qiagen magnetic bead extraction Kit (Qiagen, Valencia, California, USA) according to the manufacturer's instructions. Primers 341F (5′-CCTAYGGGRBGCASCAG-3′) and 806R (5′-GGACTACNNGGGTATCTAAT-3′) were used to amplify the V3–V4 region of the bacterial 16S rRNA gene. The resultant amplicons were purified using the Thermo Scientific GeneJET Gel Extraction Kit (Thermo Scientific, Belmont, Massachusetts, USA), and then sequenced on an Illumina NovaSeq 6000 platform to produce 250-bp paired-end reads (14).

Paired-end reads were merged into raw tags using FLASH (version 1.2.7) (18). Quality filtering (< 30 Phred score) of the raw tags was strictly performed with QIIME (version 1.9.1) to obtain high-quality clean tags (19, 20), which were compared to the SILVA database (version 138) using UCHIME algorithm to identify and remove chimeras of valid tags (21, 22). The Uparse algorithm (Uparse version 7.0.1001) was used to cluster valid tags from all samples and cluster sequences into operational taxonomic units (OTUs) with 97% identity (23). The sequence with the highest frequency of occurrence in OTUs was screened for further annotation. All analyses from clustering to determining alpha and beta diversity were performed with QIIME (version 1.9.1). The

TABLE 2 Effects of *Cyberlindnera jadinii* on the growth performance of winter fur-growing raccoon dogs.

Items	Group			*P*-value
	N	L	H	
IBW, kg	7.87 ± 0.17	7.75 ± 0.27	7.71 ± 0.24	0.868
FBW, kg	8.72 ± 0.11	9.04 ± 0.23	9.02 ± 0.26	0.495
ADG, g/d	31.48 ± 3.54^{b}	48.05 ± 2.68^{a}	48.52 ± 2.42^{a}	0.001
ADFI, g/d	606.31 ± 8.96	594.79 ± 6.23	600.21 ± 10.11	0.644
F/G	20.92 ± 2.23^{a}	12.58 ± 0.54^{b}	12.54 ± 0.52^{b}	<0.001

IBW, Initial body weight; FBW, Final body weight; ADG, Average daily gain; ADFI, average daily feed intake; F/G, ratio of feed to gain. N group, 0 CFU/g *Cyberlindnera jadinii*; L group, 1×10^9 CFU/g *Cyberlindnera jadinii*; H group, 5×10^9 CFU/g *Cyberlindnera jadinii*. Data in the same row with no letter or with the same superscripts means no significant difference ($P > 0.05$); different lowercase letters mean significant difference ($P < 0.05$).

Adonis function of the R vegan package (version 2.15.3) was used to test the significance of separation by permutation multivariate analysis of variance (PERMANOVA) (24).

Statistical analysis

The growth performance of each animal was calculated using the following formulas: ADG = (FBW– IBW)/30 days; Average daily feed intake (ADFI) = sum of daily feed intake/30 days; Feed conversion ratio (F:G) = ADFI/ADG.

Nitrogen content in diets, feces, and urine were calculated as crude protein (CP)/6.25. The nutrient apparent digestibility and nitrogen metabolism were calculated as follows: Nutrient apparent digestibility (g/Kg) = (feed nutrient intake – nutrient excretion in feces)/feed nutrient intake × 1000; Nitrogen retention = nitrogen intake – fecal nitrogen – urinary nitrogen; Net protein utilization (NPU) (%) = nitrogen retention/nitrogen intake × 100%; and Biological value of protein (BV) (%) = nitrogen retention/(nitrogen intake – fecal nitrogen) × 100%.

Data were analyzed by One-way analysis of variance (ANOVA) and Bonferroni multiple comparison test if the data were in Gaussian distribution and had equal variance or analyzed by the Kruskal-Wallis test and Bonferroni multiple comparison test if the data were not normally distributed. Data were represented as mean ± standard error. $P < 0.05$ indicates the difference is significant. STAMP software (*t*-test) was used to analyze the differences in the abundance of microbiota in each group, and the Benjamini-Hochberg FDR multiple test correction method was used to control the false positive rate.

Results

Growth performance

As shown in Table 2, there was no significant difference in IBW, FBW, or ADFI among the three groups ($P > 0.05$). ADG was increased in groups L and H compared to group N ($P < 0.05$), while F/G was decreased in groups L and H compared to group N ($P < 0.05$).

TABLE 3 Effect of *Cyberlindnera jadinii* on nutrients digestibility of winter fur-growing raccoon dogs.

Items	Group			*P*-value
	N	L	H	
Dry matter, %	85.80 ± 0.53	85.75 ± 1.71	85.71 ± 0.74	0.999
Ether extract, %	92.63 ± 0.82	91.24 ± 0.93	92.40 ± 0.88	0.500
Crude protein, %	85.61 ± 0.60	85.65 ± 1.71	85.74 ± 0.73	0.996

N group, 0 CFU/g *Cyberlindnera jadinii*; L group, 1×10^9 CFU/g *Cyberlindnera jadinii*; H group, 5×10^9 CFU/g *Cyberlindnera jadinii*. In the same row, values with no letter or with the same lowerletter superscripts indicate no significant difference ($P > 0.05$); superscripts with different lowercase letters indicate significant difference ($P < 0.05$).

TABLE 4 Effect of *Cyberlindnera jadinii* on N metabolism of winter fur-growing raccoon dogs.

Items	Group			*P*-value
	N	L	H	
Nitrogen intake, g/d	20.71 ± 0.31	20.32 ± 0.21	20.50 ± 0.35	0.642
Fecal nitrogen, g/d	2.99 ± 0.16	2.91 ± 0.34	2.92 ± 0.16	0.967
Urine nitrogen, g/d	5.22 ± 0.40	5.46 ± 0.52	4.87 ± 0.33	0.611
Nitrogen retention, g/d	12.50 ± 0.45	11.94 ± 0.70	12.71 ± 0.47	0.602
NPU, %	60.45 ± 2.21	58.75 ± 3.27	62.01 ± 2.02	0.671
BV, %	70.53 ± 2.24	68.46 ± 3.10	72.25 ± 1.91	0.563

NPU, net protein utilization; BV, biological value of protein. N group, 0 CFU/g *Cyberlindnera jadinii*; L group, 1×10^9 CFU/g *Cyberlindnera jadinii*; H group, 5×10^9 CFU/g *Cyberlindnera jadinii*. In the same row, values with no letter or the same letter superscripts indicate no significant difference ($P > 0.05$); different small letter superscripts indicate a significant difference ($P < 0.05$).

Nutrients digestibility and nitrogen metabolism

There were no significant differences among the three groups in dry matter, ether extract and crude protein digestibility ($P > 0.05$, Table 3). There was also no difference in nitrogen intake, fecal nitrogen, urine nitrogen, nitrogen retention, NPU and BV ($P > 0.05$, Table 4) among the three groups.

Serum biochemical parameters, immune levels, and antioxidant capacity of the three groups

As shown in Table 5, compared with group N, the serum GLU levels of groups L and H were decreased ($P < 0.05$), while the serum IgA levels of groups L and H was increased compared with group N ($P < 0.05$) as shown in Table 6, and the serum IgG levels was increased in group L compared with groups N and H ($P < 0.05$). The serum IgM levels was increased in group H compared with groups N and L ($P < 0.05$). The SOD activity in groups L and H was higher than that in group N ($P < 0.05$), but no significant difference was observed between groups L and H ($P > 0.05$). Serum T-AOC was increased in group H compared with groups N and L (*P*

< 0.05), but no significant difference in serum MDA and GSH-Px activities were observed among the three groups ($P > 0.05$).

Summary of high-throughput sequencing and alpha diversity

A total of 1,572,834 16S rRNA gene sequences from the three designed groups were obtained from this study. After clustering at the 97% similarity level, sequences were assigned to 3,095 OTUs. Good coverage ranged from 0.996 to 0.999 demonstrated sufficient sequencing depth for all samples. As shown in Figure 1, no difference in the observed species, Shannon, Simpson, Chao1 and ACE indices was observed among the three groups ($P > 0.05$).

TABLE 5 Effects of *Cyberlindnera jadinii* on serum biochemical indices in winter fur-growing raccoon dogs.

Items	Group			*P*-value
	N	L	H	
TP, mmol/L	80.15 ± 3.27	74.38 ± 2.07	81.37 ± 4.19	0.297
ALB, g/L	34.29 ± 1.27	31.43 ± 0.94	30.83 ± 2.87	0.401
GLU, mmol/L	3.72 ± 0.06^{a}	3.24 ± 0.11^{b}	2.89 ± 0.12^{b}	<0.001
ALP, U/L	29.96 ± 2.28	29.69 ± 1.45	29.84 ± 3.25	0.997
AST, U/L	55.17 ± 5.78	60.95 ± 2.12	65.13 ± 5.58	0.356
ALT, U/L	78.98 ± 14.29	57.21 ± 7.32	75.61 ± 15.24	0.432
TG, mmol/L	0.99 ± 0.14	0.75 ± 0.12	1.05 ± 0.13	0.264
CHO, mmol/L	4.25 ± 0.36	3.73 ± 0.34	3.31 ± 0.21	0.145
HDL, mmol/L	2.97 ± 0.24	2.62 ± 0.24	2.36 ± 0.11	0.135
LDL, mmol/L	0.25 ± 0.02	0.24 ± 0.03	0.21 ± 0.02	0.522

TP, totalprotein; ALB, albumin; GLU, glucose; ALP, alkaline phosphatase; AST, aspartate ami-notransferase; ALT, alanine aminotransferase; TG, triglycerides; CHO, cholesterol; HDL, highly density lipoprotein; LDL, low density lipoprotein. N group, 0 CFU/g *Cyberlindnera jadinii*; L group, 1×10^9 CFU/g *Cyberlindnera jadinii*; H group, 5×10^9 CFU/g *Cyberlindnera jadinii*. In the same row, values with no letter or the same letter superscripts indicate no significant difference ($P > 0.05$); different small letter superscripts indicate a significant difference ($P < 0.05$).

Composition and comparison of gut microbiota

PCoA was applied to examine differences in taxonomic community composition and structure in the gut of raccoon dogs. PCoA based on binary Jaccard distance (Figure 2A) showed that group N was separated from group L [Table 7, Adonis: $P < 0.05$ (N vs. L)]. PCoA based on binary Jaccard distance (Figure 2A) and unweighted UniFrac distance (Figure 2B) showed separation of group N from group H [Table 7, Adonis: $P < 0.05$ (N vs. H)], whereas PCoA based on binary Jaccard distance (Figure 2A), unweighted UniFrac distance (Figure 2B), and Bray–Curtis distance (Figure 2C) showed that group L was separated from group H [Table 7, Adonis: $P < 0.05$ (L vs. H)].

At the phylum level, the top 10 bacteria in relative abundance were identified, and the results indicated that Bacteroidota (N = 49.55 ± 5.49%, L = 48.90 ± 4.19%, H = 50.36 ± 7.19%), Firmicutes (N = 40.99 ± 5.56%, L = 41.93 ± 4.02%, H = 39.24 ± 7.02%), unidentified_Bacteria (N = 4.19 ± 0.34%, L = 3.58 ± 0.49%, H = 4.28 ± 0.12%), and Proteobacteria (N = 3.09 ± 0.73%, L = 2.99 ± 0.74%, H = 2.25 ± 0.87%) were the most abundant bacteria in all three groups (Figure 2D). At the genus level, *Prevotella_9* was the dominant genus in all three groups (N = 32.91 ± 4.36%, L = 30.75 ± 5.09%, H = 36.54 ± 5.91%). While *Alloprevotella* (7.65 ± 0.94%), *Prevotella* (4.50 ± 0.68%), *Faecalibacterium* (3.19 ± 0.62%), and *Streptococcus* (2.93 ± 1.90%) were the most abundant genera in group N. *Alloprevotella* (8.51 ± 1.08%), *Faecalibacterium* (6.41 ± 2.51%), *Prevotella* (5.32 ± 0.55%), and *Dialister* (4.46 ± 1.56%) were the most abundant genera in group L, and *Megamonas* (6.43 ± 5.96%), *Alloprevotella* (6.38 ± 2.43%), *Streptococcus* (4.92 ± 2.36%), and *Megasphaera* (3.70 ± 1.93%) were the most abundant genera in group H (Figure 2E).

By comparing the differences in the relative abundance of bacteria within the phylum, we found that the relative abundance of the Campylobacterota phylum was decreased in groups N and L compared with group H ($P < 0.05$) (Figures 3A, B). At the genus level, the relative abundance of *Sarcina* was increased in group L compared to group N ($P < 0.05$) (Figure 3C). The relative abundance of *Subdoligranulum* and *Blautia* was increased in group N compared with group H ($P < 0.05$) (Figure 3D). In addition,

TABLE 6 Effects of *Cyberlindnera jadinii* on serum immune and antioxidant indices in winter fur-growing raccoon dogs.

Items	Group			*P*-value
	N	L	H	
IgA (μg/ml)	40.31 ± 0.62^{b}	44.58 ± 1.08^{a}	44.43 ± 0.64^{a}	0.003
IgG (μg/ml)	355.44 ± 4.96^{b}	401.86 ± 6.97^{a}	373.08 ± 8.26^{b}	<0.001
IgM (μg/ml)	18.40 ± 0.27^{b}	18.22 ± 0.31^{b}	20.87 ± 0.38^{a}	<0.001
GSH-Px, U/mL	1,011.11 ± 90.56	1,055.56 ± 74.14	1,025.93 ± 81.88	0.928
SOD, U/mL	17.03 ± 0.86^{b}	19.58 ± 0.56^{a}	20.32 ± 0.37^{a}	0.005
T-AOC, U/mL	2.37 ± 0.25^{b}	2.73 ± 0.12^{ab}	3.26 ± 0.21^{a}	0.020
MDA, nmol/mL	9.12 ± 0.39	9.66 ± 0.99	10.34 ± 1.87	0.786

GSH-Px, Glutathione peroxidase; SOD, Superoxide dismutase; T-AOC, Total antioxidant capacity; MDA, Malondialdehyde. N group, 0 CFU/g *Cyberlindnera jadinii*; L group, 1×10^9 CFU/g *Cyberlindnera jadinii*; H group, 5×10^9 CFU/g *Cyberlindnera jadinii*. In the same row, values with no letter or the same letter superscripts indicate no significant difference ($P > 0.05$); different small letter superscripts indicate a significant difference ($P < 0.05$).

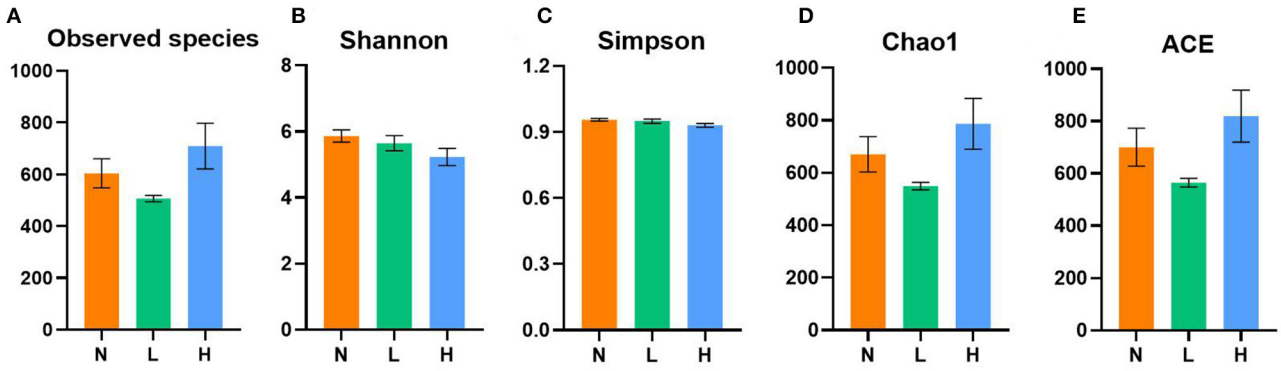

FIGURE 1
Comparisons of the alpha diversity indices of gut microbiota among the three groups of the raccoon dogs. Observed species **(A)**, Shannon index **(B)**, Simpson index **(C)**, Chao1 index **(D)**, and ACE index **(E)**. N group, 0 CFU/g *Cyberlindnera jadinii*; L group, 1×10^9 CFU/g *Cyberlindnera jadinii*; H group, 5×10^9 CFU/g *Cyberlindnera jadinii*.

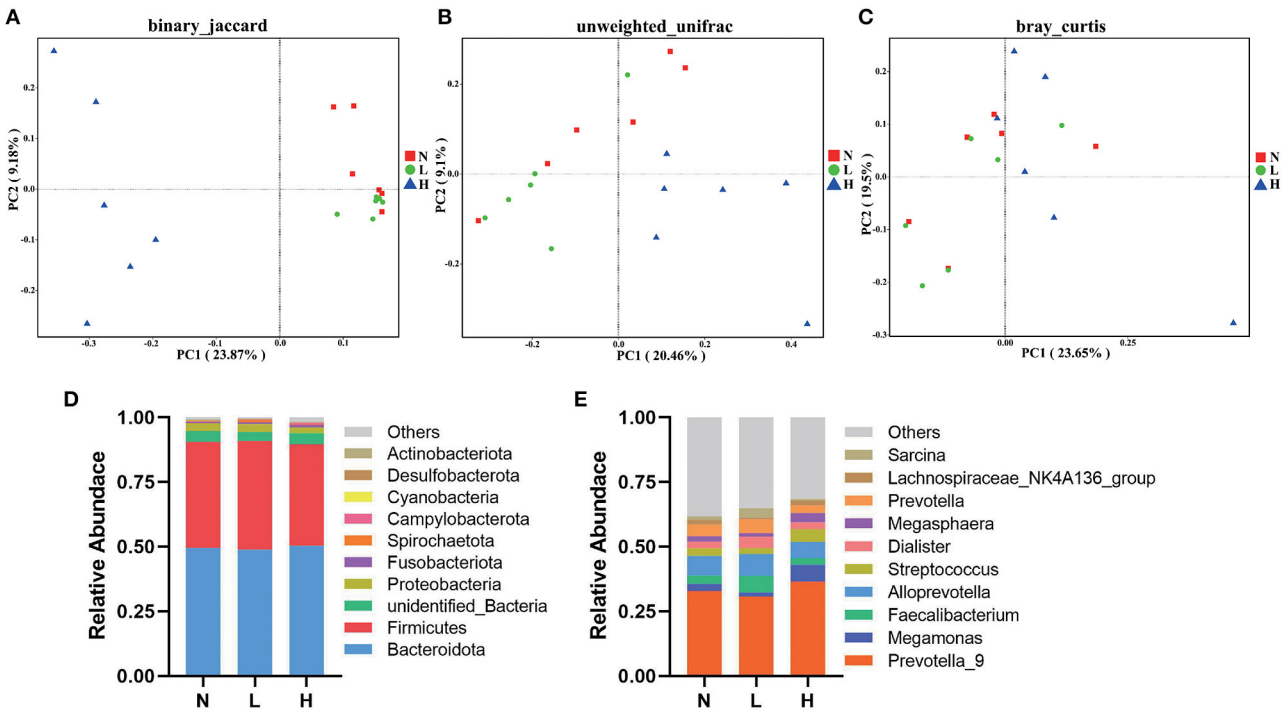

FIGURE 2
Composition and comparison of gut microbiota in three groups of raccoon dogs. PCoA revealed the separation of gut microbiota in the three groups based on binary Jaccard **(A)**, unweighted UniFrac distance **(B)**, and Bray–Curtis distance **(C)**. Gut microbial composition of the N, L and H group raccoon dogs at phylum **(D)** and genus **(E)** levels. N group, 0 CFU/g *Cyberlindnera jadinii*; L group, 1×10^9 CFU/g *Cyberlindnera jadinii*; H group, 5×10^9 CFU/g *Cyberlindnera jadinii*.

the relative abundance of *Sarcina*, *Prevotella*, *Subdoligranulum*, *Blautia*, *Sutterella* and *Catenibacterium* in group L were all increased compared with group H ($P < 0.05$) (Figure 3E).

Discussion

Cyberlindnera jadinii can improve growth performance, reduce the diarrhea rate, improve intestinal health, and increase the diversity and abundance of cecal microflora in weaned piglets (15). As shown in our previous study (14), dietary supplementation of *Cyberlindnera jadinii* can increase ADG and decrease F/G in growing raccoon dogs. In the present study, we found that *Cyberlindnera jadinii* could enhance ADG and decrease F/G in winter-growing raccoon dog, and dietary supplementation of *Cyberlindnera jadinii* at 5×10^9 CFU/g resulted in the highest ADG and the lowest F/G. In addition, our results also showed that *Cyberlindnera jadinii* supplementation had no significant effect on the digestibility of dry matter, ether extract and crude protein of raccoon dogs. Indeed, a previous study indicates that

TABLE 7 Adonis analysis of the gut bacterial communities of winter fur-growing raccoon dogs.

Group	Binary Jaccard		Unweighted UniFrac		Bray–Curtis	
	R^2	*P*-value	R^2	*P*-value	R^2	*P*-value
N vs. L	0.110	0.028	0.099	0.277	0.083	0.470
N vs. H	0.243	0.003	0.149	0.006	0.130	0.076
L vs. H	0.278	0.002	0.202	0.003	0.160	0.023

N group, 0 CFU/g *Cyberlindnera jadinii*; L group, 1×10^9 CFU/g *Cyberlindnera jadinii*; H group, 5×10^9 CFU/g *Cyberlindnera jadinii*.

dietary supplementation of *Cyberlindnera jadinii* have no obvious effects on the *in vitro* dry matter digestibility of dairy cow (6). However, the dietary supplementation of *Cyberlindnera jadinii* to Hu sheep seems to moderately improve the digestibility of CP (7). This different result might be explained by the experimental animals, appropriate doses of yeast and time of feeding. Likewise, we also observed that *Cyberlindnera jadinii* supplementation had no significant effect on nitrogen metabolism in winter-growing raccoon dogs. Only preliminary studies showed that addition of yeast to feed reduces urinary nitrogen excretion and improve nitrogen deposition and BV in minks (25). This might be because yeast cells are rich in proteins, fats, enzymes, and some coordination factors (26). These factors may lead to different effects and mechanisms of yeast products on the absorption and utilization of nutrients in the digestive tract of animals, but the specific reasons need further study.

Excessive production of free radicals can directly damage proteins, lipids, and nucleic acids, leading to cell death, and consumption of dietary antioxidants is believed to help alleviate oxidative damage and the risk of related diseases, such as cardiovascular disease, cancer, diabetes, and Alzheimer's disease (27, 28). In the present study, *Cyberlindnera jadinii* supplementation was found to increase SOD activity, and 5×10^9 CFU/g *Cyberlindnera jadinii* also increased T-AOC activity. SOD seems to play a fundamental antioxidant role in the detoxification from reactive oxygen species by dismutating superoxide radical anion to oxygen and hydrogen peroxide (29). Increased SOD activity thus plays a crucial role in the natural antioxidant defense system (30). With the improvement of SOD activity, the ability to scavenge oxygen free radicals is enhanced, thereby enhancing the antioxidant capacity (31). T-AOC is the total capacity of various antioxidants to scavenge oxygen free radicals in both enzymatic and non-enzymatic systems (32) and acts as a potent quencher and scavenger of many free radicals (33).

It has been well known that GLU is the most direct source of energy for animals. We found that *Cyberlindnera jadinii* decreased serum GLU concentrations, indicating that *Cyberlindnera jadinii* can promote the efficient use of GLU in winter fur-growing raccoon dogs. Also, oxygen free radicals may damage antioxidant defense system due to hyperglycemia (34). Combined with the antioxidant index results of this study, it seems to suggest that *Cyberlindnera jadinii* may lower serum glucose by scavenging oxygen free radicals. The extended significance of these results may be that adding *Cyberlindnera jadinii* to the diet is beneficial to the stability of blood glucose levels in canine animals, and has a more positive effect on the health of canine animals. In addition, our results showed that *Cyberlindnera jadinii* improved IgA, IgG and IgM levels in winter-growing raccoon dogs. The reason may be that mannan oligosaccharides and 1,3/1,6 β-D-glucans, the two major components of the yeast cell walls, can modulate immunity (35–37). Our results showed that *Cyberlindnera jadinii* supplementation improved the antioxidant activities and immunity in winter-growing raccoon dogs, and that supplementation with 5×10^9 CFU/g *Cyberlindnera jadinii* may be more beneficial.

Diet can alter the intestinal microbiome, which in turn can exhibit a profound impact on overall health (38). In this study, there were significant differences in the microbial community composition of the three groups of winter-growing raccoon dogs, as shown by the PCoA analysis. At the phylum level, the gut microbiota of the winter-growing raccoon dogs were dominated by representative sequences of Bacteroidota, Firmicutes, unidentified_Bacteria, and Proteobacteria. This is different from our previous study (14). The reason may be due to the differences in the living environment and growth stages of the animals. Interestingly, the relative abundance of Firmicutes and Bacteroides in the intestine of winter-growing raccoon dogs accounted for more than 90%. Interestingly, the Firmicutes phyla was found to be enriched in genes related to energy metabolism and material breakdown (39, 40), and the Bacteroidetes was associated with the degradation of proteins and carbohydrates (41, 42). Bacteroidetes are also deeply involved in nutrient metabolism, including carbohydrate and polysaccharide fermentation, and steroid metabolism, and are essential for normal physiological function of the intestine (43). Therefore, it indicates that winter-growing raccoon dogs may need to use more nutrients to meet their own needs. Moreover, the relative abundance of Campylobacterota was significantly increased in group H. Notably, Campylobacterot are recognized as important pathogens: half of the human population is colonized with the ulcer-causing stomach bacterium *Helicobacter pylori*, and *Campylobacter jejuni* is considered the leading cause of bacterial food-borne gastroenteritis worldwide (44–46). The present findings suggested that the addition of 5×10^9 CFU/g *Cyberlindnera jadinii* might increase the abundance of microbiota related to intestinal infection.

Our results showed that at the genus level, the relative abundances of *Subdoligranulum, Sarcina*, *Sutterella*, *Blautia*, *Prevotella* and *Catenibacteriumn* were significantly increased in group L. The increase of *Subdoligranulum* may be due to the presence of undigested compounds such as fibers (47). Further, *Subdoligranulum* may protect the gut through butyrate production (48). *Sarcina* has been implicated in inflammatory processes, which may be related to its ability to produce butyrate through sugar-fermenting (49–51). The relatively high abundance of *Sarcina* in gut- and mucosa-associated microbiota might contribute to a stronger immune resistance in the small intestine (52). *Sutterella* does not appear to induce substantial inflammation (53), but the

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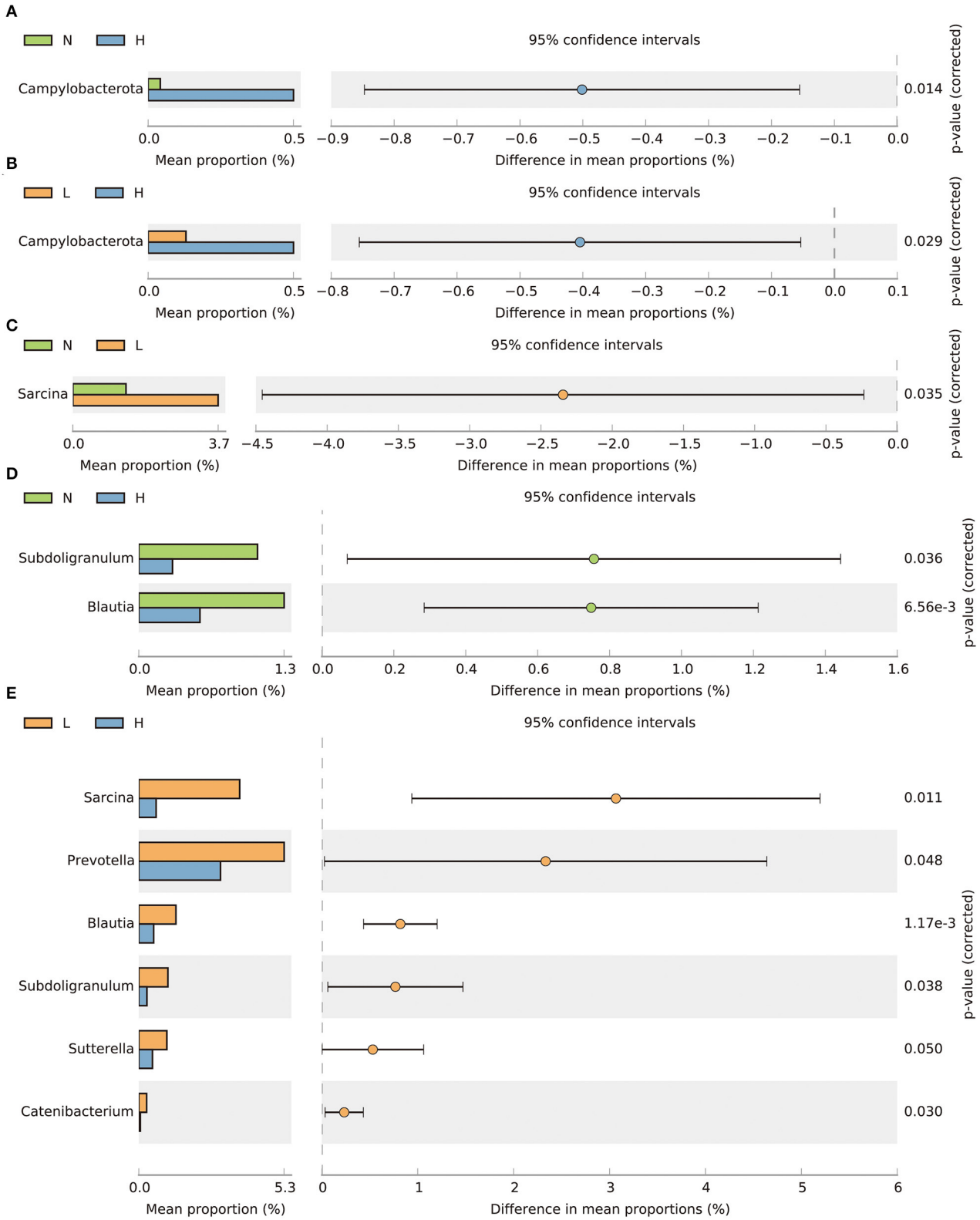

FIGURE 3
T-test bar plots showing differences in relative abundance of bacteria at the phylum **(A, B)** and genus **(C–E)** levels. N group, 0 CFU/g *Cyberlindnera jadinii*; L group, 1×10^9 CFU/g *Cyberlindnera jadinii*; H group, 5×10^9 CFU/g *Cyberlindnera jadinii*.

ability of *Sutterella* to adhere to intestinal epithelial cells indicates that they may have immunomodulatory roles (54). *Blautia* is a genus of anaerobic bacteria with probiotic characteristics widely found in the feces and intestines of mammals, and has been shown to play a role in inflammatory diseases (55). *Prevotella* favors a diet rich in sugars and complex carbohydrates (56). It has also been found to have the characteristic of decomposing starch and plant polysaccharides and is very good at catabolizing mucin (57–59). *Catenibacteriumn* and *Prevotella* are closely correlated to each other, and are strongly associated with long-term diets rich in carbohydrates but low in protein and animal fat (60). *Prevotella* and *Catenibacteriumn* could improve gut health and nutrient utilization by enhancing the fermentation of fiber to produce short chain fatty acids (SCFAs) (61). Previous studies have confirmed that higher concentrations of SCFAs in the intestine contribute to improved growth performance (62). Therefore, supplementation of *Cyberlindnera jadinii* may improve intestinal health by regulating intestinal microbiota, and this effect was greater when the supplementation level was 1×10^9 CFU/g.

Conclusions

Cyberlindnera jadinii has a certain beneficial probiotic effect on raccoon dogs. The combined analysis in this manuscript demonstrated that two feeding levels of *Cyberlindnera jadinii* (1×10^9 CFU/g or 5×10^9 CFU/g) could improve growth performance, immunity, antioxidant abilities and intestinal microbiota, and lowered blood glucose level in winter fur-growing raccoon dogs. Considering the comprehensive cost, 1×10^9 CFU/g may be the most suitable addition level under the experimental conditions.

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 at: https://www.ncbi.nlm.nih.gov/, PRJNA929307.

Ethics statement

The animal study was reviewed and approved by the Animal Care Committee of Chifeng University and conducted in strict compliance with the Committee's guidelines on animal care. Written informed consent was obtained from the owners for the participation of their animals in this study.

Author contributions

DZ: conceptualization, formal analysis, and writing—original draft. HL and HZ: funding acquisition, methodology, project administration, and writing—review and editing. DZ and YW: data curation, investigation, and supervision. KL, CM, and BZ: data curation and sample collection. All authors have read and approved the final manuscript.

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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 organic and inorganic copper on cecal microbiota and short-chain fatty acids in growing rabbits

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Introduction: Copper (Cu) is an essential trace element for the growth of rabbits. This study aimed to investigate the effects of different Cu sources on intestinal microorganisms and short-chain fatty acids (SCFAs) in growing rabbits.

Methods: The experimental animals were randomly divided into four experimental groups, each group comprised eight replicates, with six rabbits (half male and half female) per replicate. And they were fed diets was composed by mixing the basal diet with 20mg/kg Cu from one of the two inorganic Cu (cupric sulfate and dicopper chloride trihydroxide) or two organic Cu (cupric citrate and copper glycinate). Cecal contents of four rabbits were collected from four experimental groups for 16S rDNA gene amplification sequencing and gas chromatography analysis.

Results: Our results indicate that the organic Cu groups were less variable than the inorganic Cu groups. Compared with the inorganic Cu groups, the CuCit group had a significantly higher relative abundance of *Rikenella Tissierella*, *Lachnospiraceae_NK3A20_group*, *Enterococcus*, and *Paeniclostridium*, while the relative abundance of *Novosphingobium* and *Ruminococcus* were significantly lower ($p<0.05$). The SCFAs level decreased in the organic Cu groups than in the inorganic Cu groups. Among the SCFAs, the butyric acid level significantly decreased in the CuCit group than in the $CuSO_4$ and $CuCl_2$ groups. The relative abundance of *Rikenella* and *Turicibacter* genera was significantly negatively correlated with the butyric acid level in the CuCit group compared with both inorganic Cu groups. These results revealed that the organic Cu (CuCit) group had an increased abundance of *Rikenella*, *Enterococcus*, *Lachnospiraceae_NK3A20_group*, and *Turicibacter* genera in the rabbit cecum.

Discussion: In summary, this study found that organic Cu and inorganic Cu sources had different effects on cecal microbiota composition and SCFAs in rabbits. The CuCit group had the unique higher relative abundance of genera *Rikenella* and *Lachnospiraceae_NK3A20_group*, which might be beneficial to the lower incidence of diarrhea in rabbits.

KEYWORDS

gut microbiome, organic copper, rabbits, SCFAs, cupric citrate

1. Introduction

Cu is routinely supplemented to animal diets at concentrations above the nutritional requirement of the animals because the pharmacological concentrations of inorganic cupric sulfate ($CuSO_4$) have been shown to have growth-stimulatory properties, for example, 125 or 250 mg/kg of Cu in the broiler diet (1) and 242 mg/kg of Cu in the pig diet (2). However, the pharmacological concentrations of dietary Cu present an environmental concern because of its high excretion in feces (3). More studies revealed that organic Cu additive might be more environmentally friendly than $CuSO_4$ supplements, which could attenuate fecal acidity, diarrhea incidence, and fecal Cu concentration (4, 5). Cupric citrate (CuCit), an organic form of Cu, could stimulate growth at lower concentrations than $CuSO_4$ in broiler chickens (1, 6) and weanling pigs (4, 7). In China, the maximum amount of Cu in meat rabbit formula feed or fully mixed diet is 25 mg/kg (including the background value of feed raw materials). Previous studies revealed that CuCit had a beneficial effect on the average daily gain and feed weight ratio than $CuSO_4$ at the same dose (20 mg/kg of Cu) in the rabbit diet (8). Interestingly, the incidence of diarrhea was reduced when the rabbits were fed with CuCit diets compared with other Cu source diets. The incidence of diarrhea in the CuCit group was 8.3%, which was 16.7–18.7% in the other Cu source groups (8). However, studies exploring the mechanisms by which low dietary CuCit reduced the incidence of diarrhea in rabbits were few.

One of the possible mechanisms by which Cu may benefit animals was by shifting the gastrointestinal microbiota, thereby reducing the susceptibility of animals to diseases (9). Numerous studies confirmed the various properties of Cu as antibacterial, antifungal, and antiviral agents (10). Different sources of Cu have different effects on the intestinal structure and microflora (4). A pharmacological dose of Cu significantly affected the composition of microbial communities in the ileum and cecum of weaned piglets (11). The high level of Cu significantly reduced the abundance of enterococci and lactic acid bacteria (12) or lactobacilli (13) in the porcine gut. The supplementation with 36.75 mg/kg of Cu from Cu-bearing montmorillonite reduced the total viable counts of *Escherichia coli* and *Clostridia* in the small intestine and cecum of broiler chicken (14). The abundance of *Rikenella* and *Barnesiella* genera increased in Sprague–Dawley rats in the 240 mg/kg Cu group compared with the 6 mg/kg Cu group (15). Meanwhile, the changes in the intestinal microbiota composition were shown to alter the abundance of bacterial metabolites (16, 17). Short-chain fatty acids (SCFAs), mainly acetate, propionate, and butyrate, are recognized as important metabolites derived from the fermentation of indigestible dietary fibers by gut microbial species, which play fundamental roles in maintaining intestinal homeostasis and regulating energy metabolism (18). These intestinal microbial communities played an important role in the growth and gut health of animals by producing SCFAs in chickens (19) and rabbits (20). The SCFAs were the primary energy sources of the colonic epithelial cells in mice (21). Therefore, this study used 16S rDNA gene amplicon sequencing and gas chromatography, respectively, aimed to evaluate the impact of dietary supplementation of four different Cu sources at the same level on cecal microbiota and SCFAs in growing rabbits.

2. Materials and methods

2.1. Materials

In this study, cupric citrate (CuCit) was provided by Sichuan Animtech Feed Co., Ltd., Chengdu, China. CuCit is a kind of organic Cu, with a purity of approximately 98.5%, and the content of Cu was 34.5%. Cupric sulfate ($CuSO_4{\cdot}5H_2O$, Cu > 25.1%, abbreviated as $CuSO_4$), dicopper chloride trihydroxide ($Cu_2(OH)_3Cl$, Cu > 58.1%, abbreviated as $CuCl_2$), and copper glycinate (CuGly, Cu > 21%) were bought from the local market.

2.2. Collection of the cecal contents in rabbits

The animal care and experimental design were conducted as described in a previous study (8). In brief, 240 New Zealand rabbits, aged 35 days, were randomly divided into four experimental groups and one control group, each group comprised 8 replicates, with 6 rabbits (half male and half female) per replicate. The basal diet was formulated to meet the requirements for growing rabbits, the digestive energy was 10.08 MJ/kg, the crude protein level was 15.85%, and the crude fiber level was 15.08%. The basel diet added copper as the control group which contained 5.00 mg/kg Cu from the raw materials (Table 1). Four treatment groups were added the 20 mg/kg Cu from $CuSO_4$, $CuCl_2$, CuCit, and CuGly to the basel diet, respectively. Thus, the total copper content in these four experimental diets was 25 mg/kg. These animals were treated with the same nutritional management. The rabbits were raised for 49 days after 7 days of adaptation. At the end of the experiment, four rabbits were slaughtered from each group. The entire cecal contents were first blended sufficiently and then collected from each rabbit. In this study, only the cecal samples from the four Cu experimental groups were selected for 16S rDNA gene amplicon sequencing and gas chromatography. All procedures were approved by the Institutional Animal Care and Use Committee of the Sichuan Academy of Animal Science.

2.3. DNA extraction, polymerase chain reaction amplification, and sequencing

Cecal microbial genomic DNA was extracted using a Magnetic Soil and Stool DNA Kit (Qiagen, CA, United States) following the kit instructions. DNA concentration and purity were determined using a NanoDrop 2000 ultraviolet–visible spectrophotometer (Thermo Scientific, DE, United States). The primers of 341F (5′-CCTAYGGGRBGCASCAG-3′) and 806R (5′-GGACTACNNGGG TATCTAAT-3′) with the barcode were used to amply the highly variable regions V3–V4 of the bacterial 16S ribosomal RNA (rRNA) gene. The polymerase chain reaction (PCR) was carried out using the Phusion High-Fidelity PCR Master Mix (New England Biolabs), and the PCR products were purified using the Qiagen gel extraction kit (Qiagen, Germany). The sequencing libraries were generated using the TruSeq DNA PCR-free sample preparation kit (Illumina, CA, United States). Purified amplicons were paired-end sequenced on an Illumina NovaSeq 6000 platform (Illumina, CA, United States).

TABLE 1 Diet composition and nutritional composition (air-dried base).

Project	Content
Raw material composition, %	
Alfalfa grass powder	30.00
Maize	17.00
Bran	22.50
Secondary powder	5.00
Commanding chaff	7.00
Soybean meal (43% CP)	8.50
Rapeseed meal	4.60
Puffed soybeans	2.00
Calcium carbonate	1.00
Dicalcium phosphate	0.70
L–Lysine hydrochloride	0.26
DL-methionine	0.04
Table salt	0.40
Premixed feed[a]	1.00
Total	100.00
Nutrition facts[b]	
Digestive energy, MJ/kg	10.08
Crude protein, %	15.85
Crude fiber, %	15.08
Calcium, %	1.08
Phosphorus, %	0.65
Lysine, %	0.92
Methionine + cystine, %	0.64
Copper, mg/kg	5.00

[a]Premixed feed provides per kilogram of diet: vitamin A 10,000 IU, vitamin D3 1,000 IU, vitamin E 20 IU, vitamin K_3 1 mg, vitamin B_1 2 mg, vitamin B_2 2 mg, vitamin B_6 1 mg, vitamin B_{12} 10 μg, niacin 50 mg, calcium pantothenate 20 mg, folic acid 0.1 mg, Biotin 0.2 mg, choline 300 mg, iron 50 mg, zinc 80 mg, manganese 8.5 mg, selenium 0.05 mg, iodine 0.2 mg.
[b]The digestion energy in the nutrient composition is the calculated value, and the rest are measured values.

2.4. Bioinformatics analysis of sequencing data

The paired-end reads were assigned to samples based on their unique barcodes and primer sequences and merged using FLASH (v.1.2.7, http://ccb.jhu.edu/software/FLASH/). Quantitative Insights Into Microbial Ecology (QIIME, v.1.9.1, http://qiime.org/scripts/split_libraries_fastq.html) was used for the quality control of sequencing data to obtain high-quality clean tags. Operational taxonomic units (OTUs) (*de novo*) were picked using UPARSE (v7.0.1001, http://www.drive5.com/uparse/) with a 97% similarity threshold. Alpha diversity, as indicated by Observed-OTUs, Chao1, Simpson, and Shannon index, was calculated. Analyses of similarities (ANOSIM and Adonis) were used for determining the significance analysis of beta diversity. MetaStats (set to 5,000 permutations for the nonparametric *t*-test) and *t*-test were employed for differential abundance analysis between groups. The R (3.6.0) software was used to draw box plots and conduct principal components analysis (PCA), and nonmetric multidimensional scaling (NMDS).

2.5. SCFA extraction and analysis

SCFAs were extracted by Metware Biotechnology Co., Ltd. (Wuhan, China). Briefly, 20 mg of cecal contents were dissolved in 1 ml of phosphoric acid (0.5% v/v) solution, mixed by spinning, and sonicated in an ice bath for 5 min. Then, the solution was centrifuged at 12,000 rpm for 10 min at 4°C. Further, 100 μl of the supernatant was taken, and 500 μl of Methyl tert-butyl ether (MTBE) solvent containing internal standards was added and vortexed for 3 min. After sonication in an ice bath for 5 min, the mixture was centrifuged at 12,000 rpm for 10 min at 4°C. After centrifugation, 200 μl of the supernatant was pipetted off for gas chromatography–tandem mass spectrometry analysis. The analysis of variance was performed to detect the differences in the levels of SCFAs in the cecum.

2.6. Microbial combined analysis with SCFAs

Microbial and SCFA data were unit variance scaled. Spearman correlation analysis between microbial and SCFA data was calculated using the cor function of the R software, and significance tests for correlation were conducted using the corPvalueStudent function of the Weighted correlation network analysis (WGCNA) package of the R software. Spearman correlation with $|r| \geq 0.8$ and p value < 0.05 were considered to have a significant correlation coefficient. The R (3.6.0) software was used to draw the Spearman circos and correlation scatterplot.

3. Results

3.1. Microbial community composition and microbiome diversity in the cecum of the groups fed with four different Cu sources

A total of 1,360,930 reads with an average length of 407 bp per read were obtained after quality filtering using the UPARSE software. A total of 3,569 OTUs were identified at the 97% similarity level using these filtered sequences. Among these, 1,022 OTUs were common in the four groups, and the number of unique OTUs was higher in the CuCit organic Cu group than in the other groups (Figure 1A). These OTUs were taxonomically annotated to a total of 43 phyla, 102 classes, 239 orders, 333 families, 534 genera, and 224 species. The top 10 OTUs were composed of the microbial communities of cecal samples from the groups fed with four different Cu sources, and each individual at the phylum and genus levels are summarized in Figures 1B,C. As observed in all groups and individuals, the order of abundance at the phylum level was *Firmicutes* followed by *unidentified_Bacteria* and Bacteroidota (Figures 1B1,C1). The CuCit group had higher relative abundance of phyla Bacteroidota and Verrucomicrobiota compared with the other groups (Figure 1B). At the genus level, *NK4A214_group* was the most abundant, followed by the *Christensenellaceae_R-7_group* (Figures 1C1,C2). The *Rikenellaceae_RC9_gut_group* showed abundance in the cecum of one individual (M3.4), which led to the specifically higher relative

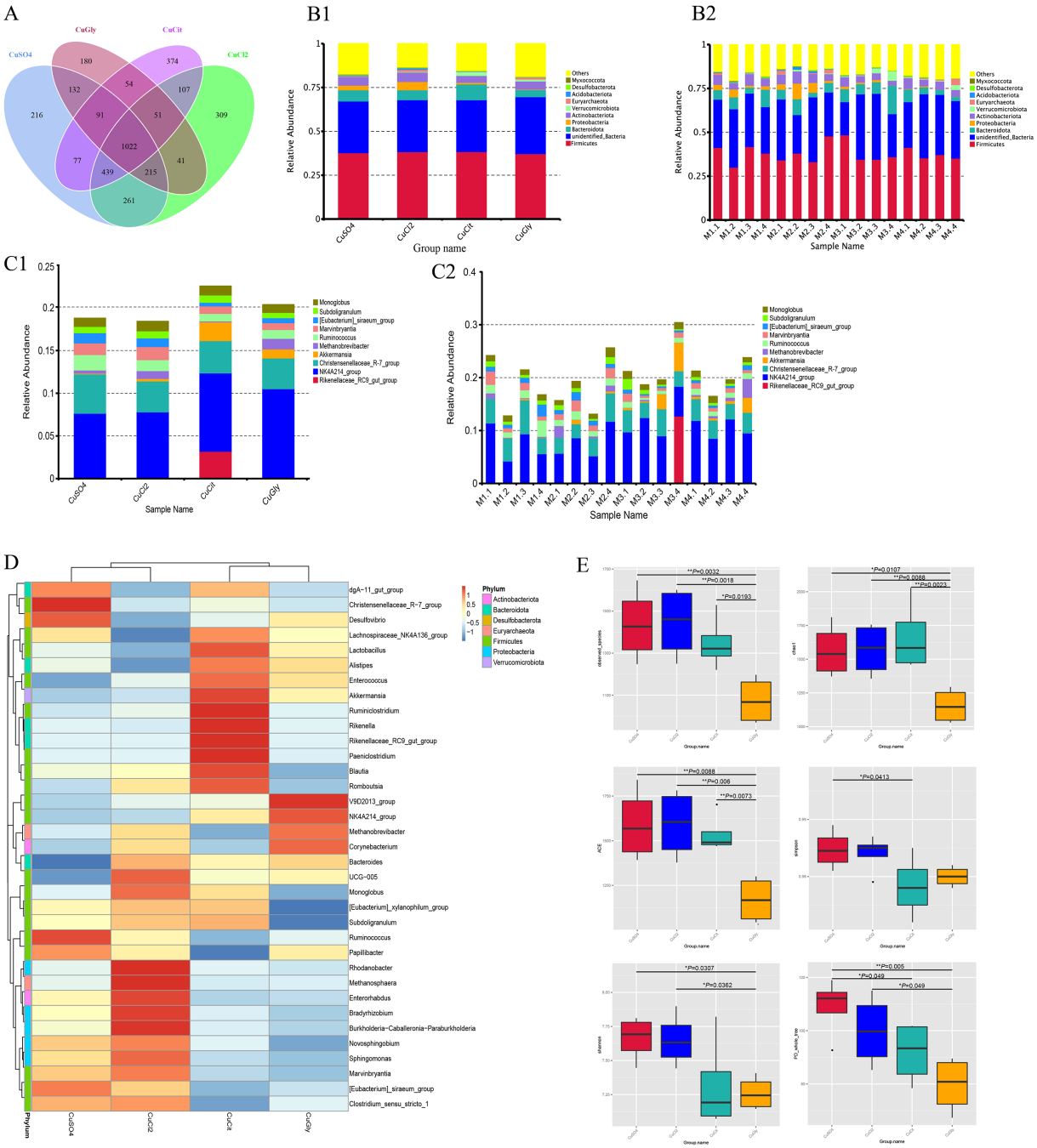

FIGURE 1
Cecal microbial community composition and alpha diversity from four different copper sources in rabbit diet. **(A)** Venn plot of the OTU. **(B)** Relative abundance of the top 10 bacterial phyla for each group **(B1)** and per individual **(B2)**. **(C)** Relative abundance of the top 10 bacterial genera in each group **(C1)** and per individual **(C2)**. **(D)** Cluster heatmap of top 35 genera. **(E)** Box plot for the analysis of differences between groups in alpha diversity index.

abundance in the CuCit group (Figures 1C1,C2). The heatmap of 35 genera showed that the CuCit group had a specific higher relative abundance of *Rikenella* and *Paeniclostridium* genera (Figure 1D). The inorganic Cu groups had higher relative abundance of *Marvinbryantia* and *[Eubacterium]_siraeum_group* genera compared with the organic Cu groups (Figure 1D).

The richness and evenness information about the microbiome diversity across the four different Cu sources, and common and unique OTU information among different groups, were obtained using alpha diversity assessment. The CuGly treatment group showed significantly lower species richness in terms of the Observed-OTUs, Chao1, and Abundance-based Coverage Estimator (ACE) compared with the other three groups ($p < 0.05$) (Figure 1E). For the Shannon index, the CuGly were less variable than inorganic Cu groups $CuCl_2$ and $CuSO_4$ ($p < 0.05$) (Figure 1E). No obvious difference was found among the four groups in terms of the Simpson index (Figure 1E). These results revealed that these organic Cu groups had lower species richness compared with the inorganic Cu groups.

3.2. Different cu sources altered the microbiota composition in the rabbit cecum

Beta diversity measures were assessed to capture the changes in the microbiome community composition among the groups. We first employed the PCA method for clustering all samples based on the OTU profile. These 16 samples were clearly clustered in the spaces of PC1 and PC2 (Figure 2A), respectively. The differences between organic and inorganic Cu groups could be explained by PC1, while the difference between two different sources of organic Cu or inorganic Cu was explained by PC2 (Figure 2A). The NMDS method also showed that these four groups were clustered in different quadrants (Figure 2B). These results indicated significant differences between organic and inorganic Cu groups.

The ANOSIM and Adonis analyses were used to learn more about the difference in the microbiome community among the four different Cu sources (Table 2). As a nonparametric test, ANOSIM could evaluate whether the variation in the cecal microbiome among the four different Cu sources was significantly larger than the variation within groups. This information helped us evaluate the reasonability of the division of groups. Our ANOSIM results showed a positive R value and p value < 0.05, which suggested that the intergroup variation was larger than the intragroup variation (Figure 2C; Table 2). Adonis is a nonparametric multivariate variance analysis method based on the Bray–Curtis distance. This method could analyze the interpretation of different grouping factors to sample differences. In total, ANOSIM and Adonis analyses suggested a significant difference between the organic and inorganic Cu groups at the OTU level (Table 2).

Several analytic methods were used to identify the specific clades of the microbiome in the four groups, including the MetaStat and the t-test analyses. In MetaStat analysis, $q < 0.05$ was confirmed as a significant difference. At the genus level, the cluster heatmap revealed that the significant difference clades of the microbiome between the organic and inorganic Cu groups were grouped in cluster 1, while the specific clades in the CuCit group were grouped in cluster 2 (Figure 2D). The PCA analysis using these top 35 genera showed that the difference between organic and inorganic Cu groups could be explained by PC1, and the difference between CuCit and $CuSO_4$ groups was explained by PC2 (Figure 2E).

In this study, we focused on the specific clades in the CuCit group to other groups. We found that the CuCit group had a significantly higher relative abundance of genera *Rikenella* and *Paeniclostridium* while the CuCit group had a significantly lower relative abundance of genera *Novosphingobium* and *Ruminococcus* (Figure 3A). The t-test analysis revealed that the CuCit group had significantly higher relative abundance of *Paeniclostridium*, *Tissierella*, *Lachnospiraceae_NK3A20_group*, *Enterococcus*, and *Turicibacter* than the $CuCl_2$ group and $CuSO_4$ group ($p < 0.05$) (Figures 3B,C).

3.3. Organic cu treatment decreased the SCFA production in the rabbit cecum

The changes in the microbiota composition have been shown to alter the levels of bacterial metabolites in mice (17). We measured the effect of the different Cu source treatments on the levels of SCFA metabolites in the rabbit cecum. The levels of seven SCFAs in these four different Cu sources are shown in Figure 4A. The levels of acetic acid (AA), butyric acid (BA), propionic acid (PA), valeric acid (VA), and hexanoic acid (HA) were lower in the organic Cu groups than in the inorganic Cu groups (Figure 4A). The order of SCFAs level was AA followed by BA, and PA. The BA and HA levels significantly decreased in the CuCit group than in the $CuCl_2$ group, and the BA and VA levels also significantly decreased in the CuCit group than in the $CuSO_4$ group (Figure 4B). The individual BA levels are shown in Figure 4C. These results revealed that the level of BA significantly decreased in the organic Cu groups than in the inorganic Cu groups.

Then, the Spearman correlation of the microbial composition with SCFA metabolites was analyzed (Figures 4D,E). At the genus level, the relative abundance of *Rikenella* and *Turicibacter* was significantly negatively correlated with the BA level in the CuCit versus $CuCl_2$ groups and CuCit versus $CuSO_4$ groups (Figures 4D,E). The relative abundance of genus *Paeniclostridium* was significantly negatively correlated with the HA and VA levels in the CuCit versus $CuCl_2$ groups and CuCit versus $CuSO_4$ groups, respectively (Figure 4D). These results revealed that the CuCit group had an increased abundance of *Rikenella* and *Turicibacter* and a decreased BA level in the rabbit cecum.

4. Discussion

Our previous study has shown that rabbits fed with CuCit diets had a lower incidence of diarrhea compared with those treated with other Cu source diets (8). Adding CuCit to feed could effectively reduce the diarrhea rate of piglets (22).The effect of CuCit on reducing animal diarrhea may be due to the extensive antibacterial properties of copper. Adding copper to feed can regulate intestinal flora and affect physiological functions (23), while copper from different sources has different effects on gut structure and microbiota (4). In this study, we found that CuCit had a specific effect on cecal microbiota composition and SCFAs in rabbits.

The important finding of this study was that the CuCit group had a unique higher relative abundance of genera *Rikenella*, *Paeniclostridium*, *Tissierella*, *Turicibacter*, *Enterococcus*, and *Lachnospiraceae_NK3A20_group* compared with the other groups. However, the relative abundance of the genera *Novosphingobium* and *Ruminococcus* decreased in the organic Cu compared with the inorganic Cu groups. *Rikenella* belongs to the phylum *Bacteroidota*. *Bacteroidota* is an important part of the mammalian intestinal flora, capable of breaking down polysaccharides and proteins in the feed, which promotes the development of the intestinal immune system (24). *Rikenella* is a great anti diarrhea probiotics, and it is negatively correlated with diarrhea index (25). This is consistent with our experimental results. In the previous experimental results, the diarrhea rate of meat rabbits in the CuCit group was reduced by 55.57, 55.57, and 50.03% compared to the $CuSO_4$, $CuCl_2$ and CuGly group (8). The significant decrease in diarrhea rate of rabbits in the CuCit group may be related to the increase of *Rikenella*. *Enterococcus*, *Lachnospiraceae_NK3A20_group*, and *Ruminococcus* belong to the phylum *Firmicutes*. A previous study showed that *Lachnospiraceae_NK3A20_group* might participate in the metabolism of amino acids and glycerophospholipids, enhance antioxidant capacity, and promote the digestion and absorption of nutrients (26). There are also reports that compared to healthy individuals, the content of *Lachnospiraceae* in patients with

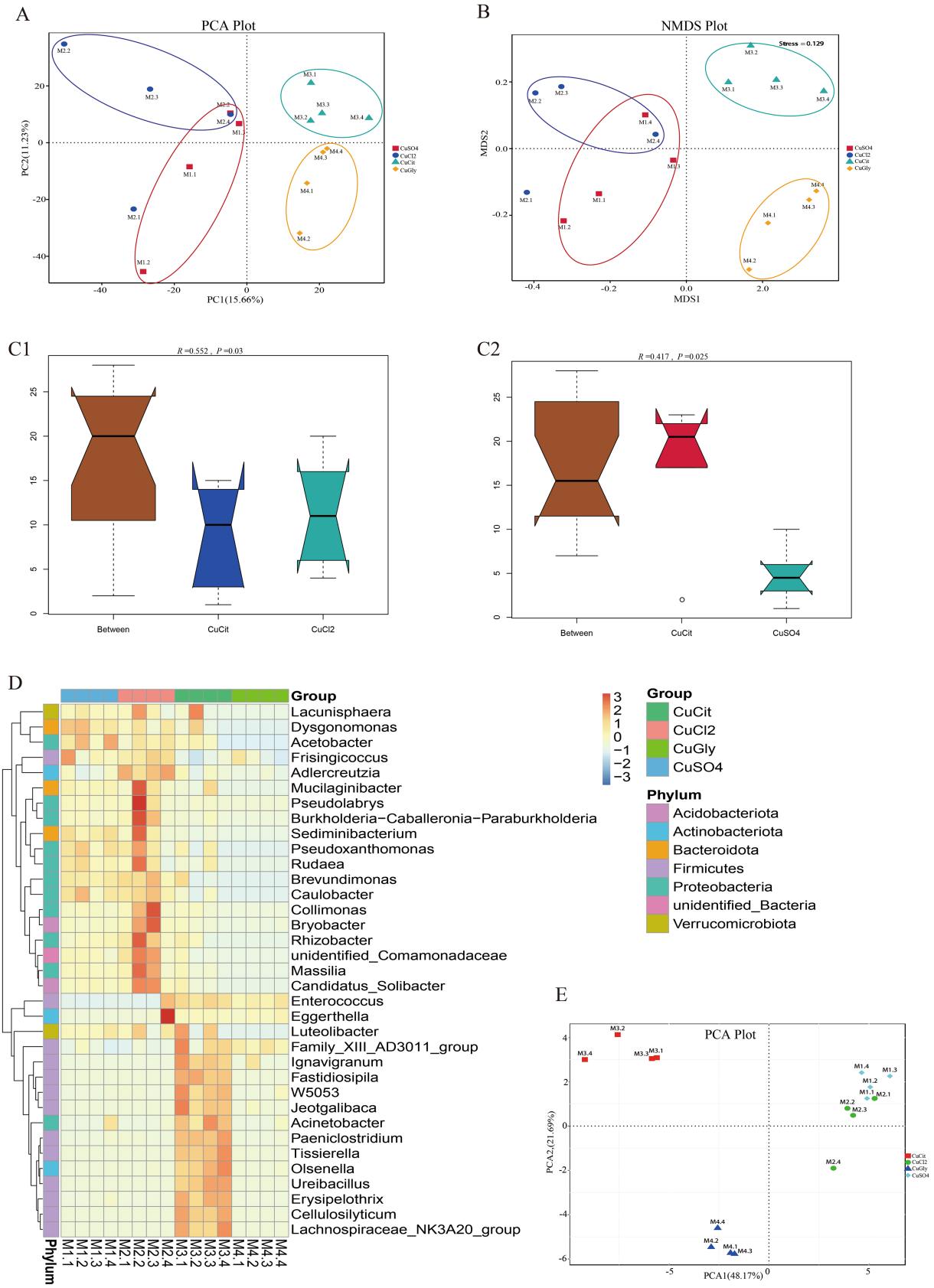

FIGURE 2
Different copper sources altered the microbiota composition in the rabbit cecum. **(A,B)** PCA score plot and NMDS score plot based on unweights. **(C1,C2)** ANOSIM plot of cupric citrate (CuCit) compared with two inorganic coppers. **(D)** MetaStat of cluster at the genus level. **(E)** PCA plot of top 35 genera from MetaStat analysis.

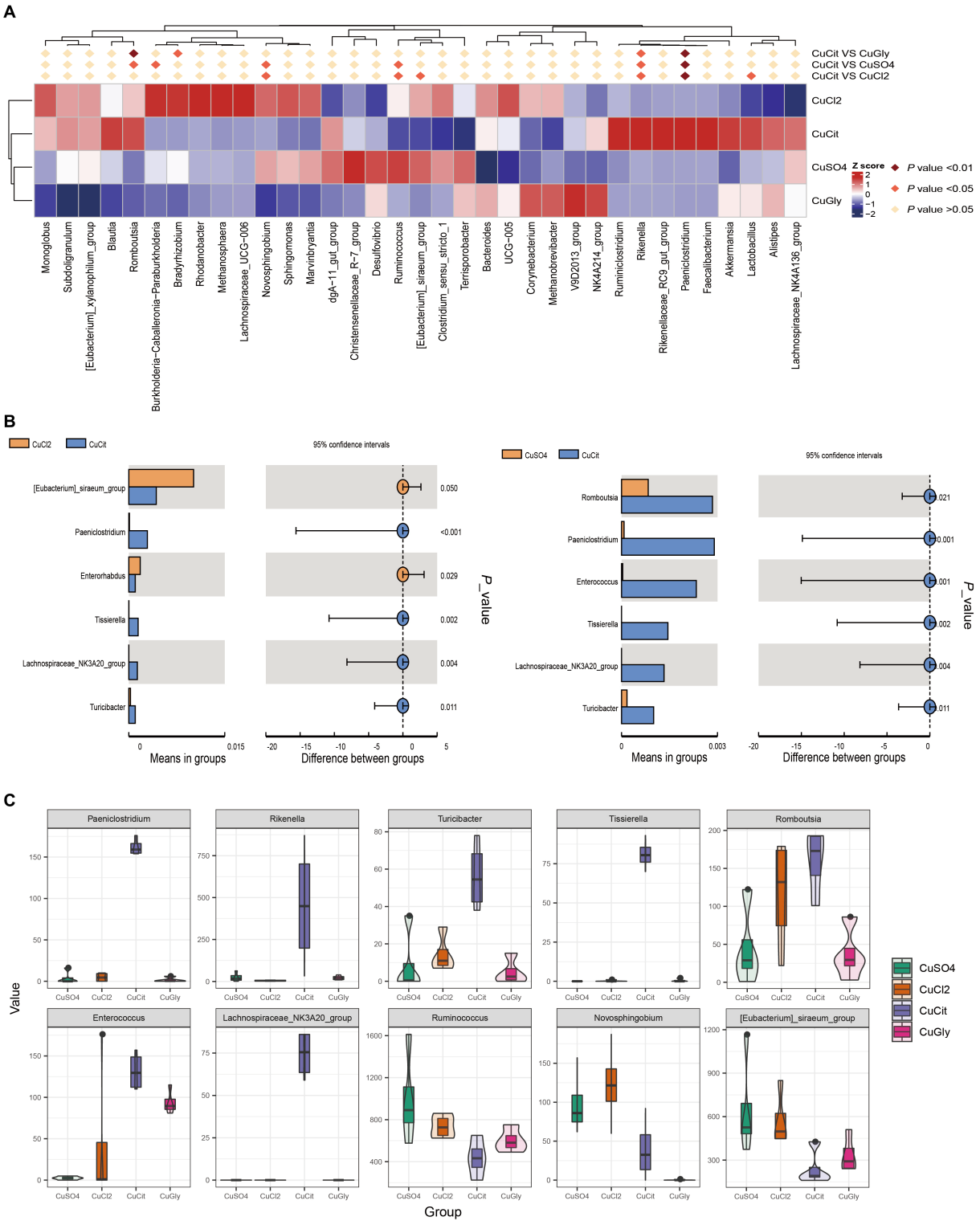

FIGURE 3
CuCit has significantly different microbial communities from the inorganic copper groups **(A)** MetaStat of differences at the genus level. **(B)** T-Test analysis of cupric citrate (CuCit) compared with two inorganic coppers. $p<0.05$ indicated a statistically significant difference. **(C)** Box plot of the 10 different genera in the four groups.

colitis is lower (27). A report also showed that the addition of active tripeptides extracted from egg white can reduce diarrhea caused by *E. coli*, reduce the expression of inflammatory factors, increase the abundance of probiotics such as *Lachnospiraceae*, and reduce the abundance of pathogenic bacteria (28). Thus, the CuCit group having the unique higher relative abundance of genera *Rikenella*, and

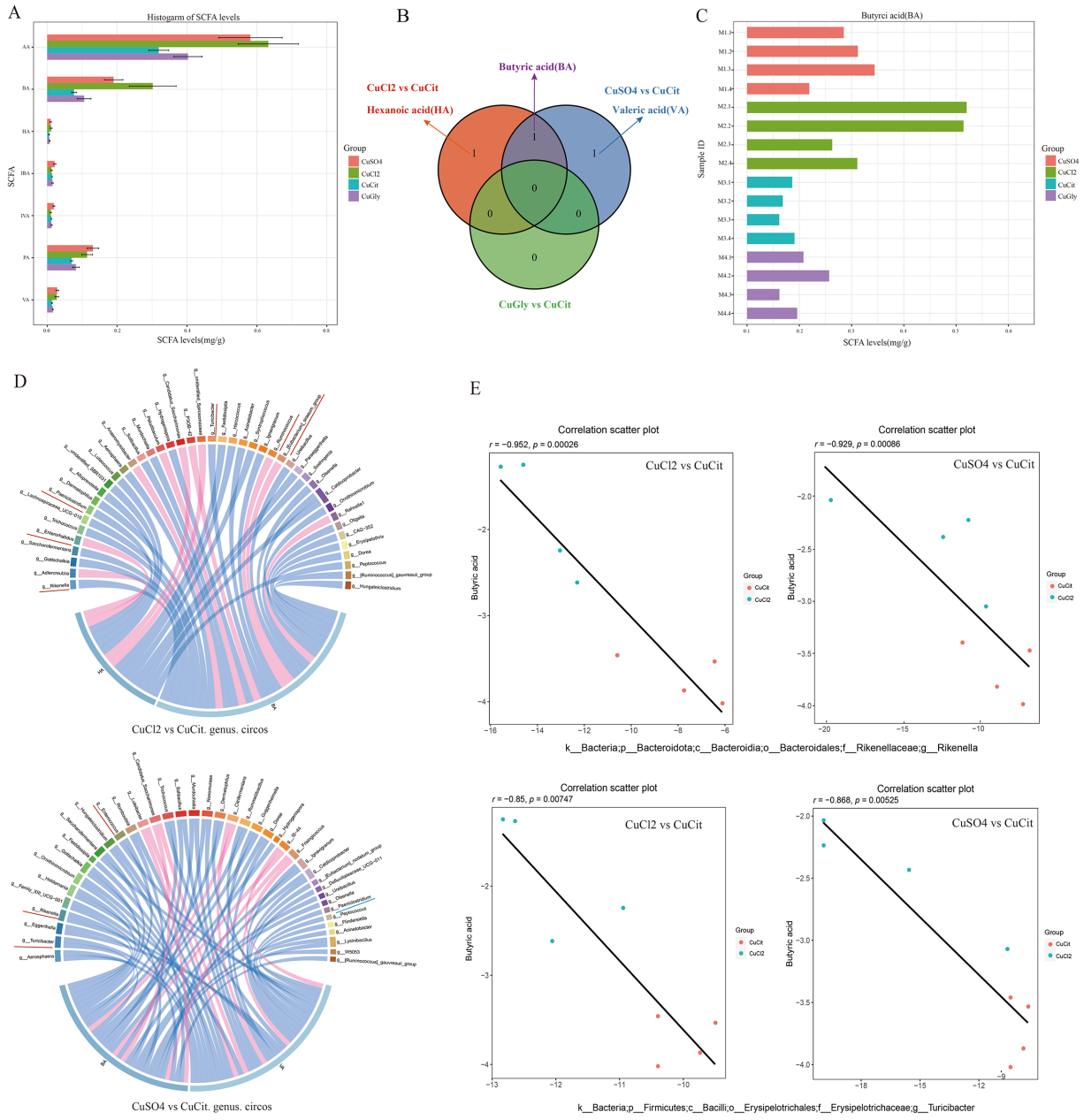

FIGURE 4
Short chain fatty acids(SCFAs) level and Spearman correlation between the butyric acid level and microbial composition in the cecum. **(A)** Histogram of SCFAs levels in different groups. **(B)** Venn of significantly different SCFAs between different groups. **(C)** Histogram of individual butyric acid (BA) levels. **(D)** Spearman correlation and circos plot of differential microbial genus abundance and differential SCFAs. **(E)** Scatter plot of Spearman correlation between microbial composition and butyric acid level.

Lachnospiraceae_NK3A20_group might be beneficial to the lower incidence of diarrhea in rabbits. *Enterococcus* plays a protective role in regulating colonic homeostasis during development, inhibiting pathogen-mediated inflammatory responses in human intestinal epithelial cells, inducing interleukin-10 expression, and reducing the secretion of pro-inflammatory cytokines (29). *Ruminococcus* is a major fiber-degrading bacterium that significantly alters the digestion and utilization of nutrients in the gut (30). The increase in the abundance of *Ruminococcus* was positively correlated with the expression of Toll-like receptor genes (31), these genes are induced by host's outer wall bacteria and bacterial products to activate the immune response to maintain homeostasis. The above results indicate that the regulation of gut microbiota may be an important factor in the anti diarrhea effect.

A phenomenon worthy of attention was that the BA level in the CuCit group was significantly lower than that in the inorganic Cu group. SCFAs played an important role in providing energy, regulating intestinal permeability, and inhibiting inflammation (32). AA, PA, and BA are important metabolites produced during intestinal microbial fermentation (33). The BA level is an important source of intestinal epithelial cells that maintain intestinal

TABLE 2 ANOSIM/adonis analyses of microbial communities in different groups.

Item	ANOSIM		Adonis	
	R	*p*	R^2	*p*
$CuCl_2$-CuCit	0.552	0.03	0.227	0.03
$CuSO_4$-CuCit	0.417	0.025	0.228	0.001
$CuSO_4$-$CuCl_2$	−0.052	0.611	0.130	0.635
CuGly-CuCit	0.187	0.03	0.181	0.022
CuGly-$CuCl_2$	0.364	0.026	0.246	0.001
CuGly-$CuSO_4$	0.427	0.057	0.223	0.022

homeostasis (34). It is generally believed that the increase in the BA level is conducive to the balance of gut microbiota (35, 36). However, high concentrations of SCFAs are more likely to have the opposite effect, possibly increasing intestinal permeability and causing diarrhea (37). It has been reported that SCFAs can promote intestinal mucosal cells to release 5-hydroxy tryptamine (5-HT), thereby increasing intracellular Ca^{2+}, activating K^+channels to induce hyperpolarization, accelerating the contraction of intestinal smooth muscle, and excreting feces faster (38). When feed quickly passes through the intestine before water is absorbed by the large intestine, there is more water in the feces. In this experiment, SCFAs were significantly reduced in the CuCit group, which may lead to slower fecal passage and dryness, which helps alleviate diarrhea. The decrease in SCFAs in the CuCit group may be due to changes in gut microbiota. In this experiment, the BA levels in the CuCit group were negatively correlated with the relative abundance of *Rikenella* and *Turiciactor*. *Rikenella* ferments propionic acid in normal cells to generate energy and promote gluconeogenesi (39). It has been reported that a positive correlation between *Turiciactor* and BA (40). This is contrary to our results. In addition, *Lachnospiraceae* and *Ruminococcus* are also the important bacteria that produce butyric acid (30, 41). However, the abundance of *Lachnospiraceae* in the CuCit group increased while the abundance of *Ruminococcus* decreased, which may be due to the greater impact of *Ruminococcus* on butyric acid, leading to a decrease in butyric acid. On the other hand, SCFAs can regulate the morphological structure of the intestine and affect the digestion and absorption of nutrients (42). The decrease in SCFAs may be due to their absorption by intestinal epithelial cells, promoting the development of intestinal epithelial cells. The specific mechanism needs further exploration.

5. Conclusion

In summary, this study found that organic Cu and inorganic Cu sources had different effects on cecal microbiota composition and SCFAs in rabbits. The CuCit group had the unique higher relative abundance of genera *Rikenell* and *Lachnospiraceae_NK3A20_group*, which might be beneficial to the lower incidence of diarrhea in rabbits. In this experiment, we recommend a copper content of 20 mg/kg for CuCit, and we can further explore the effects of different concentrations of CuCit on intestinal microbiota and SCFAs to find the most suitable amount of CuCit added to the diet in rabbits.

Data availability statement

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

Ethics statement

The animal study was reviewed and approved by the Institutional Animal Care and Use Committee of the Sichuan Animal Science Academy.

Author contributions

JY and G-WZ designed the experiments. JY supervised the research work. G-WZ, YD, and YT analyzed the data. ZZ and RH assisted in sample collection. G-WZ and YD wrote the manuscript. JY revised the manuscript. All authors contributed to the article and approved the submitted version.

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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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Raw potato starch diet supplement in weaned pigs could reduce *Salmonella* Typhimurium infection by altering microbiome composition and improving immune status

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Backgorund: *Salmonella enterica* serovar Typhimurium (ST) is one of the causative agents of gastroenteritis in pigs. Pigs fed a diet supplemented with raw potato starch (RPS) have improved gut health by the alteration of the microbiota composition and production of short-chain fatty acids (SCFAs). This study aimed to evaluate the effects of RPS supplementation in reducing infection severity and fecal shedding in ST-infected pigs.

Methods: The weaned experimental pigs were divided into two groups: CON ($n = 6$) fed a corn/soybean-based diet and TRT ($n = 6$) supplemented with 5% RPS. After 21 d, the pigs were inoculated with ST, and their body weight, clinical signs, and fecal shedding of ST were monitored for 14 d. At 14 d post-inoculation (dpi), the jejunum, cecum, ileum, and colon tissues were collected from euthanized pigs, and histopathological lesions and cytokine gene expression were compared. Additionally, blood samples at 2 dpi were analyzed for gene ontology enrichment. Moreover, the gutmicrobiome was analyzed using 16S rRNA metagenomic sequencing, and the SCFA concentration was measured using gas chromatography.

Results: The average daily weight gain was significantly higher in TRT than in CON during the ST infection period; however, histopathological lesion scores were significantly lower in TRT than in CON. The relative abundance of nine genera of butyrate- and acetate-producing bacteria significantly increased in TRT compared with that of only two acetate-producing bacteria in CON. Among the genes involved in the immune response, IL-18 expression level was significantly lower in the jejunum and colon in TRT than in CON. Furthermore, *Reg3γ* expression was significantly different in the cecum and colon of both groups.

Conclusion: The diet supplemented with RPS in weaned pigs could result in predominance of butyrate- and acetate-producing bacteria, reducing the severity of ST infection by improving the immune status.

KEYWORDS

diarrhea, microbiome, raw potato starch, resistant starch, *Salmonella* Typhimurium, weaned pig, cytokine, transcriptome

1. Introduction

Salmonella is an important foodborne pathogen that can cause human and animal infections (1). Among the various serovars of *Salmonella,* non-typhoidal *Salmonella* infections are a major public health problem worldwide (1). Pigs are an important non-typhoidal *Salmonella* infection source for humans, especially *Salmonella enterica* serovar Typhimurium (ST) (2). Intestinal inflammation caused by *Salmonella* infection can disrupt commensal microbiota and gut barriers, resulting in the bacteria colonizing the tissues of the host intestine (3, 4), leading to diarrhea, fibrinonecrotic enterocolitis, and dehydration in pigs (5).

Healthy gut microbiota can reduce the severity of *Salmonella* infection; therefore, a feed supplement diet that supports beneficial microbial populations is a potential on-farm strategy to control *Salmonella* infection in pigs (6, 7). Resistant starch (RS) is an important source of microbiota-accessible carbohydrates because it is digested in the large rather than the small intestines (8). RS feeding can increase short-chain fatty acids (SCFAs) in the intestinal tract, resulting in improved barrier functions, enhanced tolerance to commensal organisms, and reduced inflammation in the gut tissue (9, 10). Raw potato starch (RPS) is a common ingredient of RS that improves fermentation in the digestive tract and increases pro- and anti-inflammatory cytokine levels (11). Recently, studies have shown that RPS feeding could increase gene expression related to the cecal barrier function and improve the mucosal immune system in animals (8, 12).

Our previous study revealed that RPS consumption could promote the growth of beneficial microbes, promoting SCFA production in weaned pigs (13). Therefore, we hypothesized that feeding RPS as a supplement could reduce ST infection severity and fecal shedding in weaned pigs. This study aimed to determine the effect of feeding RPS in reducing ST infection severity in weaned pigs by comparing ST shedding and colonization, histopathological lesions, microbiota composition, and immunological responses in ST-inoculated pigs fed RS and non-RS diets. The findings could provide potential methods for preventing ST infection in pigs by altering microbiome composition and improving immune responses.

2. Materials and methods

2.1. Animal ethics

All experiments were approved by the Animal Ethics Committee of the National Institute of Animal Science, Republic of Korea (Approval No. NIAS 2021-503).

2.2. Animals, experimental diets, and *Salmonella* Typhimurium inoculation

Twelve castrated male piglets (Landrace × Yorkshire, aged 25 d) were obtained from the same herd in a commercial farm. The average weight of the pigs was 5.00 ± 0.8 kg. All pigs were carefully monitored daily for 3 d before the diet experiment. During the adaptation period, all pigs were confirmed to be sero-negative for foot-and-mouth disease, porcine respiratory and reproductive syndrome, classical swine fever, *Mycoplasma* spp. infection, and *Salmonella* spp. infection. In addition, *Salmonella* spp. and *Escherichia coli* were not detected in the fecal samples of the experimental pigs. Piglets (aged 28 d) were randomly divided into two groups: the treatment (TRT, $n = 6$) and negative control (CON, $n = 6$) groups. The CON diet was formulated according to the nutritional requirements suggested by the Korean feeding standard for pigs, and the TRT pigs were fed the CON diet supplemented with 5% RPS for 21 d (Table 1). After 21 d, the TRT pigs' diet (aged 49 d) was changed to the CON diet until the end of the experiment. Subsequently, all experimental pigs aged 49 d were orally inoculated with 1×10^8 colony forming units of ST LT2 strain (ATCC 19585). The ST-infected pigs were then euthanized 14 d after bacterial inoculation and immediately necropsied to collect the tissue samples.

TABLE 1 Ingredients and chemical composition of the experimental diet.

Items	TRT (%)	CON (%)
Ingredients		
Corn	68.24	73.74
Soybean meal 44%	23.25	22.20
Soybean oil	0.33	0.86
L-Lysine-HCl	0.15	0.17
Dicalcium phosphate	1.15	1.15
Limestone	0.88	0.88
Vitamin-mineral premix[a]	0.50	0.50
NaCl	0.50	0.50
Raw potato starch	5.00	-
Calculated composition		
Metabolizable energy (kcal/kg)	3,300	3,300
Crude protein	16.00	16.0
Lysine	0.95	0.95
Methionine	0.26	0.26
Calcium	0.66	0.66
Total protein	0.56	0.56

[a]Values supplied per kilogram premix feed concentrations: Vitamin A 5,000,000 IU; vitamin E, 1,000 mg; Vitamin B_1, 150 mg; Vitamin B_2, 300 mg; Vitamin B_{12}, 1,500 mg; niacin amide, 1,500 mg; DL-calcium pantothenate, 1,000 mg; folic acid, 200 mg; Vitamin H, 10 mg; choline chloride, 2,000 mg; min 3,800 mg; zinc, 1,500 mg; iron, 4,000 mg; Cu, 500 mg; I, 250 mg; Co, 100 mg; Mg, 200 mg.
TRT, piglets fed the control formula diet supplemented with 5% raw potato starch; CON, piglets fed the corn/soybean control formula diet.

2.3. Sampling and evaluating *Salmonella* shedding

Fecal samples were collected at 0 and 21 d post-feeding (dpf) and 2, 5, 8, and 11 d post-ST inoculation (dpi). The fecal and intestinal tissues (jejunum, ileum, colon, and cecum) were collected

from euthanized pigs at 14 dpi. The Rappaport–Vassiliadis R10 broth (BD, Sparks, MD, USA) containing the fecal and tissue samples (1 g) was incubated immediately at 42°C for 24 h, and one loop of the RV culture was streaked onto CHROMagar *Salmonella* Plus (CHROMagar, Paris, France). Lastly, the mauve colonies were identified as ST by polymerase chain reaction (PCR) using the *AccuPower Salmonella* spp. 3-Plex PCR Kit (Bioneer, Daejeon, Korea).

2.4. Histopathology and immunohistochemistry

Tissue samples from the jejunum, ileum, cecum, and colon of necropsied pigs (at 14 dpi) were fixed in 10% neutral-buffered formalin and embedded in paraffin wax. Subsequently, 4-μm-thick sectioned tissues were stained with hematoxylin and eosin using a standard laboratory protocol and immunohistochemically stained with anti-*Salmonella* Typhimurium (BS-4801R; Thermo Fisher Scientific, Rockford, IL, USA). Lastly, the histopathological lesions were scored (from 0 to 5) using previously described parameters, including villus shortening and erosion, presence and concentration of ST, and inflammatory cell infiltration (14).

2.5. Microbial community analysis

DNA was extracted using the DNeasy PowerSoil Kit (Qiagen, Hilden, Germany) following the manufacturer's instructions. Amplicons of the V3–V4 region were generated and sequenced following the Illumina 16S metagenomic sequencing library preparation protocol. Subsequently, paired-end sequencing of the amplicon was performed using the MiSeq platform (Illumina, San Diego, CA, USA). Afterward, bioinformatics analysis was performed as described in our previous study (13). Lastly, sequencing data were arranged according to the two experimental groups (TRT and CON) for analytical purposes.

2.6. Measurement of SCFA concentrations

Acetate, butyrate, and propionate were selected based on their specific differences reported in our previous study (13). Fecal concentrations of SCFAs were determined using an Agilent 6890 series gas chromatograph (Agilent Technologies, Santa Clara, CA, USA) coupled with mass spectrometry (13).

2.7. Quantification of cytokines in gastrointestinal tissue

The jejunum, cecum, and colon tissues from pigs at 14 dpi were analyzed for cytokine quantification. First, total RNA was isolated using the RNeasy Mini Kit (Qiagen, Hilden, Germany) and reverse-transcribed into cDNA using a High-Capacity cDNA Synthesis Kit (Applied Biosystems, Foster City, CA, USA) following the manufacturer's instructions. Additionally, the relative expression of seven genes was quantified by reverse transcription (RT) PCR (RT-PCR), including four genes related to gut barrier function [claudin (CLDN), occludin (OCLN), zonula occludens-1 (ZO-1)], and regenerating islet-derived protein 3-gamma (Reg3γ), and three genes related to the immune response against *Salmonella* infection, including interleukin (IL)-10, IL-17A, and IL-18. Notably, RT-PCR was performed using the ABI 7500 Real-Time PCR System (Applied Biosystems) under the following conditions: 10 min at 95°C, 40 cycles at 95°C for 15 s, the annealing temperature of each primer for 30 min, and 72°C for 15 s. The primers and annealing temperatures are listed in Table 2. Lastly, the expression fold change was determined using the $2^{-\Delta\Delta Ct}$ method with the beta-actin gene as the endogenous reference gene to normalize the level of target gene expression.

2.8. RNA sequencing data and differentially expressed gene (DEG) analysis

Total RNA was obtained from all experimental pigs at 2 dpi using a Tempus Blood RNA Tube (Applied Biosystems, Seoul, Korea). To produce the transcriptome, TNT Research Corporation Limited (Anyang, Korea) conducted RNA and cDNA library construction and RNA sequencing as previously described (18). Lastly, the DEGs in TRT and CON groups' blood were analyzed based on the expression level of each transcript, as previously described (18).

2.9. Statistical analysis

The Kruskal–Wallis and unpaired Wilcoxon rank-sum tests were used for comparing the alpha diversities of the fecal microbiome composition in the TRT and CON groups at 14 dpi and three time points (0, 21 dpf, and 14 dpi), respectively. All statistical analyses adopted a *P*-value of 0.05 as the cut-off value and a linear discriminant analysis (LDA) score of 2.0 using the QIIME software version 2.0. Moreover, beta diversity was visualized by principal coordinate analysis (PCoA) matrix using Bray–Curtis distance and QIIME software version 2.0. Additionally, the LDA effect size (LEfSe) was used to determine the specific effect on the relative abundance of taxa in the RS and non-RS groups. Taxa with a significant difference ($P < 0.05$) between both groups were subjected to LEfSe analysis, and those with LDA score > 2.0 were considered to have been significantly altered after *Salmonella* infection in the TRT group compared with those in the CON group. Lastly, significant changes in average daily gain (ADG), histopathological lesion scores, and SCFA concentrations between the TRT and CON groups were compared by Student's *t*-test using the SPSS software (version 26.0; IBM, Armonk, NY, USA).

TABLE 2 The primer information and PCR condition for quantitative real-time polymer chain reaction.

Primer	Target	Sequence (5′ → 3′)	Size (bp)	Annealing temp (°C)	Reference
IL-10-F	Interleukin 10	GCCTTCGGCCCAGTGAA	101 bp	62°C	(15)
IL-10-R		AGAGACCCGGTCAGCAACAA			
IL-17A-F	Interleukin 17A	CCCTGTCACTGCTGCTTCTG	57 bp	62°C	(16)
IL-17A-R		TCATGATTCCCGCCTTCAC			
IL-18-F	Interleukin 18	ACGACCAAGTCCTTTTCATTAACC	85 bp	63.6°C	(17)
IL-18-R		TGAGGTGCATTATCTGAACAGTCA			
ZO-1-F	Zonula occludin-1	GGCTCTTGGCTTGCTATTCG	98 bp	62.0°C	
ZO-1-R		TGGACACTGGCTAACTGCTCA			
OCLN-F	Occludin	CCAACGGGAAAGTGAACGAG	149 bp	63.0°C	
OCLN-R		CGCCTCCAAGTTACCACTGC			
CLDN1-F	Claudin-1	AACCCGTGCCTTGATGGTAA	127 bp	62.6°C	
CLDN1-R		AATGACAGCCATCCGCATCT			
REG3γ-F	Regenerating islet-derived 3 gamma	TGTCTCAGGTCCAAGGTGAAGA	102 bp	62.2°C	
REG3γ-R		ACAAGGCATAGCAGTAGGAAGCA			
DefB-F	Beta defensin 1	CTCTGCTTGCTGCTGCTGAC	188 bp	62.2°C	
DefB-R		CACTTGGCCTTGCCACTGTA			
ACTB-F	Beta-actin	CAAATGCTTCTAGGCGGACTGT	75 bp	-	(15)
ACTB-R		TCTCATTTTCTGCGCAAGTTAGG			

3. Results

3.1. Growth performance and *Salmonella* Typhimurium fecal shedding

We compared the pigs in the TRT group (fed with RPS supplemented diet) with those in the CON group (Figure 1A). The ADG during the feeding period (from 0 to 21 dpf) was 0.15 and 0.12 kg/day in the TRT and CON groups, respectively; however, ADG during ST infection period (from 0 to 14 dpi) in the TRT group (0.27 kg/day) was significantly ($P = 0.010$) higher than that in the CON group (0.15 kg/day) (Figure 1B). Moreover, the TRT group showed marked reductions in ST shedding at 8 and 11 dpi compared with the CON group [8 dpi: 33.3% (TRT) *vs.* 66.7% (CON) and 11 dpi: 16.7% (TRT) *vs.* 50.0% (CON)] (Figure 1C).

3.2. Histopathological lesions and *Salmonella* Typhimurium isolation in tissues

Although histopathological lesions and ST were observed in both groups (Figure 2A), the CON pigs exhibited more severe organismal damage than the TRT pigs, indicated by the total average histopathological lesion scores in the jejunum, ileum, cecum, and colon [TRT (1.8 ± 0.9) vs. CON (3.6 ± 1.3), $P < 0.001$] (Figure 2B). Additionally, the average scores in the TRT pigs' ileum (1.2 ± 0.4), cecum (1.2 ± 0.4), and colon (2.5 ± 0.5) were significantly lower ($P < 0.001$, $P = 0.001$, and $P < 0.001$, respectively) than those in the CON pigs' ileum (3.5 ± 0.8), cecum (4.5 ± 0.5), and colon (4.3 ± 0.7).

For intestinal tissues, the estimated isolation rate of ST from the TRT pigs was lower than that from the CON pigs (Figure 2C). The isolation rate from the TRT pigs' jejunum (0%, zero of six pigs) was significantly ($P = 0.046$) lower than that for the CON pigs (50.0%, three of six pigs). Moreover, ST was detected in the ileum of only one CON pig. Lastly, one TRT pig (16.7%) and four CON pigs (66.7%) harbored ST in the cecum, and the bacteria were isolated from the colon tissue in all pigs, excluding one TRT pig.

3.3. Bacterial communities and SCFA concentration

Alpha diversity in fecal microbiota was compared between TRT and CON groups and time points (0, 21 dpf, and 14 dpi) (Figure 3A). Four indices were analyzed, including the number of amplicon sequence variants (ASV), Chao1 richness indices, and Shannon and Gini–Simpson diversity indices. The Kruskal–Wallis test showed no significant differences in the four alpha diversity indices: the number of ASV ($P = 0.7$) and Chao1 richness indices ($P = 0.7$), and Shannon ($P = 0.59$) and Gini–Simpson indices ($P = 0.59$). For the TRT group, the Gini–Simpson index ($P = 0.016$) was significantly higher at 14 dpi than at 0 and 21 dpf. Moreover, the Gini–Simpson ($P = 0.0015$) and Shannon indices ($P = 0.0017$) were significantly lower in the CON group than in the TRT group at 14 dpi. Furthermore, we performed beta diversity analysis to investigate the structure of the bacterial community at 14 dpi; the results are presented as a PCoA ordination plot based on

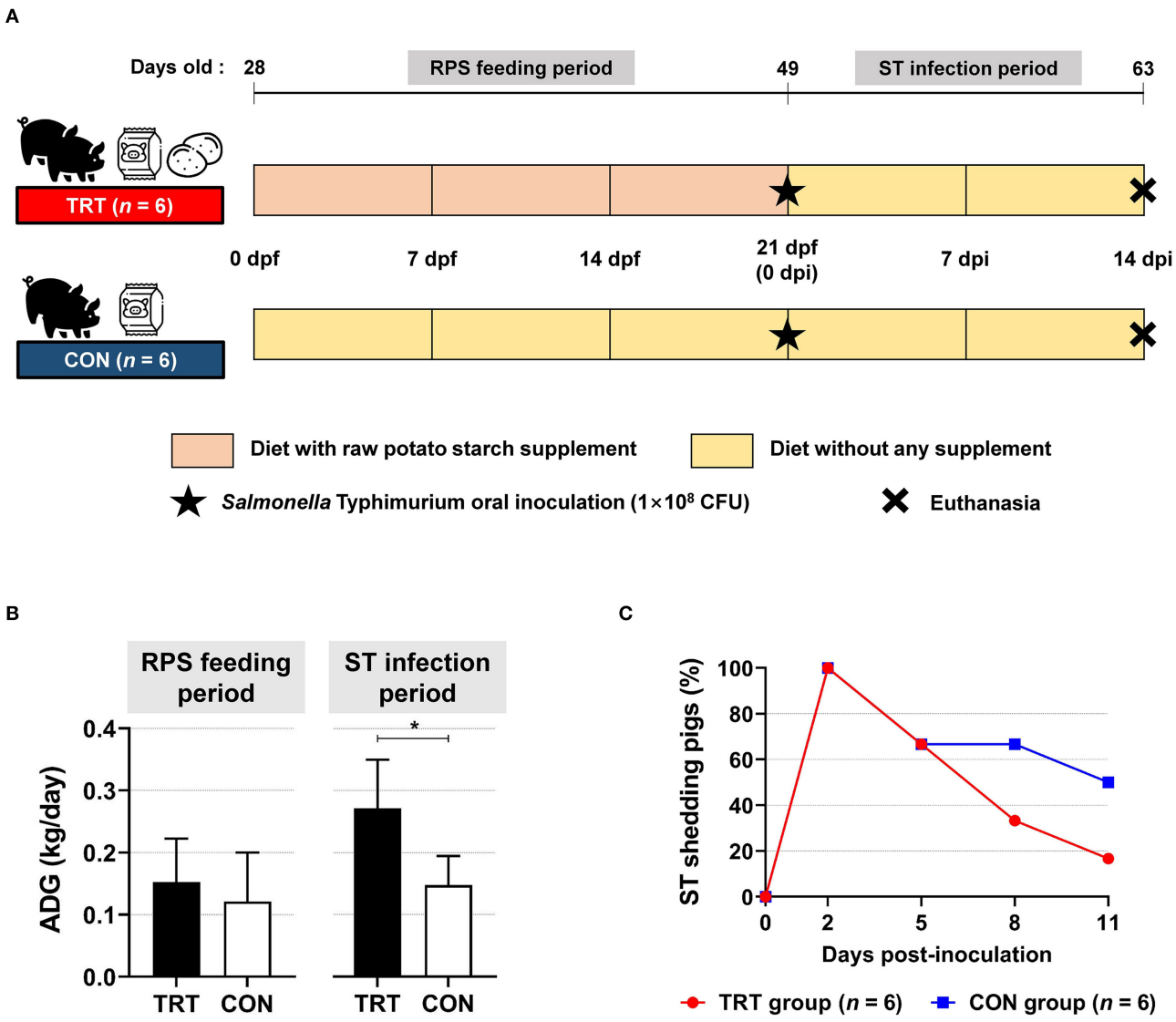

FIGURE 1
Effect of RPS diet supplement in *Salmonella* Typhimurium (ST)-infected weaned pigs. **(A)** Experimental scheme. **(B)** Average daily gains (ADG) in RS-fed (TRT) and non-RS-fed pigs (CON) during two experimental periods. *$P < 0.05$. **(C)** Isolation of ST in fecal samples from TRT and CON until 11 d post-inoculation.

Bray–Curtis distance matrices. Beta diversity differed between the TRT and CON groups at 14 dpi; however, no significant difference was observed between both groups ($P = 0.529$). The bacterial communities during ST infection in the TRT group shifted leftward along the PC1 axis in the opposite direction to those in the CON group (Figure 3B). Moreover, LEfSe analysis showed that 9 bacterial genera and 11 species of fecal microbes were significantly ($P < 0.05$) increased in the TRT group; however, the abundance of only two genera and three species significantly increased in the CON group (Figure 3C).

The concentrations of three SCFAs (acetate, butyrate, and propionate) were evaluated in all experimental pig fecal samples (14 dpi) to investigate the effect of altered bacterial communities in the TRT and CON groups (Figure 3D). The levels of the three SCFAs were higher in the TRT group than in the CON group. Additionally, the concentrations of acetate, butyrate, and propionate were 1,090.8 ± 170.3 μg/g, 843.3 ± 110.6 μg/g, and 773.6 ± 93.8 μg/g, respectively, in the TRT group, and 1,033.4 ± 120.4 μg/g, 789.3 ± 81.6 μg/g, and 697.3 ± 49.6 μg/g, respectively, in the CON group.

3.4. Cytokine expression

The relative mRNA expression of the proinflammatory cytokine IL-18 was significantly lower in the jejunum [0.00010 ± 0.00014 (TRT) vs. 0.41 ± 0.11 (CON); $P = 0.0139$] and colon of the TRT group [0.00003 ± 0.00002 (TRT) vs. 0.73 ± 0.05 (CON); $P = 0.00001$] than in those of the CON group (Figure 4A). Additionally, the anti-inflammatory cytokine IL-10 was less expressed in the jejunum, cecum, and colon. Moreover, the expression of the proinflammatory cytokine IL-17A was higher in the TRT group's colon than in the CON group's colon; however, the differences were insignificant due to the large deviations between samples.

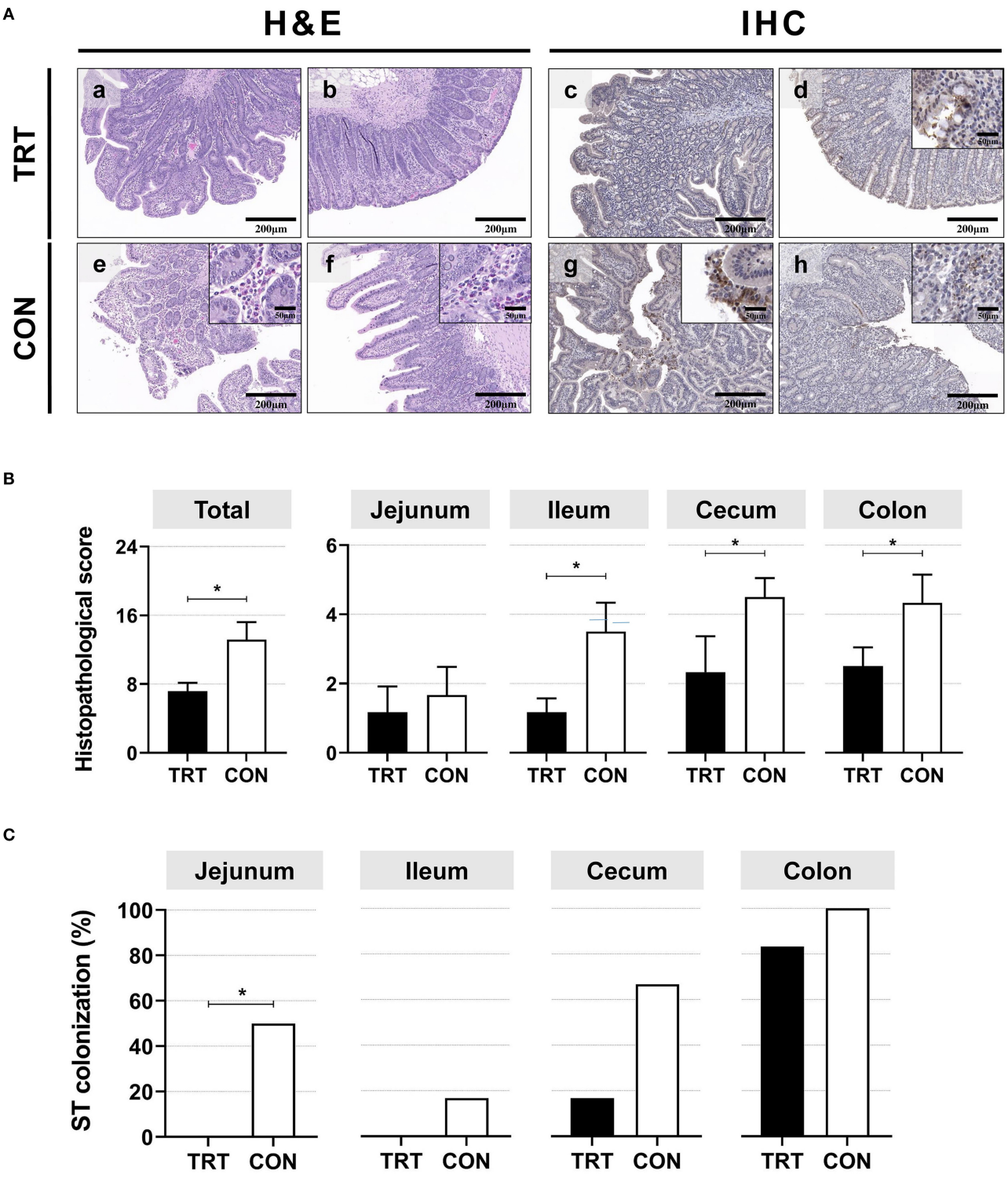

FIGURE 2
Histopathological lesions in experimental pigs and *Salmonella* Typhimurium (ST) isolation from intestinal organ tissues. **(A)** Representative hematoxylin and eosin and immunohistochemical (anti-ST) staining of the ileum (a, e), cecum (b, f), jejunum (c, g), and colon (d, h). **(B)** Histopathological scores of the intestinal organs (jejunum, ileum, cecum, and colon). *$P < 0.05$. **(C)** Isolation of ST from the intestinal organs (jejunum, ileum, cecum, and colon). *$P < 0.05$.

Among the genes related to the gut barrier, the expression of the antimicrobial peptide gene *Reg3γ* was significantly higher in the cecum [1.120 ± 0.535 (TRT) *vs.* 0.023 ± 0.004 (CON); $P = 0.0309$] but lower in the colon of the TRT group [0.073 ± 0.012 (TRT) vs. 0.148 ± 0.036 (CON); $P = 0.0190$] than in those of the CON group (Figure 4B). Furthermore, the relative mRNA expression levels of

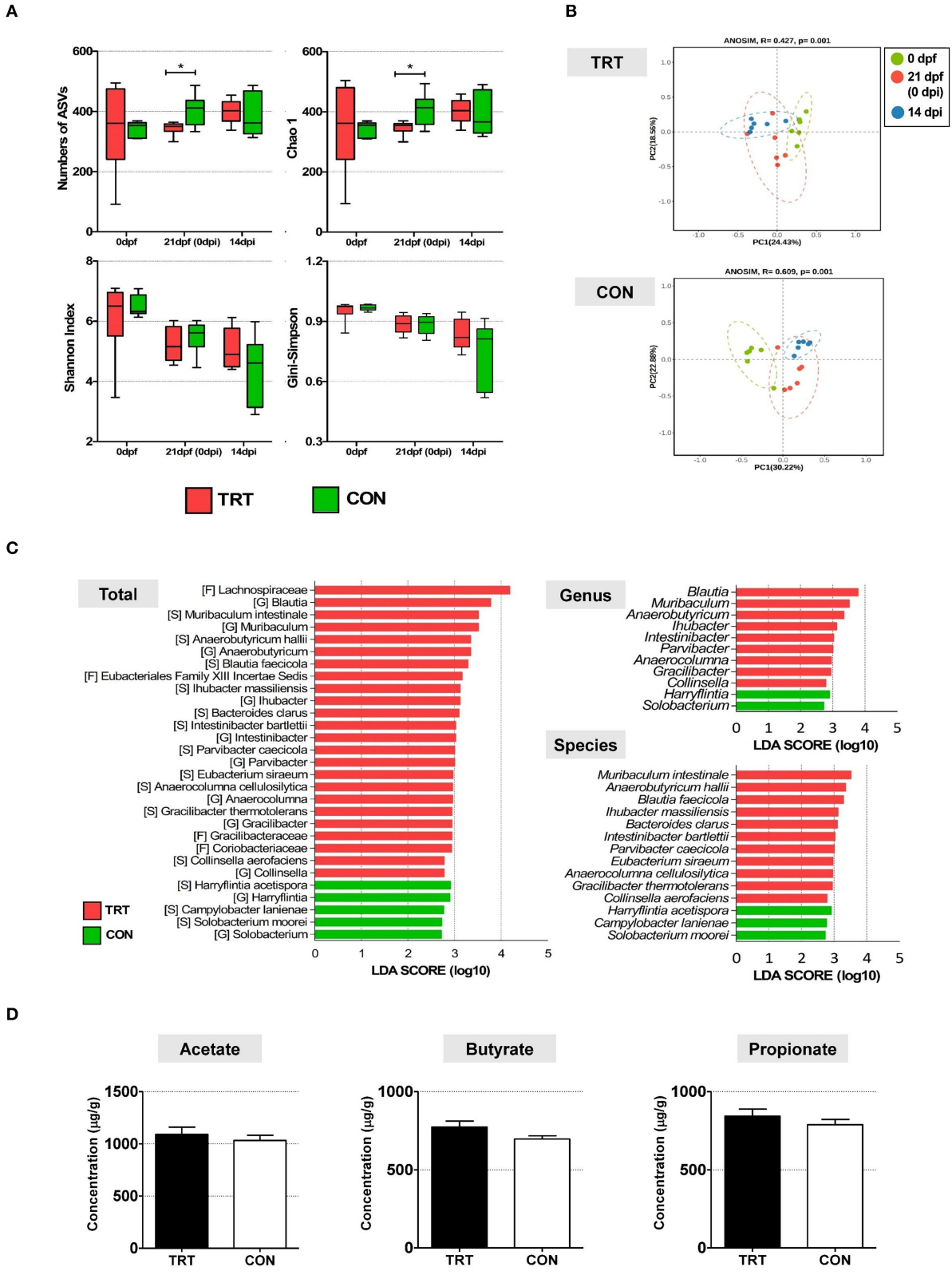

FIGURE 3
The comparison of alteration of microbiota diversity and composition and SCFA concentration in RPS-fed pigs (TRT) and non-RPS-fed pigs (CON) after *Salmonella* Typhimurium inoculation (at 14 dpi). **(A)** Comparison of alpha diversity of microbiome from feces between TRT and CON. The four indices included the number of ASV, Chao1 richness indices, and Shannon and Gini –Simpson diversity indices. **(B)** PCoA of beta diversity analysis based on the Bray–Curtis dissimilarity matrix. **(C)** LEfSe revealed predicted biological effect sizes of differential taxa in fecal microbiota between TRT and CON. The LDA scores show a significant difference in the abundance and consistency of the detected bacterial taxa at the genus and species levels. **(D)** Concentrations of three SCFAs (acetate, propionate, and butyrate) (μg/g) in TRT and CON fecal samples at 14 dpi. *$P < 0.05$.

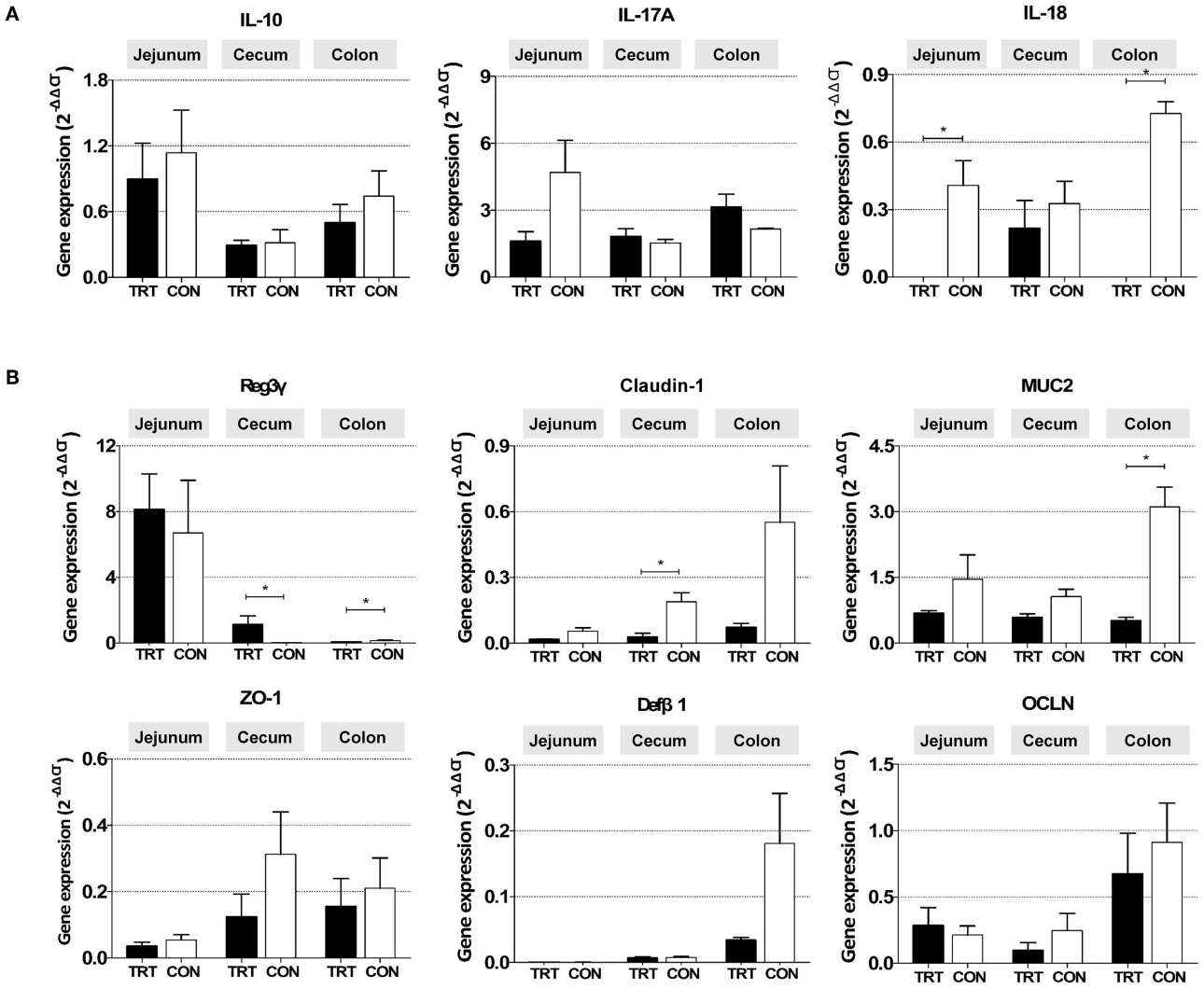

FIGURE 4
Gene mRNA expression in three intestinal organs (jejunum, cecum, and colon) from RPS-fed (TRT) and non-RPS-fed pigs (CON) after *Salmonella* Typhimurium inoculation (at 14 dpi). **(A)** Genes associated with inflammatory response (*IL-10, IL-17A*, and *IL-18*). **(B)** Genes associated with gut barrier function (*Reg3γ, Claudin-1, MUC2, Zo-1, Defβ1*, and *OCLN*). *$P < 0.05$.

CLDN-1 in the cecum [0.216 ± 0.124 (TRT) *vs.* 0.327 ± 0.099 (CON); $P = 0.0254$] and *MUC2* in the colon [0.51 ± 0.08 (TRT) vs. 3.11 ± 0.45 (CON); $P = 0.0095$] were significantly lower in the TRT group than in the CON group. However, insignificant difference was observed in *CLDN-1* expression in the jejunum and colon, *MUC2* in the jejunum and cecum, and the other three genes (*ZO-1, OCLN,* and *DefB1*) in the jejunum, cecum, and colon between the two feeding groups.

3.5. Transcriptome analysis of the blood sample

An average of 6.7 Gb raw data for each sample were collected from paired-end transcriptome sequencing using the Illumina NovaSeq 6000 platform. Raw data were subjected to quality control using Trimmomatic (ver. 0.38), and the trimmed data were mapped using HISAT2 ver. 2.1.0 (Bowtie2 aligner). Next, the mapped reads were assembled using the StringTie-e option ver. 1.3.4d. Afterward, the obtained genes were filtered by excluding those with at least one zero count, leaving 12,189 genes for DEG analysis. Overall, 424 genes were considered differentially expressed based on the threshold level (fold change [$\log_2$] ≥ 2 and $P < 0.05$) (Figure 5A). Lastly, the biological function of 424 DEGs was determined by gene ontology (GO) and Kyoto Encyclopedia of Genes and Genomes pathways using the DAVID 6.8 tool. Figure 5B shows the GO functional analysis of the biological process (top 20), cellular component (top 12), and molecular function (top 6).

4. Discussion

Salmonella is a major causative agent of diarrhea in pigs, threatening food safety and human health. Local inflammation in *Salmonella*-infected pigs can reportedly cause changes in the

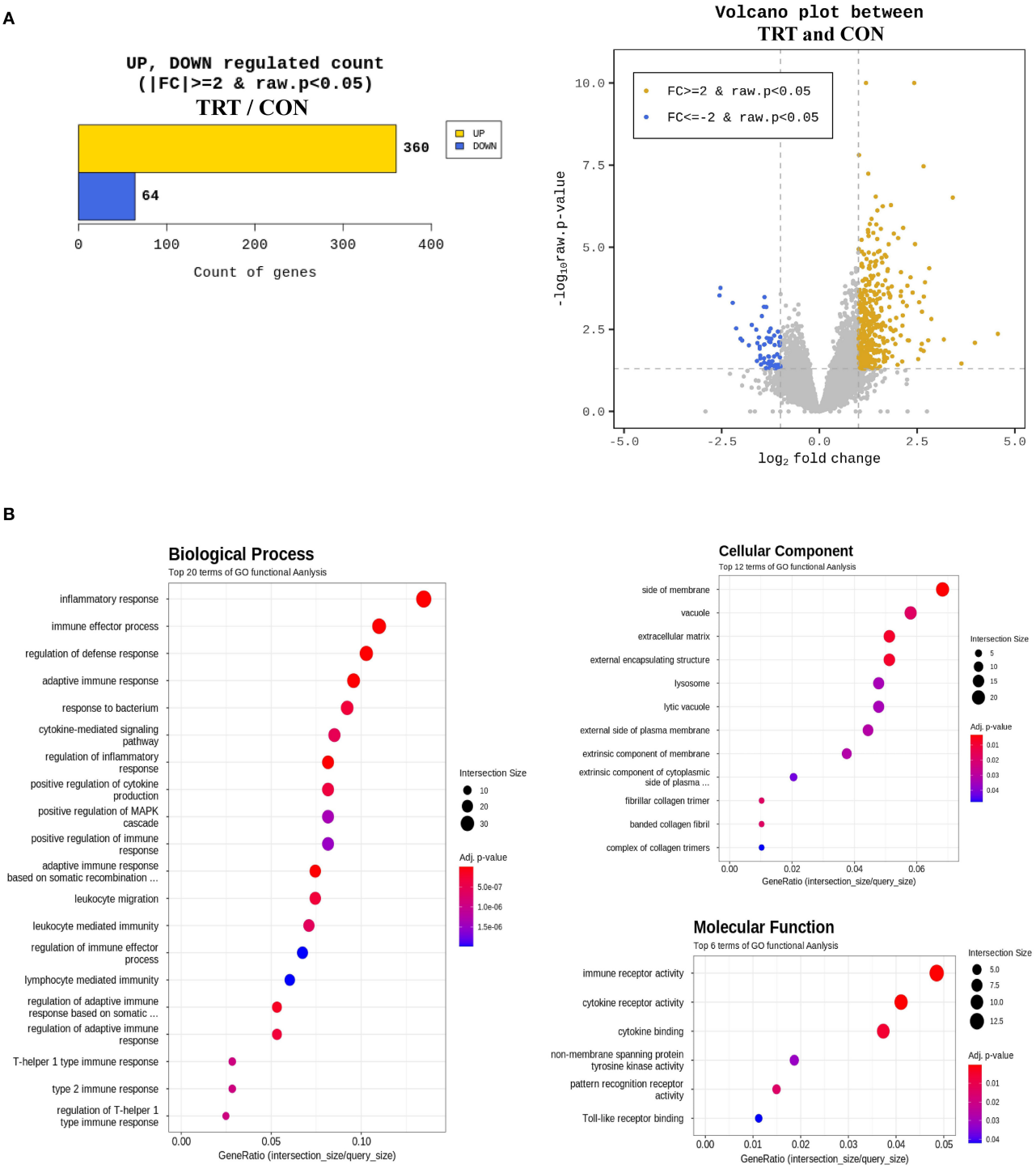

FIGURE 5
Differentially expressed genes (DEG) and gene ontology (GO) enrichment analysis in blood samples from RPS-fed (TRT) and non-RPS-fed pigs (CON) at 2 d after *Salmonella* Typhimurium inoculation. **(A)** Numbers of up- and down-regulated genes after comparison of normalized values using the DESeq2 package. **(B)** GOTERM_Biological process, GOTERM_Cellular Component, and GOTERM_Molecular Function.

gut microbiome, favoring the survival of *Salmonella* (4, 19). We previously revealed that RPS feeding of weaned pigs could improve gut health by maintaining the balance of beneficial bacteria and promoting SCFA production (13). Therefore, we investigated the gut microbiota composition and immunological response for preventing ST infection in RPS-fed pigs.

RPS is a type II RS that can decrease body weight (BW) in humans and animals. However, the ADG of TRT pigs was not significantly different from that of CON pigs during the RPS feeding period (until 21 dpf), consistent with the finding in our previous study (13). Although *Salmonella* infection in pigs reduces BW and ADG (20), the ADG of TRT pigs was

significantly higher than that of CON pigs during the ST infection period. These findings could explain the gut health-promoting effect in RPS-fed pigs because ADG in pigs is strongly related to intestinal morphology (21). Additionally, healthy gut microbiota and its derived SCFAs could prevent the colonization of pathogenic bacteria by decreasing gut mucosal permeability (13, 22). The histopathological lesions in the intestinal organ tissues of TRT pigs were significantly milder than those of CON pigs. Moreover, ST fecal shedding was reduced in RPS-fed pigs (TRT) at 8 dpi. These results suggest improved gut health after the post-weaning diet supplemented with RPS.

The abundance of nine bacterial genera significantly increased in TRT pigs, among which *Blautia* ($P = 0.0374$), *Muribaculum* ($P = 0.0104$), *Anaerobutyricum* ($P = 0.0374$), and *Anaerocolumna* ($P = 0.0247$) were the main butyrate-producing bacteria (23–26). *Blautia* is considered as a novel potential probiotic due to its ability to produce bacteriocin (sactipeptide and lanthipeptide), inhibit pathogenic bacterial colonization, and regulate inflammatory responses (25). Thus, we speculated that the increased abundance of *Blautia* might have contributed to the mild histopathological lesions and reduced ST colonization in the TRT pigs' intestinal organ tissues. Yuan et al. (27) showed that metabolites from *Muribaculum* could improve gut barrier function and integrity, preventing leakage of inflammatory mediators into the systemic circulation. Moreover, herein, the genus *Anaerobutyricum* and its subtaxon *A. hallii* ($P = 0.0374$) increased more in the TRT group than in the CON group. *A. hallii* is a potential next-generation probiotic bacterium because of its capacity to produce propionate and butyrate (28). Additionally, the four main acetate-producing bacteria in TRT, *Intestinibacter* ($P = 0.0250$), *Anaerocolumna* ($P = 0.0247$), *Gracilibacter* ($P = 0.0374$), and *Collinsella* ($P = 0.0250$), were more prevalent in the TRT group than in the CON group. *Anaerocolumna* reportedly decomposes cellulose, oligosaccharides, polysaccharides, and organic acids into energy sources (29). Moreover, *Gracilibacter* can degrade glucose, and *Intestinibacter* is involved in mucin consumption by degrading fucose (30). Lastly, *Collinsella* is significantly and positively correlated with most bile acids and is related to lipid metabolism (31). Conversely, the abundance of only two genera, *Harryflintia* ($P = 0.0463$) and *Solobacterium* ($P = 0.0278$) increased in the CON group, which can only produce acetate (32, 33). Lawhon et al. (34) reported that unbalanced SCFA ratio (e.g., high acetate and low butyrate/propionate concentration) could cause a more invasive ST infection. Further, *Harryflintia* abundance reduced when mice were fed high concentrations of RPS (0–10%) (35). Although the abundant species *Campylobacter lanienae* ($P = 0.0222$) and *Solobacterium moorei* ($P = 0.0278$) are common in the gastrointestinal tract of pigs, they have emerged as a potential cause of human gastroenteritis (32, 36).

Our previous study showed that feeding 5% RPS resulted in significantly higher concentrations of acetate, butyrate, and total SCFAs in healthy pigs (13). In the present study, however, the three SCFAs in the TRT and CON groups showed no significant difference, despite the increased numbers of butyrate-producing bacteria. ST reportedly uses and decreases microbiota-derived butyrate by altering the gut microbiota composition; consequently, the intestinal epithelium shifts to lactate fermentation (37, 38). In addition, sufficient concentrations of butyrate and propionate reportedly enabled the abrogation of ST-induced gut inflammation by regulating the expression of genes responsible for ST invasion and pathogenesis; moreover, they increased the sensitivity of the pathogens to butyrate-mediated repression of invasion-related gene expression (34, 37). Herein, butyrate consumption by ST might not significantly increase butyrate and propionate concentrations in the gut of TRT pigs; however, this may affect the severity and bacterial colonization results in RS-fed pigs.

In the present study, the mRNA expression of *Reg3γ* (antimicrobial peptide gene) markedly increased in the cecum and colon of the TRT pigs. *Reg3γ* restricts bacterial colonization of the intestinal mucosal surface and maintains spatial segregation between bacteria and intestinal epithelium (39). Therefore, the poor colonization of ST in TRT pigs could result from the enhanced *Reg3γ* expression in the cecum and colon. Furthermore, IL-18 mRNA expression levels and histopathological scores were significantly reduced in TRT pigs' colons. Previous studies have suggested that IL-18 is necessary to initiate mucosal inflammation (40, 41). Moreover, the upregulation of IL-18 is central to the pathogenesis of tissue destruction and the severity of gastroenteritis in humans and mice (41, 42). Therefore, the high *Reg3γ* expression and low IL-18 expression in TRT are related to the reduced ST colonization and histopathology results.

In this study, GO analysis revealed that upregulated DEGs were primarily involved in immune and inflammatory responses at 2 dpi. The top five biological processes were the inflammatory response, immune effector process, defense response regulation, adaptive immune response, and response to bacteria. Furthermore, GO enrichment analysis of the upregulated DEG included immune receptor activity, cytokine receptor activity, and cytokine binding. These results are consistent with previous findings that inflammatory features peaked, and inflammatory infiltration significantly increased in the small intestinal tissues of ST-infected piglets at 2 dpi (43, 44). Considering these results together with qPCR results, the GO analysis revealed that ST infection triggered immune responses in the early phase. At 2 dpi, transcript levels of the tight junction proteins claudin and occludin (*CLDND1* and *OCEL1*) in blood increased by 22.33 and 1.21 folds, respectively. However, at 14 dpi, the genes were less expressed in the TRT group than in the CON group. This result suggests that *CLDN and OCLN* might have been expressed earlier, making it unnecessary by 14 dpi. Additionally, at 2 dpi, the cytokine genes related to inflammation, IL-10 subunit alpha and subunit beta (IL10Rα and IL10RB) showed 1.62- and 2.23-fold increases, respectively, and the IL-17 gene (IL17B) showed a 41-fold increase. Moreover, we observed 4.84-, 2.49-, and 1.91-fold increases in IL-18 receptor (IL18R1), binding protein (IL18BP), and IL18 genes, respectively. However, excluding IL-17A in the colon, IL-10 and IL-17A were lower in all tissues examined in this study, and IL-18 was significantly lower in the jejunum and colon of the TRT group than in those of the CON group. *Reg3γ* overexpression reportedly induces high immunosuppression (45). Therefore, the dramatic reduction in cytokine genes in our study might be caused by a significant increase in *Reg3γ* at 14 dpi.

Overall, the results demonstrate that the RS-supplement diet could prevent ST infection in weaned pigs and provide insights

into the mechanisms underlying the immune responses of RS-fed pigs against ST infection. However, there are some limitations to providing a general conclusion of using RS feeding as a preventative measure for ST in pigs. First, this study was conducted on a limited number of pigs; therefore, the results may not represent the general pig population. Further studies with larger sample sizes and different breeds of pigs are required to validate these findings. Second, the animal experiment was conducted under controlled conditions; therefore, the results may be difficult to replicate immediately on a pig farm. An application experiment in actual farm units is required in the future.

5. Conclusion

The study results suggest that feeding weaned pigs with RPS—a type II RS—could improve gut health and reduce ST infection. The TRT group showed higher ADG during the infection period and milder histopathological lesions in the intestinal organs than the CON group. In addition, TRT pigs exhibited marked reduction in ST shedding compared with that in CON pigs. These results suggested that RPS feeding in weaned pigs could reduce economic losses in farm due to ST infection. The gut microbiota of the TRT group showed an increased abundance of four main butyrate-producing bacteria and four main acetate-producing bacteria. The increased levels of these beneficial bacteria could have contributed to promoting gut health and reducing ST colonization in the TRT group. Moreover, *Reg3γ* expression was markedly increased in the TRT pigs, preventing ST colonization in RPS-fed pigs. Overall, our findings highlight the potential use of RPS as a dietary intervention to improve gut health and reduce *Salmonella* infections in pigs.

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 at: https://www.ncbi.nlm.nih.gov/bioproject; PRJNA881483, PRJNA952690.

Ethics statement

All the experiments were approved by the Animal Ethics Committee of the National Institute of Animal Science, Republic of Korea (Approval No. NIAS 2021-503).

Author contributions

S-IO made substantial contributions to the conception and design of the work and revised the manuscript prior to the submission. S-WY was responsible for laboratory analyses, data curation, and interpretation of experimental data. S-WY, HL, EK, and S-IO were responsible for animal experiments and investigations. S-WY, Y-HJ, E-YB, AC, YD, T-YH, and S-IO were responsible for data validation and resources. S-WY and S-IO wrote the original draft. All authors read and approved the final manuscript.

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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.

Publisher's note

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Effects of *Lacticaseibacillus casei* (*Lactobacillus casei*) and *Saccharomyces cerevisiae* mixture on growth performance, hematological parameters, immunological responses, and intestinal microbiome in weaned pigs

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Introduction: This study was conducted to evaluate the effects of *Lacticaseibacillus casei* (*Lactobacillus casei*) and *Saccharomyces cerevisiae* mixture on growth performance, hematological parameters, immunological responses, and gut microbiome in weaned pigs.

Methods: A total of 300 crossbred pigs [(Landrace × Yorkshire]×Duroc; 8.87±0.34 kg of average initial body weight [BW]; 4 weeks of age) were divided into two dietary treatments (15 pigs/pen, 10 replicates/treatment) using a randomized complete block design (block=BW): control (CON) and the effective microorganism (MEM). The CON was not treated, while the MEM was treated with the mixture of *L. casei* (1×10^7CFU/mL) and *S. cerevisiae* (1×10^7CFU/mL) at 3mL/pig/day for 4 weeks *via* the drinking water supply. Two feces and one blood sample from the randomly selected pigs in each pen were collected on D1 and D28 after weaning. Pigs were individually weighed, and pen feed intakes were recorded to evaluate pig growth performance. For the gut microbiome analysis, 16S rRNA gene hypervariable regions (V5 to V6) were sequenced using the Illumina MiSeq platform, and Quantitative Insight into Microbial Ecology (QIIME) and Microbiome Helper pipeline were used for 16S rRNA gene sequence analysis.

Results and Discussion: The daily weight gain and feed efficiency of MEM were significantly higher than those of CON ($p<0.001$). There were no significant differences in hematological parameters and immune responses between CON and MEM. However, MEM had significantly lower *Treponema* genus, whereas significantly higher *Lactobacillus* and *Roseburia* genera compared to CON. Overall, our data showed that *L. casei* and *S. cerevisiae* mixture could promote growth performance through the modulation of gut microbiota in pigs. This study will help to understand the correlation between the growth performance and the gut microbiome.

KEYWORDS

mixed effective microorganisms, growth performance, gut microbiome, weanling pigs, hematology

Introduction

Weaning is a very important period in which the piglets have to deal with separation from the sow, along with the feed changes from milk to solid feed (1, 2). Weaning imposes enormous stress on the piglets and causes the marked microbiological and physiological changes in the pig (3). As a result, the poor growth performance including slow growth rate, reduced feed intake and reduced feed conversion of post-weaning piglets can be induced by the biological stresses after weaning (4). Consequently, the weaning transition generally causes huge economic losses in the pig industry (2, 5). To overcome the weaning stresses, antibiotic growth promoters (AGPs) have previously been used to improve growth rate and to prevent diseases. However, the use of AGPs have been banned in the numerous countries including the United States and the European Union because of the increased public health concerns, such as antimicrobial resistance (6). Therefore, there has been a need for alternatives to feed antibiotics to reduce mortality and improve gut health in pigs during the pivotal weaning period.

The various alternatives to AGPs have been evaluated to enhance pig growth performance and to prevent diseases. Among them, beneficial microorganisms have been widely used in swine industry worldwide because they are known to improve the gut microbiome and the livestock productivity.

The porcine gastrointestinal tract (GIT) harbors a highly diverse microbial ecosystem (7), which, once developed, remains stable over time (8). This variety of microbiota has the profound effects on the development of immunological, morphological, and physiological development of the GIT (8). The microbiota contributes to the digestion of nutrients and forms a layer on mucosal surfaces that protects from overgrowth of pathogens (9). As advanced metagenomics using next generation sequencing have revealed high correlations between the gut microbiome and the health of animals, the roles of intestinal microbiome to improve livestock productivity and to prevent diseases are being emphasized. Various studies are being conducted on effective microorganisms that can improve the gut microbiome and the livestock productivity. Beneficial microorganisms are also commonly known to improve intestinal health as well (10). One of the commonly used beneficial bacteria in the swine industry is *Lacticaseibacillus casei* (*L. casei*) that was formerly known as *Lactobacillus casei*. *L. casei* is a dominant beneficial bacterium in the pig intestine, and is known as a strain that has been recognized for its immunomodulatory and harmful bacteria suppression functions (11). Another commonly used beneficial bacteria in the swine industry is *Saccharomyces cerevisiae* (*S. cerevisiae*) that is a yeast. *S. cerevisiae* is not a naturally occurring strain in the pig intestinal tract, but it has effects such as adsorption of toxins from β-glucan present in the cell wall, stimulation of the immune system, and suppression of pathogen attachment in the intestinal tract (12). Probiotics that contain multiple strains of different species are generally more effective than those with multiple strains of the same species because the mixed strains of different probiotic species can work together in the host gut environmental conditions (13). As an example, Giang et al. reported that a combination of yeast with lactic acid bacteria (LAB) had better probiotic effects on growth performance and digestion over a longer time period than the single use of LAB alone (14). Therefore, the combined use of these two strains (*L. casei* and *S. cerevisiae*) is expected to have a synergistic effect on the development of the intestinal tract and the establishment of the immune system during weaning. With these backgrounds, this study was conducted to determine the effects of a mixture of effective microorganisms (*L. casei* and *S. cerevisiae*) on growth performance, hematological parameters, immunological responses, and intestinal microbiome in weaned pigs.

Abbreviations: ADFI, Average daily feed intake; ADG, Average daily gain; BW, Body weight; EDTA, Ethylenediaminetetraacetic acid; FB ratio, ratio of Firmicutes to Bacteroidetes; GIT, Gastrointestinal tract; HCT, Hematocrit; HGB, Hemoglobin; MCH, Mean corpuscular hemoglobin; MCHC, Mean corpuscular hemoglobin concentration; MCV, Mean corpuscular volume; PLT, Platelets; SEM, Standard error of mean; TNF-α, Tumor necrosis factor-α; RBC, Red blood cell.

Materials and methods

Animal ethics statement

The protocol used in this experiment was reviewed and approved by the Institutional Animal Care and Use Committee of Dankook University, Cheonan, South Korea (approval no. DKU-21-040). The experiment was conducted at the Saeiri-Farm, Jincheon, South Korea (Coordinates: 36° 51′ 24.00″ N / 127° 26′ 36.00″ E) in where the average temperature and precipitation during the experimental period were 20.2°C and 6.4 mm, respectively.

Experimental design, animals, and housing

A total of 300 crossbred piglets [(Landrace × Yorkshire) × Duroc; 8.87 ± 0.34 kg of average initial body weight [BW]; 4 weeks of age] were used in this experiment. These pigs were randomly allotted to two treatments (15 pigs/pen, 10 replicates/treatment) using a randomized complete block design (block = BW): control (CON) and the effective microorganism (MEM). The animals used in the experiment were female and castrated male pigs, and both genders were randomly assigned into the respective dietary treatments. The liquid form of MEM mixture containing 1×10^7 CFU/mL of each *L. casei* and *S. cerevisiae* was provided by the government institution, Korean Agricultural Technology Center (Jincheon, South Korea). The optimal dose of MEM was determined by Korean Agricultural Technology Center (Jincheon, South Korea), and pigs in the MEM treatment

TABLE 1 Composition of the basal diets for weaning pigs (as-fed basis).

Item[a]	CON
Ingredient, %	
Corn	46.91
Whey powder	15.00
Soybean meal, 44%	20.00
Soy protein concentrate	11.50
Soybean oil	2.90
Limestone	1.34
Monocalcium phosphate	0.95
Vitamin premix[a]	0.40
Mineral premix[b]	0.40
L-Lys•HCl	0.34
DL-Met	0.18
L-Thr	0.08
Total	100.00
Calculated energy and nutrient	
Metabolizable energy, kcal/kg	3,465
Crude protein, %	22.36
Ca, %	0.85
P, %	0.65
Lys, %	1.54
Met, %	0.47

[a]Vitamin premix provided the following quantities of vitamin per kilogram of complete diet: vitamin A, 12,000 IU; vitamin D_3, 2,500 IU; vitamin E, 40 IU; vitamin K_3, 3 mg; D-pantothenic acid, 15 mg; nicotinic acid, 40 mg; choline, 400 mg; and vitamin B_{12}, 12 μg.
[b]Mineral premix provided the following quantities of mineral per kilogram of complete diet: Fe, 90 mg from iron sulfate; Cu, 8.8 mg from copper sulfate; Zn, 100 mg from zinc oxide; Mn, 54 mg from manganese oxide; I, 0.35 mg from potassium iodide; Se, 0.30 mg from sodium selenite.

received the optimal dose of *L. casei* (1×10^7 CFU/mL) and *S. cerevisiae* (1×10^7 CFU/mL) at 3 mL/pig/day for 4 weeks *via* the drinking water supply using a proportional dosing pump for treatments (Dosatron®, Tresses, France) according to the manufacturer's instructions. All pigs were fed a corn soybean meal based commercial basal diet formulated to meet the nutrient requirements proposed by the National Research Council (Table 1) (15). Pigs were housed in the room with slatted plastic floors, automated ventilation and heating systems. Pigs had free access to feed and water for the entire duration of the experiment.

Sample and data collection

For the evaluation of pig growth performance, pigs were individually weighed on day 1 (D1) and 28 (D28), and the average daily weight gain (ADG) was calculated. Feed consumption was also recorded at the same time (D1 and D28), and the average daily feed intake (ADFI) and feed efficiency (gain:feed) were calculated. Two fecal samples from the randomly selected pigs in each pen (a total of 20 samples/treatment/time point) were collected directly from the rectum on D1 and D28 after weaning. Diarrhea scores in pigs were assessed visually on a pen basis by 2 evaluators for 2 weeks after the start of the experiment as follows: 0, very hard, often pellet-like faeces; 1, well-formed faeces firm to cut; 2, formed faeces but soft to cut; 3, faeces falling out of shape upon contact with surfaces, sloppy; 4, pasty diarrhea; 5, liquid diarrhea (16). The blood samples (a total of 10 samples/treatment/time point) were collected from the jugular veins of the randomly selected pigs using vacutainer tubes of 5 mL containing with or without ethylenediaminetetraacetic acid (EDTA) as an anticoagulant on D1 and D28 of the experiment (17). The blood samples collected with EDTA were stored at 4°C until usage, then they were used for the complete blood count analysis. The blood samples collected without EDTA were left to clot at room temperature for 2 h. Then the serum samples were prepared by centrifuging the blood samples at 3,000 × g at 4°C for 15 min, and stored at −80°C until they were used for Enzyme-Linked Immunosorbent Assay (ELISA).

Blood sample analysis

Hematological parameters were analyzed using the Scil Vet abc hematology analyzer (Scil Animal Care Company, Altorf, France) that was calibrated for porcine blood. The concentrations of serum cortisol, tumor necrosis factor-α (TNF-α) (R&D Systems, Inc., Minneapolis, United States), and immunoglobulin G, M, and A (Abnova Corp., Taipei City, Taiwan) were determined using the ELISA kits according to the manufacturer's protocol.

16S rRNA gene analysis

For the total DNA extraction from the feces, 200 mg of feces per sample was used and the DNA extraction was conducted using the QIAamp Fast DNA Stool Mini Kit (QIAGEN, Hilden, Germany) following the manufacturer's protocol. The Colibri Microvolume Spectrometer (Titertek Berthold, Pforzheim, Germany) was used to measure the concentrations of DNA, and the samples with OD260/280 ratios of 1.80–2.15 were utilized further. To amplify the V5 to V6 hypervariable regions of the 16S rRNA genes, the PCR primer sets consisted of 799F-mod6 (5' CMGGATTAGATACCCKGGT-3′) and 1114R (5'-GGGTTG CGCTCGTTGC-3′) were used. Briefly, 25 ng of DNA in a reaction volume of 50 μL was used, and the amplification mix contained 5X PrimeSTAR Buffer (Mg2+) (Takara Bio, Inc., Shiga, Japan), 2.5 mM concentrations of each deoxynucleotide triphosphates, 2.5 U/μL of PrimeSTAR HS DNA Polymerase, and 10 pmol of each primer. The polymerase chain reaction cycling conditions were as follows: initial denaturation was at 98°C for 3 min, followed by 30 cycles of 98°C for 10 s, 55°C for 15 s, and 72°C for 30 s, and a final 3-min extension at 72°C. Then, the PCR product was purified using Wizard® SV Gel and PCR Clean Up System purification kit (Promega, Madison, United States) according to the manufacturer's instructions. The sequencing of the 16S rRNA gene amplicons was conducted using the Illumina MiSeq chemistry at BRD Inc. (Dongtan, Republic of Korea) according to the manufacturer's instructions. All the raw sequence data generated from the Illumina MiSeq platform were quality checked utilizing FastQC (18, 19). Then, 16S rRNA gene sequences were analyzed using the Deblur algorithm implemented in the QIIME2 and Microbiome Helper pipeline.

Statistical analysis

Data were analyzed using the General Linear Model Procedure of SAS (version 9.0, SAS Inst. Inc., Cary, United States) in a randomized complete block design with the initial BW as a block, considering the pen as the experimental unit. The statistical model for growth performance, hematological parameters and immunological responses included the effect of dietary treatments as a fixed effect. The statistical significance was set as the p value of less than 0.05 ($p < 0.05$). The results were presented as the mean ± standard error of the mean (SEM). Alpha diversity indices, such as Shannon, Simpson, observed OTUs, and Chao1 of each dietary treatment were calculated utilizing the MicrobiomeAnalystR. Significant differences in alpha diversity indices between dietary treatments were calculated based on ANOVA. To determine significant differences in beta diversity, statistical comparisons of weighted and unweighted UniFrac distances between dietary treatments were performed using analysis of similarities (ANOSIM).

TABLE 2 Effect of mixture of effective microorganisms on growth performance parameters[a] and diarrhea score of piglets.

Item[b]	CON	MEM	SEM	p-value
Initial BW, kg	9.11	8.53	0.88	0.102
Final BW, kg	15.55	16.61	0.40	0.087
ADG, g/d	222	279	4.97	< 0.001
ADFI, g/d	428	447	7.17	0.094
Feed efficiency, g/g	0.52	0.62	0.01	< 0.001
Diarrhea score[c]	3.49	3.45	0.34	0.746

[a]Pigs were individually weighed, and pen feed intakes were recorded to evaluate pig growth performance on D1 and D28. Each value is the mean of 10 replicates.
[b]CON, a control diet without any treatments; MEM, a diet treated with mixture of effective microorganisms; SEM, standard error of mean.
[c]Diarrhea scores in pigs were assessed visually on a pen basis by 2 evaluators for 2 weeks after the start of the experiment. Diarrhea score: 0 = very hard, often pellet-like faeces; 1 = well-formed faeces firm to cut; 2 = formed faeces but soft to cut; 3 = faeces falling out of shape upon contact with surfaces, sloppy; 4 = pasty diarrhea; 5 = liquid diarrhea.

Results and discussion

Growth performance

There were no differences on final BW and ADFI of pigs during the experimental period between dietary treatments (Table 2). However, the daily weight gain (279 g/d vs. 222 g/d) and feed efficiency (0.62 g/g vs. 0.52 g/g) of weanling pigs were significantly higher in MEM than those in CON ($p < 0.001$). During the first 14 days after weaning, no treatment effects were observed in the diarrhea scores between CON and MEM (Table 2). The growth performance observed in this study agrees with previous results, and showed that the overall growth performance was improved when useful microorganisms were fed. In the previous study, the *L. casei* fed group showed a higher weight gain than the control group, and a pattern similar to that of the antibiotic (colistin sulfate) group was observed for growth performance (20). Jang et al. reported that there was no significant difference in diarrhea frequency compared to the control group when probiotics (*L. casei*, *Bacillus subtilis*, *Lactobacillus crispatus*) were fed, but the daily weight gain showed a tendency to improve by 4% and feed efficiency by 6% (21). However, Francisco Tortuero reported that there was no difference in the growth of weaned pigs compared to the control group when mixed with *Streptococcus faecium* M74 (22). Because of these discrepancies between results from previous studies, it was suggested that the growth promoting and diarrhea preventing effects of probiotic bacteria should be observed when pigs were exposed to stress or had health problems, resulting in a greater effects (23, 24). Kritas et al., reported that it was effective in improving prevalence and growth performance in pigs infected with porcine reproductive and respiratory syndrome virus, although it did not show weight improvement under normal conditions (24). Similarly, in a previous study, *L. casei* treatment was reported to improve the diarrhea index in pigs infected with pathogenic bacteria (23). Wang et al., also reported that *L. casei* plays an important role in contributing to intestinal morphological development by preventing intestinal pathological damage and reducing inflammation (11).

Hematological parameters and immunological responses

It has been known that the frequency of diarrhea is correlated with the hematocrit values (25), and the serum hematological parameters are also associated with the stress and health status of the animals (26, 27). In addition, Bhattarai et al. (28) reported that red blood cells (RBC) and hemoglobin (HGB) were positively correlated with ADG in weaned pigs (28). However, there were no significant differences between dietary treatments of weaned piglets in all hematological parameters including RBC and HGB (Table 3). The blood parameter data for this study were within the normal range for the hematologic characteristics of weaned pigs as reported by previous studies (29).

In addition, no differences were observed between dietary treatments in the serum TNF-α, cortisol, and Immunoglobulin G, M, and A of weanling pigs during the experiment (Table 4). A number of previous studies have reported that cortisol concentrations, an indicator of weaning stress, increased over 7 days after weaning (30–32). In addition, pro-inflammatory cytokines such as TNF-α, which are closely related to weaning stress, are associated with transient inflammation in the intestine. Therefore, pro-inflammatory cytokines could cause negative effects for intestinal development and nutrient absorption in weaning piglets (33, 34). However, in this experiment, no differences were observed between dietary treatments in the serum TNF-α, and cortisol of weanling pigs during the experiment (Table 4). Similarly, no significant differences in immunoglobulin G, M, and A were found between dietary treatments in this experiment (Table 4), although, several other studies have reported that certain LABs, such as *Lactobacillus casei*, *Lactobacillus rhamnosus* and *Lactobacillus plantarum*, stimulated immune responses (35, 36). We speculated

TABLE 3 Effects of mixture of effective microorganisms on hematological parameters of weaning pigs.[a]

Item[b]	CON	MEM	SEM	*p*-value
WBC, 10^3/μL				
Pre-weaning	19.01	22.13	1.76	0.226
Post-weaning	29.89	25.25	2.66	0.240
RBC, 10^6/μL				
Pre-weaning	7.01	6.69	0.18	0.218
Post-weaning	7.12	7.20	0.22	0.965
HGB, g/dL				
Pre-weaning	11.24	10.47	0.38	0.174
Post-weaning	11.48	11.75	1.58	0.335
HCT, %				
Pre-weaning	41.83	40.84	1.22	0.572
Post-weaning	52.31	52.39	3.82	0.420
Lymphocyte, %				
Pre-weaning	48.51	45.58	2.92	0.487
Post-weaning	47.13	44.82	4.61	0.996
Monocyte, %				
Pre-weaning	3.41	4.34	0.58	0.268
Post-weaning	2.58	5.29	3.29	0.669
Eosinophil, %				
Pre-weaning	1.38	1.10	0.23	0.393
Post-weaning	0.32	0.55	2.33	0.347
Basophil, %				
Pre-weaning	0.43	0.53	0.08	0.374
Post-weaning	0.67	0.76	0.19	0.633

[a]Each value is the mean of 5 replicates.
[b]CON, a control diet without any treatments; MEM, a diet treated with mixture of effective microorganisms; SEM, standard error of mean; RBC, red blood cell; HGB, hemoglobin; HCT, packed cell volume (Hematocrit); PLT, platelets; MCV, mean corpuscular volume; MCH, mean corpuscular hemoglobin; MCHC, mean corpuscular hemoglobin concentration; Pre-weaning, the start of the experiment (D1); Post-weaning, the end of the experiment (D28).

TABLE 4 Effects of mixture of effective microorganisms on serum immune status of weanling pigs.[a]

Item[b]	CON	MEM	SEM	*p*-value
Cortisol, ng/mL				
Pre-weaning	5.12	3.14	0.52	0.053
Post-weaning	4.45	3.45	1.19	0.584
TNF-α, pg/mL				
Pre-weaning	78.51	89.70	4.97	0.187
Post-weaning	80.25	72.50	6.56	0.451
IgG, mg/mL				
Pre-weaning	639.22	401.18	76.73	0.093
Post-weaning	967.24	672.67	86.38	0.073
IgM, mg/mL				
Pre-weaning	1.58	3.58	0.48	0.069
Post-weaning	9.89	7.15	2.56	0.492
IgA, mg/mL				
Pre-weaning	7.77	9.14	2.36	0.703
Post-weaning	50.28	32.15	7.68	0.171

[a]Each value is the mean of 3 replicates.
[b]CON, a control diet without any treatments; MEM, a diet treated with mixture of effective microorganisms; SEM, standard error of mean; Pre-weaning, the start of the experiment (D1); Post-weaning, the end of the experiment (D28).

that these discrepancies between our results and others might be caused by the sampling time point.

Swine fecal microbial diversity

We performed 16S rRNA gene analysis on the fecal samples collected on the first and final day of the experiment (post weaning D1 and D28). The microbial diversity indices are presented in Figure 1. No differences in Chao1, Shannon, and Simpson diversity indices were observed between the MEM and CON. The visualization of the relative distances of microbial communities between the CON and MEM using a principal coordinate analysis (PCoA) plot are shown in Figures 2, 3.

At the first day of the experiment, the PCoA plots based on the unweighted and weighted UniFrac distances revealed no differences of microbial communities between dietary treatments (Unweighted: $R = 0.09567$. $p < 0.05$; Weighted: $R = 0.060086$, $p < 0.05$) (Figure 2). However, microbial populations were clustered into two distinct groups at the end of the experiment (Unweighted: $R = 0.243$. $p < 0.001$; Weighted: $R = 0.29356$, $p < 0.001$) (Figure 3). Because the pigs were housed under the same conditions and fed the same feed, it is reasonable to speculate that the differences of microbial communities between dietary treatments could be the effects of the MEM.

Taxonomic classification of the sequences

The relative abundances of the bacterial taxa at the phylum and genus levels for all sequences are shown in Figures 4, 5, respectively. There were no differences in the relative fecal microbial compositions between dietary treatments at the phylum level during the experiment (Figure 4). The three phyla Firmicutes, Bacteriodetes and Proteobacteria were sequentially dominant in both dietary treatments, of which Firmicutes, Bacteriodetes accounted for approximately 90% of the total sequence reads. This result is consistent with previous studies on the swine gut microbiota, the two most abundant taxa at the phylum level were Firmicutes and Bacteroidetes (37, 38). The result of this experiment showed that the ratio of Firmicutes to Bacteroidetes (FB ratio) of the two dietary treatments on the first day of the weaning was similar, however, the FB ratio of the

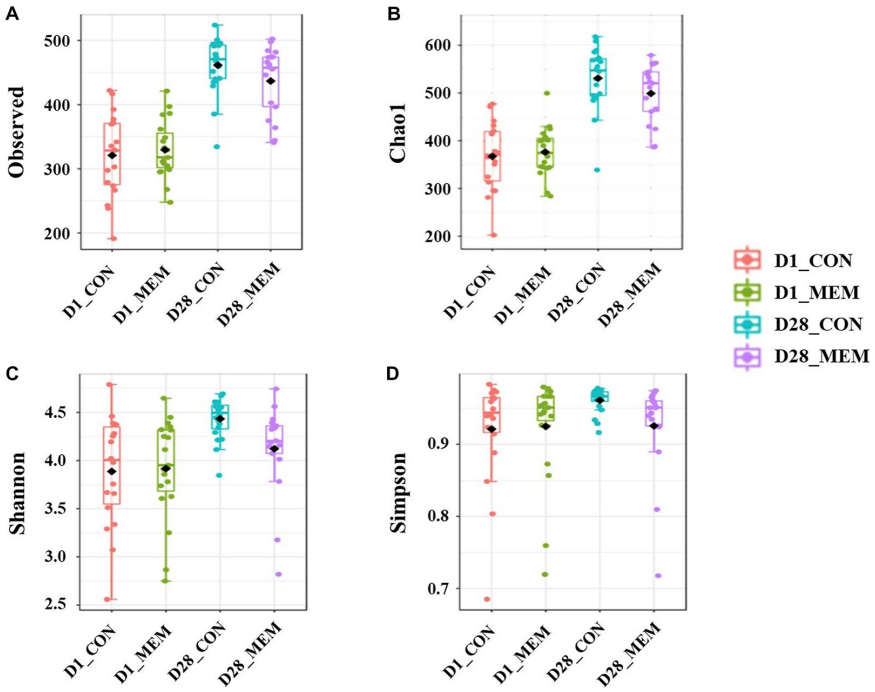

FIGURE 1
Box plots showing the alpha diversity indices of the pigs in CON and MEM on D1 and D28. **(A)** Number of observed OTUs, **(B)** Chao1 index, **(C)** Shannon index and **(D)** Simpson index. CON, a control diet without any treatments; MEM, a diet treated with mixture of effective microorganisms.

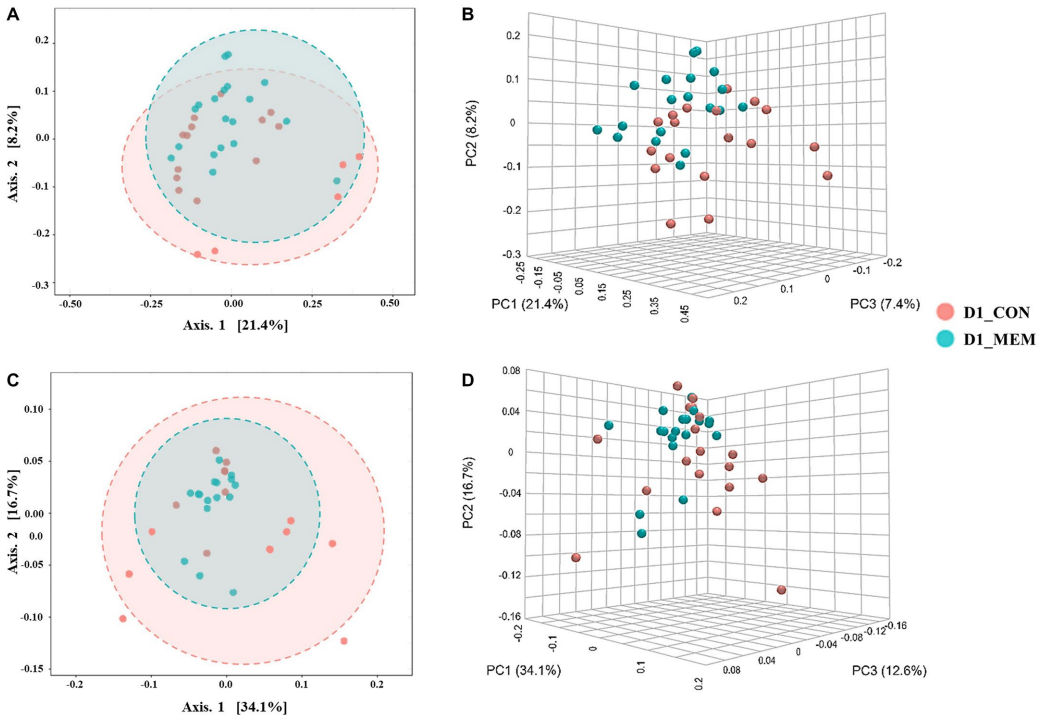

FIGURE 2
Beta diversity analysis of the pig gut microbiota of the pigs in CON and MEM on D1. Principal co-ordinates analysis (PCoA) plots based on the unweighted **(A, B)** and weighted **(C, D)** UniFrac distances of gut microbial communities (unweighted: R=0.093567, $p<0.05$; weighted: $R=0.060086$, $p<0.05$). Each symbol represents the microbiota from individual pig sample and was color coded according to dietary treatments (CON and MEM). The axes show the percent variation. CON, a control diet without any treatments; MEM, a diet treated with mixture of effective microorganisms.

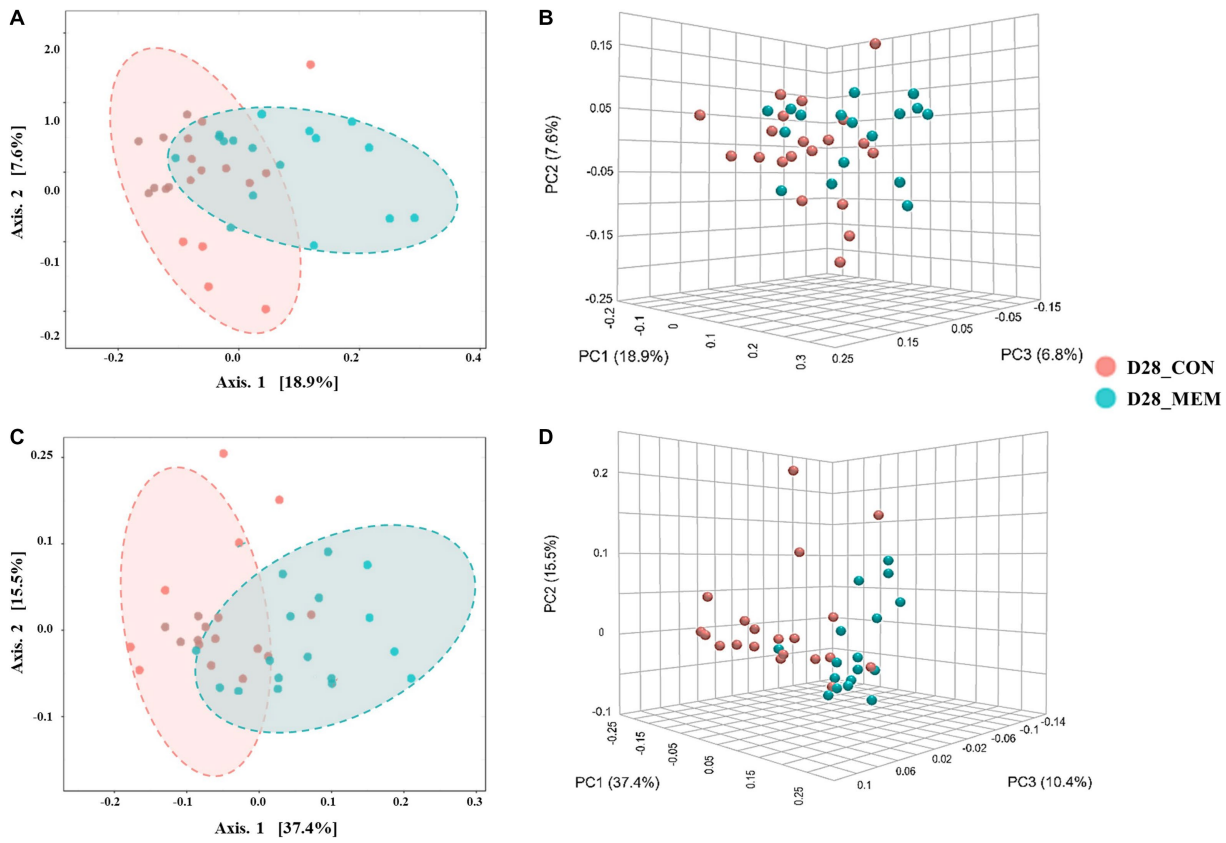

FIGURE 3
Beta diversity analysis of the pig gut microbiota of the pigs in CON and MEM on D28. Principal co-ordinates analysis (PCoA) plots based on the unweighted **(A, B)** and weighted **(C, D)** UniFrac distances of gut microbial communities (unweighted: R=0.243, p<0.001; weighted: R=0.29356, p<0.001). Each symbol represents the microbiota from individual pig sample and was color coded according to dietary treatments (CON and MEM). The axes show the percent variation. CON, a control diet without any treatments; MEM, a diet treated with mixture of effective microorganisms.

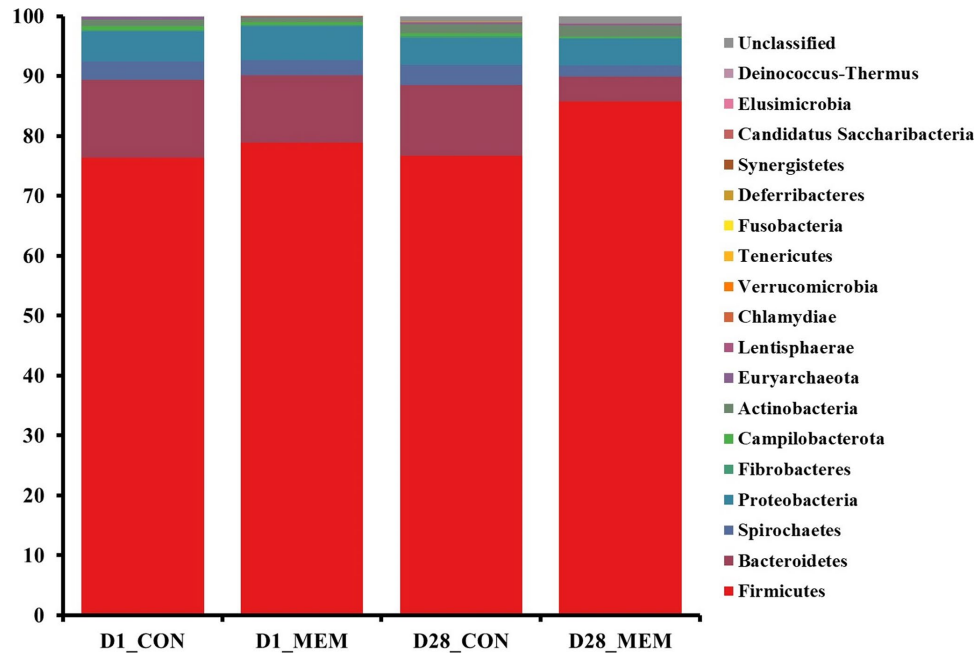

FIGURE 4
Stacked bar plots of the relative abundance of gut microbial communities at the phylum levels in weanling pigs on D1 and D28. CON, a control diet without any treatments; MEM, a diet treated with mixture of effective microorganisms.

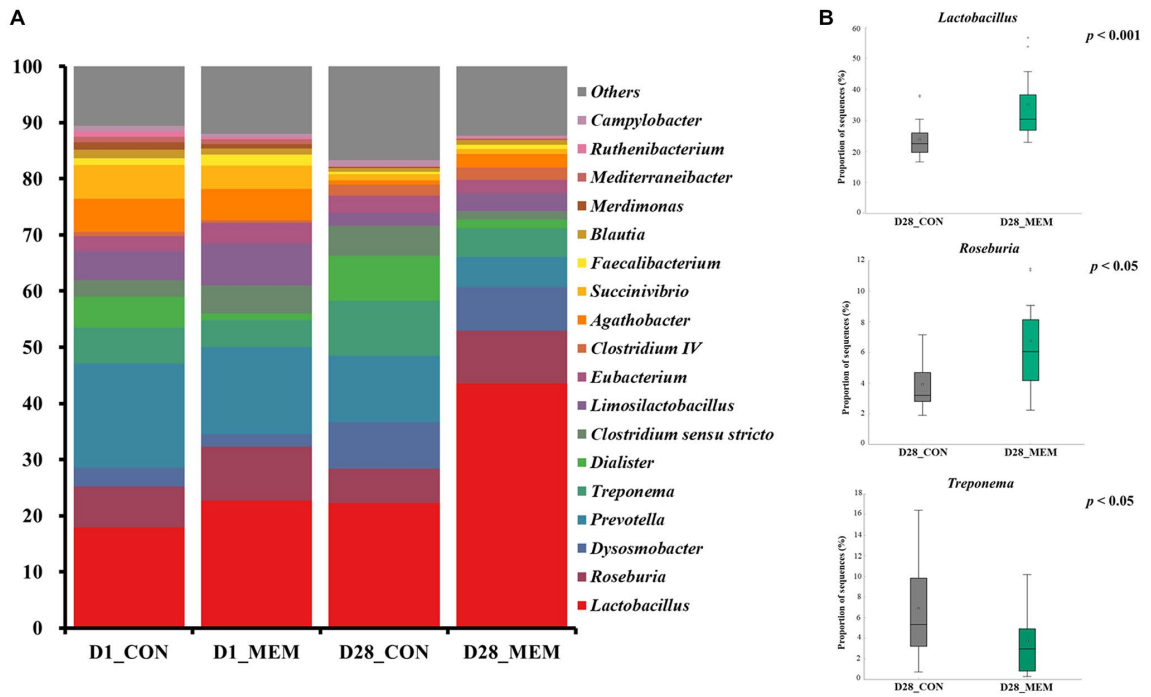

FIGURE 5
Stacked bar plots of the relative abundance of gut microbial communities at the genus levels in weanling pigs on D1 and D28. Relative abundances of the sequences at the genus level **(A)**, Relative abundances of *Treponema, Lactobacillus,* and *Roseburia* on d28 **(B)**. CON, a control diet without any treatments; MEM, a diet treated with mixture of effective microorganisms.

MEM at 4 weeks after treatment was 2 times higher than that of the CON. This is consistent with a previous study that reported an association between improved weight gain and the higher FB ratio (39). They reported that the higher the FB ratio produced the more short chain fatty acids during microbial metabolism, which exhibit anti-inflammatory and antioxidant properties in the porcine intestinal tract, which is associated with weight gain (40, 41). The difference in fecal microbial compositions between CON and MEM was confirmed at the genus level (Figure 5). The relative abundances of *Treponema, Lactobacillus,* and *Roseburia* were not significantly differ between dietary treatments on D1. Regarding the relative abundance of *Treponema* that is known as a potential pathogen and is mainly responsible for intestinal inflammation, the MEM had significantly lower ($p < 0.05$) *Treponema* than CON on D28 (Figure 5B). Whereas MEM had significantly higher ($p < 0.001$) *Lactobacillus* which helps prevent infection and improve growth performance (42), compared to the CON on D28 (Figure 5B). Also, it was confirmed that the relative abundance of *Roseburia*, which produces short chain fatty acids and acts as an anti-inflammatory agent was significantly higher in the MEM than the CON ($p < 0.05$) on D28 (Figure 5B) (43–46).

The results of alpha diversity analyses showed no significant changes in the gut microbial richness between dietary treatments. However, the PCoA plot presented differences in the gut microbial composition and their relative abundances between CON and MEM on D28. As such, relative abundances of *Treponema, Lactobacillus,* and *Roseburia* shifted over time. Overall, these results indicate that the MEM shifted the gut microbial communities in the studied pigs.

Conclusion

Overall, our data showed that *L. casei* and *S. cerevisiae* mixture improved the growth rate of weaned pigs by shifting their gut microbiome. Mixture of *L. casei* and *S. cerevisiae* increased the abundances of beneficial microbes including *Lactobacillus* and *Roseburia*. The findings of this study suggested that the mixed effective microorganisms could promote growth performance through modulation of gut microbiota in pigs.

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/, PRJNA905856.

Ethics statement

The animal study was reviewed and approved by Institutional Animal Care and Use Committee of the Dankook University,

Cheonan, South Korea (approval no. DKU-21-040). Written informed consent was obtained from the owners for the participation of their animals in this study.

Author contributions

SaK, MS, JC, and HK: conceptualization. MS: data curation. HD, JK, SP, SR, YI, and ShK: formal analysis. EK, ShK, and YI: methodology. HD: resources. GK: software. JC: supervision. SaK, YI, and MS: validation. JHC: visualization. ShK, JK, JC, and MS: writing – original draft. HK: writing – review and editing. All authors contributed to the article and approved the submitted version.

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Conflict of interest

SaK was employed by BRD Korea Corp.

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

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Exploring the temporal dynamics of rumen bacterial and fungal communities in yaks (*Bos grunniens*) from 5 days after birth to adulthood by full-length 16S and 18S rRNA sequencing

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The rumen of ruminants is inhabited by complex and diverse microorganisms. Young animals are exposed to a variety of microorganisms from their mother and the environment, and a few colonize and survive in their digestive tracts, forming specific microflora as the young animals grow and develop. In this study, we conducted full-length sequencing of bacterial and fungal communities in the rumen of pastured yaks of different ages (from 5 days after birth to adulthood) using amplified sequencing technology. The results showed that the rumen microflora of Zhongdian yaks changed gradually from 5 to 180 days after birth and tended to stabilize at 2 years of age. The rumen of adult yaks was the most suitable for the growth and reproduction of most bacteria. Bactria diversity of the yak rumen increased gradually from 5 days after birth to adulthood. With the growth of yaks, different dominated bacteria were enriched in different groups, but *Prevotella* remained highly abundant in all groups. The yak rumen at 90 days of age was the most suitable for the growth and reproduction of most fungi, and 90days of age could be a cut-off point for the distribution of fungal communities. Fungal *Thelebolus* was the firstly reported in yak rumen and was enriched in the yak rumen of 90 days after birth. The most abundant and balanced fungal genera were found in adult yaks, and most of them were only detected in adult yaks. Our study reported on the rumen bacterial and fungal communities of Zhongdian yaks grazed at different ages and provided insights into the dynamic changes of dominant microflora with yak growth.

KEYWORDS

rumen microbiology, yak, microbiome, bacteria, fungi

Introduction

Gut microbiomes are inseparable from their animal host (1, 2), affecting the health and proper functioning of their host. Ruminant rumen microbes degrade complex plant polysaccharides into compounds which can be absorbed and utilized by ruminants (3). This critical process not only converts plant fiber in forage grass into milk and meat for human consumption but also promotes the reproduction and growth of rumen microorganisms. In young ruminants, microflora plays an important role in the development of the rumen wall and in nutrient absorption processes. Zhongdian yak (*Bos grunniens*) is a characteristic breed of cattle of Chinese Yunnan Province, playing a vital role in the economic, genetic, and ecological diversity of the Qinghai Tibet Plateau area (4, 5). They live in a low-temperature hypoxic high-altitude environment and have a higher quality production of milk than that of dairy cows (6). Investigation of yak rumen microbes is important to understand how rumen microbiome are associated with their specific phenotypes.

It has been well-proven that the rumen microbiome is related to altitude adaptation and milk performance traits. The rumen microbiome diversity of Zhongdian yak is significantly higher than that of cattle and buffalo, who live at an altitude of 1,100 m (7). Interactions of Proteobacteria-choline and Firmicutes-myristic acid in the rumen cause to changes in milk fat percentage in yaks (6). Furthermore, the rumen microflora of ruminants undergoes gradual changes from birth, primarily marked by an increase in anaerobic microorganisms and a decrease in aerobic and facultative anaerobic microorganisms (8). The rumen microflora of calves undergoes changes with growth and dietary structure, while the rumen physiological environment facilitates the interaction between microorganisms (9). Facultative anaerobic flora typically exhibit higher levels during the first day of a calf's life and then decrease to a stable abundance after 6–8 weeks (10). Anaerobic fungi appear in the rumen of ruminants shortly after birth, and communities of bacteria, fungi, and archaea form in the rumen of animals at 7 days of age (11, 12). The microflora of young ruminants was similar, but the abundance and diversity of the microflora also changed with the change in dietary structure and the increase in feed intake (13). Bacterial and fungal changes in the calve rumen are also affected by the natural grazing patterns of calves. Although researchers have explored the effects of the rumen microbiome on plateau adaptation and milk-production performance, an understanding of the microbial colonization process in the life of grazing yak calves remains elusive.

Exploring the temporal dynamics in microbial communities in yaks from birth to adulthood can aid to establish microbial interactions during rumen development. Hence, in this study, the full length of bacterial 16S rDNA and fungal ITS2 of rumen fluids from grazing Zhongdian yaks at 5, 45, 90, 180, and 720 days of age were sequenced to investigate the temporal dynamics of rumen microbiome. Our results described rumen bacterial and fungal compositions and their temporal dynamics (newborn to adulthood) in Zhongdian yaks, which allow us to understand the importance of rumen microbiome in yaks. Rumen fluid was collected from 3 yaks at 5, 45, 90, and 180 days after birth. Rumen fluid was collected from 6 yaks 720 days after birth. Bacterial 16S rDNA and fungal ITS2 sequencing were performed to investigate the changes in rumen microbial composition, diversity, core microorganisms, and anti-inflammatory characters in different ages of Zhongdian yak. Our results described rumen bacterial and fungi compositions and the dynamics occurring with ages (newborn to adulthood) in Zhongdian yaks.

Materials and methods

Experimental animals

The animal trial was conducted in the natural pasture of Tiancheng Lun Zhu Agricultural Products Development Co., Ltd. in the north of Shangri-La County, which is a double-row fully open barn with free grazing open feeding mode for the experimental yaks. There are drinking water supply points in the pasture, which can ensure the yaks' drinking water. In July 2019, 10 female yaks were selected from the Zhongdian yak herd which were simultaneously estrous and inseminated in 2018 (average gestation period of 271 days). After the calves were born, six yak calves in similar conditions were selected for sampling. The calves were separated from their dams on day 5 after birth. Rumen fluids were collected from each yak at 5, 45, 90, and 180 days after the birth. The experimental yaks were divided into five groups according to their age: D5 (5-day after birth), D45 (45 days after birth), D90 (90 days after birth), D180 (180 days after birth), and D720 (adult yaks; Supplementary Table S1). The nutrient compositions of the fed diets are shown in Supplementary Table S2.

An oral stomach tube was used for collection of rumen fluid samples. In brief, 2 h after morning grazing, a tube was inserted into the rumen, and a vacuum sampler was used to pump rumen fluid. For each animal, 30 ml of rumen fluid was collected, and divided into three parts which were placed in 10 ml polypropylene tubes. Then, all samples were rapidly stored in liquid nitrogen, and brought back to the laboratory for storage in a refrigerator at −80°C. Due to some calves dead during the experiment, the number of yaks was reduced to three. Six adult yaks were randomly collected from naturally grazing yaks that were birthed in 2017, and the rumen fluid was collected using the same method.

DNA extraction and sequencing

The EZNA Stool DNA Kit (Omega Bio-Tek, Norcross, GA, USA) was used to extract microbial community DNA according to the manufacturer's instructions. DNA was quantified using the Qubit dsDNA BR Assay kit (Invitrogen, USA) and quality was assessed by running an aliquot on a 1% agarose gel. To amplify variable regions V1-V9 of the bacterial 16S rRNA gene, degenerate PCR primers 27F (5′-AGRGTTYGATYMTGGCTCAG-3′) and 1492R (5′-RGYTACCTTGTTACGACTT-3′) were used (14). Additionally, degenerate PCR primers ITS3 (5′-GCATCGATGAA GAACGCAGC-3′) and ITS4 (5′-TCCTCCGCTTATTGATATGC-3′) were used to amplify the ITS2 of the internal transcribed spacer (ITS) region (15). Both forward and reverse primers were tagged with Illumina adapter, pad, and linker sequences. PCR enrichment was carried out in a 50 µl reaction containing 30 ng template, fusion PCR primer, and linker sequences, using PCR master mix. The PCR cycling conditions were as follows: 94°C for 3 min, 30 cycles of 94°C for 30 s, 56°C for 45 s, and 72°C for 45 s, and a final extension for 10 min at

72°C. PCR products were purified using AmpureXP beads and eluted in elution buffer. The Agilent 2100 Bioanalyzer (Agilent, USA) was used to qualify the libraries. Validated libraries were sequenced using the Illumina MiSeq platform (BGI, Shenzhen, China) following Illumina's standard pipelines. Bacterial and fungal sequences from this project have been deposited in the National Center for Biotechnology Information (NCBI) Short Read Archive (SRA) under the BioProject number PRJNA630991.

Bioinformatics and data analyses

The raw paired-end reads from the sequencer were merged and filtered to eliminate adapter pollution and low-quality readings, obtaining clean reads. Further processing and quality controls were performed using mothur v1.42.1 according to the most recent versions of our lab's standard analysis pipelines, adapted from the Schloss lab protocol (16). Chimeric sequences were identified and removed using the UCHIME algorithm in mothur. Subsequently, tags were clustered into operational taxonomic units (OTUs) with a 97% sequence similarity. Taxonomic ranks were assigned to the representative sequences of the OTUs using the Ribosomal Database Project (RDP) Naive Bayesian Classifier v2.2. Alpha diversity, beta diversity, and the screening of different species were then analyzed based on the OTUs and taxonomic ranks.

Tags were clustered into operational taxonomic units (OTUs) using USEARCH (v7.0.1090) software. The taxonomic classification of OTU representative sequences was performed using Ribosomal Database Project (RDP) Classifier v.2.2, trained on the Greengene_2013_5_99 database. A 0.5% confidence value was used as the cutoff. Filtered tags were then clustered into OTUs at 97% similarity, where the OTU number per sample primarily represented the degree of sample diversity. The OTUs of each group were listed, and Venn diagrams were generated using Venn Diagram Software R (v3.1.1). Common and specific OTU IDs were summarized. Based on the abundance of OTUs, the relative abundance of each OTU in each sample was calculated. Principal component analysis (PCA) of the OTUs was performed using the relative abundance values. Good's coverage, alpha diversities (including Inverse Simpson and Shannon indices), richness (observed number of OTUs), and evenness (Shannon evenness) were calculated using Mothur V.1.31.2. Beta diversity analysis was also performed. Since there were differences in sequencing depth among samples, normalization was introduced by randomly extracting sequences according to the minimum sequence number for all samples. The extracted sequences were used to generate a new "OTU table biom" file, and the beta diversity distance was calculated based on this file. OTUs were annotated based on the Kyoto Encyclopedia of Genes and Genomes (KEGG) version 2018.01. The outputs of diversity analysis were visualized using the "ggplot2" package in R (v3.6.0), which is available online at https://www.r-project.org/.

The Linear Discriminant Analysis (LDA) Effect Size (LEfSe 1.1.01), an analytical tool used to discover and interpret biomarkers in high-dimensional data, was employed to identify the signature bacteria associated with growth stages and intestinal segments. A criterion of LDA score > 2 was used to determine significant effect size. The signature bacteria were visualized in a heatmap using the "pheatmap" function in R.

For assessing microbial interactions within treatments, network analysis was conducted by calculating all possible Pearson rank correlation coefficients (ρ) between microbial pairs. To minimize spurious associations, we considered a valid co-occurrence between two different taxa if the correlation coefficient was above 0.6 or below −0.6 and statistically significant. The subnetworks within the regimes were generated based on betweenness clustering calculated using the Girvan-Newman algorithm.

Results

Sequencing of the yak rumen at different ages

To explore the rumen microbial community of grazing yaks at different growth stages, we sequenced the full length of 16S rDNA and ITS for each sample using the Illumina HiSeq 2500 sequencing platform. A total of 164,397 16S rDNA sequences were detected from all samples, with an average of 9,133 sequences per sample, and were clustered into 1,709 OTUs with 97% similarity. With the increase of age, the number of OTUs detected in the rumen increased linearly. By comparing with the D720, the number of overlapped OTUs was 408 (72.21%) at the D5, 624 (86.31%) at the D45, 742 (86.78%) at the D90, and 944 (86.05%) at the D180, indicating that a large number of microorganisms existed stably in the rumen from 45 days until adulthood (Figure 1A; Supplementary Table S3).

For the ITS analysis, 1,009,208 ITS sequences were detected from all samples, with an average of 56,067 ITS sequences per sample, and clustered to 2,525 fungal OTUs. By comparing with the D720, the number of overlapped OTUs was calculated, which was low in each group, indicating that the fungi of the yak rumen were variable during their growth (Figure 1B; Supplementary Table S3). The detected OTUs were labeled to construct changes in the rumen microflora of Zhongdian yaks at different growth stages.

Bacterial community diversity and the temporal dynamics of the rumen signature bacteria in yak

According to the alpha diversity analysis of 16S rDNA data, the biodiversity of bacteria in the yak rumen gradually increased from the D5 and reached the richest in the adult (Figure 2A). The Sobs, Richness, Chao, Ace, and Shannon indices were the highest in the adult (D720) and the lowest at D5 which gradually increased with the growth of the yak. In contrast, the Simpson index was the lowest in the adult group and the highest at D5, which gradually decreased with the growth of the yak, indicating that the rumen fluid of adult yaks was more suitable for the growth and reproduction of bacteria. Beta diversity analysis of PCoA and cPCoA showed significant differences in community distribution among different ages, indicating that there were notable differences in rumen bacterial community compositions in different yak growth stages (Figure 2B).

The dominant rumen phyla (relative abundance of all groups >10) were Bacteroidetes and Firmicutes across all ages, followed by Proteobacteria, Verrucomicrobia, Planctomycetes, Kiritimatiellaeota, Lentisphaerae, and Cyanobacteria (Figure 2C). Notably, Proteobacteria

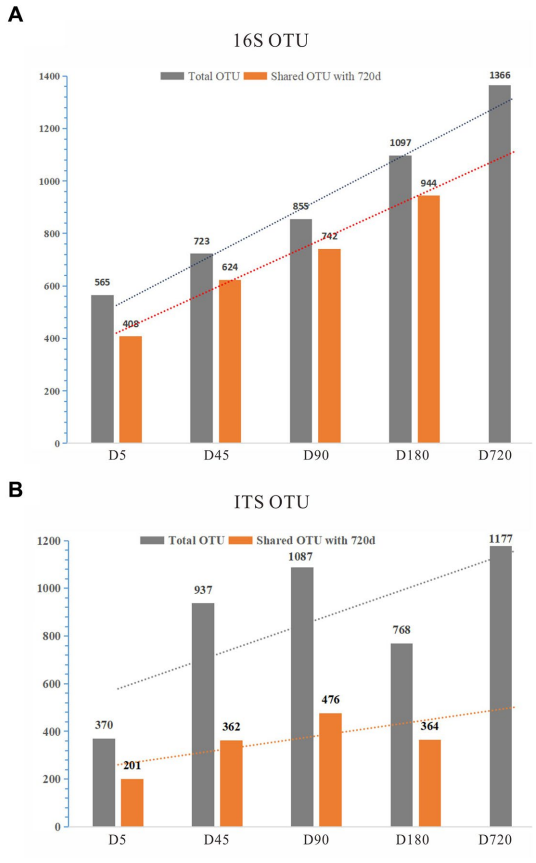

FIGURE 1
The statistics of OTUs of 16S and ITS. **(A)** OTUs of 16S; **(B)** OTUs of ITS.

was enriched at D5, while Verrucomicrobia was higher at D90. At the genus level, *Prevotella* was the absolute dominant genus (relative abundance of all groups >50). Other genera with higher abundance at a specific age were observed. For example, *Bacteroides* was high at D5, while *Luteolibacter* was enriched at D90.

Linear discriminant analysis Effect Size (LEfSe) analysis was performed to identify the signature bacteria differentiating ages at both the phylum and genus level. At the phylum level, a total of 11 significantly different abundance phyla were detected among different ages (Figure 2D). Proteobacteria and Fusobacteria(6.69%) were higher at D5 and kept lower abundances at other ages. Planctomycetes and Kiritimatiellaeota had the highest abundance at D45 and gradually decreased from D45 to D720. Verrucomicrobia and Cyanobacteria were highly abundant at D90. The relative abundance of Synergistetes reached the peak at D180. The relative abundance of Lentisphaerae, Elusimicrobia, Fibrobacteres, and Tenericutes gradually increased from D5 to adulthood. At the genus level, *Prevotella* and *Butyrivibrio* were highly abundant in D45(15.94 and 4%), D90(19.06 and 2.7%), and D720(15.4 and 3.03%), and *Luteolibacter* (14.20%) was only highly abundant at the D90. The abundance of *Kiritimatiella* was the highest in the D45 and declined from D45 to D720, and *Streptococcus* was only highly abundant at the D5. Some signature bacterial genera were highly enriched in a specific age, including *Porphyromonas*, *Fusobacterium*, *Mannheimia*, and *Peptostreptococcus* at the D5, and *Christensenella*, *Barnesiella*, and *Ornithobacterium* at the D45, and *Megamonas*, *Millionella*, and Coraliomargarita at the D90, and *Propionispira* at the D160. In addition, the abundance of *Victivallis*, *Paraprevotella*, *Fibrobacter*, *Vampirovibrio*, and *Paludibacter* also increased gradually with the growth of the yak.

Fungal community diversity and the temporal dynamics of fungi in the rumen of the yak

With the increasing age of the yak, the diversity of fungi gradually increased from D5 to the D90, and then decreased until D180, and slightly increased in adult yaks (Figure 3A). According to the *alpha* diversity analysis of ITS data, the Sobs, Richness, Chao, and Ace indices were the highest on D90 and the lowest at the D5, which gradually increased from D5 to D90, then decreased from D90 to D180, and finally increased in the D720 group. The Shannon index was also the lowest at the D5 but quite high in both D45 and D180, and the Simpson index was low in both D45 and D180 but the highest at the D5. The *alpha* diversity results showed that the rumen fluid of the D90 was the most suitable period for the growth and reproduction of most fungi and had the richest fungi biodiversity. In contrast, the rumen fluid of the D5 was the least suitable period for fungi. *Beta* diversity analysis of PCoA and cPCoA showed that rumen fluid samples of yak at different ages were separated except the D90 group and D180 were extremely similar, indicating that the changing of fungal community experienced variousness periods across the whole development of yak (Figure 3B).

The predominant fungal phyla included Ascomycota across all ages, followed by Basidiomycota, and Chytridiomycota (Figure 3C). At the same time, the dominant fungal phyla were influenced by ages, such as higher abundance of Basidiomycota at D5 and greater abundance of Chytridiomycota at D720. The dominant fungal genera mainly included *Cryptococcus Cladosporium*, *Plenodomus*, *Penicillium*, *Thelebolus*, *Saccharicola*, *Preussia*, and *Neocallimastix*, which were associated with ages. For example, *Cryptococcus* was abundance value at D45(29.3%), *Plenodomus* was over-represented at D180(18.36%), and *Penicillium* was abundance value at D5(26.7%; Figure 3C).

LEfSe analysis was also performed to detect the fungal phyla and genera differentiating ages (Figure 3D). At the phylum level, Ascomycota had the highest abundance from D45(53.29%) to D180(87.86%), and Chytridiomycota was only highly abundant at D5(34.49%). At the genus level, *Cladosporium* had a high abundance at D45(8.89%), D180(8.62%), and D720(8.93%). *Plenodomus* had the highest abundance at D720(18.36%) and followed at D90(5.7%), and *Penicillium* only had a high abundance at D5(26.7%). The abundance of *Thelebolus* was the highest at D180(15.47%) and gradually increased from D5(0.27%) to D90(3.4%), and that of *Preussia* was the lowest at D45(0.16%). *Neocallimastix* only had a high abundance at D5(9.31%). Other non-dominant fungal genera were only highly enriched in one specific age, including *Collophora* and *Rachicladosporium* at D90, and *Ascochyta*, *Fusarium*, and *Hormonema* at D90, and *Pyrenochaetopsis*, *Ascochyta*, *Phaeosphaeria* at D180, and *Orpinomyces*, *Piromyces*, *Cyllamyces*, *Mrakiella*, and *Acrostalagmus* at D720.

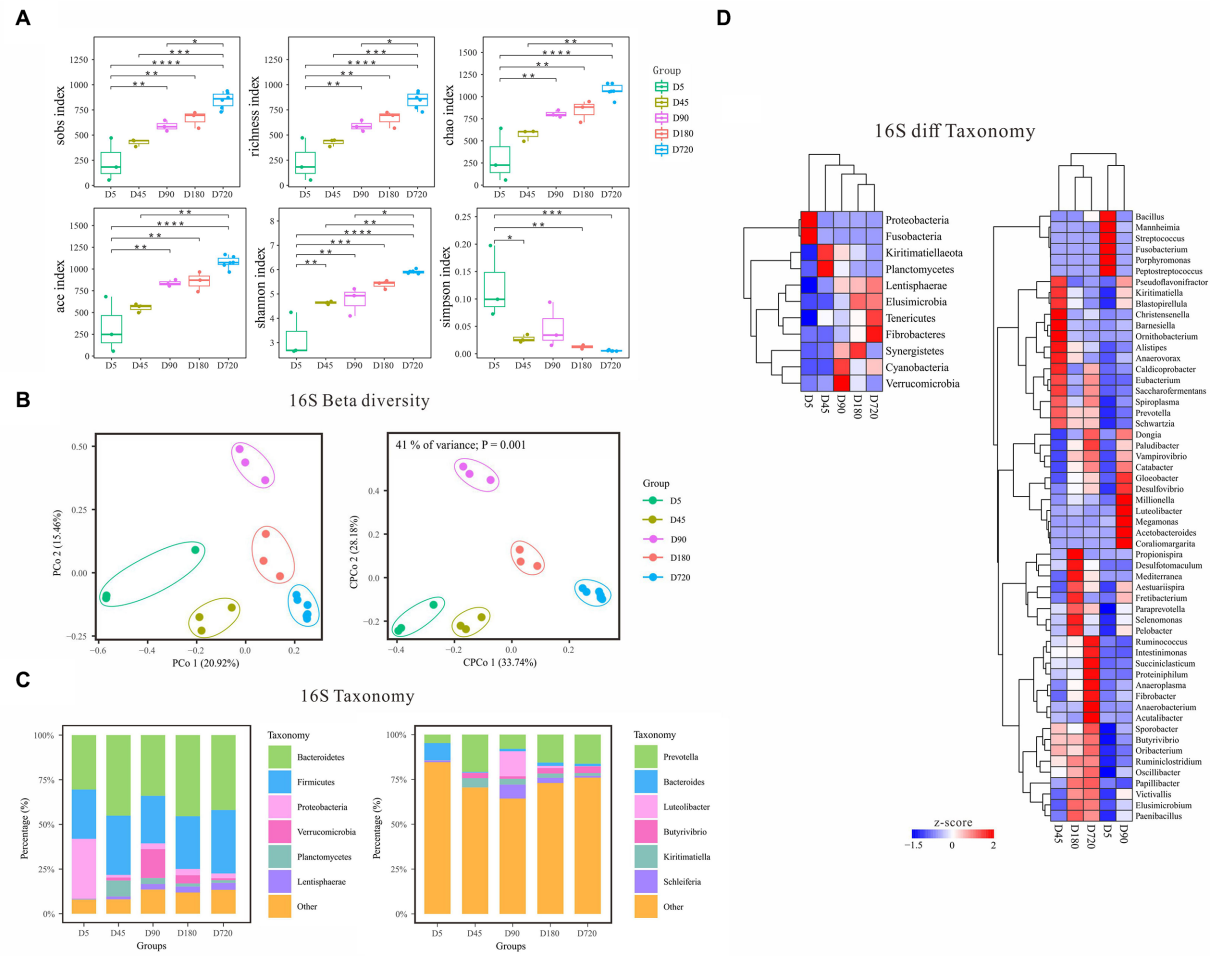

FIGURE 2
Characteristics of rumen bacterial communities of Zhongdian yaks at different ages. **(A)** Alpha diversity of bacterial communities; **(B)** Beta diversity of bacterial communities; **(C)** Composition of bacterial communities; **(D)** Phylum and genus of bacteria with significantly different abundance in different groups (ns p >0.05, *p =0.01–0.05,**p <0.01,***p <0.001,****p <0.0001).

Network analysis for bacterial and fungal communities in the yak rumen

Network analysis was performed at both the phylum and genus level (Figure 4). At the phylum level, the fungal Ascomycota was negatively correlated with fungal Chytridiomycota, and the bacterial Planctomycetes was positively correlated with bacterial Kiritimatiellaeota. At the genus level, there were four genus clusters consisting of bacteria and fungi, the clusters dominated by "*Selenomonas* and *Plenodomus*" and "*Luteolibacter* and *Ascochyta*" were all positively correlated with other genera in the same cluster, and the clusters dominated by "*Kiritimatiella* and *Preussia*" and "*Acutalibacter* and *Cyllamyces*" were negatively correlated with other genera in the same cluster. In the cluster dominated by "*Acutalibacter* and *Cyllamyces*," the negative correlations were caused by both *Cyllamyces* and *Acutalibacter*. There were five clusters consisting of 21 bacteria, with the *Prevotella*-dominated cluster containing the most genera, followed by the *Ruminococcus*- and *Paraprevotella*-dominated clusters. Both *Succiniclasticum*- and *Butyrivibrio*-dominated clusters had negatively correlated with other genera. In addition, 16 clusters were formed by two genera, including five clusters with a positive correlation between bacteria and bacteria, two clusters with a positive correlation between bacteria and fungi, two clusters with a negative correlation between bacteria and fungi, five clusters with a positive correlation between fungi and fungi, and two clusters with a negative correlation between fungi and fungi (Figure 4).

Temporal dynamics of metabolic characteristics in the yak rumen microbiome

The functions of the rumen microbiome in yaks were predicted by using PICRUSt, and functions with significantly different abundance among ages were detected. There was no metabolic that only significant enrichment in one or several groups, and some metabolites with significant differences in the abundance between different groups were also smaller in a multiplicity of differences. The abundance clustering results of differential metabolism and enzyme showed no difference between D180 and D720, and most of the differences existed with the D5, such as pyruvate, ascorbate, inositol phosphate, geraniol degradation nitrogen, lysine degradation, and

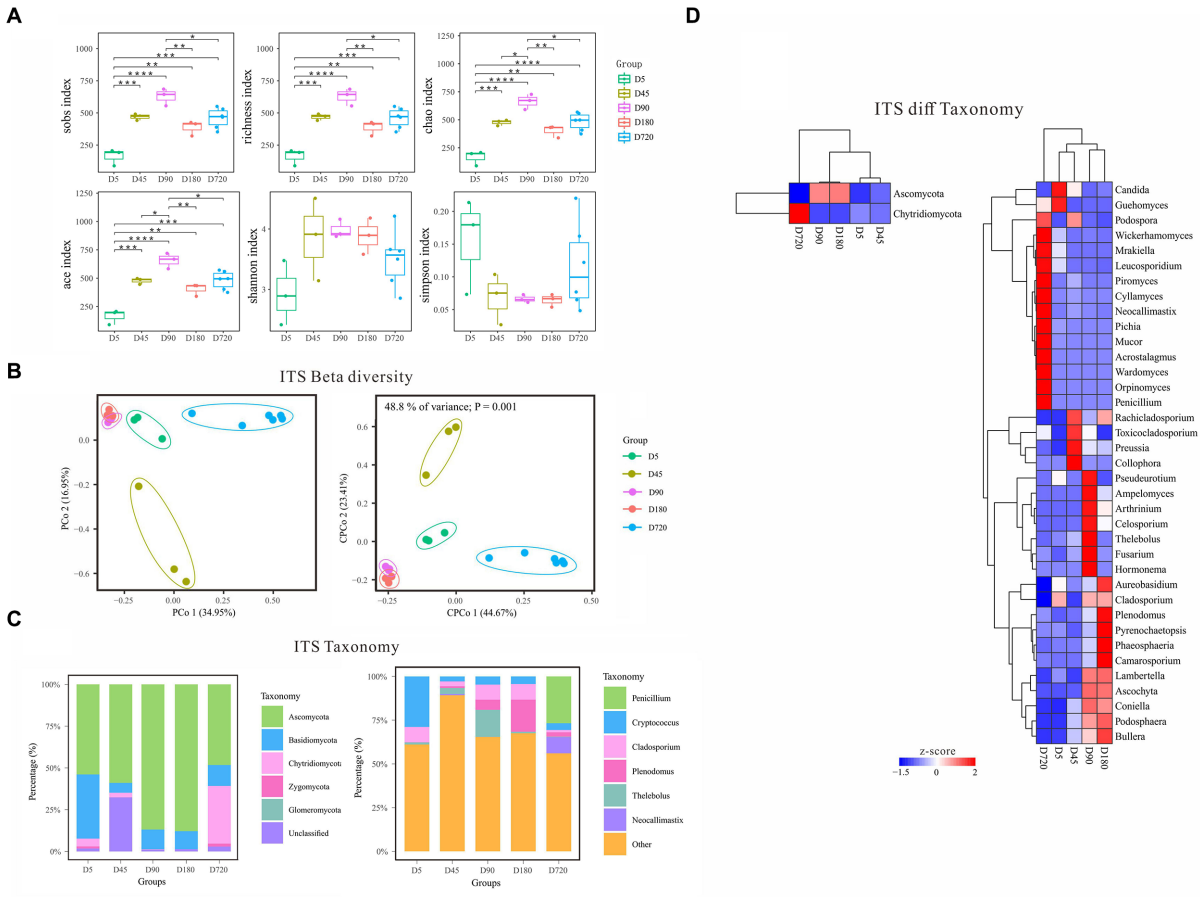

FIGURE 3
Characteristics of rumen fungal communities of Zhongdian yaks at different ages. **(A)** Alpha diversity of fungal communities; **(B)** Beta diversity of fungal communities; **(C)** Composition of fungal communities; **(D)** Phylum and genus of fungi with significantly different abundance in different groups (ns $p > 0.05$, *p =0.01–0.05,**p <0.01,***p <0.001,**** p <0.0001).

toluene degradation metabolisms had a high abundance at the D5, while methane, nicotinate and nicotinamide, and glycerolipid, novobiocin biosynthesis, polyketide sugar unit biosynthesis, histidine and pyrimidine metabolisms were in high abundance in the other groups. Some metabolisms were also enriched at D45 and D90 groups, such as microbiome related to amino acid metabolism was abundant at the D45, and biotin, sulfur, fatty acid biosynthesis, and selenocompound metabolisms were more abundant at the D90. For enzymes, bacteria related to the iron complex outer-membrane receptor protein (K02014) were more abundant at the D5, and bacteria related to ubiquinone/menaquinone biosynthesis methyltransferase (K03183), acetyl-CoA carboxylase (K01961) and uracil reductase (K11752) were more abundant in the D90 group (Figure 5).

Discussion

To evaluate the composition and temporal dynamics of rumen bacteria and fungi in Zhongdian yaks, the full length of microbiome of yaks at different ages were sequenced in this study. Our findings showed that yaks underwent a gradual process of change from birth to adulthood and that the bacterial and fungal community diversity in the rumen stabilized at 2 years of age. A rait-based method to explore the succession mechanism of the rumen microbial communities and their influence on host immunity to help better understand the functional roles of the microbiome.

Rumen microbial and fungal structure varies with the age, which may relate to diet digestion, altitude adaptability, and immunity strength of the yak. There were over 100 species shared between the rumen bacterial and fungal communities of young ruminants and the rumen microbiota of adult yaks (Figures 1A,B), suggesting that the rumen microbiota of calves were active and involved in the digestion of fibrous material. With the growth of yaks, the number of gut microorganisms with oxygen tolerance and flagella decreased gradually, while the number of microorganisms with slow growth and spore formation increased gradually (14). The diversity and richness of bacteria increased with age, and the horizontal component of the rumen fungal phylum in calves was the same as that in adult yaks, but the proportion was significantly different. This indicates that the rumen environment of grazing adult yaks, although more functional, is also a closed and independent ecosystem with more specific and homogeneous bacterial and fungal communities, compared to primary communities with more heterogeneity among the younger ages. The gradual increases in bacterial diversity in Holstein cattle and

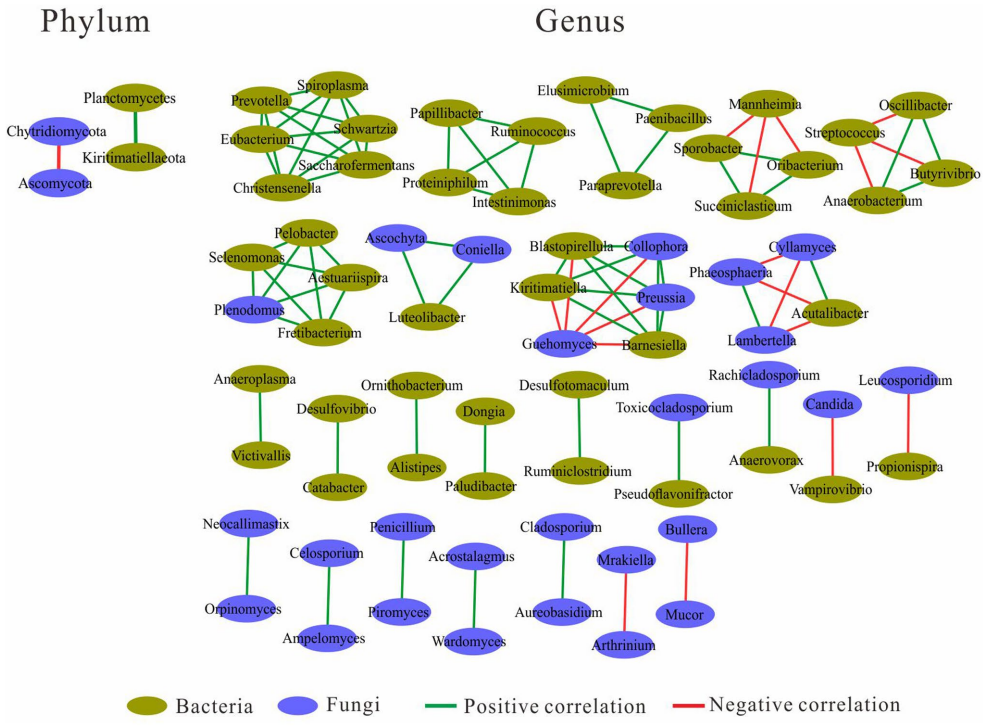

FIGURE 4
Correlation between bacterial and fungal communities.

goats from birth to adulthood are associated with a gradual change in community diversity (8, 15). Interestingly, similar studies have been reported in recent years on the gut microbes of human infants. It has been found that the intestinal flora of infants starts from the early colonizers who are variable and good at rapid proliferation, but the functional traits gradually converge and stabilize in the first year of life; the gut microbiota adapts to the anoxic environment in the intestine and spreads among individuals through spores, while the taxonomic composition of the flora continues to change (16).

Dominant microbiome changed with the growth of yaks. At the D5, phylum bacterial Proteobacteria and fungal Basidiomycota were significantly higher than that in other ages, which was consistent with previous studies that facultative anaerobes *Proteobacteria* is more suitable for a fluid diet of earliest stage life (17). Xufeng et al. found the abundance of Basidiomycota increased with the proportion of dietary concentrate, suggesting that Basidiomycota are also suitable for a fluid diet (18). In addition, *Prevotella* was found in all age but varied in number and composition. With the decrease of Proteobacteria and Basidiomycota, the abundance of Prevotella and Thelebolus was increased at the D45, and *Prevotella* carries plenty of Carbohydrate-active enzyme (19), which may help Zhongdian yaks digest plant fiber and produce certain volatile fatty acids, enabling them to adapt to the high-altitude environment and high-fiber diet (20).

Fungal *Thelebolus* was the first reported in yak rumen, and its abundance reached a peak at the D90. The previous report showed that *Thelebolus* has the ability to produce anti-inflammatory exopolysaccharide (21), suggesting *Thelebolus* assists host immunity promotion. The abundance of *Luteolibacter* was also increased, which was also the first time reported in yak rumen. Marine bacterium *Luteolibacter algae* H18 can degrade fucoidan, which may enrich the feed degradation ability of Zhongdian yaks (22), while its positive partner ascochyta contains some disease-causing species (23). After 90 days, the diet was obtained entirely from free grazing and had more exposure to pathogens in the environment, like black leg causer *Plenodomus* was increased at 180 days age (24). *Plenodomus* is positively related to *Fretibacterium* and is also an opportunistic pathogen. There was no significant change in rumen bacterial structure between 180 and 270 days after birth, suggesting that the bacterial ecosystem tended to be stable after 180 days, while the fungal structure was changed as the proportion of *Penicillium* had increased significantly. *Penicillium* isolated from the gastric juice of cow rumen has a high capacity for cellulose degradation (25), assisting digest high altitude diet.

The strong functional correlation between rumen genes and microorganisms is evident in the synergistic effect observed during the early stages of rumen development (26). This study demonstrates an up-regulation of Transport and Catabolism in the 5-day-old and 180-day-old groups, indicating the importance of enhancing these processes for the maintenance of overall calf health. Newborn calves require strengthened transport and catabolism to ensure their well-being during this critical stage (27). Similarly, calves at 180 days of age face challenges such as cold winter conditions and a lack of forage, necessitating an increase in transport and catabolic activities. Concurrently, the Day5 group, representing early rumen development, exhibits a robust capacity for heterogenesis and metabolism. However, with advancing age, the rumen-based heterogenesis and related metabolism in Zhongdian yaks experience downregulation. Notably, the down-regulation of immune system-related genes observed may be associated with passive immunity acquired through colostrum intake (28).

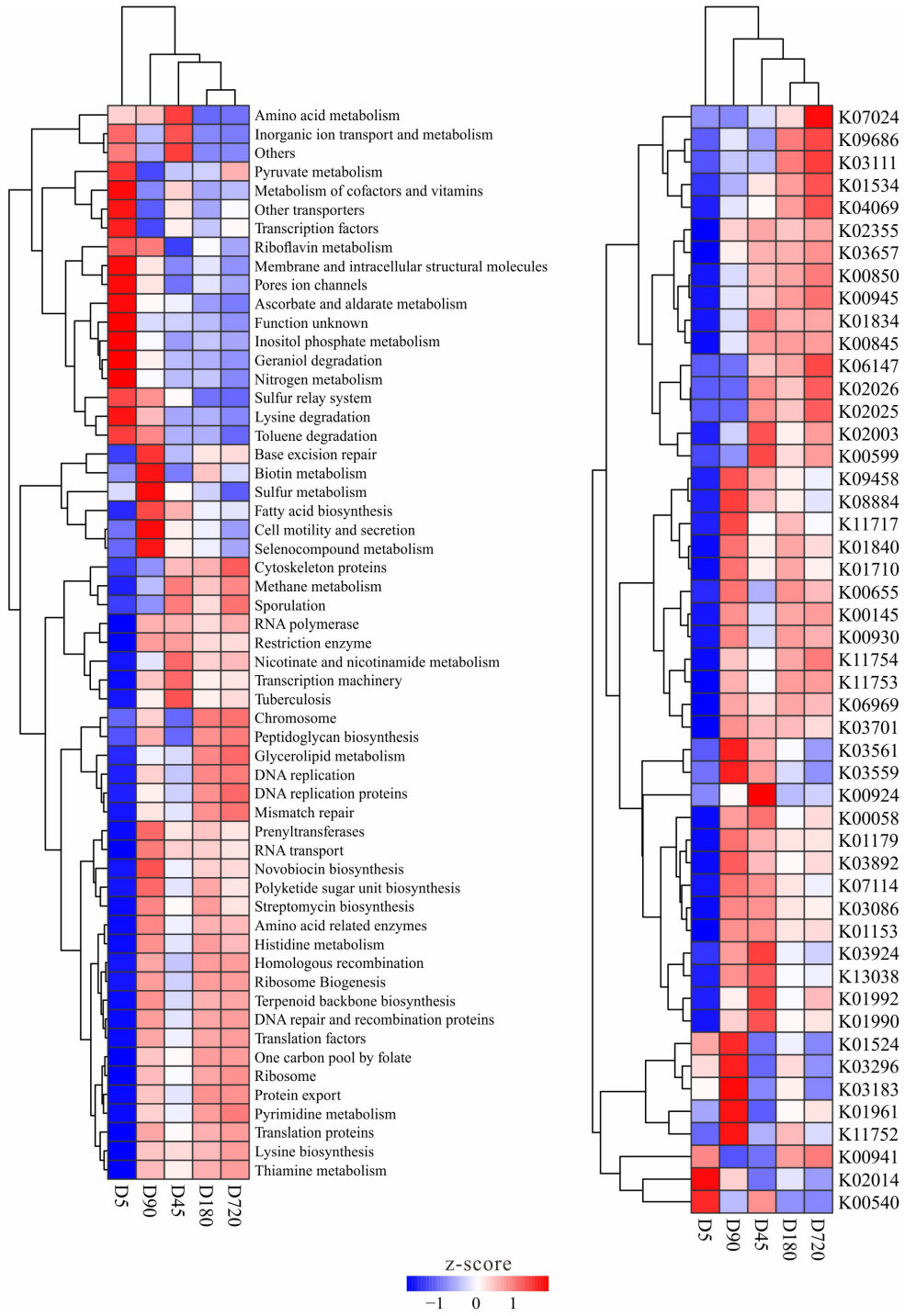

FIGURE 5
Metabolism and enzymes with significantly different abundance in the yak rumen at different ages.

With the change of diet, rumen microbial richness and diversity of calves changed to a mature ruminant state. The animals in this study were grazed in the wild for a long time without artificial feeding. Newborn calves began to eat the plant fiber slowly at an early stage to promote the development of the rumen and affect microbial colonization at the early stage of rumen development. The trend of diversity change was different between bacteria and fungi. The diversity index and number of bacterial OTUs communities increased with age, while the fungus reached the first peak at day 90, which was the time of weaning. The change in bacterial diversity is consistent with that in previous studies of Mongolian cattle data (29) and dairy calf data (13).

A limitation of this study might be related to the sample size. In the beginning, six underage yaks and six adult yaks were included in this study, but three underage yaks died during the experiment since a series of reasons. Although the difficulties of sampling collection of the wild animals were usually met, small sample size is a factor that might cause

individual variation of our result. However, the basic dynamics of rumen microbiome from birth to adulthood are well investigated. More yaks and samples should be included in future studies to verify our results.

Conclusion

Based on the full-length analysis of 16S rDNA and ITS sequences, we studied the dynamic changes of rumen microorganisms in grazing yaks at different growth stages. The rumen microbial community of yaks remained stable during growth and development, and we identified the signature rumen bacteria and fungi at each growth stage. Furthermore, we observed temporary changes in the characteristic bacteria and fungi in the two age compartments, which were linked to diet changes, rumen wall development, and microbial interaction. The colonization of rumen microflora in the early stage may influence the microbial community of yaks upon sexual maturity. Additionally, rumen fungi exhibited associations with bacteria at different growth stages. This study highlights the significance of rumen fungi and bacteria in grazing yaks.

Data availability statement

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

Ethics statement

The animal study was reviewed and approved by All animals used in this experiment were approved by the animal protection and utilization committee of Yunnan Agricultural University, China (protocol # 2017-0081), and there was compliance with the guidelines of the Laboratory Animal Ethics Committee in experimental animal handling. Written informed consent was obtained from the owners for the participation of their animals in this study.

Author contributions

DW, HM, and SY made substantial contributions to the conception or design of the experiments. DW, GZ, JZ, and SY performed the experiments. DW and SH analyzed the data. JC and SY wrote the paper. All authors contributed to the article and approved the submitted version.

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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.

Publisher's note

Supplementary material

The Supplementary material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fvets.2023.1166015/full#supplementary-material

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Swine gut microbiome associated with non-digestible carbohydrate utilization

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Non-digestible carbohydrates are an unavoidable component in a pig's diet, as all plant-based feeds contain different kinds of non-digestible carbohydrates. The major types of non-digestible carbohydrates include non-starch polysaccharides (such as cellulose, pectin, and hemicellulose), resistant starch, and non-digestible oligosaccharides (such as fructo-oligosaccharide and xylo-oligosaccharide). Non-digestible carbohydrates play a significant role in balancing the gut microbial ecology and overall health of the swine by promoting the production of short chain fatty acids. Although non-digestible carbohydrates are rich in energy, swine cannot extract this energy on their own due to the absence of enzymes required for their degradation. Instead, they rely on gut microbes to utilize these carbohydrates for energy production. Despite the importance of non-digestible carbohydrate degradation, limited studies have been conducted on the swine gut microbes involved in this process. While next-generation high-throughput sequencing has aided in understanding the microbial compositions of the swine gut, specific information regarding the bacteria involved in non-digestible carbohydrate degradation remains limited. Therefore, it is crucial to investigate and comprehend the bacteria responsible for the breakdown of non-digestible carbohydrates in the gut. In this mini review, we have discussed the major bacteria involved in the fermentation of different types of non-digestible carbohydrates in the large intestine of swine, shedding light on their potential roles and contributions to swine nutrition and health.

KEYWORDS

pigs, non-digestible carbohydrates, bacteria, fermentation, nutrition

1. Introduction

Dietary carbohydrates (DCs) are principal substrates for maintaining physiological health and serve as an energy source for animals. In the diets of pigs, carbohydrates contribute to the majority of feed energy, accounting for approximately 60–70% of overall energy intake. In addition, the digestion of carbohydrates has significant impacts on various aspects of colonic functions, including the metabolism, balance of commensal flora and the health of large intestine epithelial cells (1).

DCs encompass a group of chemical substances and can be classified based on molecular sizes, ranging from simple mono- and disaccharides to complex compounds with intricate structures (2). Moreover, carbohydrates can be divided into two nutritional categories based on chemical classification. The first category is digestible carbohydrates (DGCs), which are metabolized by the host's endogenous enzymes and absorbed in the small intestine. This category includes monosaccharides, disaccharides, and polysaccharides such as starches. The second category is dietary fiber, which consists of non-digestible soluble and insoluble carbohydrates and lignin. These components have the potential to be degraded through microbial fermentation in the large intestine (3). Non-starch polysaccharides (NSPs) are a component of dietary fiber. American Association of Cereal Chemists (AACC) defined the dietary fiber in 2000 as edible plant parts or comparable carbohydrates that are resistant to digestion and absorption in the small intestine but can be completely or partially fermented in the large intestine (4). Dietary fiber contains a substantial amount of energy, but the majority of enzymes required for its breakdown are not encoded in the mammalian genome.

Starch is the principal source of energy for monogastric animals and cereals are the primary source of starch in animal feeds (5). Starch is a polysaccharide composed of polymers amylose and amylopectin. Resistant starch (RS), on the other hand, refers to starches that resist digestion in the small intestine by amylases and instead reach the large intestine, where they are available for bacterial fermentation (6).

NSPs (cellulose, pectin, and hemicellulose), RS and non-digestible oligosaccharides (NDOs) (Fructo-oligosaccharide and Xylo-oligosaccharide) are the major types of non-digestible carbohydrates (NDCs). The large intestine serves as an anaerobic digestive environment for complex molecules, such as NDCs. It is predominantly colonized by obligate anaerobic microorganisms, although a small number of aerobic and facultative microorganisms are also present (7, 8). These microorganisms within the large intestine utilize NDCs as their primary energy sources. As a result, they produce Short Chain Fatty Acids (SCFAs), vitamins, and participate in various metabolic processes. Moreover, these microorganisms engage in intricate interactions with host cells and the host immune system (9–11).

With the aid of next-generation high-throughput sequencing, researchers have been able to comprehend the gut microbial composition of swine. However, knowledge concerning the gastrointestinal tract microbiome that facilitates the fermentation of NDCs remains limited, despite several research endeavors aimed at understanding such bacterial species. Therefore, this mini review aims to consolidate information regarding the major bacterial species involved in the fermentation of different types of NDCs in the large intestine of swine.

2. Degradation of NDCs by the swine gut microbiota

DGCs are such carbs that can be digested by host's enzymatic system (3), whereas NDCs are those carbs that resist the action of salivary and intestinal digestive enzymes and hence are fermented by microbes in the large intestine (12).

The carbohydrates in the swine feed like cereal grains, legumes, oil seeds, and potato are also composed of DGCs and NDCs (13). The legumes and oil seeds are source of protein however the cell wall of these crops contains NDCs (14). NDCs have a significant role in pig diets, and it is necessary to add a certain amount to ensure proper physiological functioning (15).

As summarized in Figure 1A, NDCs are a distinct group of carbohydrates found almost entirely in plants and is generally classified as NSPs (cellulose, hemicellulose, pectin), RS (potato starch) and NDOs (Fructo-oligosaccharide, Xylan-oligosaccharide, soybean Oligosaccharide) (16).

NDCs are either water soluble or insoluble based on its solubility in water. Insoluble NDCs includes cellulose, hemicellulose, lignin, whereas soluble NDCs includes pectin, β-glucan, fructan, mucilage, gum, and psyllium fiber (17, 18). The most commonly present NDCs are cellulose, hemicellulose and pectic substances.

Digestion of simple carbs and starch occurs predominantly by enzymatic digestion, whereas the complex carbs that are resistant to host's digestive enzymes are degraded by microbial fermentation after reaching large intestine (19).

Even though a host's genome does not encode the enzymes required to break down the linkage between the monomers in NSPs and NDOs, 20 to 25% of NSPs and 40 to 95% of NDOs is found to be degraded while passing through the small intestine (Figure 1B). This breakdown is facilitated by the microbial enzymes of the microflora present in this part of the gut and not by the host's enzymatic system (20). Nevertheless, the major types of carbs reach the large intestine, and those available for fermentation are plant cell wall polysaccharides, also known as NSPs, RS, and NDOs. Those carbohydrates are fermented by the swine gut microbiota.

Gastro-intestinal tract microbiota is defined as the ecological community made up of commensal, symbiotic and potentially pathogenic microorganisms that harbors the gut (17). The gut microbial composition of the swine is of great significance, as it affects the overall physiology and health, along with the feed conversion ratio. The swine gut microbiota are mainly made up of anaerobic & facultative anaerobic bacteria, and more than 90% of these bacteria belong to the phyla Firmicutes, Proteobacteria and Bacteroidetes (Figure 1C) (21–24). Several studies have shown 'core' genera consisting of *Prevotella, Clostridium, Ruminococcus, Lactobacillus, Faecalibacterium, Bacteroides, Fusobacterium,* and *Alloprevotella* in a larger portion of studied healthy pigs (25, 26). In this review, we will discuss major bacterial species involved in fermentation of different types of NDCs in the large intestine of swine.

2.1. Fermentation of NSPs by swine gut microbiota

NSPs comprise plant cell wall polysaccharides (Cellulose, hemicellulose, Pectin), structural non-polysaccharide (lignin) and non-structural polysaccharides. (7, 27). Numerous parameters, including the animal species, solubility, chemical composition, and consumption amount, influence the ease of digestion of NSPs. The order of microbial degradation in the large intestine is sugar residues = NDOs > Starch residues > Soluble NSP > RS=Insoluble NSP

Abbreviations: DCs, Dietary carbohydrates; DGCs, Digestible carbohydrates; NSPs, Non-starch polysaccharides; RS, Resistant starch; NDOs, Non-digestible oligosaccharides; NDCs, Non-digestible carbohydrates; SCFAs, Short Chain Fatty Acids..

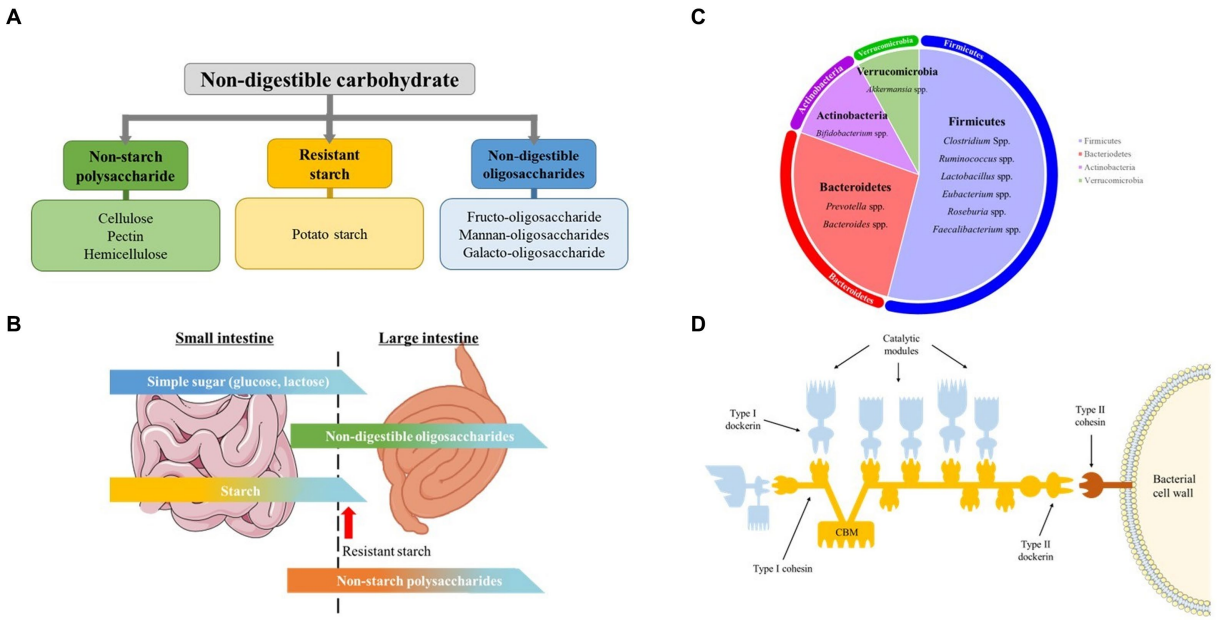

FIGURE 1
Non-digestible carbohydrates, pig gut microbiome, carbohydrate digestion in the intestine, and celluosome structure. **(A)** Classification and examples of non-digestible carbohydrate addressed in this mini review. **(B)** Schematic illustration of digestion of different carbohydrates in small and large Intestine. The figure gives a rough idea on the amount of carbohydrates digested in the small and large intestine. Modified from Bach Knudsen et al. **(C)** Predominant genus of swine gut microbiota. Each area of the circle signifies the domination of the respective phyla in the swine gut. **(D)** Cellulosome structure. The Type I dockerins, attached to the catalytic subunit (blue) interacts with the cohesin (yellow) of the primary CipA scaffoldin protein forming cellulosome complex. The cellulosome is attached to the bacterial surface through interaction of Type II dockerin in CipA with Type II cohesin module of a membrane-bound protein (red). The Cellulosome complex binds to cellulose through cellulose-binding module (CBM) of the CipA primary scaffoldin protein. Modified from Gilbert H.J.

(3). Lignin, a component of plant cell wall, is however not digested by the enzymes of the small intestine and neither fermented by the gut bacteria. It is however supposed to impact the fermentability of other components in the diet (12, 28).

The degradation of complex fiber by the fibrolytic bacterial community is mainly carried out by several anaerobic gut microbes that possess the ability to produce enzymes. They belong to the dominant bacteria groups including *Bacteroides*, *Roseburia*, *Ruminococcus* or *Bifidobacterium* species (Table 1). Several specific as well as multi-carbohydrate degrading bacteria is found in swine gut. The anerobic bacteria mainly degrade cellulose through cell-bound organelle-like structure, cellulosome (46). Cellulosome is a large multi-enzyme complex bound to the bacterial cell wall, that helps degrade plant cell wall polysaccharides into usable sugars (47, 48). It basically consists of 2 major subunits: the enzymes and the noncatalytic subunit scaffoldin (Figure 1D). Scaffoldin possess 6 cohesin domains which bind with the dockerin module present in the enzymes and hence forms the functional cellulosomal-complex. Scaffoldin also has one another module, the cellulose-binding domain (CBM) that helps bind to the cellulosic substrates.

Ruminococcus flavefaciens (*R. flavefaciens*), one of the predominant and important cellulolytic bacteria, degrades cellulose and a variety of plant cell walls (30) using a high-molecular-weight enzyme complex, cellulosome (49). It is essential for the *R. flavefaciens* to bind to the cellulose in order to break it down. Another bacteria, *Ruminococcus albus* (*R. albus*), is also a well-known specialist cellulose-degrading bacterium found in rumen and gastro-intestinal tract of herbivores, but it has also been isolated from swine gut (8). It is also known to produce a cellulosome-like complex. However, it is supposed to employ another mechanism for adhesion to cellulose. It has been found that a cellulose-binding protein belonging to the Pil-protein family is involved in attachment (50). *Ruminococcus champanellensis* (*R. champanellensis*), a recently identified cellulolytic strain from human feces, ferments cellulose and xylase, while metabolizing cellobiose to acetate, succinate, ethanol, dihydrogen and small quantities of formate and lactate (31, 32).

Clostridium (*C. leptum* and *C. herbivorans*), which are normal inhabitants of the pig's intestine, also possess multi-enzyme system including cellulosome and xylanosome, which aid in the breakdown of complex cellulosic polymers and several cellulosic by-products (29). However, it should be noted that not all polysaccharidase activities in *R. flaveciens* or the cellulolytic *Clostrdia* are linked to a cellulosome (51).

Bacteroides, another highly prevalent genus in swine gut, possess a high concentration of the carbohydrate-active enzyme (CAZyme) genes. These enzymes enable *Bacteroides* to degrade various components of plant cell wall, like glucronylxylans, xyloglucans and pectin (52). Moreover, a unique feature of *Bacteroidetes* is the presence of polysaccharide utilization locus (PUL), which consists of linked genes involved in the saccharification of complex carbohydrates, such as glycans (53, 54). *Bacteroides ruminicola* (*B. ruminicola*) and *Bacteroides xylanisolvens* (*B. xylanisolvens*) are significantly involved in the degradation of xylan (36, 37). Another species, *Bacteroides succinogens* (*B. succinogens*), isolated from the swine's large intestine, also possesses the ability to degrade cellulose (34). Metagenome-assembled genomes (MAGs) have identified several members of the

TABLE 1 Gut bacteria involved in fermentation of non-digestible carbohydrates.

Non-Digestible Carbohydrates (NDCs)		Genus	Species	References
NSP[1]	Cellulose	*Clostridium*	*leptum, herbivorans*	(29)
		Ruminococcus	*flaveciens, albus,*	(8, 30)
			champanellensis	(31, 32)
		Fibrobacter	*intestinalis, succinogenes*	(33)
		Bacteroides	*succinogenes*	(34)
	Hemicellulose	*Butyrivibrio*	*fibrisolvens*	(8, 35)
		Bacteroides	*ruminicola, xylanisolvens*	(36, 37)
		Ruminococcus	*champanellensis*	(31, 32)
	Pectin	*Bacteroides, Prevotella*	–	(38, 39)
Resistant Starch	–	*Ruminococcus*	*bromii*	(40, 41)
		Bifidobacterium	*adolescentis*	
		Bacteroides	*thetaiotaomicron*	
		Eubacterium	*rectale*	
NDO	FOS	*Bifidobacterium*	–	(42, 43)
	GOS			
	MOS			
	SBOS	*Lactobacillus*	*plantarum, acidophilus*	(44, 45)
	IMO			

NSP, Non-Starch Polysaccharide; NDO, Non-Digestible Oligosaccharide; FOS, Fructo-oligosaccharide; GOS, Galacto-oligosaccharide; MOS, Mannan-oligosaccharide; SBOS, Soybean-oligosaccharide; IMO, Isomalto-oligosaccharide.

Bacteroidaceae family, including *Bacteroides fragilis (B. fragilis)*, *Bacteroides heparinolyticus (B. heparinolyticus)*, *Bacteroides stercoris (B. stercoris)*, *Bacteroides thetaiotaomicron (B. thetaiotaomicron)*, *Bacteroides uniformis (B. uniformis)*, and *Bacteroides xylanisolvens (B. xylanisolvens)*. These bacteria have been found to possess the carbohydrate-active enzyme (CAZyme) genes involved in the degradation of starch, pectin, fucose oligosaccharides, rhamnose oligosaccharides, and other complex carbohydrates. Additionally, using metagenome-assembled genomes (MAGs), it has been predicted that *B. thetaiotaomicron* and *Bacteroides ovatus* (*B. ovatus*) possess PULs.

Several species of *Fibrobacter*, including *Fibrobacter intestinalis* (*F. intestinalis*) and *Fibrobacter succinogenes* (*F. succinogenes*), possess the ability to ferment NSPs. These species, found in the caeca of pigs, have gained significant attention due to their relatively higher fibrolytic activity (33). *F. succinogenes,* in particular, is known for its efficient degradation of cellulose. The specific mechanism by which it utilizes cellulose is still not fully understood, but it has been proposed that *F. succinogenes* binds to cellulose through a protein present in its outer membrane, facilitating the degradation of cellulose (55, 56). *Bacteroides*, along with *Prevotella*, is known to contain carbohydrate-active enzymes (CAZymes) and play a major role in the degradation of pectin, a component of plant cell walls. However, it is important to note that while these bacterial genera contribute to the breakdown of various dietary carbohydrates, cellulose degradation is mainly carried out by other cellulolytic bacteria such as *Ruminococcus, Fibrobacter*, and certain species of *Clostridium* (38, 39). It is also widely known that *Butyrivibrio* spp., found in swine colon, can hydrolyze hemicellulose (8). *Butyrivibrio fibrisolvens* is a ruminal hemicellulose- degrading bacteria but also show modest cellulolytic activity (35).

2.2. Fermentation of RS by swine gut microbiota

Starch is the principal source of energy for monogastric animals, and cereals are the primary source of starch in animal feeds (5). Starch is a polysaccharide composed of polymers: amylose and amylopectin. RS, on the other hand, refers to starches that escape digestion in small intestine by the amylases and reach the large intestine, where they become available for bacterial fermentation (6).

Based on their physiochemical characteristics, resistant starch (RS) is classified into five different types. RS1 refers to starches that are physically inaccessible as they are located inside a fiber-protein matrix and are resistant to breakdown even with normal cooking. RS2 is a type of starch found in green bananas and raw potatoes, which can be reduced by thermal treatment. RS3 refers to retrograded starches that occur when starchy foods like bread or potatoes are gelatinized through heating and then undergo retrogradation upon cooling. RS4 is a group of resistant starches that are generated through chemical modifications such as esterification, etherification, and cross-linking. RS5 is predominantly associated with amylose-lipid V-type complexes, such as starch-monoglycerides and starch-fatty acids (57, 58). Given that starchy ingredients consumed by pigs typically contain significant quantities of RS1, RS2, and RS3, these three types of resistant starch are commonly regarded as the primary ones in the swine industry.

Several types of gut bacteria are involved in fermenting resistant starch in the hindgut, which leads to the production of SCFAs (such as acetate, butyrate, propionate, and valerate), gasses (including CO_2, H_2, and CH_4), as well as lesser amounts of organic acids (like lactate, succinate, and formate), branched SCFAs, and alcohols (such as methanol and ethanol) (57).

The main three phyla involved in starch fermentation are Firmicutes, Bacteroidetes, and Actinobacteria, which collectively account for 95% of the total gut bacteria in mammals. Numerous studies have indicated the interactions between resistant starch and microorganisms in the gut. It has been observed that as the levels of RS increase, there is an increase in the populations of the *Bifidobacteria* and *Lactobacillus* genera. Specifically, *Lactobacillus sobrius* and *Lactobacillus amylovorus* have been identified as the major amylolytic genera in the digestive system of swine. It is important to note that not all *Bifidobacteria* species are involved in the degradation of RS. However, *Bifidobacterium breve, Bifidobacterium dentium*, and *Bifidobacterium pseudolongum* have shown extracellular starch-degrading activities (59). A human study also evaluated the roles of four dominant amylolytic bacteria in the human colon, namely *Bacteroides thetaiotaomicron (B. thetaiotaomicron), Ruminococcus bromii (R. bromii), Eubacterium rectale (E. rectale)*, and *Bifidobacterium adolescentis (B. adolescentis)*, in the breakdown and utilization of RS. The findings of the study indicated that *R. bromii* exhibited a much greater capacity for RS degradation compared to the other three bacteria. However, all four bacteria demonstrated the ability to utilize RS (40, 60).

The starch-utilization system of *B. thetaiotaomicron* has been thoroughly investigated and found to possess a starch-utilization-structure (sus) gene clusters, which play a role in binding and utilization of starch (41, 61). On the other hand, the remarkable starch-degrading capacity of *R. bromii* is believed to be attributed to cohesion (Coh)-dockerin interactions, which are particularly significant in cellulosomal enzyme systems (62).

E. rectale depends on a large extracellular amylase attached to its cell wall, along with some membrane-associated binding proteins and hydrolases to use resistant starch, however it is not a primary degrader (63, 64) (Table 1).

2.3. Fermentation of NDOs by swine gut microbiota

NDOs are a type of oligosaccharides that cannot be broken down by mammalian endogenous enzymes (65). The terms "resistant oligosaccharides," "NDOs" and "resistant short chain carbohydrates (RSCC)" are interchangeable and refer to the same compound. NDOs, such as fructo-oligosaccharides (FOS), transgalacto-oligosaccharides (TOS), xylo-oligosaccharides (XOS), and soybean oligosaccharides, occur naturally in legume seeds and cereals. They can also be artificially synthesized. These examples represent some common types of NDOs. Numerous studies have demonstrated that the presence of NDOs leads to an increase in *Bifidobacterium* species compared to other bacteria (42, 43). While *Bifidobacterium* shows a strong preference for fermenting NDOs, other bacteria such as *Lactobacillus, Bacteroides,* and *Clostridium* also have the ability to ferment NDOs, albeit at lower levels.

Lactic acid bacteria, such as *Lactobacillus* species can utilize simple carbohydrates broken down by other bacteria (66). However, their ability to utilize complex carbohydrates is generally limited, with only certain species such as *Lactobacillus acidophilus* and *Lactobacillus plantarum* having the capability to utilize NDOs (44, 45).

Bifidobacterium species are known for their production of glycolytic enzymes, which enable them to efficiently utilize NDOs. These enzymes allow *Bifidobacterium* strains to hydrolyze various monosaccharides and glycosidic linkages, providing them with a broader range of carbohydrate substrates. In contrast, other enteric bacteria such as *Escherichia coli, Streptococcus*, and *Lactobacillus* generally exhibit less diversified enzyme activities and lower levels of activity compared to *Bifidobacterium* (67) (Table 1).

3. SCFAs from complex carbohydrate fermentation

NDCs play a crucial role in the overall health of pigs at all stages of life. When these carbohydrates undergo fermentation in the gut, they contribute to the production of SCFAs, gasses, and organic acids.

The anaerobic fermentation of complex carbohydrates in the large intestine primarily produces SCFAs, which are small organic monocarboxylic acids (68, 69). SCFAs have several beneficial effects on the host's gut health. They contribute to the maintenance of intestinal barrier integrity, promoting a healthy gut lining and preventing the entry of harmful substances into the bloodstream. SCFAs also stimulate the production of mucus, which forms a protective layer in the gut and aids in the proper functioning of the digestive system. Furthermore, SCFAs have anti-inflammatory properties and help regulate the immune response in the gut, reducing the risk of inflammation-related conditions. Overall, the production of SCFAs through the fermentation of complex carbohydrates plays a crucial role in supporting gut health and protecting against intestinal inflammation (70). The major SCFAs produced in the gut are acetate, propionate, and butyrate (71). Among these, butyrate plays a crucial role as it serves as the primary energy source for colonocytes (72). Propionate, on the other hand, is transported to the liver where it contributes to gluconeogenesis. Acetate, being the most abundant SCFA, has various important functions including improving cholesterol metabolism and lipogenesis, regulating the immune system, and exhibiting anti-inflammatory responses (73–75). SCFAs cause a decrease in the pH of the large intestine, which helps inhibit the growth of pathogenic microorganisms and facilitates the absorption of calcium and magnesium (71). Furthermore, SCFAs can function as signaling molecules by binding to G protein-coupled receptors (GPCRs) present in gut epithelial cells and immune cells (76). In both anaerobic ecosystems and the alimentary canal, an inevitable byproduct of microbial fermentation is gas, with representative gasses being H_2 and CO_2.

4. Conclusion

In addition to the energy production through fermentation of NDCs, they also play a crucial role in maintaining gut health by producing a diverse range of metabolites, such as SCFAs. Gut microbes contribute to host health through SCFA production, lowering pH, and synthesizing vitamins.

The specific gut anaerobes involved in the breakdown of complex carbohydrates in the large intestine are still not fully understood. Advancing our knowledge in this area is crucial to better understand the microbial ecosystem and its impact on swine health. Prioritizing research efforts to identify the bacterial species responsible for fermenting various dietary fibers will not only contribute to

maintaining overall health but also promote optimal growth and well-being of swine. This knowledge can aid in developing targeted interventions and strategies to optimize gut health and maximize the benefits of complex carbohydrate fermentation in swine. Identifying and understanding the functional roles of different bacterial species involved in the fermentation of dietary fibers can greatly enhance swine production. By optimizing diets and developing targeted interventions based on this knowledge, we can promote efficient fermentation and maximize the utilization of dietary fibers by beneficial gut bacteria. This can lead to improved nutrient absorption, enhanced gut health, and ultimately, better swine production outcomes. Additionally, such advancements can contribute to more sustainable and efficient farming practices by reducing feed waste and improving the overall utilization of available resources.

Author contributions

JC, MS, and HK: conceptualization. SP, EK, HD, SK, GK, JiK, SR, YC, JuK, and JL: resources. HK: supervision. SP, EK, and HK: writing – original draft. JC, MS, and HK: writing – review & editing. All authors contributed to the article and approved the submitted version.

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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.

Publisher's note

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Effects of stimbiotic supplementation on gut health, immune response, and intestinal microbiota in weaned piglets challenged with *E. coli*

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In order to make piglet diets more effective, it is necessary to investigate effective methods for breaking down xylan in cereal. The objective of this study was to determine the effects of dietary stimbiotic (STB) supplementation on growth performance, intestinal morphology, immune response and intestinal microbiota in weaned piglets. A total of 24 (Duroc × Yorkshire × Landrace) weaned pigs (initial body weight of 8.01 ± 0.38 kg and 28 ± 3 d old), were assigned to 4 treatments with 6 replicates per treatment. Pigs were housed in individual pens for 17 days, including 5 days adaption period and 12 days after the first *Escherichia coli (E. coli)* challenge. The experiment was conducted in a 2 × 2 factorial arrangement of treatments consisting of two levels of challenge (challenge and non-challenge) and two levels of STB (0 and 0.5 g/kg diet). Supplementations of STB 0.5 g/kg improved the gain to feed ratio (G:F) ($P < 0.05$) in piglets challenged with shiga toxigenic *E. coli* (STEC). STB supplementation decreased ($P < 0.05$) white blood cells, neutrophils, lymphocytes, and expression levels of tumor necrosis factor-alpha and interleukin-6. Supplementation of STB improved ($P < 0.05$) the lymphocytes and neutrophils in piglets challenged with STEC on 12 dpi. Supplementation of STB also improved ($P < 0.05$) the villus height to-crypt depth ratio of ileum in piglets challenged with STEC. Supplementation of STB increased ($P < 0.05$) the expression levels of claudin-1 of ileum. In genus level, supplementation of STB increased ($P < 0.001$) the abundance of *Prevotella* compared to non-supplementation of STB groups in pre-inoculation period. Also, supplementation of STB decreased ($P < 0.05$) the abundance of *Faecalibacterium* and *Eubacterium_coprostanoligenes_*group compared to non-supplementation of STB groups in post-inoculation period. In phylum level, supplementation of STB increased ($P < 0.05$) the abundance of *Desulfobacterota* and *Fibrobacterota* in pre-inoculation period. *E. coli* challenge increased the abundance of *Fibrobacterota* compared to non-challenged group in post-inoculation period. In conclusion, these findings indicated that STB supplementation could alleviate a decrease of the performance, immune response, and inflammatory response in piglets induced by the STEC challenge.

KEYWORDS

stimbiotic, immune response, gut health, *E. coli*, intestinal microbiota

Introduction

Commonly, grains in feed contain variable amounts of non-starch polysaccharides (NSP), which can reduce absorption, decrease digestibility of nutrients, and increase digesta viscosity in the small intestines (1). Xylan, the most abundant fiber source in pig diets, is resistant to digestion by endogenous digestive enzymes (2). Additionally, xylan can result in gut leakage and inflammation by viscous digesta (3). To make piglet diets more effective, it is necessary to investigate effective methods for breaking down xylan into cereal.

Xylanase (XYL), a carbohydrase that can degrade NSP, has been used in the diets of monogastric animals to mitigate the growth performance (4). Zheng et al. (5) reported that xylanase supplementation reduced digesta viscosity and improved nutrient digestibility. According to Petry and Patience (6), supplementation of XYL improved growth performance and villus height in weaned piglets challenged with *Escherichia coli (E. coli)*. Similarly, a mixture of XYL and protease supplementation mitigated the proliferation of coliforms in the ceca and improved the growth performance of the broilers (7).

Xylo-oligosaccharides (XOS) are derived from the hydrolysis of xylan and are made up of xylose monomers bonded together with β-(1, 4) linkages (8). In addition, XOS are considered prebiotics which are non-digestible dietary components that selectively fermented in the intestine (9). Such selective fermentation can change the activity of gut microbiome and promote short-chain fatty acid (SCFA) production (10).

Stimbiotic (STB), a complex of XYL and XOS, means "non-digestible but fermentable additive that obviously activates fiber-degrading microbiota to improve fiber fermentability at an insufficient amount" (11). Hence, STB has multi-function that reduces the antinutritive effects of NSP in feed and stimulates the microbiota to produce more SCFA (12). It may be possible to increase the fermentability of NSP by the supplementation of STB.

In the weaning period, piglets face new environments and experience intestinal morphological changes because of solid diet feeding (13). Post-weaning diarrhea (PWD) caused by these stress factors can lead to changes in gastrointestinal microbiology and immunology (14). Weaning stress has a negative impact on reduced feed intake, poor growth performance, and disease susceptibility (15). Especially, *E. coli* is the main cause of diarrhea, and gut microbiome is associated with diarrhea (16). For an experiment to be successful, it is crucial to develop a model that mimics an outbreak of PWD in a commercial setting. According to our previous studies (11, 14), we determined the optimal dosage of *E. coli*. Our previous study showed that supplementation of STB 0.5 g/kg and 1 g/kg improved gut health compared with non-supplementation of STB (11). However, supplementation of STB 0.5 g/kg showed a higher improvement in immune response such as pro-inflammatory cytokines compared with supplementation of STB 1 g/kg. However, we conducted the experiment and the effects of dietary STB supplementation of 0.5 g/kg on growth performance, intestinal health, and immune response in weaned piglets challenged with the PWD infection model. Therefore, we hypothesized that (1) experimental induction of PWD could increase the damage of intestinal mucosa and inflammatory response and (2) supplementation of STB 0.5 g/kg could reduce the antinutritive effects of NSP and provide beneficial bacteria which improve gut health and immunity. To test these hypotheses, we induced inflammation and gut damage through oral inoculation of *E. coli* and then investigated the effects of STB supplementation on gut health.

TABLE 1 Compositions of basal diets (as-fed-basis).

Items	Content
Ingredients, %	
Corn	34.43
Extruded corn	15.00
Lactose	10.00
Dehulled soybean meal, 51% CP[a]	13.50
Soy protein concentrate, 65% CP[a]	10.00
Plasma powder	6.00
Whey	5.00
Soy oil	2.20
Monocalcium phosphate	1.26
Limestone	1.40
L-Lysine-HCl, 78%	0.06
DL-Methionine, 50%	0.15
Choline chloride, 25%	0.10
Vitamin premix[b]	0.25
Trace mineral premix[c]	0.25
Salt	0.40
Total	100.00
Calculated value	
ME, Kcal/kg	3433
CP, %	20.76
Lysine, %	1.35
Methionine, %	0.39
Ca	0.82
P	0.65
Analyzed value	
ME, kcal/kg	3512
CP, %	20.92

[a]CP, crude protein.
[b]Provided per kg of complete diet: vitamin A, 11,025 IU; vitamin D_3, 1103 IU; vitamin E, 44 IU; vitamin K, 4.4 mg; riboflavin, 8.3 mg; niacin, 50 mg; thiamine, 4 mg; d-pantothenic, 29 mg; choline, 166 mg; and vitamin B_{12}, 33 mg.
[c]Provided per kg of complete diet without Zinc: Cu (as $CuSO_4 \bullet 5H_2O$), 12 mg; Mn (as MnO_2), 8 mg; I (as KI), 0.28 mg; and Se (as $Na_2SeO_3 \bullet 5H_2O$), 0.15 mg.

Materials and methods

Ethics approval and consent to participate

The protocol for this study was reviewed and approved by the Institutional Animal Care and Use Committee of Chungbuk

National University, Cheongju, Korea (approval no. CBNUA-1697-22-01).

Bacterial strains, culture, and challenges

Shiga toxin-producing *E. coli* F18 was provided in stock form. The F18 *E. coli* expressed heat-labile toxin (LT) and Shiga toxin type 2e (stx2e). In total, 10 μl of thawed *E. coli* stock was inoculated into 10 ml of nutrient broth and cultured at 37°C for 24 h and then subcultured. Thereafter, the subcultured *E. coli* was smeared on MacConkey agar to confirm the bacterial enumeration. A final concentration of 1.2×10^{10} CFU/ml was used in this study.

Animals, experimental design, and diets

A total of 24 (Duroc × Yorkshire × Landrace) weaned pigs (initial body weight of 8.01 ± 0.38 kg and 28 ± 3 days old) were assigned to 4 treatments with 6 replicates per treatment. Pigs were housed in individual pens for 17 days, including 5 days of adaptation period and 12 days after the first *E. coli* challenge (d 0). The experiment was conducted in a 2 × 2 factorial arrangement of treatments consisting of two levels of challenge (challenge and non-challenge) and two levels of STB (0 and 0.5 g/kg diet). Corn and soybean meal basal diets were formulated to meet or exceed the nutrient requirements for the weaned piglets as recommended by NRC (Table 1) (17). STB used in this study was obtained by a commercial company (Eugene-Bio, Suwon, South Korea). The pigs were fed daily at 8:30 and 17:00 h and had *ad libitum* access to water. Feed residues were removed before the next meal and considered in the calculations. In the *E. coli* challenge treatments, all pigs were orally inoculated by dividing 10 ml of *E. coli* F18 for 3 consecutive days. Challenged piglets and non-challenged piglets were housed in a separate room. Strict biosecurity procedures were followed to avoid *E. coli* contamination of the non-challenged piglets.

Growth performance

All piglets were weighed every week during the experimental period, and feed consumption was recorded to calculate average daily gain (ADG), average daily feed intake (ADFI), and gain-to-feed ratio (G:F).

Diarrhea scores

The diarrhea scores were individually recorded at 08:00 and 17:00 h by the same person during the entire experimental period. The diarrhea score was scored using a method used by Zhao et al. (18). The diarrhea scores were assigned as follows: 0, normal feces; 1, soft feces; 2, mild diarrhea; and 3, severe diarrhea.

Nutrient digestibility

To estimate the digestibility, 0.2% chromium oxide (Cr_2O_3) was supplemented with diets as an indigestible marker. Pigs were fed diets mixed with chromium oxide for 4 consecutive days from days post-inoculation (DPI) 4 and 12, and fresh excreta samples were collected in that period. At the end of the experiment, fecal samples were stored at −20°C and dried at 70°C for 72 h and then ground to pass through a 1 mm screen. All analysis items (feed and fecal) were analyzed for DM and CP. The procedures utilized for the determination of dry matter (DM) and crude protein (CP) digestibility were conducted with the AOAC methods (19). Chromium was analyzed with an ultraviolet absorption spectrophotometer (UV-1201, Shimadzu, Kyoto, Japan). The digestibility was calculated using the following formula: digestibility (%) = [1–(Nf × Cd)/(Nd × Cf)] × 100, where Nf is the nutrient concentration in feces (% DM), Nd is the nutrient concentration in diet (% DM), Cd is the chromium concentration in diet (% DM), and Cf is the chromium concentration in feces (% DM).

Blood profile

Blood samples were obtained from the jugular vein of 6 pigs, each treatment at dpi 0, dpi 2, dpi 4, dpi 7, and dpi 12. At the time of collection, blood samples were collected into vacuum tubes containing K_3EDTA for CBC analysis and non-heparinized tubes for serum analysis, respectively. After collection, blood samples were centrifuged (3,000 × g for 15 min at 4°C). The white blood cells (WBC), basophils, neutrophils, and lymphocyte levels in the whole blood were measured using an automatic blood analyzer (ADVIA 120, Bayer, NY, USA).

Morphological analysis of small intestine

At the end of the experiment (dpi 12), pigs were anesthetized with carbon dioxide gas after blood sampling and euthanized by exsanguination. Intestinal tissues of approximately 10 cm from the ileum (close to the ileocecal junction) were collected and fixed in 10% neutral buffered formalin (NBF; Sigma–Aldrich, St. Louis, MO, United States). After cutting the intestine sample, it was dehydrated and dealcoholized. The samples were, then, installed on slides, treated with paraffin, and stained with hematoxylin and eosin. Villus height (VH) and crypt depth (CD) were measured under the light microscope (OLYMPUS DP71, BX50F-3, Olympus Optical Co. Ltd., Tokyo, Japan). VH was determined by measuring the distance between the tip of the villi to the villus crypt junction, and CD was determined by measuring the distance between adjacent villi.

The hematoxylin-eosin-stained slides were also used for goblet cell counting. In crypts, goblet cells were counted in the five best-oriented crypts/intestinal tract, from crypt mouth to base (adjacent to submucosa). The number of goblet cells is expressed as the mean number per crypt per tract and mean number of goblet cells/100 μm of crypts (mean data of crypt length). This

TABLE 2 Effects of stimbiotic supplementation on growth performance in pigs challenged with STEC.

Items	-C		+C		SE[1]	C		STB		*P*-value		
	0	0.05	0	0.05		-	+	0	0.05	C	STB	C×STB
BW, kg												
Initial (D-5)	8.01	8.03	8.00	8.01	0.170	8.02	8.00	8.01	8.02		0.938	
D0	8.28	8.23	8.23	8.37	0.157	8.25	8.30	8.25	8.30		0.762	
D7	10.12^{a}	9.89^{ab}	9.36^{b}	10.09^{a}	0.149	10.01	9.72	9.74	9.99	0.073	0.106	0.005
Final (D12)	11.13^{a}	11.16^{a}	10.35^{b}	11.17^{a}	0.108	11.14	10.76	10.74	11.16	0.002	0.001	0.002
Pre												
d - 5 to 0												
ADG, kg	0.05	0.04	0.05	0.07	0.013	0.05	0.06	0.05	0.06		0.592	
ADFI, kg	0.17	0.16	0.15	0.18	0.021	0.16	0.16	0.16	0.17		0.708	
G:F	0.31	0.25	0.26	0.37	0.047	0.28	0.32	0.29	0.31		0.620	
Post												
d 0 to 7												
ADG, kg	0.26^{a}	0.24^{a}	0.16^{b}	0.25^{a}	0.017	0.25	0.20	0.21	0.24	0.014	0.112	0.005
ADFI, kg	0.34^{ab}	0.34^{ab}	0.33^{b}	0.35^{a}	0.005	0.34	0.34	0.34	0.35	0.510	0.028	0.006
G:F	0.76^{a}	0.70^{a}	0.49^{b}	0.70^{a}	0.044	0.73	0.59	0.62	0.70	0.006	0.124	0.007
d 7 to 12												
ADG, kg	0.25	0.32	0.25	0.27	0.018	0.28	0.26	0.25	0.29	0.177	0.028	0.277
ADFI, kg	0.40	0.40	0.40	0.40	0.001	0.40	0.40	0.40	0.40	0.717	0.039	0.717
G:F	0.64	0.79	0.62	0.68	0.045	0.71	0.65	0.63	0.73	0.172	0.034	0.291
d 0 to 12												
ADG, kg	0.26^{a}	0.27^{a}	0.19^{b}	0.25^{a}	0.011	0.26	0.22	0.23	0.26	0.002	0.005	0.020
ADFI, kg	0.36^{ab}	0.36^{ab}	0.35^{b}	0.37^{a}	0.003	0.36	0.36	0.36	0.37	0.543	0.022	0.009
G:F	0.71^{a}	0.73^{a}	0.55^{b}	0.69^{a}	0.026	0.72	0.62	0.63	0.71	0.001	0.005	0.034

-C, non-challenge with STEC; +C, challenge with STEC; 0 and 0.05, supplementation of STB 0, 0.05%; BW, body weight; ADG, average daily gain; ADFI, average daily feed intake; G:F, gain-to-feed ratio; Pre, pre-inoculation period; Post, post-inoculation period.
[1]SE, standard error.
[a,b]Values within a row with different superscripts are significantly different.

was determined in order to supply the number of cells/crypt (anatomo-functional unit), flanked by number of goblet cells/unit length of epithelium (linear density), that is more comparable with bibliographic data (Obj. 40X). The equation to determine the number of goblet cells/100 μm was: goblet cells/100 μm = number of goblet cells × 100/(crypt depth × 2) (20). In villi, goblet cells were counted in the five best-oriented villi/intestinal tract, from villus tip to base (adjacent to crypt mouth). The number of goblet cells is expressed as the mean number/villus per tract and the mean number of goblet cells/100 μm of villi epithelium (mean data of villus height).

Measurements of pro-inflammatory cytokine and immunoglobulin

The inflammatory biomarkers such as interleukin-6 (IL-6) and tumor necrosis factor α (TNF-α) were measured using commercially available ELISA kits, according to the manufacturer's instructions (R&D Systems, Minneapolis, MN). Immunoglobulin G (IgG) and immunoglobulin A (IgA) levels were gauged using an automatic biochemistry blood analyzer (Hitachi 747; Hitachi, Tokyo, Japan).

Expression of tight junction proteins

The intestinal sample stored at −80°C after sampling was homogenized and used for calprotectin and claudin-1 (CLDN-1) concentration analysis. The concentration of total protein was quantified using a Pierce BCA protein assay kit (#23225, Thermo Fisher Scientific, Waltham, MA, USA). After the homogenized intestinal sample was diluted to reach a working range of 20–2,000 μg/ml, the absorbance was measured at 562 nm. The total protein concentration was calculated as a standard curve and used to normalize the concentrations of calprotectin and CLDN-1. The relative protein expression of calprotectin and CLDN-1 was determined by using commercially available ELISA kits

(Cat no. MBS707210, MBS025129; Mybiosource, San Diego, CA, USA). Homogenized intestinal samples were diluted to reach a working range of 0.312–20 ng/ml for calprotectin and 0.5–16 ng/ml for CLDN-1. Both absorbances were measured at 450 nm. The concentrations of calprotectin and CLDN-1 were calculated by the standard curve and described as ng/mg of protein.

16S metagenomic data analysis

Bacterial 16S rRNA sequencing data of the two different metagenomics sequencing methods were analyzed using QIIME2 next-generation microbiome bioinformatics pipeline for comparative metagenomics study. The samples were sent to Sanigen (Anyang, South Korea) for microbial sequencing using the 16s rRNA technique. All raw input data were transformed in the form of QIIME2 artifacts, which contain information about the data types and sources for the downstream processing. From raw sequence data, the amplicon sequence variants (ASVs) were obtained using the Divisive Amplicon Denoising Algorithm 2 (DADA2) within QIIME 2 plugin, which detected and corrected amplicon errors and filtered out the potential base error and chimeric sequences (21, 22). The relative classification frequency table represented differential abundance tests at specific taxonomic levels was created using collapse and feature-table within the QIIME2 plugins. The "diversity" QIIME2 plugin was used to estimate alpha diversity measurements and plots using the R bioinformatics packages. This microbial diversity analysis pipeline was designed to use the ASV table (a higher resolution analog than the traditional OTU table) of the ASV picking step as necessary input data. Analyzing the differences in species richness and evenness scores considering the sampling depth was measured using the observed OTUs and Chao1, Shannon, and Simpson indices. Each index estimates the V3-V4 hypervariable region of the bacterial 16S rRNA gene. In addition, a difference in the relative abundance was analyzed by comparing the average bacterial proportion and composition investigated in each taxonomic ranking. Additionally, according to the different amplicon regions, the bacterial classification accuracy was cross-checked by comparing the taxonomy matching rate of each ASV taxonomy and NCBI bacterial reference genome database at the phylum and genus levels.

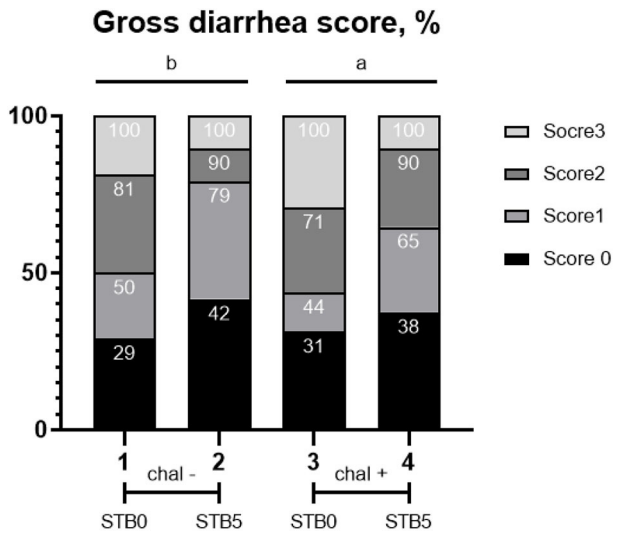

FIGURE 1
Effects of STB in weaned piglets challenged with *E. coli* on diarrhea score. $\chi 2 = 19.571$, $P = 0.0208$. Numbers inside the bar indicate the percentage of score out of total (100%) as shown in the legend. [a,b]Mean scores followed by different superscripts in the bar graph indicate statistical significance by Student's *t*-test ($P < 0.05$).

Statistical analysis

Statistical analyses and graph construction were performed using JMP Pro 16 (SAS Institute Inc., Cary, NC, United States) and GraphPad Prism (Version 9.1.0; GraphPad Software, San Diego, CA), respectively. Parametric data (growth performance,

TABLE 3 Effects of stimbiotic supplementation on digestibility in pigs challenged with STEC.

Items, %	-C		+C		SE[1]	C		STB		*P*-value		
	0	0.05	0	0.05		-	+	0	0.05	C	STB	C×STB
Post												
1 W												
DM	89.92	89.62	89.96	89.81	0.308	89.77	89.89	89.94	89.72	0.705	0.486	0.803
CP	70.19	69.30	66.62	68.57	0.774	69.74	67.60	68.40	68.93	0.012	0.501	0.081
GE	71.13	70.78	69.94	70.39	1.276	70.96	70.17	70.54	70.58	0.543	0.970	0.761
2 W												
DM	90.81[a]	90.30[ab]	89.28[b]	91.05[a]	0.384	90.56	90.16	90.05	90.68	0.316	0.117	0.008
CP	71.40[a]	70.92[a]	65.65[b]	70.81[a]	1.319	71.16	68.23	68.53	70.86	0.018	0.052	0.022
GE	73.49[a]	74.10[a]	67.52[b]	74.86[a]	1.325	73.79	71.19	70.50	74.48	0.057	0.006	0.017

-C, non-challenge with STEC; +C, challenge with STEC; 0 and 0.05, supplementation of STB 0, 0.05%; Post, post-inoculation period.
[1]SE, standard error.
[a,b]Values within a row with different superscripts are significantly different.

TABLE 4 Effects of stimbiotic supplementation on blood profile in pigs challenged with STEC.

Items	-C		+C		SE[1]	C		STB		*P*-value		
	0	0.05	0	0.05		-	+	0	0.05	C	STB	C×STB
D 0												
WBC, $10^3/\mu L$	15.65	15.69	15.07	15.13	0.761	15.67	15.09	15.36	15.41		0.947	
Bas, %	0.07	0.10	0.07	0.03	0.026	0.08	0.50	0.07	0.07		1.000	
Neu, %	48.20	50.73	53.00	47.00	3.406	49.47	50.00	50.60	48.87		0.616	
Lym, %	46.53	43.67	40.60	46.97	3.466	45.10	43.78	43.57	45.32		0.619	
N:L	1.04	1.19	1.47	1.07	0.170	1.12	1.27	1.26	1.13		0.472	
Post												
D 2												
WBC, $10^3/\mu L$	16.78	16.53	25.58	25.07	1.486	16.53	25.32	21.18	20.80	<0.001	0.803	0.931
Bas, %	0.03	0.03	0.07	0.07	0.021	0.03	0.07	0.05	0.05	0.130	1.000	1.000
Neu, %	50.53	51.57	55.00	57.17	2.125	51.05	56.08	52.77	54.37	0.028	0.460	0.793
Lym, %	41.27	44.97	35.23	33.13	2.502	43.12	34.18	38.25	39.05	0.002	0.753	0.260
N:L	1.31	1.15	1.57	1.82	0.138	1.23	1.69	1.44	1.48	0.003	0.757	0.152
D 4												
WBC, $10^3/\mu L$	15.89	15.69	28.25	26.11	1.187	15.78	27.18	22.07	20.90	<0.001	0.336	0.421
Bas, %	0.07	0.07	0.07	0.07	0.028	0.07	0.07	0.07	0.07	1.000	1.000	1.000
Neu, %	47.67	48.13	62.53	55.73	3.651	47.90	59.13	55.10	51.93	0.006	0.396	0.332
Lym, %	45.37	46.60	30.47	39.97	3.509	45.98	35.22	37.92	43.28	0.006	0.142	0.253
N:L	1.14	1.11	2.27	1.47	0.255	1.12	1.87	1.70	1.29	0.009	0.123	0.145
D 7												
WBC, $10^3/\mu L$	15.74	15.38	26.13	20.02	1.922	15.56	23.07	20.94	17.70	0.001	0.108	0.150
Bas, %	0.10	0.07	0.07	0.10	0.030	0.08	0.08	0.08	0.08	1.000	1.0000	0.277
Neu, %	44.93	42.17	51.83	47.40	4.024	43.55	49.62	48.38	44.78	0.147	0.382	0.838
Lym, %	45.70	48.90	40.67	43.73	3.268	47.30	47.20	43.18	46.32	0.134	0.349	0.984
N:L	1.08	0.90	1.30	1.13	0.153	0.99	1.22	1.20	1.02	0.154	0.274	0.990
D 12												
WBC, $10^3/\mu L$	15.76^b	16.48^b	21.38^a	17.59^b	0.925	16.12	19.48	18.57	17.03	0.002	0.112	0.024
Bas, %	0.10	0.10	0.07	0.07	0.024	0.10	0.07	0.08	0.08	0.173	1.000	1.000
Neu, %	40.87^b	40.80^b	47.23^a	40.70^b	0.906	40.83	43.97	44.05	40.75	0.003	0.002	0.002
Lym, %	53.13^a	52.47^a	47.10^b	53.00^a	0.903	52.80	50.05	50.12	52.73	0.006	0.009	0.002
N:L	0.77^b	0.78^b	1.01^a	0.77^b	0.033	0.78	0.89	0.89	0.77	0.002	0.002	0.001

-C, non-challenge with STEC; +C, challenge with STEC; 0 and 0.05, supplementation of STB 0, 0.05%; WBC, white blood cell; Bas, basophils; Neu, neutrophils; Lym, lymphocytes; N:L, neutrophils to lymphocytes ratio; Post, post-inoculation period.

[1]SE, standard error.

[a,b]Values within a row with different superscripts are significantly different.

ileal morphology, blood profile, cytokine level, and TJ proteins) were submitted to two-way ANOVA using the Standard Least Squares model. The statistical model included the effect of the *E. coli* challenge (chal -, chal +), the effect of STB supplementation (0, 0.5 g/kg), and the interaction between *E. coli* and STB, and initial body weight at the start of the trial (d 0) was also included as a covariate. The richness and alpha diversity were calculated with raw counts based on Shannon estimators. For quantitative beta diversity measurement, each treatment group was placed as the control group, and treatment groups were compared by using PROC MIXED with Dunnett's *post-hoc* test. Non-parametric data (diarrhea score) were analyzed using contingency analysis to test the relationship between categorical variables (scores) and the different combinations tested in this study. A chi-square test was performed to determine if the different combinations had an effect on the

TABLE 5 Effects of stimbiotic supplementation on cytokine in pigs challenged with STEC.

Items	-C		+C		SE[1]	C		STB		*P*-value		
	0	0.05	0	0.05		-	+	0	0.05	C	STB	C×STB
Pre												
D 0												
TNF-α, pg/mL	70.65	67.22	66.91	64.14	4.372	68.94	65.52	68.78	65.68		0.486	
IL-6, pg/mL	1178.78	1182.68	1177.88	1174.74	56.355	1180.73	1176.31	1178.33	1178.71		0.995	
IgG, mg/dL	227.00	224.00	223.33	223.00	6.206	225.50	223.17	225.17	223.50		0.791	
IgA, mg/dL	1.00	1.33	1.17	1.17	0.158	1.17	1.17	1.08	1.25		1.000	
Post												
D 2												
TNF-α, pg/mL	69.72	61.27	125.49	89.62	8.519	65.50	107.55	97.60	75.45	<0.001	0.017	0.123
IL-6, pg/mL	1233.76	1246.47	2469.85	2064.38	146.923	1240.11	2267.11	1851.80	1655.42	<0.001	0.196	0.170
IgG, mg/dL	213.67	219.33	163.00	173.00	11.349	216.50	168.00	188.33	196.17	<0.001	0.498	0.851
IgA, mg/dL	1.67	1.67	1.33	1.00	0.316	1.67	1.17	1.50	1.33	0.130	0.604	0.604
D 4												
TNF-α, pg/mL	68.32	80.22	143.95	118.13	21.948	74.27	131.04	106.13	99.17	0.018	0.754	0.400
IL-6, pg/mL	1200.32	1064.56	2729.34	1983.21	102.468	1132.44	1356.28	1964.83	1523.89	<0.001	<0.001	0.007
IgG, mg/dL	205.00	209.00	138.00	153.33	5.054	207.00	145.67	171.50	181.17	<0.001	0.071	0.276
IgA, mg/dL	1.33	1.67	1.33	1.33	0.279	1.50	1.33	1.33	1.50	0.557	0.557	0.557
D 7												
TNF-α, pg/mL	60.87	59.60	118.66	93.81	10.961	60.24	106.23	89.76	76.71	<0.001	0.248	0.295
IL-6, pg/mL	1166.43^{c}	1152.87^{b}	2334.19^{a}	1575.26^{b}	52.438	1159.65	1954.73	1750.31	1364.07	<0.001	<0.001	<0.001
IgG, mg/dL	200.00	211.33	170.33	193.33	10.515	205.67	181.83	185.17	202.33	0.035	0.118	0.585
IgA, mg/dL	1.33	1.67	1.33	1.33	0.279	1.50	1.33	1.33	1.50	0.557	0.557	0.557
D 12												
TNF-α, pg/mL	59.03^{bc}	53.28^{c}	100.90^{a}	67.08^{b}	2.598	56.16	83.99	79.97	60.18	<0.001	<0.001	<0.001
IL-6, pg/mL	1040.77^{b}	1036.96^{b}	1826.10^{a}	1075.99^{b}	76.957	1038.87	1451.05	1433.43	1056.48	<0.001	<0.001	<0.001
IgG, mg/dL	219.33	217.00	177.33	196.67	10.252	218.17	187.00	198.33	206.83	0.007	0.417	0.303
IgA, mg/dL	1.33	1.33	1.67	1.00	0.258	1.33	1.33	1.50	1.17	1.000	0.211	0.211

-C, non-challenge with STEC; +C, challenge with STEC; 0 and 0.05, supplementation of STB 0, 0.05%; TNF-α, tumor necrosis factor alpha; IL-6, interleukin-6; IgG, immunoglobulin G; IgA, immunoglobulin A; Pre, pre-inoculation period; Post, post-inoculation period.

[1]SE, standard error.

[a,b,c]Values within a row with different superscripts are significantly different.

TABLE 6 Effects of stimbiotic supplementation on villus height and counts of goblet cell in pigs challenged with STEC.

Items	-C		+C		SE[1]	C		STB		*P*-value		
	0	0.05	0	0.05		-	+	0	0.05	C	STB	C×STB
VH, μm	358.05	400.23	317.68	364.12	15.270	379.14	340.90	337.87	382.18	0.021	0.009	0.891
CD, μm	153.01	156.68	164.13	137.20	10.271	154.85	150.67	158.57	146.94	0.688	0.271	0.152
HDR	2.43	2.57	1.98	2.69	0.173	2.50	2.33	2.21	2.63	0.344	0.024	0.110
Goblet cell												
Villus	21.17	19.67	32.17	25.00	2.574	20.42	28.58	26.67	22.33	0.005	0.108	0.284
Crypt	19.83	19.33	21.00	20.83	0.908	19.58	20.91	20.42	20.08	0.158	0.718	0.856

-C, non-challenge with STEC; +C, challenge with STEC; 0 and 0.05, supplementation of STB 0, 0.05%; VH, villus height; CD, crypt depth; HDR, height to depth ratio.

[1]SE, standard error.

10.3389/fvets.2023.1187002

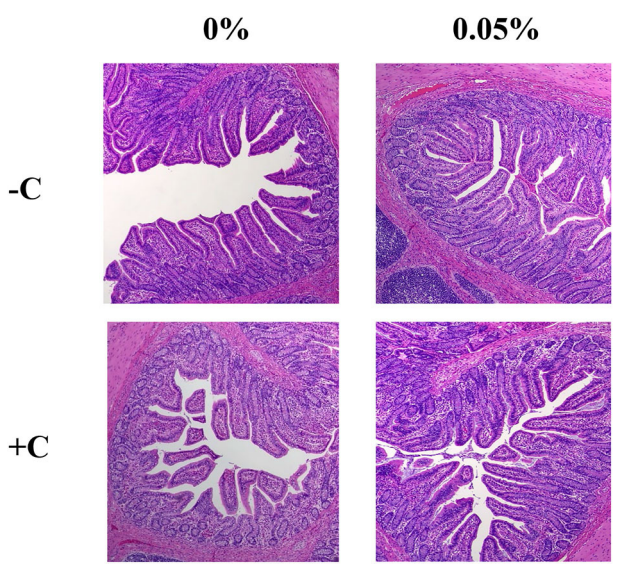

FIGURE 2
Histological analysis of the intestinal morphology of weaned piglets. Figures display the morphology of the ileum tissue from pigs in four dietary treatments.

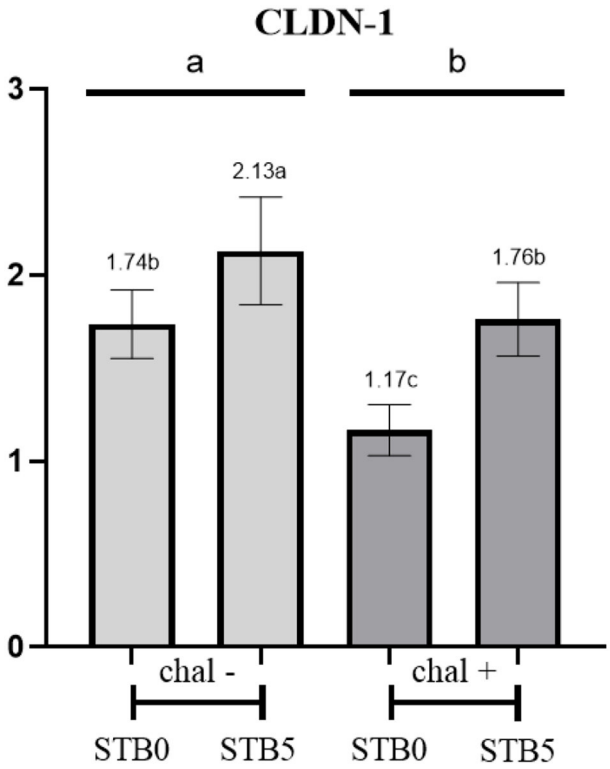

FIGURE 3
Effects of STB in weaned piglets challenged with *E. coli* on CLDN-1. [a,b,c]Mean scores followed by different superscripts in the bar graph indicates statistical significance by Student's *t*-test ($P < 0.05$).

categorical variables, repartition with significance accepted at $P < 0.05$.

Results

Growth performance

The effects of STB supplementation on the growth performance of piglets are presented in Table 2. There was an interaction ($P < 0.05$) between the supplementation of STB and *E. coli* challenge in BW, ADG, ADFI, and G:F. Piglets supplemented STB5 with *E. coli* challenge had higher BW on 7 dpi ($P = 0.005$) and final ($P = 0.002$) compared with piglets fed STB0 with *E. coli* challenge.

Incidence of diarrhea

An overview of the incidence of diarrhea is shown in Figure 1. After the *E. coli* challenge, there were differences in diarrhea scores from 1 to 7 dpi. *E. coli* challenge increased ($P = 0.0004$) the average diarrhea scores compared with the non-challenged group. Supplementation of STB5 decreased ($P < 0.001$) the average diarrhea scores compared with supplementation of STB0. There was an interaction between supplementation of STB and *E. coli* challenge in diarrhea score on days 0 to 12.

Nutrient digestibility

E. coli challenge decreased the CP digestibility on 1w and 2w compared with the non-challenged groups (Table 3). There was an interaction between STB and *E. coli* challenge in digestibility on 2w. Piglets supplemented with STB5 with *E. coli* challenge had higher digestibility of CP, DM, and GE on 2w compared with piglets supplemented with STB0 with *E. coli* challenge.

Blood profile

E. coli challenge increased ($P < 0.05$) WBC and neutrophils and decreased ($P < 0.05$) lymphocytes compared with the non-challenged group during the post-inoculation period (Table 4). The piglets fed STB5 had improved ($P < 0.05$) neutrophils and lymphocytes in 12 dpi compared with STB0. There was an interaction between STB and *E. coli* challenge in WBC, neutrophils, and lymphocytes. Piglets supplemented STB5 with *E. coli* challenge improved WBC ($P = 0.024$), neutrophils ($P = 0.002$), and lymphocytes ($P = 0.002$) compared with piglets fed STB0 with *E. coli* challenge on 12 dpi.

Measurements of pro-inflammatory cytokine

E. coli challenge increased ($P < 0.05$) TNF-α and IL-6 and decreased ($P < 0.05$) IgG compared with the non-challenged group during the post-inoculation period (Table 5). Supplementation of STB5 improved ($P < 0.05$) TNF-α on 2 and 12 dpi compared with STB0. In addition, supplementation of STB5 improved (P

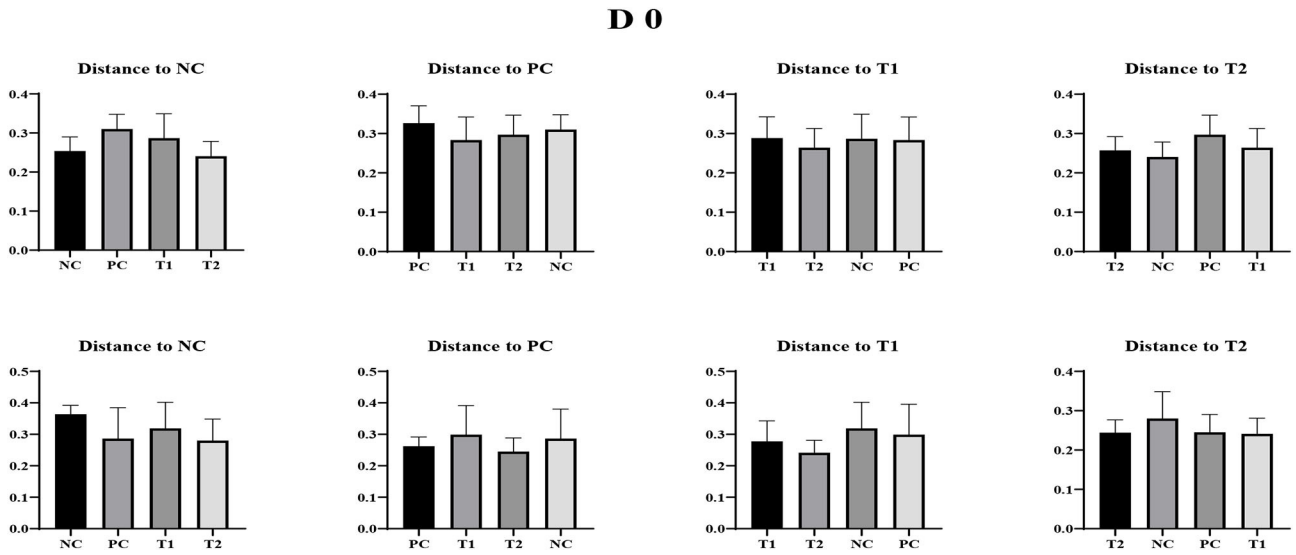

FIGURE 4
Weighted Unifrac measurement in negative control (NC): basal diet; positive control (PC): NC + *E. coli* challenge; treatment 1 (T1): NC + stimbiotic 0.5 g/kg; treatment 2 (T2): PC + stimbiotic 0.5 g/kg. Each treatment group was placed as the control group, and treatment groups were compared by using one-way PROC MIXED with Dunnett's *post-hoc* test.

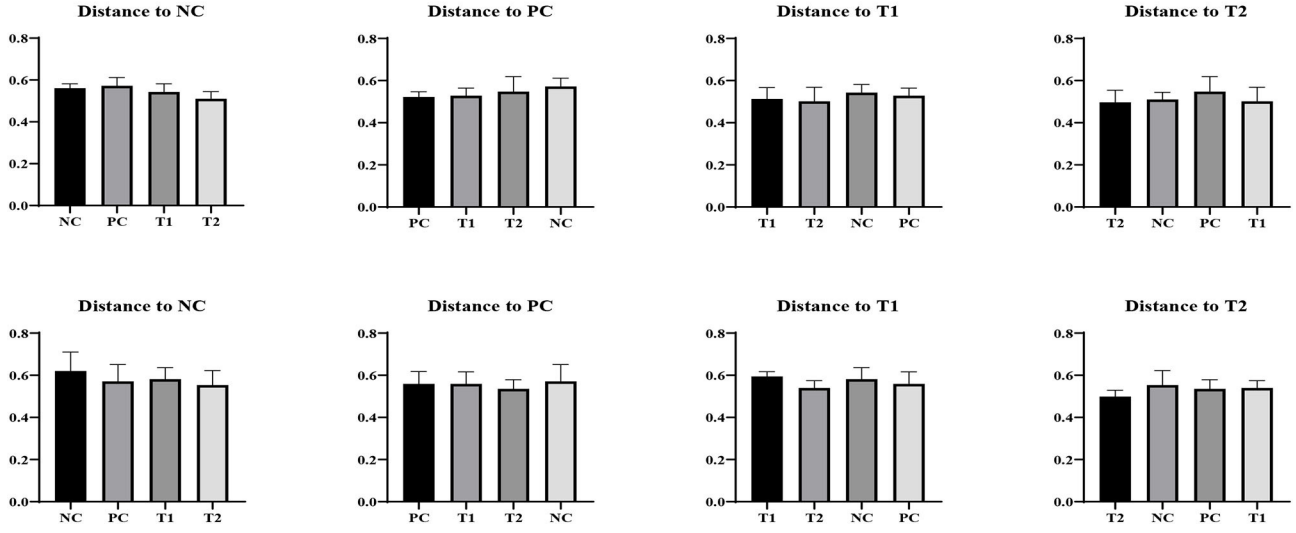

FIGURE 5
Unweighted unifrac measurement in negative control (NC): basal diet; positive control (PC): NC + *E. coli* challenge; treatment 1 (T1): NC + stimbiotic 0.5 g/kg; treatment 2 (T2): PC + stimbiotic 0.5 g/kg. Each treatment group was placed as the control group, and treatment groups were compared by using one-way PROC MIXED with Dunnett's *post-hoc* test.

< 0.05) IL-6 on 4, 7, and 12 dpi compared with STB0. There was an interaction between STB and *E. coli* challenge in TNF- α and IL-6. Piglets supplemented with STB5 with *E. coli* challenge improved ($P < 0.05$) IL-6 on 4, 7, and 12 dpi compared with piglets fed STB0 with *E. coli* challenge. In addition, piglets supplemented STB5 with *E. coli* challenge improved ($P < 0.05$) TNF-α compared with piglets fed STB0 with *E. coli* challenge on 12 dpi.

Morphological analysis of the small intestine

There was no interaction between supplementation of STB and *E. coli* challenge in villus height, crypt depth, and height-to-depth ratio. *E. coli* challenge decreased ($P = 0.021$) villus height of the ileum (Figure 2, Table 6). Supplementation of STB increased ($P > 0.05$) VH and HDR. However, there was no difference between *E.*

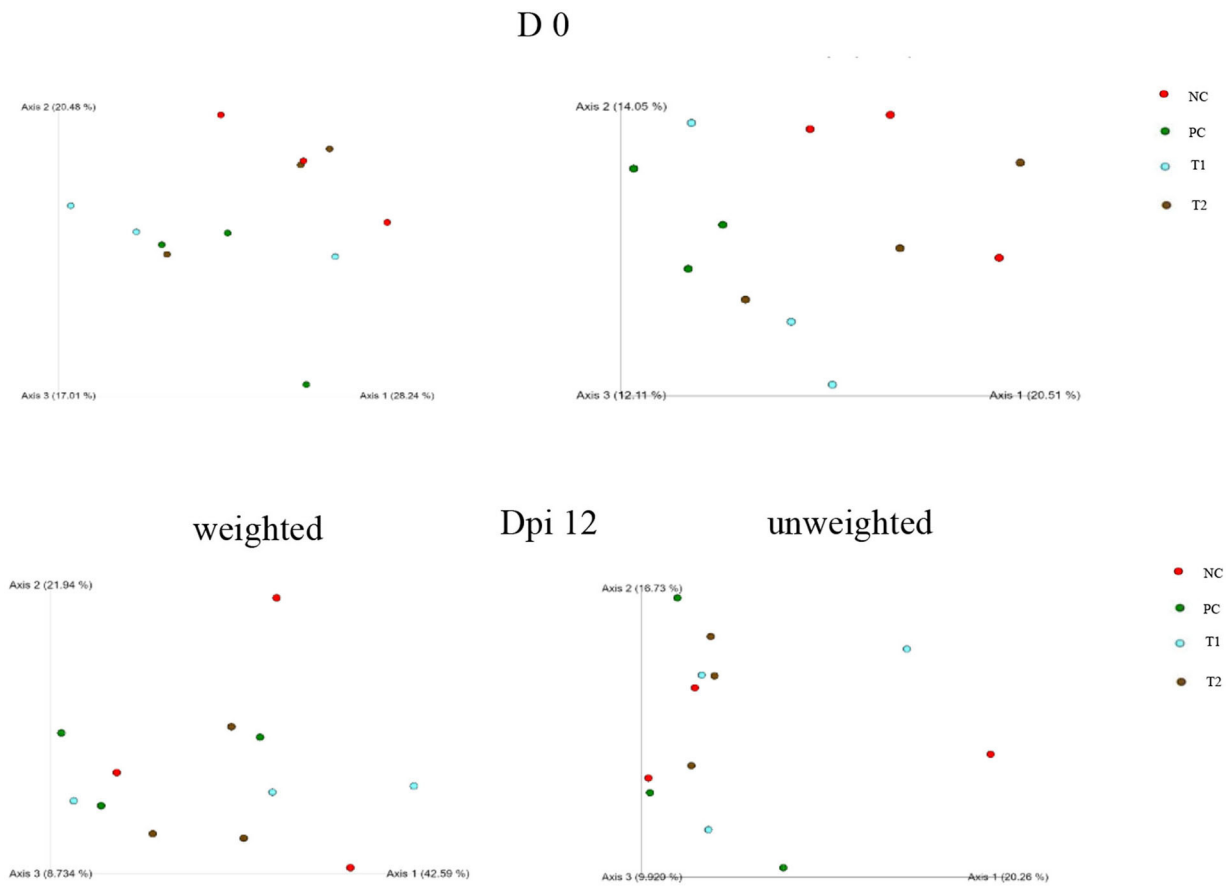

FIGURE 6
Visualized beta diversity indices including unweighted and weighted emperor in negative control (NC): basal diet; positive control (PC): NC + *E. coli* challenge; treatment 1 (T1): NC + stimbiotic 0.5 g/kg; treatment 2 (T2): PC + stimbiotic 0.5 g/kg.

coli and supplementation of STB in CD. There was no interaction between supplementation of STB and *E. coli* challenge in counts of goblet cells. *E. coli* challenge increased ($P = 0.005$) the counts of goblet cells in the villus.

Expression of tight junction proteins

There was no interaction between the supplementation of STB and *E. coli* challenge in CLDN-1 and calprotectin (Figure 3). *E. coli* challenge downregulated ($P < 0.05$) the expression of CLDN-1 while supplementation of STB upregulated ($P < 0.05$) the expression of CLDN-1. In addition, the *E. coli* challenge upregulated ($P < 0.001$) the expression of calprotectin compared with the non-challenged group. However, supplementation of STB did not affect ($P > 0.05$) the expression of calprotectin.

Alpha diversity of the fecal microbiome

No differences were observed in the alpha diversity parameters including Chao 1, Simpson, and Shannon indices on d 0 and dpi 12.

Beta diversity of the fecal microbiome

No differences were observed in unweighted and weighted unifrac distance to each treatment both on d 0 and dpi 12 ($P < 0.05$; Figures 4–6).

Relative abundance

At the genus level, supplementation of STB increased ($P < 0.001$) the abundance of *Prevotella* compared with non-supplemented STB groups in the pre-inoculation period (Table 7; Figure 7). In addition, supplementation of STB decreased ($P < 0.05$) the abundance of the *Faecalibacterium* and *Eubacterium_coprostanoligenes*_group compared with the non-supplemented STB groups in the post-inoculation period. *E. coli* challenge decreased ($P < 0.05$) the abundance of *Clostridium_sensu_stricto*_1 and *Faecalibacterium* compared with the non-challenged groups in the post-inoculation period. There was an interaction between the supplementation of STB and *E. coli* challenge in the abundance of *Muribaculaceae* and *Faecalibacterium* in the post-inoculation period. Piglets supplemented STB5 with *E. coli* challenge decreased ($P < 0.05$)

TABLE 7 Relative abundance of fecal microbiota at the genus level in pigs challenged with STEC on d 0 and 12 dpi and fed diets supplemented with stimbiotic.

Items, %	-C		+C		SE[1]	C		STB		*P*-value		
	0	0.05	0	0.05		-	+	0	0.05	C	STB	C×STB
Pre (d 0)												
Prevotella	17.62	21.40	8.56	20.79	2.576	19.51	14.68	13.09	21.10		0.006	
Lachnospiraceae	8.05	5.09	6.29	7.72	1.403	6.57	7.00	7.17	6.41		0.593	
Anaerovibrio	2.16	2.67	4.13	3.65	1.017	2.41	3.89	3.14	3.16		0.985	
Muribaculaceae	4.61	1.96	3.25	2.94	1.034	3.29	3.10	3.93	2.45		0.469	
Alloprevotella	3.08	3.96	2.17	3.08	0.758	3.52	2.62	2.62	3.52		0.253	
Prevotellaceae _NK3B31_group	4.40	1.52	2.94	3.66	1.061	2.96	3.30	3.67	2.59		0.320	
Treponema	1.49	3.22	4.88	1.09	1.086	2.36	2.98	3.18	2.16		0.355	
Prevotellaceae_UCG-003	2.52	1.62	2.31	4.29	0.607	2.07	3.30	2.41	2.95		0.386	
Succinivibrio	1.06	2.65	4.47	1.68	0.820	1.85	3.08	2.77	2.16		0.471	
Rikenellaceae _RC9_gut_group	1.44	3.54	2.69	2.48	0.464	2.49	2.59	2.07	3.01		0.055	
Rest	53.57	52.37	58.32	48.62	1.808	52.97	53.47	55.94	50.49		0.007	
Post (12 dpi)												
Prevotella	8.50	8.28	10.08	11.95	1.502	8.39	11.01	9.29	10.11	0.096	0.588	0.496
Lachnospiraceae	7.43	7.79	10.75	8.06	1.042	7.61	9.40	9.09	7.93	0.101	0.279	0.160
Lactobacillus	10.39	3.39	9.51	10.05	3.038	6.89	9.78	9.95	6.72	0.353	0.300	0.229
Muribaculaceae	5.12	8.69	8.96	4.30	1.640	6.91	6.63	7.04	6.50	0.087	0.744	0.021
Prevotellaceae _NK3B31_group	3.38	5.34	4.77	6.21	1.885	4.36	5.49	4.07	5.78	0.555	0.377	0.894
Clostridium_sensu _stricto_1	7.21	4.09	1.85	3.63	1.200	5.65	2.74	4.53	3.86	0.025	0.583	0.055
Alloprevotella	3.94	4.09	1.62	3.07	1.452	4.01	2.35	2.78	3.58	0.265	0.586	0.660
Faecalibacterium	1.91	1.52	6.55	2.16	0.900	1.72	4.35	4.23	1.84	0.008	0.015	0.038
Eubacterium_ coprostanoligenes_group	2.56	3.07	3.52	2.13	0.460	2.81	2.83	3.04	2.60	0.974	0.035	0.052
Clostridia_UCG-014	3.01	2.15	2.74	2.89	0.865	2.58	2.81	2.87	2.52	0.790	0.687	0.569
Rest	46.56	51.58	39.66	45.55	4.486	49.07	42.60	43.11	48.56	0.165	0.238	0.924

-C, non-challenge with STEC; +C, challenge with STEC; 0 and 0.05, supplementation of STB 0, 0.05%.
[1]SE, standard error.

the abundance of *Muribaculaceae* and *Faecalibacterium* compared with piglets fed STB0 with *E. coli* challenge.

In the phylum level, supplementation of STB increased ($P < 0.05$) the abundance of *Desulfobacterota* and *Fibrobacterota* in the pre-inoculation period (Figure 7, Table 8). However, supplementation of STB decreased ($P = 0.040$) the abundance of *Fibrobacterota* compared with the non-supplemented group in the post-inoculation period. *E. coli* challenge increased the abundance of *Fibrobacterota* compared with the non-challenged group in the post-inoculation period. There was an interaction between the supplementation of STB and *E. coli* challenge in the abundance of *Fibrobacterota*. Piglets supplemented STB5 with *E. coli* challenge decreased ($P = 0.010$) the abundance of *Fibrobacterota* compared with piglets supplemented STB0 with *E. coli* challenge.

Discussion

Weaned piglets are commonly affected by PWD, which decreases their growth performance and increases their mortality (23). *E. coli* F18 is a primary pathogen associated with PWD in weaned piglets (24). It is known that *E. coli* F18 attaches to a specific receptor on the epithelium of the pig's intestinal tract through their fimbriae (25). As a result of colonization of the gut, toxins are produced, causing diarrhea (26). In many research facilities, experimental induction of PWD has been used to test the efficacy of functional additives under commercial PWD conditions (27–29). Furthermore, these studies have evaluated the gut health and immune response in weaned piglets challenged with *E. coli*.

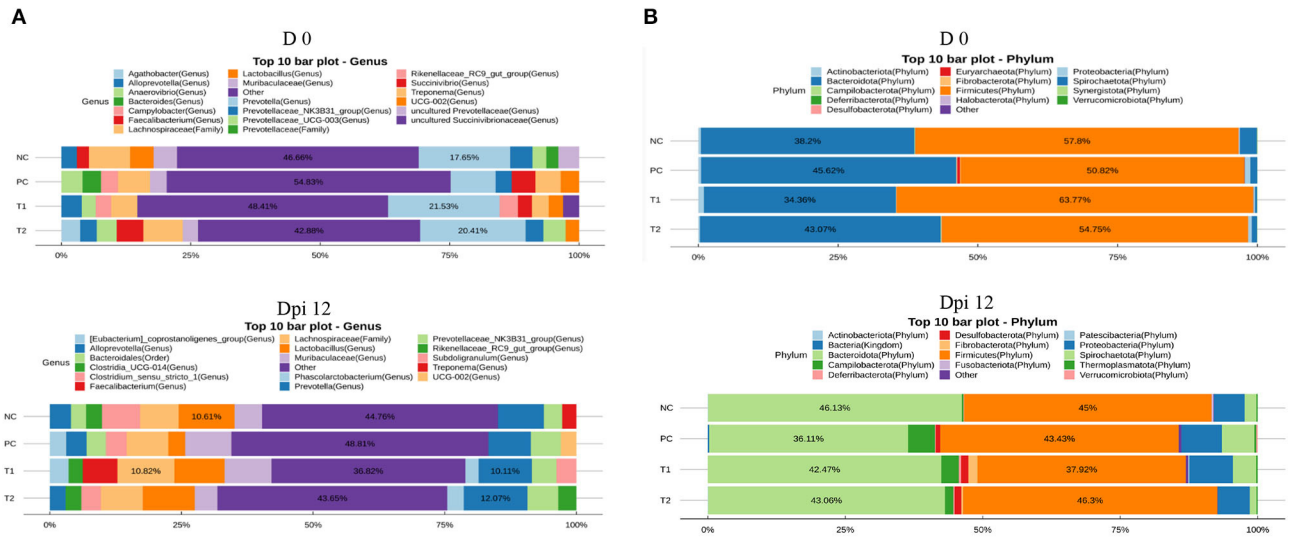

FIGURE 7
(A) 16S rRNA gene analysis revealed the relative abundance of fecal bacterial community structure at the genus level in piglets challenged with *E. coli* in negative control (NC): basal diet; positive control (PC): NC + *E. coli* challenge; treatment 1 (T1): NC + stimbiotic 0.5 g/kg; treatment 2 (T2): PC + stimbiotic 0.5 g/kg. **(B)** 16S rRNA gene analysis revealed the relative abundance of fecal bacterial community structure at the phylum level in piglets challenged with *E. coli* in negative control (NC): basal diet; positive control (PC): NC + *E. coli* challenge; treatment 1 (T1): NC + stimbiotic 0.5 g/kg; treatment 2 (T2): PC + stimbiotic 0.5 g/kg.

The results obtained from this study showed that supplementation of 0.5 g/kg of STB could mitigate growth performance, diarrhea rate, intestinal morphology, and index of inflammation of weaned piglets challenged with *E. coli* infection. These results were consistent with other types of research, indicating an improved immune response after supplementation of STB (11, 30).

In the current study, F18 *E. coli* infection was accomplished in agreement with our previous studies using an *E. coli* challenge model in weaned piglets (9, 12). According to Kim et al. (31), heat-labile toxin and Shiga toxin induce gut permeability and inflammation, which, in turn, increase cytokines.

The integrity of the intestinal barrier is strictly regulated by Tight junction (TJ) proteins including CLDN, occludin, and zonula occludin which are continuously threatened by pro-inflammatory stimuli (32). Especially, CLDN-1, a representative TJ protein in mammals, enhances gut barriers to stop the loss of electrolytes (33). As a marker of neutrophilic inflammation in the gut, calprotectin concentrations are associated with the histological activity of inflammatory bowel disease (34). In the present study, supplementation of STB5 decreased the gross diarrhea incidence in *E. coli*-challenged piglets. This result is similar to those of previous studies showing that supplementing carbohydrase and XOS could alleviate diarrhea incidence in pigs (35, 36). It would be possible that increased expression of CLDN-1 in ileal mucosa fortified the intestinal barrier function in pigs fed STB infected with *E. coli*. TNF-α is a crucial pro-inflammatory cytokine in reaction to an infection by bacteria which is produced by neutrophils (31). IL-6 is also a pro-inflammatory cytokine expressed widely across vertebrates and plays multiple physiological roles involved in inflammation (37). TNF-α and IL-6 are served as biological indicators of intestinal inflammation in pigs (34, 38). Our observation indicated that supplementation of STB decreased the concentration of TNF-α and IL-6 in piglets infected with *E. coli*. Similar research reported that feeding STB improved TJ proteins between epithelial cells and reduced the production of pro-inflammatory cytokines such as TNF-α and IL-6 (39). Serum IgG and IgA are known to play an important role in humoral immunity.

The immune system depends on WBC, which includes neutrophils, lymphocytes, basophils, monocytes, and eosinophils, to control infections in the body, and the count of WBC is considered a marker of infection (40). The primary line of protection against bacterial infection is provided by neutrophils, and lymphocytes provide particular cellular and humoral immune responses (41). In the present study, *E. coli* infection increased counts of WBC and neutrophils, but supplementation of STB decreased the count of WBC and neutrophils in piglets infected with *E. coli*. In addition, the ratio of neutrophils to lymphocytes as a biomarker of inflammation was increased by *E. coli* infection. Similarly, the previous study reported that supplementation of mannan-oligosaccharide (MOS) decreased the neutrophils in broilers (42).

VH and CD are markers of enterocyte proliferation and villus damage (43). Shorter VH and deeper CD may indicate the presence of toxins (44). The villus contains enterocytes, goblet cells, and enteroendocrine cells that line the space, and the crypt contains undifferentiated cells and a subset of differentiated secretory cells (5). In our current study, *E. coli* infection decreased the VH, but supplementation of STB increased the VH, VH:CD, and goblet cells in the villi of piglets infected with *E. coli*. It has been reported that supplementation of MOS increased

TABLE 8 Relative abundance of fecal microbiota at the phylum level in pigs challenged with STEC on d 0 and 12 dpi and fed diets supplemented with stimbiotic.

Items, %	-C		+C		SE[1]	C		STB		*P*-value		
	0	0.05	0	0.05		-	+	0	0.05	C	STB	C×STB
Pre (d 0)												
Firmicutes	44.94	38.04	43.93	46.16	2.459	41.49	45.05	44.44	42.10		0.354	
Bacteroidota	46.48	42.40	35.49	43.68	2.993	44.44	39.59	40.99	43.04		0.500	
Proteobacteria	5.48	7.87	7.35	5.52	1.659	6.67	6.44	6.41	6.69		0.868	
Spirochaetota	2.01	4.22	5.87	1.21	1.161	3.12	3.54	3.94	2.72		0.306	
Campilobacterota	0.30	3.26	5.03	1.47	1.157	1.78	3.25	2.66	2.37		0.799	
Desulfobacterota	0.01	1.37	0.76	1.18	0.424	0.69	0.97	0.39	1.28		0.049	
Fibrobacterota	0.03	1.56	0.03	0.27	0.373	0.79	0.15	0.03	0.92		0.027	
Thermoplasmatota	0.21	0.28	0.34	0.17	0.046	0.24	0.26	0.28	0.22		0.248	
Deferribacterota	0.09	0.39	0.19	0.19	0.085	0.24	0.19	0.14	0.29		0.097	
Fusobacteriota	0.32	0.16	0.02	0.01	0.104	0.24	0.01	0.17	0.09		0.431	
Rest	0.12	0.44	0.98	0.12	0.128	0.28	0.55	0.55	0.28		0.049	
Post (12 dpi)												
Firmicutes	57.84	63.66	51.06	54.46	4.156	60.75	52.76	54.45	59.06	0.069	0.281	0.775
Bacteroidota	37.90	34.48	45.31	43.36	4.281	36.19	44.33	41.61	38.92	0.072	0.538	0.865
Spirochaetota	3.28	0.37	1.29	0.93	0.969	1.82	1.11	2.29	0.65	0.469	0.107	0.203
Actinobacteriota	0.47	0.99	0.47	0.28	0.251	0.73	0.37	0.47	0.63	0.172	0.522	0.180
Proteobacteria	0.17	0.29	1.04	0.70	0.328	0.23	0.87	0.61	0.49	0.064	0.729	0.487
Euryarchaeota	0.04	0.00	0.39	0.02	0.111	0.03	0.20	0.22	0.01	0.122	0.080	0.153
Campilobacterota	0.16	0.06	0.08	0.12	0.051	0.11	0.10	0.12	0.09	0.884	0.544	0.192
Desulfobacterota	0.00	0.07	0.16	0.08	0.050	0.03	0.12	0.08	0.08	0.088	0.915	0.166
Fibrobacterota	0.00	0.02	0.12	0.00	0.024	0.01	0.06	0.06	0.01	0.040	0.040	0.010
Synergistota	0.04	0.03	0.02	0.04	0.021	0.04	0.03	0.03	0.03	0.609	0.945	0.552
Rest	0.09	0.04	0.06	0.02	0.026	0.06	0.04	0.07	0.30	0.362	0.115	0.977

-C, non-challenge with STEC; +C, challenge with STEC; 0 and 0.05, supplementation of STB 0 and 0.05%.
[1]SE, standard error.

the villus height in weaned piglets (45). However, our results showed that supplementation of STB did not affect ileal CD. According to Luise et al. (46), XYL supplementation did not affect jejunal CD in weaned piglets which are genetically susceptible to ETEC. Zhang et al. (47) reported that the morphology of intestinal villi is tightly associated with the absorption of nutrients. Consistent with the results of the morphology of the small intestine, the digestibility of CP was also decreased by *E. coli* infection. There was an interaction effect between STB and *E. coli* infection. These findings support that supplementation of STB might mitigate inflammation and improve CP digestibility in *E. coli*-infected piglets.

Pathogenic challenges impair pig intestinal integrity by disturbing intestine microbial balance (48, 49). At the phylum level, *Fibrobacterota* is known for degrading lignocellulosic materials in the gut (47). Our study indicated that supplementation of STB increased the relative abundance of *Fibrobacterota* in the post-inoculation period. *Desulfobacterota* is associated with inflammation and increased relative abundance of *Desulfobacterota*, reducing VH and epithelial cells and downregulating the expression of TJ proteins (50). In the current study, the relative abundance of *Desulfobacterota* increased after *E. coli* infection. Similar research indicated that the abundance of *Desulfobacterota* is increased in broilers challenged with *C.perfrigens* (51).

At the genus level, *Prevotella* is associated with the production of acetate and butyrate in the small intestine (52). In the current study, supplementation of STB increased the relative abundance of *Prevotella*. Similar research indicated that the fermentation of xylan leads to the increased production of short-chain fatty acids such as butyrate (53).

Conclusion

The results of this study support the hypothesis that supplementation of STB is capable of alleviating the growth performance and intestinal morphology,

immune response, and gut microbiota in weaned piglets infected with *E. coli*. Our results supported that STB supplementation might increase the fermentability of NSP and reduce the antinutritive effects of NSP. Therefore, STB could be used as an antidiarrheal growth stimulator in weaned piglets.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: https://www.ncbi.nlm.nih.gov/, SUB12953593.

Ethics statement

The animal study was reviewed and approved by Institutional Animal Care and Use Committee of Chungbuk National University.

Author contributions

DS, WK, and JL conducted the experiment and wrote the manuscript. HO, SC, JA, HC, SP, and KJ helped to conduct animal trial and laboratory work and helped to revise the manuscript. JC was the principal investigator and wrote the last version of the manuscript. All authors read and approved the final manuscript.

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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.

Publisher's note

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In vitro ruminal fermentation and cow-to-mouse fecal transplantations verify the inter-relationship of microbiome and metabolome biomarkers: potential to promote health in dairy cows

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Introduction: There are differences in the gut microbiome and metabolome when the host undergoes different physical or pathological conditions. However, the inter-relationship of microbiome and metabolome biomarkers to potentially promote the health of dairy cows needs to be studied. Further, the development of next-generation probiotics for dairy cattle health promotion has not been demonstrated.

Objective: In the present study, we identified the microbiome and metabolome biomarkers associated with healthy cows.

Methods: We analyzed the relationships of the ruminal microorganism profile and metabolites between healthy and mastitis lactating dairy cows. The roles of bacterial biomarker were further verified by in vitro fermentation and cow-to-mouse fecal microbiota transplantation (FMT).

Results: Two species, *Ruminococcus flavefaciens* and *Bifidobacterium longum* subsp. *longum*, and six rumen metabolites were positively correlated with healthy cows by Spearman's correlation analysis. Through in vitro ruminal fermentation, inoculating *R. flavefaciens* and *B. longum* subsp. *longum* showed the upregulation of the levels of putrescine, xanthurenic acid, and pyridoxal in the mastitis ruminal fluid, which confirmed the inter-relationships between these microbiota and metabolites associated with healthy cows. Further, we verified the role of *R. flavefaciens* and *B. longum* subsp. *longum* in promoting health by FMT. The administration of *R. flavefaciens* and *B. longum* subsp. *longum* reduced the death rate and recovered the bodyweight loss of germ-free mice caused by FMT mastitis feces.

Discussion: We provided evidence that the bacterial biomarkers alter downstream metabolites. This could indirectly indicate that the two bacterial biomarkers have the potential to be used as next-generation probiotics for dairy cattle, although it needs more evidence to support our hypothesis. Two species, *R. flavefaciens* and *B. longum* subsp. *longum*, with three metabolites, putrescine, xanthurenic acid, and pyridoxal, identified in the ruminal fluid, may point to a new health-promoting and disease-preventing approach for dairy cattle.

KEYWORDS

Ruminococcus flavefaciens, *Bifidobacterium longum* subsp. longum, metabolites, biomarkers, dairy cows

1. Introduction

Bovine rumen possesses a highly diverse population of microorganisms, including bacteria, protozoa, archaea, and fungi, which degrade and ferment the plant materials into digestible compounds (1). Proteobacteria, Firmicutes, and Bacteroidetes are the dominant phyla of the Kingdom Eubacteria in dairy cattle rumen (2, 3). However, the bacterial abundance in the digestive tract may fluctuate because of age, nutrients, and other factors related to lifestyle (4–6) or immune status (7). Changes in the gut microbiota play an influential role in the performance of the animals (3, 8) as well as contribute to the development of diseases including mastitis (7, 9). Gut dysbiosis also plays an important role in the health of dairy cows due to the difference and diversity of the gastrointestinal microbiota that competes for nutrients, regulate the immune system, and produce metabolites (10). Different microbiota found in feces (9) and ruminal fluid (7) of healthy and mastitis cows, and induction of mastitis in germ-free (GF) mice by fecal microbiota transplantation (FMT) suggested that bovine mastitis is not necessary a local infection of the mammary glands (9). Many evidences have shown that the stress factors such as high concentrate feeding or heat stress, could disturb the rumen microbiota and upregulate the level of lipopolysaccharide (LPS) (11), resulting in changes in the permeability of the rumen epithelial layer (12). The rumen-derived LPS could enter the mammary gland via blood circulation and further impair the blood-milk barrier, leading to inflammation of the mammary gland in cows (12, 13). Additionally, Zhao et al. (13) suggested that ruminal dysbiosis-derived low-grade endotoxemia could cause mastitis and worsen pathogen-induced mastitis by damaging host anti-inflammatory enzymes.

The metabolites, derived from fermentation by rumen microorganisms, are considered as a downstream outcome, illustrating the interaction between microorganisms, hosts, and the microenvironment. Metabolomics has been employed to evaluate the quality of milk (14) and search for new biomarkers for disorders (7). Chuang et al. (7) identified seven rumen fluid metabolites that changed between healthy and mastitis cows, which could be used as potential biomarkers for the diagnosis of mastitis.

The probiotics currently available to farm animals are generally limited to a narrow range of organisms. Characterizing the gut microbiota and metabolites is a novel preventive or therapeutic approach for the development of next-generation probiotics (15, 16). Therefore, the comprehensive description of the ruminal microbiota and metabolome and their roles in health and disease is crucial. Although the ruminal microbial and metabolomic structure in lactating dairy cows with mastitis has been studied (7), the inter-relationship of the microbiome and metabolome biomarkers in promoting health in dairy cows has never been confirmed. The role of bacterial biomarkers in health promotion and disease prevention also remains unknown. Thus, this study first identified the microbiome and metabolome biomarkers associated with healthy cows by evaluating the relationships among the ruminal microbial profile, metabolites, and mastitis outcomes. We then verified the inter-relationship of the microbiome and metabolome biomarkers and the role of bacterial biomarkers in health promotion and disease prevention through *in vitro* ruminal fermentation and cow-to-mouse fecal microbiota transplantation, respectively.

2. Materials and methods

2.1. Potential ruminal microorganism biomarkers and related metabolites for healthy dairy cows

2.1.1. Animals and sample collection

Thirty lactating Holstein dairy cows with 120–240 milk production days and an average age of 3.53 ± 0.67 years from a commercial farm were involved in the present study. All cows were under the same management, receiving total mixed ration (TMR) feeding and water *ad libitum*, and milked twice per day. After the outcomes of veterinary diagnosis, raw milk test with California mastitis test (CMT) and somatic cell counts (SCC), and serum proinflammatory cytokines, 15 healthy cows and 15 cows with clinical mastitis were selected for microbiomic and metabolomic analysis. Cows with one quarter milk showed positive reaction by CMT, SCC ≥ 1,000,000 cells/mL, and elevated cytokines in serum were defined as mastitis cows. On the other hand, cows with negative CMT reaction, SCC < 200,000 cells/mL, and no specific cytokines were included in health group (17). Milk, ruminal fluid, and blood samples were collected 2 h after morning feeding (4 h after morning milking), according to previously described methods (7).

2.1.2. Analysis of somatic cell counts and N-acetyl-β-D-glucosaminidase in milk

The California mastitis test kit (ImmuCell Corp., Portland, ME, United States) was used to analyze milk CMT reaction on the farm and was followed the manufacturer's instructions. The SCC of quarter milk samples was conducted by a Fossomatic FC instrument (Foss Electric, Hillerød, Denmark). Milk N-acetyl-β-D-glucosaminidase (NAGase) activity was measured with a fluoro-optical method described by Kalmus et al. (18).

2.1.3. Analysis of serum cytokines

The commercial enzyme-linked immunosorbent assay kits (Bovine TNF-alpha and IL-6 DuoSet ELISA kit, R&D Systems, Minneapolis, MN, United States) were used to measure the levels of tumour necrosis factor (TNF)-α and interleukin (IL)-6.

2.1.4. Microbiome analysis

Total genomic DNA was extracted from ruminal fluid samples using the bead-beating method (19). The microbiome analysis adopted the method described by Chuang et al. (7) using the Illumina

HiSeq 2,500 PE250 platform (20–25). The representative sequence for each operational taxonomic units (OTUs) was analyzed through taxonomic annotation (26, 27) and determined the alpha diversity (Chao1 richness estimator and Shannon's diversity index). We used partial least squares discriminant analysis (PLS-DA), and the linear discriminant analysis (LDA) effect size (LEfSe) algorithm to analyze the data (28). The false discovery rate (FDR) was used to carry out multiple testing for the correction of the *p* value using the Benjamini–Hochberg procedure.

2.1.5. Metabolite analysis

The ruminal fluid sample preparation and metabolite analysis adopted the method described by Chuang et al. (7). The orthogonal PLS-DA (oPLS-DA) model with MetaboAnalyst 5.0[1] (29) and the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways using the KEGG database were also analyzed.

2.2. Verification of potential biomarkers related to the health of dairy cows

2.2.1. Bacterial preparation

Bifidobacterium longum subsp. *longum* (BCRC 14664) and *Ruminococcus flavefaciens* (DSM 25089) were purchased from the Bioresource Collection and Research Center (BCRC, Food Industry Research and Development Institute, Hsinchu, Taiwan) and Deutsche Samulung von Mikroorganisem und Zelkultruen (DSMZ, Braunscheig, Germany), respectively. *B. longum* subsp. *longum* was activated three times using the Lactobacilli MRS broth (Lactobacilli de Man, Rogosa and Sharpe broth, Acumedia, Lansing, MI, United States) at 37°C before subsequent analysis. *R. flavefaciens* was cultured under an anaerobic environment at 37°C using the medium formulated by Wang (30).

2.2.2. *In vitro* ruminal fermentation

After the outcomes of veterinary diagnosis, CMT, SCC, NAGase, and serum proinflammatory cytokines, 5 healthy cows (H group) and 5 cows with clinical mastitis (M group) under a similar milk production stage (the first 40- to 60 day lactation) and an average age of 2.75 ± 0.71 years old from same commercial farm mention above were selected as rumen fluid donors. After the morning feeding, 1,500 mL of rumen fluid was obtained as described above. The samples were filtered with four layers of cheesecloth and placed in a flask with thermal insulation (39°C) before being transported to the laboratory. The artificial saliva, prepared according to the description of Menke and Steingass (31), was combined with rumen fluid in a non-oxygen atmosphere. The 40 mL mixture was filled into a 100 mL serum bottle with CO_2 containing 0.4 mg of feed subtract (fresh TMR prepared by the farm) and bacterial culture. Both the H and M groups of rumen fluid were further divided into 4 sub-groups defined as followed: A, with 1 mL of sterile ddH_2O as the control; B, with 1 mL of *B. longum* subsp. *longum* (10^6 CFU/mL) bacterial culture; C, with 1 mL of *R. flavefaciens* (10^6 CFU/mL) bacterial culture; D, with 1 mL of each bacterial culture. For fermentation, the bottles were capped and incubated in a shaking incubator (120 rpm) at 39°C. The fermented fluid was collected at 0, 3, and 12 h.

2.2.3. Qualitative metabolites

The ruminal fluid samples were centrifuged at 13,400 × g for 15 min. The supernatants were analyzed using a Shimadzu LC-20A high-performance liquid chromatography (HPLC) system (Shimadzu, Kyoto, Japan) coupled to a linear ion trap-Orbitrap mass spectrometer (LTQ Orbitrap Velos, Thermo Fisher Scientific, Waltham, MA, United States). The standards of metabolites were used for the qualitative analysis.

2.2.4. Fecal microbiota transplantation

Fresh fecal samples from 15 mastitis and 15 healthy cows which were the same as Section 2.1.1. mentioned were, respectively, collected. The preparation procedure followed the method described by Ma et al. (9). GF mice were obtained and housed according to animal care regulations in the germ-free animal facility at the Animal Resource Center, National Taiwan University (Taipei, Taiwan). A total of 15 female adult (8 week-old) C57BL/6 J mice were randomly divided into three groups, which received 0.3 mL fecal supernatant from (i) healthy cows (Control group), (ii) mastitis cows (Mastitis group), or (iii) mastitis cows, plus 10^8 CFU per day of *B. longum* subsp. *longum* and *R. flavefaciens* administration (M + BR group) for 4 weeks. The animals had measured bodyweight per week to determine the changes in body weight during the experiment period. The three groups of mice were caged in different gnotobiotic isolators after FMT to prevent cross-contamination.

2.3. Statistical analysis

All phenotypic and next-generation sequencing (NGS) data were analyzed with a nonparametric Mann–Whitney U test to identify significant differences between groups. Spearman's correlation analysis was used to conduct the correlation between the relative abundance of biomarkers and metabolites. Statistical Analysis System v9.4 (SAS Institute Inc., Cary, NC, United States) and R software were used for all statistical analysis.

3. Results

3.1. Healthy status of tested dairy cows

Since the healthy status is crucial for this study, the mastitis cows were selected not only by veterinary diagnosis and CMT, but also by the milk SCC and NAGase as well as serum proinflammatory cytokines. The selected 15 mastitis cows demonstrated significantly higher milk SCC ($p < 0.05$) and NAGase ($p < 0.05$) than those of the 15 healthy counterparts (Figure 1). The serum IL-6 in the mastitis cows was also higher than that of healthy cows, which provided a solid foundation for the current study.

3.2. Beta diversity illustrates the dissimilar gut microbiota harbored in ruminants between healthy and mastitis cows

After verifying the healthy status of the cows, we analyzed the ruminal microbiota by NGS. 16S rRNA analysis revealed a total of

1 https://www.metaboanalyst.ca

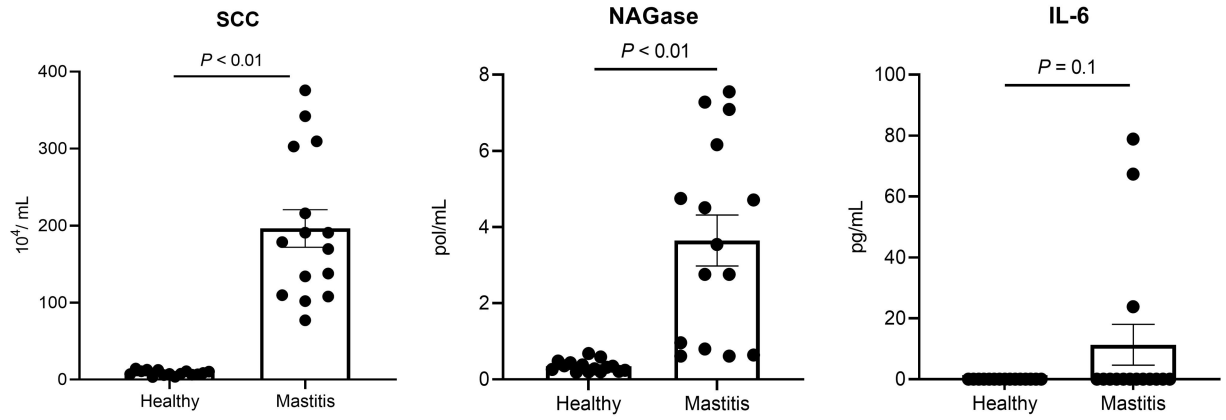

FIGURE 1
Milk somatic cell counts (SCC), NAGase, and serum IL-6 of healthy and mastitis cows.

778,270 and 794,717 effective tags from 1,200,157 and 1,233,089 raw paired-end reads from healthy and mastitis groups, respectively. The Venn diagram in Figure 2A showed that 2,307 OTUs were identical between the groups, with 75 and 95 unique OTUs for the healthy and mastitis groups, respectively. The alpha diversity (Chao1 richness estimator and Shannon's diversity index) revealed no significant difference ($p > 0.05$) between the two groups (Supplementary Figure S1). The top 10 dominant taxa at the genus level, which covered 58% of the total genus level results, were identical between groups but with different proportions (Supplementary Figure S2). Additional beta diversity analysis separated the healthy and mastitis groups using the PLS-DA plot (Figure 2B). PLS1 and PLS2 explained 6.59 and 5.78%, respectively, of the variation in gut microbiota composition, illustrating the dissimilar gut microbiota harbored in ruminants. The predicted phenotypes showed that the healthy group possessed higher relative abundance in stress-tolerant, anaerobic, and Gram-positive bacteria, and lower abundance in Gram-negative and potentially pathogenic bacteria compared to the mastitis counterpart (Figure 2C).

3.3. Identification of the critical ruminal bacterial biomarkers

The beta diversity analysis and predicted phenotypes revealed the dissimilar ruminal bacteria existing between the two groups. Thus, the critical taxa associated with healthy and mastitis groups were then analyzed using the LEfSe algorithm with LDA > 2.5 as the bacterial biomarkers. The results identified 37 influential taxonomic clades, including 17 genera and 3 species (Figure 2D). The most impacted taxa in the healthy group were 12 genera (*Ruminococcaceae* UCG 014, *Eubacterium coprostanoligenes* group, *Eubacterium ruminantium* group, *Ruminococcus* 1, *Syntrophococcus*, *Dialister*, *Pseudobutyrivibrio*, *Desulfovibrio*, *Lachnoclostridium* 12, *Ruminococcaceae* UCG 007, *Peptostreptococcus*, *Mitsuokella*), and 2 species (*R. flavefaciens* and *B. longum* subsp. *longum*). Four genera (*Prevotella* 1, *Prevotellaceae* UCG001, *Prevotellaceae* UCG003, *Fibrobacter*) and one species (unidentified rumen bacterium RNF82) were the critical taxa in the mastitis group.

3.4. Correlation of mastitis parameters with the bacterial biomarkers

After identifying the bacterial biomarkers in both groups, we illustrated the correlation of mastitis parameters (SCC, NAGase, IL-6) with the bacterial biomarkers at the species level (Figure 2E). The species enriched in the healthy group, *R. flavefaciens* and *B. longum* subsp. *longum*, were negatively correlated with the levels of SCC and NAGase. Conversely, the species enriched in the mastitis group, the unidentified rumen bacterium RNF82, demonstrated positive correlations with the levels of SCC and NAGase. The relative abundance of the bacterial biomarkers related to the mastitis group was paralleled with the above findings. The mastitis group demonstrated a significantly lower relative abundance in the *R. flavefaciens* and *B. longum* subsp. *longum* ($p < 0.05$) (Figure 2F).

Phylogenetic investigation of communities by the reconstruction of unobserved states (PICRUSt) was applied to investigate the mastitis-associated functional profiles of microbiome communities (Figure 3). In the mastitis group, 13 functions were enriched, including xenobiotics biodegradation and metabolism, energy metabolism, amino acid metabolism by cysteine and methionine metabolism, neurodegenerative and infectious diseases, replication and repair, transcription and translation. The remaining 18 pathways were depleted, including nucleotide metabolism, lipid metabolism, glycan biosynthesis and metabolism, environmental adaptation, and vitamin B-related metabolic pathways.

3.5. Metabolomics analysis revealed a dissimilar ruminal metabolite composition

In total, 1,181 compounds were identified through blasting, matching the mzCloud online database among 12,709 practicable peaks. A volcano plot showing the metabolite profile of the statistical significance (VIP > 1, FDR adjusted $p < 0.05$ threshold) against fold change revealed the ruminal metabolites with significant differences between the two groups (Figure 4A). Additionally, the oPLS-DA plot showed a clear separation between the healthy and mastitis groups, suggesting a dissimilar ruminal metabolite conformation (Figure 4B).

FIGURE 2
Ruminal bacteria and archaea composition identified by 16S rRNA sequencing of healthy and mastitis cows. **(A)** Venn diagram illustrating 2,307 operational taxonomic units (OTUs) of core microbiota identified both in healthy and mastitis cows. **(B)** Partial least squares discriminant analysis (PLS-DA) plot based on the relative abundance of OTUs indicates a significantly different composition of healthy versus mastitis cows. Ellipses represent 95% confidence intervals for each group. **(C)** Predicted phenotypes. **(D)** Significant differential biomarkers were identified using the LEfSe algorithm. **(E)** Spearman's correlation test between mastitis markers and gut microbial biomarkers at the species level. Each cell was colored corresponding to the Spearman's correlation results. Significant difference: $*p < 0.05$ and $**p < 0.01$. **(F)** Significant relative abundance of differential biomarkers.

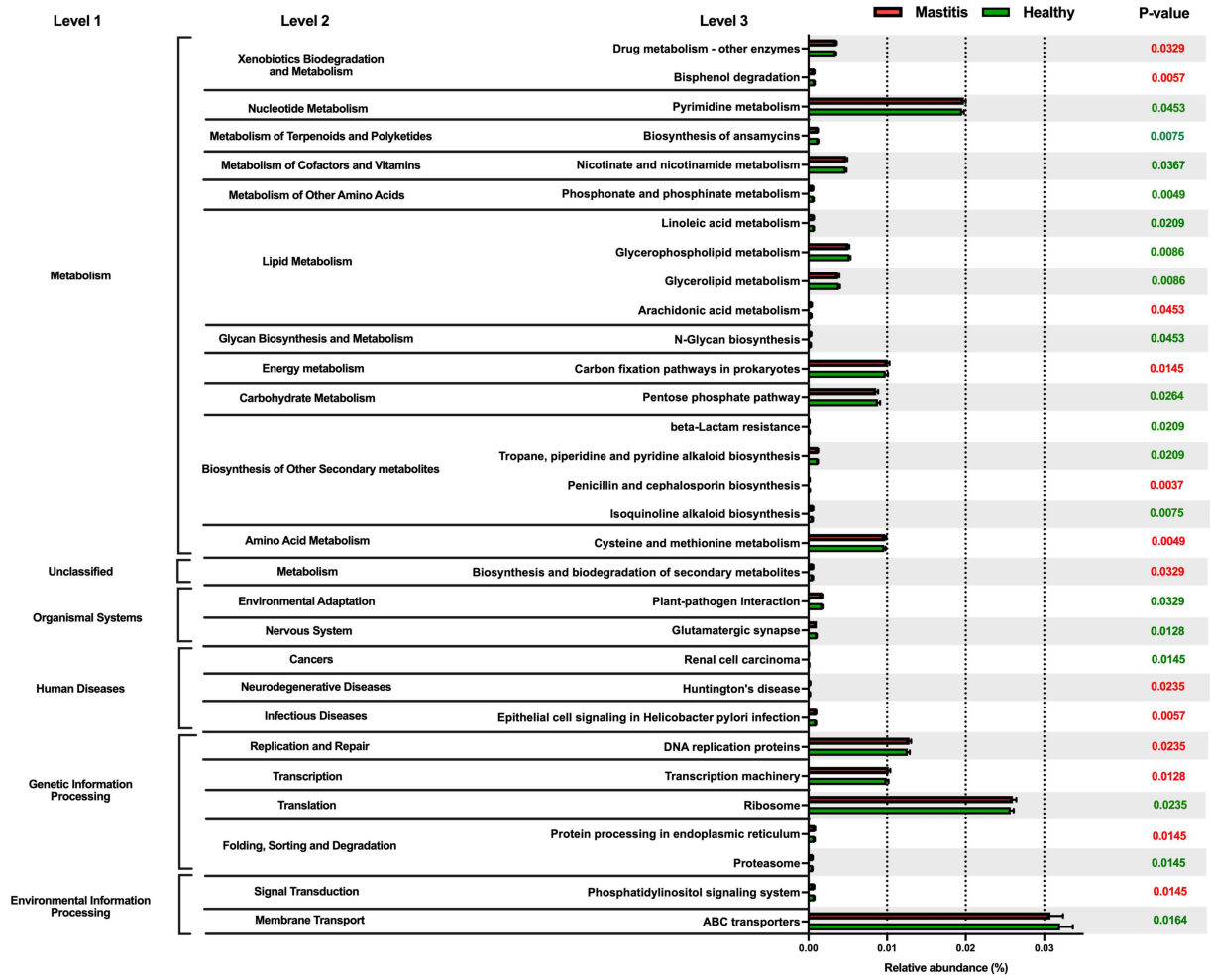

FIGURE 3
Comparison of the relative abundance of the PICRUSt functional prediction of the ruminal microbiota between healthy and mastitis groups. The results are presented as mean ± SEM (n = 15). Distinct gene categories were selected according to significant differences in the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway level 2 (Mann–Whitney U test, $p < 0.01$).

Among identified metabolites, 65 metabolites were significantly different between the two groups (VIP > 1, FDR adjusted $p < 0.05$), of which, 29 were significantly lower in the mastitis group than those in the healthy group (Supplementary Table S1). Further analysis of the metabolic pathway using KEGG (the second level) identified 21 (methionine, putrescine, proline, piperidine, 5-hydroxyindoleacetic acid, N-(2-phenylethyl)-acetamide, 1-pyrroline, 3-acetamidopropanal, nervonyl carnitine, asparaginyl-alanine, triphenylsilanol, 4-aminophenylalanine, 2-phenylbutyramide, pyrophaeophorbide, linoleoyl ethanolamide, 6-pentadecyl salicylic acid, tyr-OEt, 1-(3-aminopropyl)-4-aminobutanal, hexamethylene bisacetamide, 1,5-diphenylcarbohydrazide, and ansamitocin P3) and 16 (carnitine, (alpha)-JWH 073 N-(3-hydroxybutyl) metabolite-d5, (6aR, 11aR)-3-hydroxy-8,9-dimethoxypterocarpan, 2-ethylpyrazine, erucamide, 2-amino-octadecanoic acid, Corey PG-lactone diol, juvenile hormone I, 2,3-dinor-8-iso-PGF2a, 2-acetylpyrazine, hydrocortamate, 9-hydroperoxy-10E, 12-octadecadienoic acid, ethyl 2-furanpropionate, oleoyl ethyl amide, osmundalactone, and 9-decynoic acid) metabolites involved in amino acid and lipid metabolism, respectively. Other pathways such as the metabolism of cofactors and vitamin, nucleotide metabolism, xenobiotic biodegradation metabolism, nicotinate metabolism, carbohydrate metabolism, energy metabolism, nervous system, and replication and repair were also identified.

3.6. Correlation of the ruminal microbiota and metabolome

The reciprocal inter-relationships between 20 ruminal microbial biomarkers and 65 metabolites, which were significantly different between the two groups, were analyzed with the Spearman's correlation test. All the metabolites were significantly correlated with some of the critical bacterial biomarkers ($p < 0.05$) (Supplementary Figure S3). Among them, 3-methylcytosine, 1-(3-aminopropyl)-4-aminobutanal, putrescine, pyridoxal, xanthurenic acid, and Tyr-OEt, are related to amino acid metabolism, replication and repair, and metabolism of cofactors and vitamin (Supplementary Table S1) were significantly positively correlated with the bacterial species biomarkers in the healthy group, *R. flavefaciens* and *B. longum* subsp. *longum* ($p < 0.05$) (Figure 4C). The unidentified

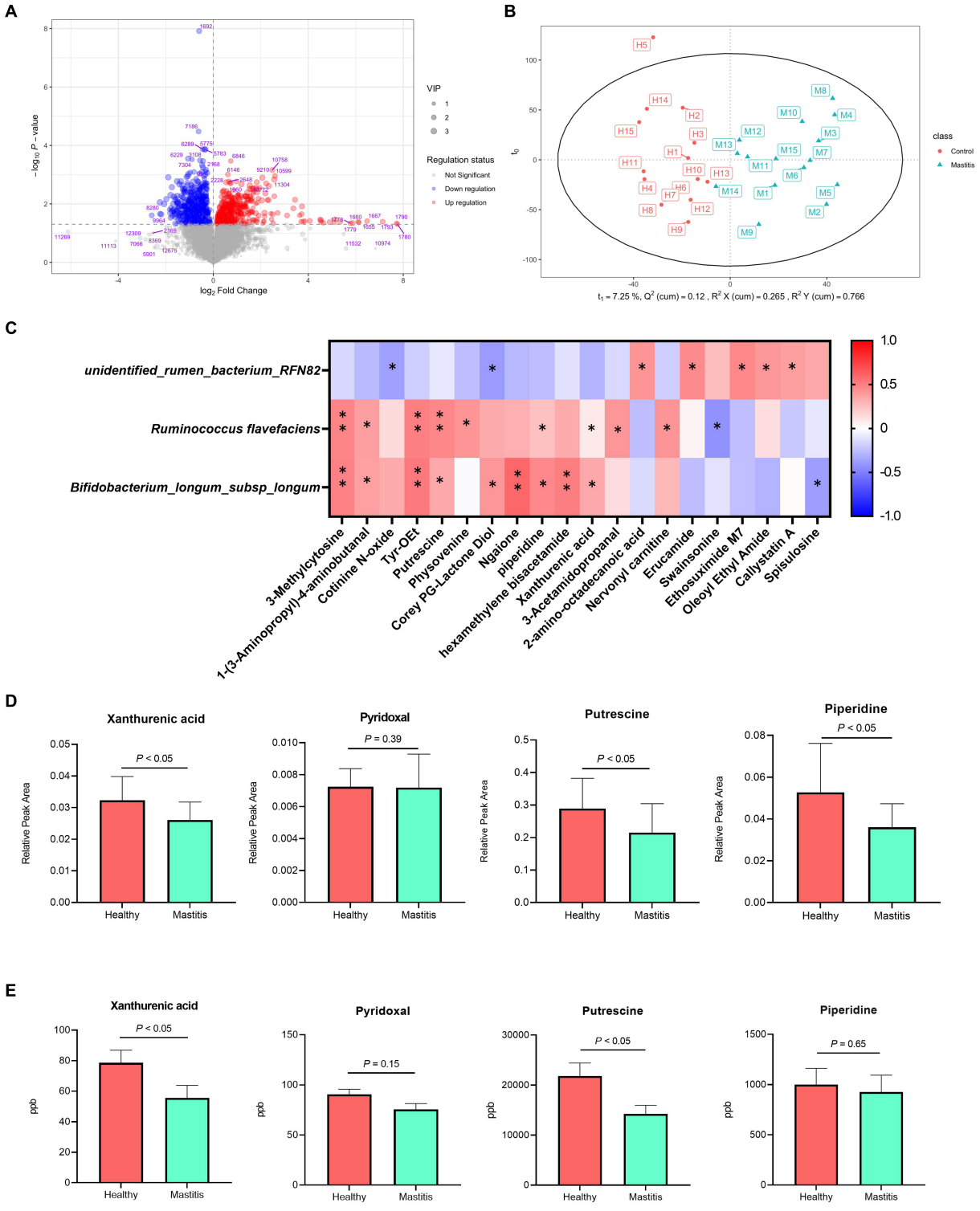

FIGURE 4
Different compositions of ruminal metabolites of healthy and mastitis cows. **(A)** Volcano plot of 1,181 compounds with log-transformed adjusted *p*-values and fold change. The green and red dots indicate significantly higher metabolites in the healthy and mastitis groups, respectively. **(B)** Orthogonal PLS-DA (oPLS-DA) plot based on the 1,181 compounds indicates significantly different metabolite compositions of the healthy and inflammatory groups. Ellipses represent 95% confidence intervals for each group. Every dot represents a single individual cow. **(C)** Reciprocal interrelationships between the ruminal microbiota and metabolome by Spearman's correlation test shown at the species level. Orange and blue colors indicate positive and negative correlation coefficients, respectively. Symbols indicate the significant correlation between metabolites and biomarkers (*$p < 0.05$ and **$p < 0.01$). **(D)** The significant relative peak area and **(E)** concentration of metabolites positively correlated with *Ruminococcus flavefaciens* and *Bifidobacterium longum* subsp. *longum*.

rumen bacterium RNF82, the bacterial biomarkers in the mastitis group, were negatively correlated with cotinine N-oxide, and Corey PG-lactone diol ($p < 0.05$) and positively correlated with 2-amino-octadecanoic acid, erucamide, and ethosuximide M7 (Figure 4C). By further quantifying the ruminal metabolites, the higher relative peak area of three metabolites, xanthurenic acid, pyridoxal, and putrescine in the healthy group (Figure 4D) demonstrated significantly higher concentrates in the ruminal fluid samples compared with the mastitis counterpart, verifying the ruminal metabolomic finding (Figure 4E).

3.7. Verification of the health-promoting effect of *Ruminococcus flavefaciens* and *Bifidobacterium longum* subsp. longum by FMT GF mice

To verify the healthy promoting effect of *R. flavefaciens* and *B. longum* subsp. *longum*, fecal microbiota from the 15 mastitis and 15 healthy cows were, respectively, pooled and inoculated into GF mice. The results showed that the mice transplanting gut microbiota from mastitis cows could decrease the survival rate and bodyweight gain compared with that from healthy cows (Figure 5). Administration of *R. flavefaciens* and *B. longum* subsp. *longum* could increase body weight gain and reduce mortality rate.

3.8. Verification of inter-relationships between microbiota and metabolome *in vitro*

We further investigated the inter-relationships between microbiota and metabolome by *in vitro* ruminal fermentation. First, the ruminal fluid pH value during a 12 h *in vitro* fermentation was above 6.0 in both the healthy and mastitis group. The mean pH was 6.72 (HA), 6.84 (HB), 6.83 (HC), 6.87 (HD), 6.95 (MA), 6.91 (MB), 7.00 (MC), and 7.02 (MD). The basic composition of the ruminal fluid in all groups had no significant change except NH_3-N (Table 1). *R. flavefaciens* and *B. longum* subsp. *longum* addition demonstrated the trend to upregulate the levels of putrescine, xanthurenic acid, and pyridoxal in the mastitis ruminal fluid (Figure 6), which confirmed the inter-relationships between microbiota and metabolome. Additionally, the levels of total volatile fatty acids (VFA) were upregulated after inoculation with *R. flavefaciens* (HC and MC groups) and *R. flavefaciens* + *B. longum* subsp. *longum* (HD and MD groups) (Table 2). As supplement of *B. longum* subsp. *longum*, the relative amount of lactate would increase (HB and MB groups).

4. Discussion

In the present study, we verified the inter-relationship of microbiome and metabolome biomarkers to potentially promote the health of dairy cows. First, a stable and resilient core microbiota in the ruminal fluid of the lactating cow w/wo mastitis was observed based on the results of the Venn diagram, Shannon, Chao1, and the relative abundance of the taxa at the different levels. The genera *Prevotella* 1, *Ruminococcaceae* NK4A214 group, and *Christensenellaceae* R7 group, the top three predominant genera in both healthy and mastitis groups, were also the abundant genera in the rumens of lactating cows, dry period cows (32), yak (33), and mastitis cows (7). These three genera play important roles in protein degradation and lipid biohydrogenation (3), SCFA production by the breakdown of fibrous plants (34), and microbial inhibiting activities (35). The *Ruminococcaceae* NK4A214 group was also positively correlated to milk total solids in lactating cows (36).

Although the bovine rumen demonstrated a highly similar microbial composition, the differences in taxa and individual bacterial abundance still existed between the lactating cows w/wo mastitis, which could effectively distinguish these two groups using the PLS plot and unweighted UniFrac. The ruminal microbiota of mastitis cows was characterized by high Gram-negative and potentially pathogenic bacteria. KEGG pathways with higher abundance in mastitis cows, including xenobiotics acid biodegradation and metabolism, energy metabolism, replication and repair, transcription, translation, and infectious disease, can be related to the inflammation and mucosa repairing in cows (37). The genera *Prevotella* (*Prevotella* 1, *Prevotellaceae* UCG001, *Prevotellaceae* UCG003) and *Fibrobacter* were the biomarkers associated with mastitis cows. *Prevotella* with

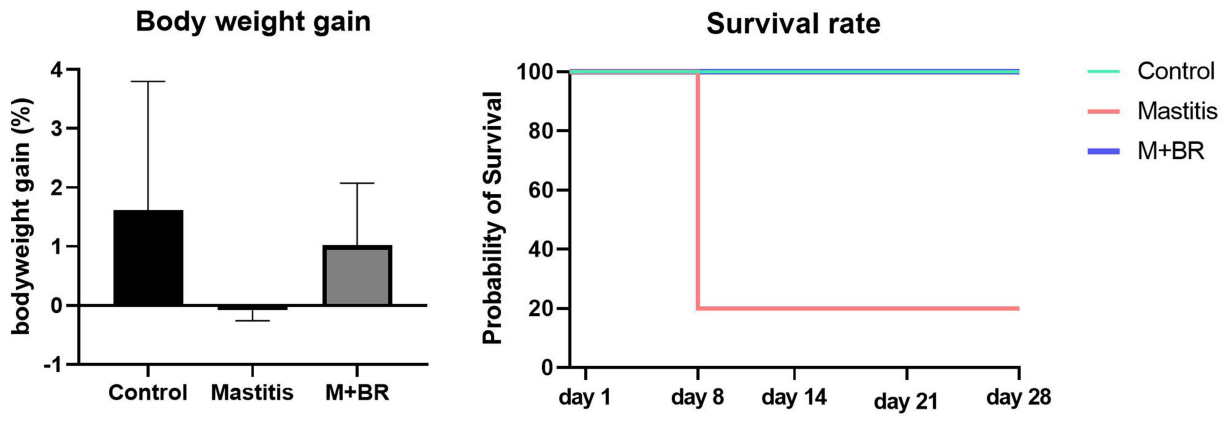

FIGURE 5
Survival rate and body weight gain after the fecal microbiota transplantation (FMT) test and bacterial biomarker supplements.

diverse isoforms is crucial for ruminal fermentation (38); however, the high abundance of this genus was associated with high-grain feed (39) as well as acidosis (40). The studies in humans and animals have connected the increased abundance of *Prevotella* species at mucosal sites with localized and systemic inflammation disease due to enhancing T helper type 17 (Th17)-mediated mucosal inflammation via IL-8, IL-6, and CCL20 stimulation (41). The increasing serum IL-6 in mastitis cows in this study may partially support the ruminal dysbiosis leading to systemic inflammatory effect.

Conversely, the ruminal microbiota associated with healthy cows was categorized by high-stress tolerant, Gram-positive, and SCFA producing bacteria. This finding was later confirmed by *in vitro* ruminal fermentation. Increasing VFA and SCFA with healthy ruminal liquid were due to the high SCFA producing bacteria. The reduction of SCFA producing bacteria in ruminal fluid (7) and feces (9) of mastitis cows has been reported. Higher KEGG pathway in lipid and carbohydrate metabolisms in healthy cows also suggested reduced lipid and carbohydrate metabolic activities of the gut microbiota in mastitis cows. Our finding was in line with a study indicating that carbon metabolism was less abundant in mastitis cows (9). Downregulation of carbohydrate metabolism may alter the glucose and carbohydrate balance in the body (42), which affects the energy for maintenance, growth, and production in farm animals (43).

The biomarkers identified in the healthy group, including the genera *Ruminococcus*, *Eubacterium*, *Lachnoclostridium*, and *Pseudobutyrivibrio*, known as cellulose and fiber degraders (44), associated with high-yield cows (45). The genus *Syntrophococcus* has been reported to utilize sugars and H_2-CO_2-using methanogens as electron donors to produce acetate and as an electron acceptor, respectively (46). At the species level, *R. flavefaciens* could modify the abundance of other cellulolytic bacterial populations (47) and improve the feed efficacy for ruminants (48). Another species, *B. longum* subsp. *longum*, a biomarker in the healthy group, was also reported to stabilize gut microbiota and improve the intestinal environment (49). Both species recognized in the healthy cow could be potential probiotics to promote animal health, which warrants further investigation.

From the *in vitro* ruminal fermentation, additional inoculation of *R. flavefaciens* and *B. longum* subsp. *longum* could significantly impact the levels of NH_3-N, total VFA, and VFA profiles in both healthy and mastitis groups. NH_3-N is the main nitrogen source used by microbes to synthesize amino acid and peptide bonds for growth (50). *Ruminobacter* spp. is a hyper-ammonia producing (HAP) bacteria (51). The increase in NH_3-N could be explained by the inoculation of *R. flavefaciens*, which leads to an increased population of HAP bacteria and deaminase activity. Total VFA and VFA profiles are important products of the bacterial fermentation activity in the rumen, which have emerged as key regulators in intestinal and energy homeostasis regulation (52). *R. flavefaciens* participates in the butyrate metabolic pathway (53, 54). Upregulating the VFA concentration has been reported in repeated ruminal dosing of *R. flavefaciens* in dairy cows (47). The increase in VFA suggested an upregulating deamination activity (55) by the addition of *R. flavefaciens*. The increase in lactate is expected with additional *B. longum* subsp. *longum*, a lactic acid producer. The administration of lactic acid bacterial probiotics is thought to help rumen microbiota adapt to the presence of lactic acid (56) and prevent lactate accumulation in the rumen (57). Nevertheless, the increase in VFA did not have much physiological impact as the rumen pH due to shifting the microbiota to lactate-consuming bacteria.

TABLE 1 Supplement of *Ruminococcus flavefacians* and *Bifidobacterium longum* subsp. *longum* on the digestibility of dietary nutrients in *in-vitro* fermentation.

Items[a]	HA[b]	HB	HC	HD	SE[c]	*p*-value
DM (%)	66.15	66.90	66.65	66.47	0.60	0.384
NDF (%)	59.76	60.45	60.58	60.02	0.52	0.153
ADF (%)	56.44	57.90	57.27	55.80	1.13	0.094
NH_3-N (mg/dL)	17.50[c]	17.79[c]	30.97[b]	32.32[a]	2.86	0.001

	MA[d]	MB	MC	MD	SE	*p*-value
DM (%)	67.22	67.03	66.27	67.28	0.93	0.424
NDF (%)	59.81	59.23	58.25	58.58	1.94	0.683
ADF (%)	56.32	56.35	54.74	54.46	1.85	0.355
NH_3-N (mg/dL)	16.39[d]	23.03[c]	29.03[b]	33.13[a]	4.15	0.001

[a]DM, dry matter; NDF, neutral detergent fibre; ADF, acid detergent fibre.
[b]HA, healthy group, without additional bacterial culture; HB, healthy group, with *B. longum* subsp. *longum*; HC, healthy group, with *R. flavefaciens*; HD, healthy group, with *B. longum* subsp. *longum* and *R. flavefaciens*.
[c]SE, standard error.
[d]MA, mastitis group, without additional bacterial culture; MB, mastitis group, with *B. longum* subsp. *longum*; MC, mastitis group, with *R. flavefaciens*; MD, mastitis group, with *B. longum* subsp. *longum* and *R. flavefaciens*.
[a–d]Values within a row with different superscripts differ significantly at $p < 0.05$.

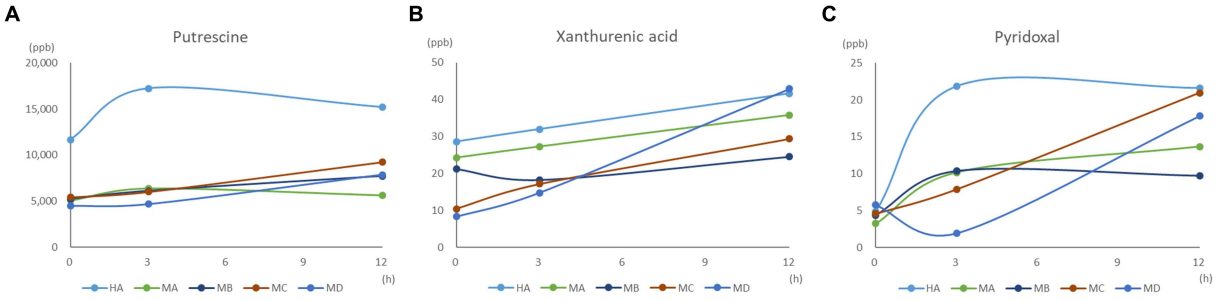

FIGURE 6
Effect of bacterial biomarker supplements on **(A)** putrescine, **(B)** xanthurenic acid, and **(C)** pyridoxal concentration during the 12 h *in vitro* fermentation.

TABLE 2 Effects of bacterial biomarker supplements on the concentration of volatile fatty acids (VFA) and lactic acid in healthy and mastitis groups after 12 h of *in vitro* fermentation.

Items[a]	HA[b]	HB	HC	HD	SE[c]	*p*-value
VFA (mM)						
Acetate	23.26[b]	24.57[b]	30.28[a]	31.89[a]	1.27	0.001
Propionate	7.86	7.61	8.31	8.95	0.74	0.109
Butyrate	4.16	4.23	4.53	4.97	0.48	0.120
Isobutyrate	0.10[b]	0.13[b]	0.14[b]	0.18[a]	0.03	0.010
Valerate	0.29	0.44	0.46	0.48	0.13	0.191
A/P	2.96[c]	3.23[b]	3.66[a]	3.61[a]	0.31	0.026
Total VFA	39.79[d]	42.73[c]	47.09[b]	50.64[a]	1.85	<0.0001
Lactate	3.70[b]	5.34[a]	2.86[c]	3.67[b]	0.60	0.001
	MA[d]	MB	MC	MD	SE	*p*-value
VFA (mM)						
Acetate	22.32	28.57	29.08	28.63	3.64	0.065
Propionate	7.85[b]	7.93[b]	10.81[a]	8.38[b]	1.27	0.019
Butyrate	4.67	4.20	5.26	5.93	0.88	0.079
Isobutyrate	0.08[b]	0.14[ab]	0.15[ab]	0.17[a]	0.02	0.001
Valerate	0.30	0.34	0.32	0.33	0.05	0.693
A/P	2.94	3.72	2.68	3.46	0.93	0.1797
Total VFA	37.69[c]	44.93[b]	48.13[a]	46.84[ab]	4.14	0.017
Lactate	2.12[c]	3.35[a]	2.11[c]	2.93[b]	0.42	0.003

[a]VFA, volatile fatty acid; A/P, acetate/propionate.
[b]HA, healthy group, without additional bacterial culture; HB, healthy group, with *B. longum* subsp. *longum*; HC, healthy group, with *R. flavefaciens*; HD, healthy group, with *B. longum* subsp. longum and *R. flavefaciens*.
[c]SE, standard error.
[d]MA, mastitis group, without additional bacterial culture; MB, mastitis group, with *B. longum* subsp. *longum*; MC, mastitis group, with *R. flavefaciens*; MD, mastitis group, with *B. longum* subsp. *longum* and *R. flavefaciens*.
[a–d]Values within a row with different superscripts differ significantly at $p < 0.05$.

FMT verified the inter-relationship among gut dysbiosis, systemic inflammatory effect, and health-promoting ability of two microbial biomarkers, *R. flavefaciens* and *B. longum* subsp. *longum*. Ma et al. (9) found that FMT from diseased cows caused mastitis-like symptoms in mice by shifting the murine intestinal microbiota. Although the acute inflammation led to mice mortality after FMT with mastitis feces, which could not provide solid evidence between mastitis and the dysbiosis of ruminal microbiota, the findings confirmed the impact of gut microbiota as one potential parameter affecting dairy cow health. The increase in the survival rate after FMT of mastitis feces with *R. flavefaciens* and *B. longum* subsp. *longum* also supported the potential efficacy of microbial biomarkers as probiotic treatment, which may point to a new health-promoting and disease-preventing approach.

Besides the microbiota, bacterial products, in turn of metabolome, were also a key factor involved in bovine health and systemic disease outcomes. Three metabolites (i.e., putrescine, xanthurenic acid, and pyridoxal) were verified by further *in vitro* ruminal fermentation and HPLC qualitative analysis, which were positively correlated with two species biomarkers, *R. flavefaciens* and *B. longum* subsp. *longum*. Putrescine, a biogenic amine produced from the decarboxylation of amino acids by decarboxylase in certain intestinal microorganisms (58), has been found in rumen fluid of healthy animals, which was in line with our findings. Putrescine has antioxidant and anti-inflammatory attributes (59) and is involved in the growth of tissues and organs (60). Xanthurenic acid, a non-indolic catabolite of tryptophan and a metabolite of the kynurenine pathway (61), demonstrated profound effects on the gut microbial composition, host-microbiome interface, and host immune system–intestinal microbiota interactions. Pyridoxal is one of the natural forms available of vitamin B6, supplied either in the diet or by rumen or intestinal symbiosis for bovine species. Vitamin B6 participates in DNA, RNA and protein synthesis. Mastitis is associated with a vitamin B metabolism disorder in intestinal microbiota (9). The upregulated putrescine, xanthurenic acid, and pyridoxal in ruminal fluid after adding *R. flavefaciens* and *B. longum* subsp. *longum* suggested a health effect on modulating intestinal homeostasis and damage repair.

5. Conclusion

Although the bovine rumen possesses a strong core microbial composition, we proved that minor microbiota shifting caused by mastitis could affect the health of dairy cows. This influence is not only because of the rumen microbiota but the downstream microbiome produced by microbiota also plays an important role in health. To the best of our knowledge, this study is the first to verify the inter-relationship of microbiome and metabolome biomarkers for the potential to promote health in dairy cows. Two species, *R. flavefaciens* and *B. longum* subsp.

longum, with three metabolites, putrescine, xanthurenic acid, and pyridoxal, were identified in the ruminal fluid, which may point to a new direction to promote health and prevent disease in dairy cattle.

Data availability statement

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

Ethics statement

The animal studies were approved by Institutional Animal Care and Use Committee of National Taiwan University (IACUC approval no: NTU-107-EL-00221). 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

S-TC and M-JC conceived and designed the experiments. J-CH, S-TC, K-YL, Y-TH, and S-TH performed the experiments. J-CH, K-YL, Y-TH, and S-HC analyzed the data. J-CH, S-TC, and M-JC wrote and revised the manuscript. All authors contributed to the article and approved the submitted version.

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Conflict of interest

S-HC was employed by Biotools 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.

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Supplementary material

The Supplementary material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fvets.2023.1228086/full#supplementary-material

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The effects of differential feeding on ileum development, digestive ability and health status of newborn calves

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Pre-weaning is the most important period for the growth and development of calves. Intestinal morphology, microbial community and immunity are initially constructed at this stage, and even have a lifelong impact on calves. Early feeding patterns have a significant impact on gastrointestinal development and microbial communities. This study mainly analyzed the effects of three feeding methods on the gastrointestinal development of calves, and provided a theoretical basis for further improving the feeding mode of calves. it is very important to develop a suitable feeding mode. In this study, we selected nine newborn healthy Holstein bull calves were randomly selected and divided into three groups ($n = 3$), which were fed with starter + hay + milk (SH group), starter + milk (SF group), total mixed ration + milk (TMR group). After 80 days of feeding Feeding to 80 days of age after, the ileum contents and blood samples were collected, and the differences were compared and analyzed by metagenomic analysis and serum metabolomics analysis. Results show that compared with the other two groups, the intestinal epithelium of the SH group was more complete and the goblet cells developed better. The feeding method of SH group was more conducive to the development of calves, with higher daily gain and no pathological inflammatory reaction. The intestinal microbial community was more conducive to digestion and absorption, and the immunity was stronger. These findings are helpful for us to explore better calf feeding patterns. In the next step, we will set up more biological replicates to study the deep-seated reasons for the differences in the development of pre-weaning calves. At the same time, the new discoveries of neuro microbiology broaden our horizons and are the focus of our future attention.

KEYWORDS

calves, feeding patterns, metagenomics, metabolomics, neuro microbiology

1. Introduction

Pre-weaning is the most important period for the growth and development of calves. Intestinal morphology, microbial community and immunity are initially constructed at this stage, and even have a lifelong impact on calves (1, 2). The living environment of the calf changed from a sterile uterus to the outside world, and the nutritional conditions changed from maternal provision to the calf's own feeding of feed and milk. Due to the incomplete development of the immune ability and digestive system of calves, newborn calves are very sensitive to external stimuli, and any external interference will seriously affect the growth of calves (3, 4). If the

appropriate feeding mode is not adopted at this time, it will lead to low daily weight gain, diarrhea (5). It is reported that 30% of the deaths of pre-weaning cows are caused by diarrhea, which highlights the importance of intestinal health (6). The development of the digestive tract of calves is a unique process. With the development of the gastrointestinal tract and the settlement of the microbial community, calves gradually change from pseudo-ruminant animals to functional ruminants in physiology. The morphology of intestinal epithelial villi, the development of intestinal smooth muscle and rumen volume are basically completed at this stage which was ac-companied by the development of salivary organs and the development of rumination behavior. These factors directly affect the feed intake, nutrient absorption rate and digestive ability of calves after weaning (7).

Studies have shown that early feeding patterns have a significant impact on gastrointestinal development and microbial communities. Differences in dietary composition and feed physical morphology will stimulate intestinal structure development and microbial community composition (8–10). For example, alfalfa hay can promote the development of intestinal epithelial villi; the feeding of high carbohydrate and low fiber feeding will be detrimental to the rumen development of calves; additives can directly or indirectly increase the proportion of probiotics (11–13). Research shows that intestinal microbial community can be directed to change, adjusting feeding patterns can help animals form beneficial microbial communities before weaning (14). This can not only promote the development of calf digestive ability, but also is indispensable for the development of intestinal immune function. Deng's research on giant pandas has proved that *Streptococcus alactolyticus* can promote the dietary adaptation of giant pandas by participating in protein metabolism (15). Recent studies have also found that microbial communities also communicate biological information with the nervous system, it points to a new field: neuro microbiology, this further illustrates the huge impact of gut microbes on the host (16).

There is no fixed feeding mode for newborn calves. Appropriate feeding mode should be comprehensively formulated according to their nutritional needs, breed characteristics, feeding plan and other factors (17). For calves at this stage, growth is important, but more importantly, the healthy development of the digestive system based on the gastrointestinal tract. This will have a long-term impact on the subsequent growth and production of calves (18, 19). Therefore, it is very important to develop a suitable feeding mode. In this study, three different feeding modes were used to explore the effects of different feeding modes on the growth and development of ileum and blood metabolism of calves from the perspective of metagenomics and metabolomics. The relationship between intestinal development, digestive function and microbial community composition was explored to provide a theoretical basis for further improving the feeding pattern of calves. At the same time, it inspired us to explore the relationship between intestinal microorganisms and the nervous system from a new perspective of neurobiology.

2. Materials and methods

2.1. Ethics statement

This study was approved and conducted in accordance with the ethical standards of the Institutional Animal Care and Use Committee of the College of Animal Science and Technology, Sichuan Agricultural University, Sichuan, 611130, China.

2.2. Animals and feeding management

Nine 7-day-old healthy male Holstein calves with a body weight of about 41.6 kg (standard deviation = 0.563) and similar physical condition were selected. Raised in Sichuan Xuebao Dairy Group HonSFeng cattle farm. Nine calves were randomly divided into three groups, SH group: starter feed + hay + milk, SF group: starter feed + milk, TMR group: total mixed ration + milk. The feed composition and nutrient composition of the starter feed are shown in Table 1. The hay in the SH group was composed of alfalfa and oat grass in a ratio of 3:2, cut into 1.5 cm long, mixed with starter and fed. TMR feed (starter: alfalfa: oat grass: water = 0.30: 0.12: 0.08: 0.50), the starter was crushed into powder, the hay was cut to about 1 cm, and mixed with water to paste. Other feeding and management methods were carried out according to the existing methods of cattle farms, and calves were free to feed and drink water during the experimental period. Continuous feeding to 80 days of age, using electric shock bloodletting method to slaughter and record the slaughter weight.

2.3. Serum sample collection and partial physiological index detection

After the calves were fasted for 24 h, the external jugular vein blood collection method was adopted (June 10, 2022), and 5 mL blood collection vessels (EDTAK2, Jiangsu Kangjian Medical Device Co., Ltd., Nanjing, China) were used to collect some calf blood, and

TABLE 1 The feed composition and nutrient composition of the starter feed.

Component	Proportion (%)
Corn	61.16
Soybean meal	30.05
Compound vitamins	0.03
Trace elements	0.4
CaHCO3	0.67
CaCO3	1.65
50% choline chloride	0.1
Soybean oil	4.02
L-lysine	0.11
DL-methionine	0.43
Mineral additives	1.39
Nutritional ingredient	
NE,Mcal/kg	8.49
CP	17.73
Ca	3.10
Available phosphorus	0.79
Lys	0.99
Met	0

heparin sodium was used for anticoagulation. Some blood samples were taken to determine blood routine indexes such as red blood cell count and white blood cell count (Chengdu Li Lai Biotechnology Co., Ltd., Chengdu). The blood samples were centrifuged at 4°C, 3000 r / min for 5 min to obtain the upper serum. Some serum samples were sent to Novogene Bioinformatics Technology Co., Ltd. (Beijing, China) for metabolomics analysis, and then some serum samples were selected to determine digestive and immune indexes such as α-amylase (C016-1-1), lysozyme (A050-1-1) and trypsin (A080-2; the kits were provided by Nanjing Jiancheng Bioengineering Institute).

2.4. Ileum morphological section analysis and sample collection

Slaughter of calves by electric bloodletting. The calves get up in the morning, use electric shock anesthesia after fasting blood collection, and then slaughter by carotid artery bloodletting. After slaughter, the contents of the ileum of the calf were quickly placed in a 2 mL frozen tube and stored in liquid nitrogen at −80°C. The unsealed ileum tissue was collected and fixed with 10% neutral formaldehyde solution. After dehydration, pruning, embedding, slicing, staining with hematoxylin and eosin (HE), sealing and other steps, the Pannoramic 250 digital slice scanner produced by Hungary 3DHISTECH company was used to collect images of the slices.

2.5. Microbial metagenomic sequencing and functional annotation analysis

According to the Tiangen Magnetic Bead Kit (Tiangen Biotech, Beijing, China) instructions, microbial DNA was extracted from ileum content samples, and the purity and integrity of DNA were detected by agarose gel electrophoresis. The qualified DNA samples were broken into fragments of about 350 bp by ultrasonic crushing instrument. After repair, purification, PCR amplification and other steps, the preliminary database was completed. Qubit2.0 was used for quantification, and then NEBNext ® Ultra DNA Library Prep Kit for Illumina (NEB, USA) was used to construct a metagenomic library and sequenced on the Illumina HiSeq Xten platform. The library construction and sequencing work was completed by Novogene Bioinformatics Technology Co., Ltd. (Beijing, China). The clean date was obtained by filtering the raw date obtained by sequencing, and the clean date of each sample was assembled and analyzed by MEGAHIT software (20, 21) (v1.0.4-beta[1]). Then MetaGeneMark software (22) (V2.10[2]) was used for gene prediction, and the genes predicted by each sample were put together to construct gene catalogue. Starting from the gene catalogue, the clean data of each sample was synthesized to obtain unigenes for subsequent analysis (23).

The obtained unigenes were compared with the NCBI's NR database (24) (Version 2018-01-02[3]) to determine the species annotation information of each unigenes. Combined with the gene abundance table, the abundance information of each sample in phylum, genus and species was obtained. PCA (25) (RADE4 package, version 2.15.3) and LEfSe (26) (LDA score default to 3) analysis were used to compare the different species between groups. Finally, unigenes were compared with KEGG database (27, 28) (version 2018-01-01[4]), CAZy database (29) (version 201,801[5]) and eggNOG database (30) (version 4.5[6]) to obtain the relative abundance and functional annotation differences between groups (31).

2.6. Serum metabolomics analysis

The metabolites in the ileum were studied based on LC–MS technology. After preliminary treatment, the supernatant was injected into the ultra-performance liquid chromatography–tandem mass spectrometry (UHPLC–MS/MS) system for analysis (32). Firstly, the raw data of mass spectrometry were imported into Compound discoverer 3.1 software for spectral processing and database retrieval, and the qualitative and quantitative results of metabolites were obtained. Then, the quality of data was controlled to ensure the accuracy and reliability of the data. Using high-resolution mass spectrometry (HRMS) technology, we can make the non-target metabolic group as much as possible to detect the molecular characteristic peaks in the sample. The raw data after offline is preprocessed by CD3.1 data processing software. In order to make the identification accurate, we extract the peaks according to the set of ppm, signal-to-noise ratio (S/N), additive ions, and other information and quantify the peak area. Then mzCloud, mzVault, and MassList databases were compared to identify metabolites. Finally, metabolites with a coefficient of variation of less than 30% in QC samples were retained as the final result. The metabolites were compared with KEGG, HMDB, and other databases to obtain the annotation results. Then, a multivariate statistical analysis of metabolites was performed, including principal component analysis (PCA), partial least squares discriminant analysis (PLS-DA) to establish the relationship between the expression of metabolites and samples (33). According to the results of Q2 and R2, the model was judged to reveal the differences in metabolic patterns between different groups. KEGG enrichment pathway analysis was performed on the differential metabolites to obtain clearer and more detailed differential analysis results.

3. Results

3.1. HE staining section of ileum tissue

The results of HE staining are shown in Figure 1. Compared with the other two groups, the epithelial villi in the SH group were relatively complete, the intestinal epithelial villi were high and well developed, the goblet cells were closely arranged, and some inflammatory cells were seen in the lamina propria. There were a large number of epithelial mucosal shedding in the SF group, and the inflammatory cells in the lamina propria were also more than those in the SH group, accompanied by congestion; the mucosal integrity of the TMR group

1 https://github.com/voutcn/megahit
2 http://topaz.gatech.edu/GeneMark/
3 https://www.ncbi.nlm.nih.gov/
4 http://www.kegg.jp/kegg/
5 http://www.cazy.org/
6 http://www.plob.org/tag/eggnog/

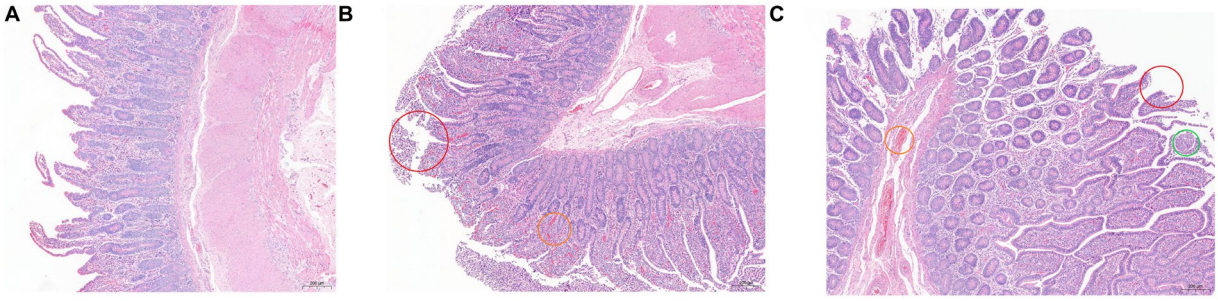

FIGURE 1
Hematoxylin and eosin (100×) -stained ileal tissue samples of Holstein calves fed different diets. **(A)** SH group; **(B)** SF group; **(C)** TMR group. Red circle marked mucosal epithelial shedding; the orange circle marked congestion; green circle marked as parasitic lesions.

was the worst, accompanied by inflammatory cells and congestion, and even obvious parasitic infection was observed in some sections.

3.2. Daily weight gain and some physiological indexes test results

In this experiment, 9 newborn, healthy and healthy male calves with no significant difference in birth weight were selected. The birth weight was about 41 kg. When the calves were slaughtered at 80 days of age, there were significant differences in weight among the three groups ($p = 0.002$). The average weight of SH group and SF group TMR group was 100.62 ± 1.94, 100.07 ± 1.92 and 90.77 ± 2.42. And the daily weight gain was 0.745 kg / day, 0.740 kg / day and 0.604 kg / day, respectively.

In Figure 2, some blood routine data. The results showed that the number of white blood cells SH was significantly less than that of SF group and TMR group, which may be due to the more severe inflammatory response in SF group and TMR group. The number of red blood cells in the TMR group was the highest. The hemoglobin content in the TMR group was also the highest in the three groups. The results of digestive enzymes and immunity showed that the contents of amylase, trypsin and lysozyme in SH were the highest in the three groups. The IgG content of SH group was also the highest in the three groups. These indicators show that the digestive ability and immune ability of SH group are higher than the other two groups.

3.3. Ileum microbial metagenomic data processing

As shown in Table 2, a total of 112,964.83 raw data were measured in 9 samples, and 112,791.07 clean data were obtained after Illumina pretreatment and filtering, with an average CG content of 45.87% and an effective value of more than 99.85%. The average length of scaftigs of 9 samples was 1434.85.

3.4. Analysis of correlation between groups and differences in microbial community

Based on the number of genes, correlation analysis was performed on 8 samples. The results are shown in Figure 3. The correlation between SF group and SH group was high, but the correlation between TMR group and SF group and SH group was low.

The processed genes were compared with the database to obtain the species annotation information of each sequence and compared after classification at each level. The results are shown in Figure 4. The dominant bacteria in the three groups were Firmicutes under the phylum level classification. The subdominant bacteria in group SF were *Proteobacteria* and *Chlamydiae*. The subdominant flora in SH group was *Actinobacteria*, more than the other two groups. The subdominant flora in the TMR group was *Proteobacteria*. Under the genus level classification, the dominant flora of group SF was *Clostridium* and *Chlamydia*; the dominant flora in SH group was *Olsenella*, which was more than that in TMR group and SF group, and the secondary dominant flora was *Clostridium*. The dominant flora in the TMR group was *Clostridium* secondary dominant flora *Sarcina* and more than that in the SF and SH groups.

The results of PCA analysis showed that under the classification of phylum level and genus level, the intra-group difference of SH group was the smallest, the intra-group difference of SF group was slightly larger than that of SH group, and the intra-group difference of TMR group was the largest. There were significant differences among the three groups, and the intra-group difference of TMR was greater than the inter-group difference, which indicate that after feeding the total mixture, the ileum microbes of calves were greatly different. In order to screen species with significant differences between groups, the results of LDA analysis (Figure 5) showed that the main differences between SH group and TMR group were p _ Firmicutes and o _ *Mycoplasmatales* in SH group and p _ *Sarcina* in TMR group. The main differential flora between SF group and TMR group were o _ *Veillonellaceae*, g _ *Prevotella* in SF group and p _ *Sarcina* in TMR group.

3.5. Functional analysis of differential species

The relative abundance of KEGG pathway enrichment in the three groups was analyzed (Figure 6). It was found that among the 45 pathways under the second level classification, the SH group was mainly enriched in carbohydrate metabolism, followed by amino acid metabolism, translation and membrane transport; the dominant

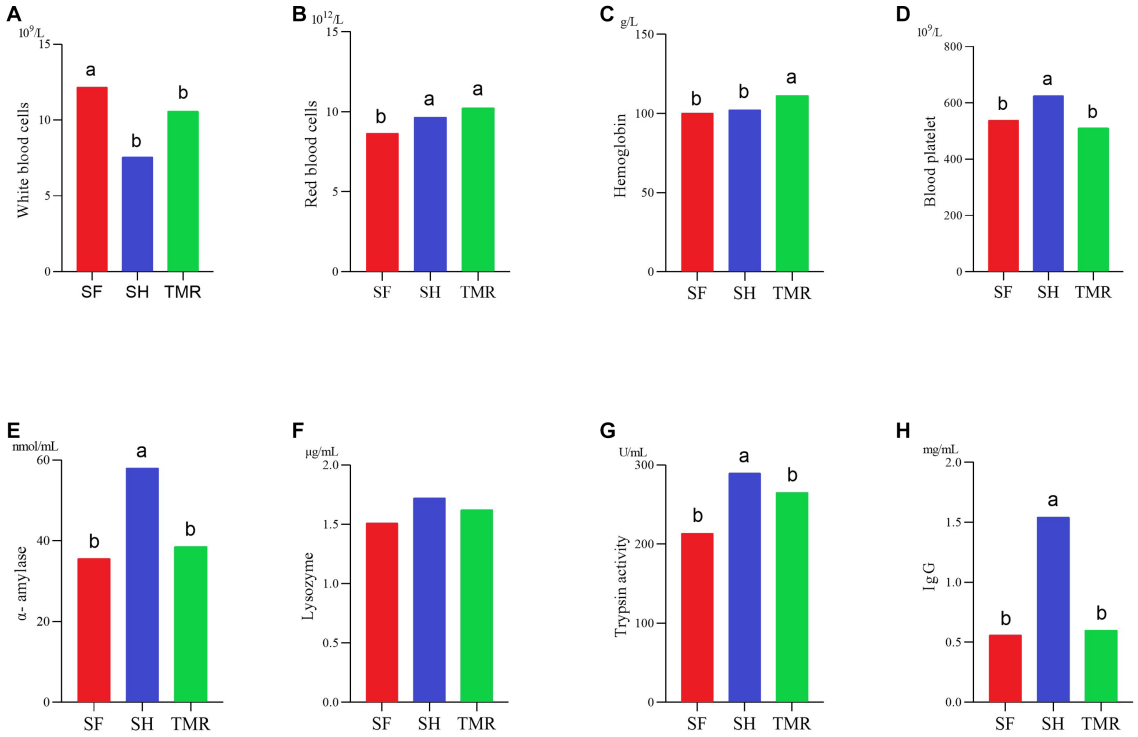

FIGURE 2
Some physiological indexes test results. **(A–D)** It's blood routine determination results. **(E–H)** It's digestive enzyme and immunoassay results.

TABLE 2 Preprocessing of sequencing data.

Sample ID	Raw date	Clean date	Clean_ GC(%)	Effective (%)	Total Len.	Num	Average Len.
SH	12,203.11	12,190.28	50.27	99.895	161,322,099	112,565	1,433.15
	12,496.78	12,475.11	47.34	99.827	217,508,616	134,445	1,617.83
	12,651.70	12,609.24	43.09	99.664	153,544,642	102,299	1,500.94
SF	12,451.10	12,439.42	50.13	99.906	156,100,365	94,575	1,650.55
	12,084.00	12,062.04	47.41	99.818	1,778,426	2,347	757.74
	12,573.19	12,546.90	38.51	99.791	177,806,423	124,453	1,428.70
TMR	13,345.45	13,323.13	44.12	99.833	344,856,565	217,392	1,586.34
	12,432.55	12,423.68	50.65	99.929	297,219,715	189,565	1,567.90
	12,726.95	12,721.27	41.27	99.955	177,848,979	129,771	1,370.48

pathways in the TMR group were carbohydrate metabolism and amino acid metabolism. It is worth noting that the TMR group was enriched in cell growth and death and drug resistance: antimicrobial pathway higher than SH group and SF group. These genes were further compared in the metabolic pathways of the third level, and were enriched in 302 pathways. The three groups all enriched more genes in ABC transporters (pathway ID: ko 02010), ribosome (pathway ID: ko03010), purine metabolism (pathway ID: ko 00230) and pyrimidine metabolism (pathway ID: ko00240), but the SF group was more and the TMR group was the least. Interestingly, the number of genes enriched in Cell cycle-Caulobacter (pathway ID: ko04112), necroptosis (pathway ID: ko04217) and other pathways under the classification of cell growth and death pathway was the least in SH group, and more in TMR group and SF group. The Cationic antimicrobial peptide (CAMP) resistance (pathway ID: ko01503) under the Drug resistance: Antimicrobial classification was similar.

The results of EggNOG analysis (Figure 7) showed that the most abundant genes were enriched in Replication, recombination and repair, followed by Amino acid transport and metabolism and Translation, ribosomal structure and biogenesis. After comparing the relative abundance of genes annotated in eggNOG in the three groups, it was found that the main enrichment pathways of the three groups were similar, with the most in SF group and the least in TMR group. The relative abundance of secondary metabolites biosynthesis, transport and catabolism, Cell motility and RNA processing and modification in TMR group was higher than that in the other two groups.

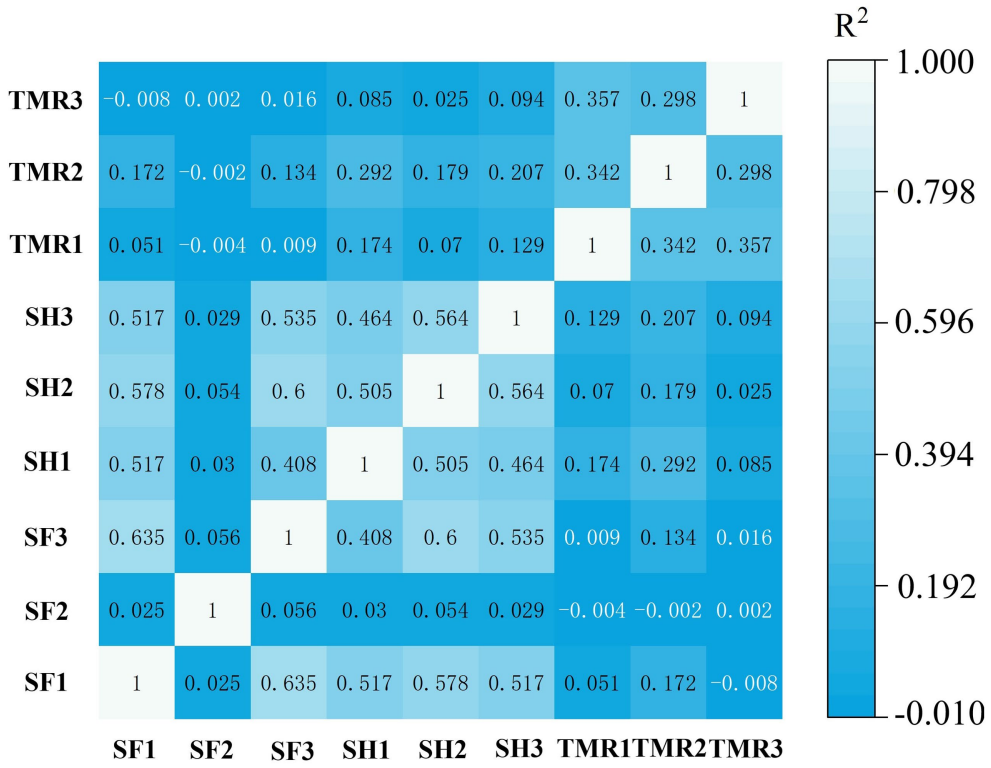

FIGURE 3
Correlation analysis between groups of samples.

After comparing the relative abundance of CAZy genes in the three groups (Figure 8), it was found that Glycoside Hydrolases (GH) were the main ones, and there were differences in CAZy family under the second level classification. The CAZy family of SH group was more evenly distributed, and the most enriched families were GT2 family and GH1 family, among which GH1, GH3 and GH25 family were higher than the other two groups. GH19, GT2 and GH13 family were more enriched in group SF than in the other two groups. There were more GH19, GH24 and GT2 family in TMR group, and GH24 was more than the other two groups. The CAZy families that attract our attention are GH1 and GH19. The differences between the three groups are also mainly reflected in these two families. The enrichment of GH1 in SH group was more than that in SF group and TMR group, mainly due to the β-glucosidase (EC 3.2.1.21) of GH1 family under level 3 classification. GH19 was more enriched in SF group and TMR group than in SH group, mainly chitinase (EC 3.2.1.14) of GH19 family under level 3 classification.

3.6. Analysis of serum differential metabolites

After analyzing the blood metabolites of the three groups, a total of 403 cathode metabolites were found. PLS-DA analysis (Figure 9) of these metabolites between groups showed that the separation between groups was obvious, the degree of polymerization in the group was high, and the model parameters of each group also met the standard, indicating that the results were stable and reliable. Further analysis and screening of differential metabolites (Table 3) showed that there were 87 differential metabolites in the cathode of SF group and SH group, 17 were significantly up-regulated and 70 were significantly down-regulated. There were 152 differential metabolites in the cathode of SF group and TMR group, 27 were significantly up-regulated and 125 were significantly down-regulated. There were 108 differential metabolites in the cathode of SH group and TMR group, 26 were significantly up-regulated and 82 were significantly down-regulated.

3.7. KEGG enrichment analysis of differential metabolites

The selected differential metabolites were subjected to KEGG enrichment analysis to determine the effects of differential metabolites on key blood metabolic pathways. The results showed that the differential metabolites between SF group and SH group were mainly enriched in cortisol synthesis and secretion (value of $p = 0.032$). The differential metabolites between SF group and TMR group were mainly enriched in cAMP signaling pathway (value of $p = 0.043$), dopaminergic synapse (value of $p = 0.043$), Taste transduction (value of $p = 0.043$). The differential metabolites between SH group and TMR group were mainly enriched in cAMP signaling pathway (value of $p = 0.024$), dopaminergic synapse (value of $p = 0.024$), taste transduction (value of $p = 0.024$), tyrosine metabolism (value of $p = 0.046$). The specific pathway information and differential metabolites enriched in the pathway are shown in the Table 4.

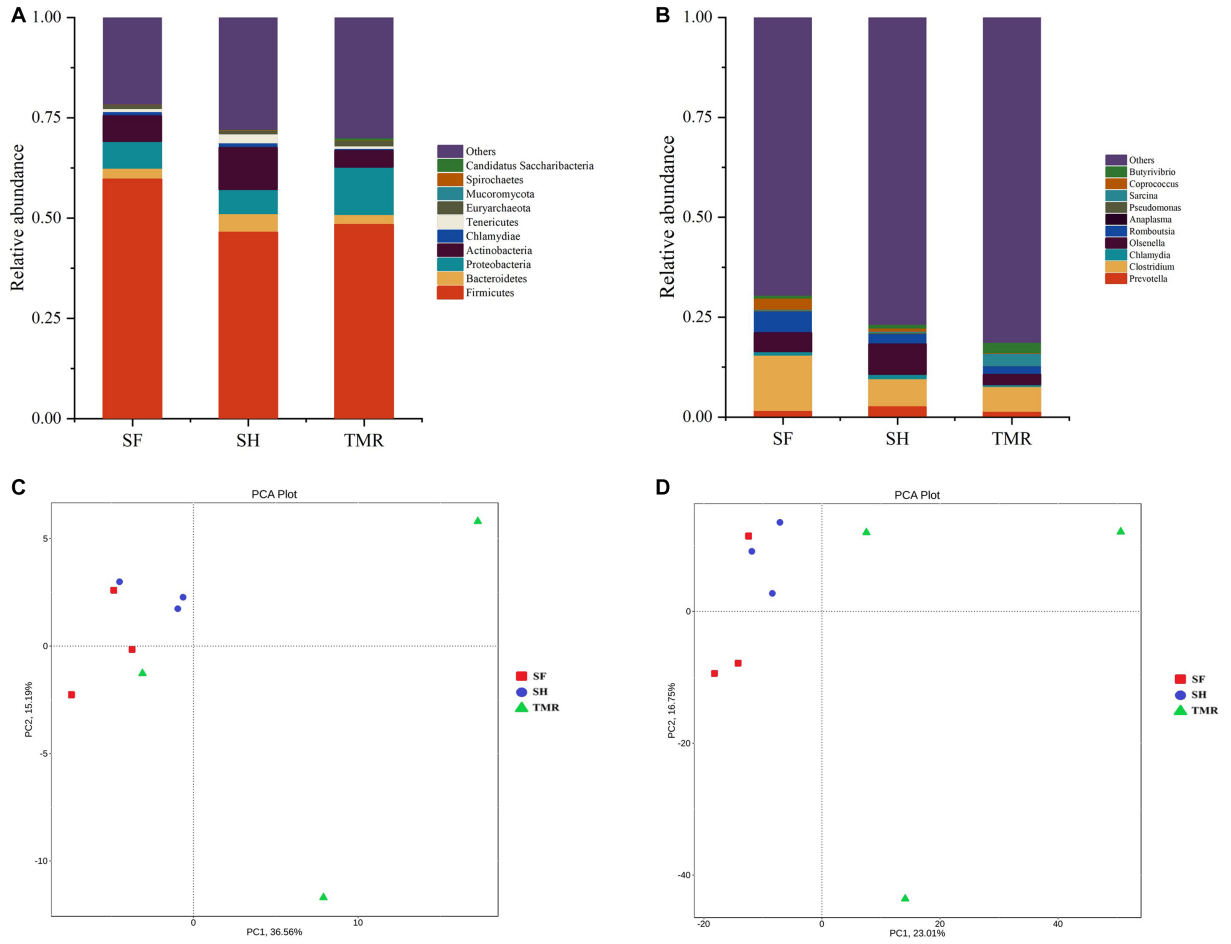

FIGURE 4
Analysis of microbial community differences between groups. **(A)** Relative abundance of species at phylum level TOP10. **(B)** Relative abundance of genus level species TOP10. **(C)** Phylum level species PCA analysis. **(D)** PCA analysis of genus-level species.

4. Discussion

The ileum is located in the posterior part of the small intestine and is the main place for digestion and absorption of nutrients. Therefore, the development of ileal function is essential for the health and growth of calves. We observed from the sections after HE staining that the intestinal epithelial villi in the SF and SH groups were better developed, the villus height was higher, and the goblet cells in the villi were more closely arranged. The goblet cells can secrete mucus and form a physical barrier on the surface of the intestinal mucosa, which is the main component of the intestinal barrier (34). These characteristics mean that the ileum of SF group and SH group has a larger absorption and digestion area, and the intestinal morphology is more conducive to the absorption of nutrients, and has a stronger barrier function, which is the characteristic of healthy development of calf intestine, which is conducive to the development of digestive ability and gastrointestinal health of calves (35, 36). The research of Ma showed that the intestinal tract of calves must undergo development, physical and physiological changes, before they can better adapt to the transition from milk to solid feed (37). Studies have shown that the healthy development of intestinal epithelial environment is conducive to the development of intestinal probiotics and the stability of microbial communities (38). We observed a more obvious inflammatory response in the TMR group, and the intestinal wall in the SH group was relatively complete. After subsequent analysis, we believe that it may be due to the digestive ability of calves and maturity, which cannot adapt to the inflammatory response caused by TMR feed. The parasite phenomenon may be caused by the invasion of pathogens. Whether it is related to the total mixed feed needs further study.

Metagenomic analysis compared the microbial community differences among the three groups. The results showed that SH group was enriched in *Actinobacteria* and more than the other two groups. In previous studies, except for a small number of pathogenic bacteria, *Actinobacteria* is usually a probiotic that is conducive to the digestion and absorption of organic matter. In some farms, probiotics are used to selectively stimulate the growth of *Actinobacteria* (39). Moreover, *Actinobacteria* can also produce secondary metabolites such as natural drugs, enzymes, and active factors, and is considered to be a potential probiotic (40). Bifidobacterium under the classification of *Actinobacteria* plays an important role in regulating the immune system and preventing intestinal diseases. It can reduce the inflammatory response by producing short-chain fatty acids and regulating inflammatory factors. The proliferation of *Actinobacteria*

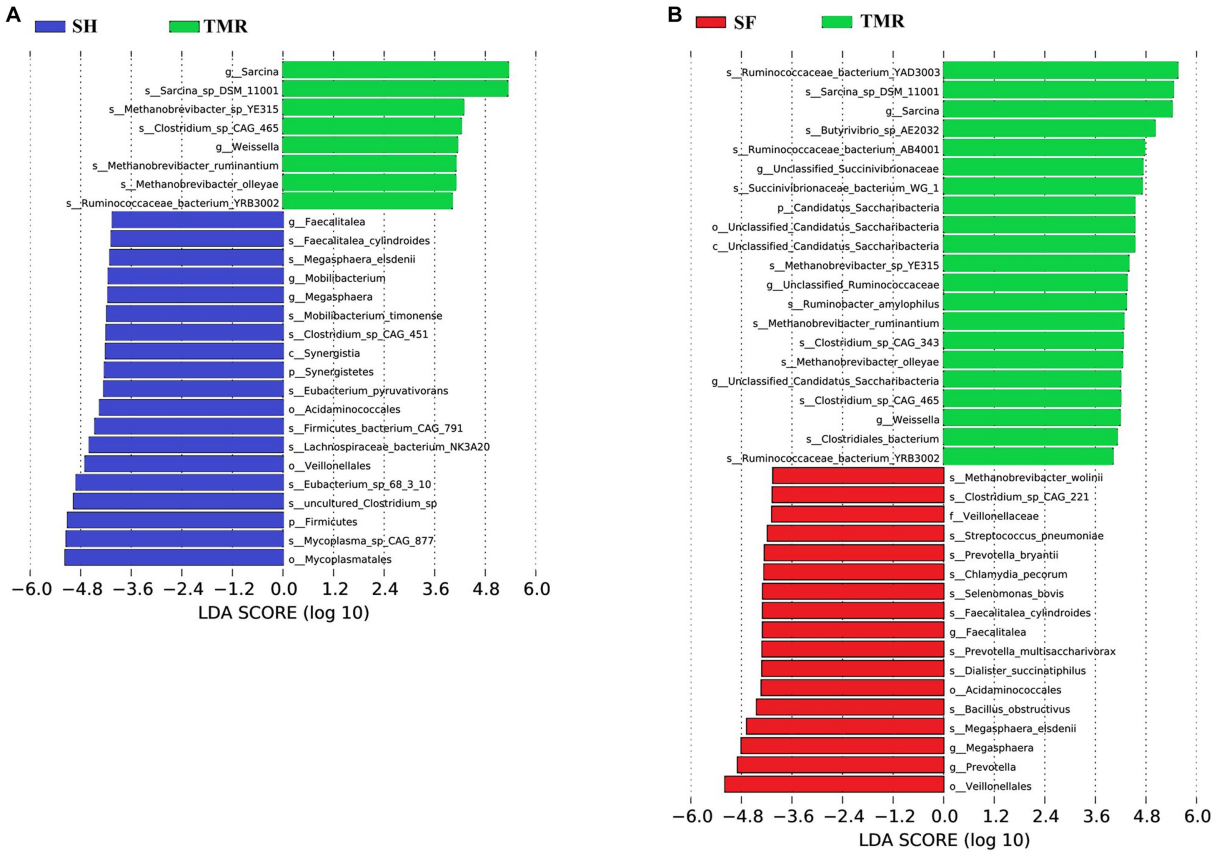

FIGURE 5
LDA score of LEfSe-PICRUSt. **(A)** LDA score analysis diagram of different species between SH group and TMR group. **(B)** LDA score analysis diagram of different species between SF group and TMR group.

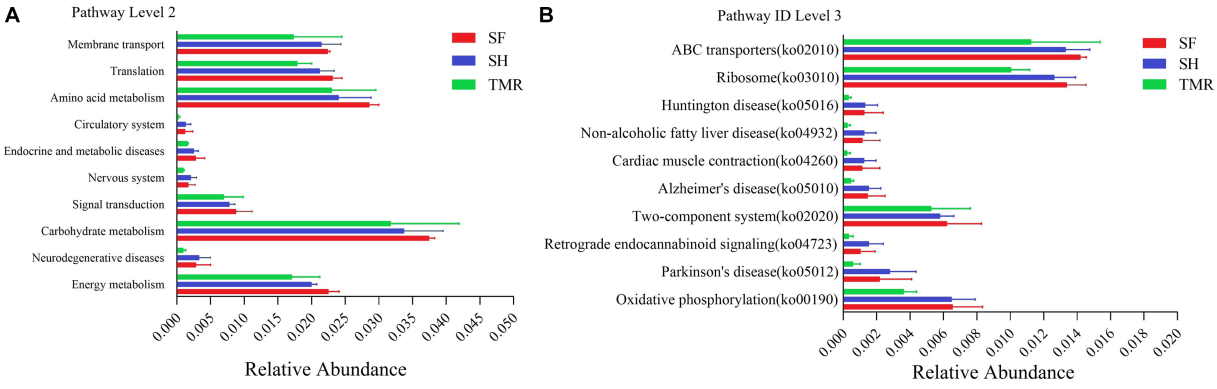

FIGURE 6
KEGG pathway enrichment analysis. **(A)** KEGG level 2 enrichment analysis top 10 between groups. **(B)** KEGG level 3 enrichment analysis top 10 between groups.

may be the key to regulating immunity and reducing inflammatory response in SH group. Some studies have shown that *Actinobacteria* also plays a role in reducing pathogens such as Escherichia-Shigella, indicating that it can also have a positive effect on preventing diarrhea (41). *Olsenella* has obvious advantages in SH enrichment. *Olsenella* belongs to probiotics under the classification of lactic acid bacteria. Studies have shown that *Olsenella* is positively correlated with butyrate. Butyrate is one of the main volatile fatty acids produced by rumen fermentation, and ileum is also the main part of absorbing butyrate. Butyrate can provide energy for *Olsenella* and can strengthen the intestinal barrier function, which is conducive to intestinal health (42). The study of Zhang also showed that individuals with higher relative abundance of *Olsenella* have a stronger ability to produce short-chain fatty acids, which can effectively affect the structure of the intestinal epithelial mucus layer, stimulate the development of intestinal epithelial tissue, nourish the development of intestinal

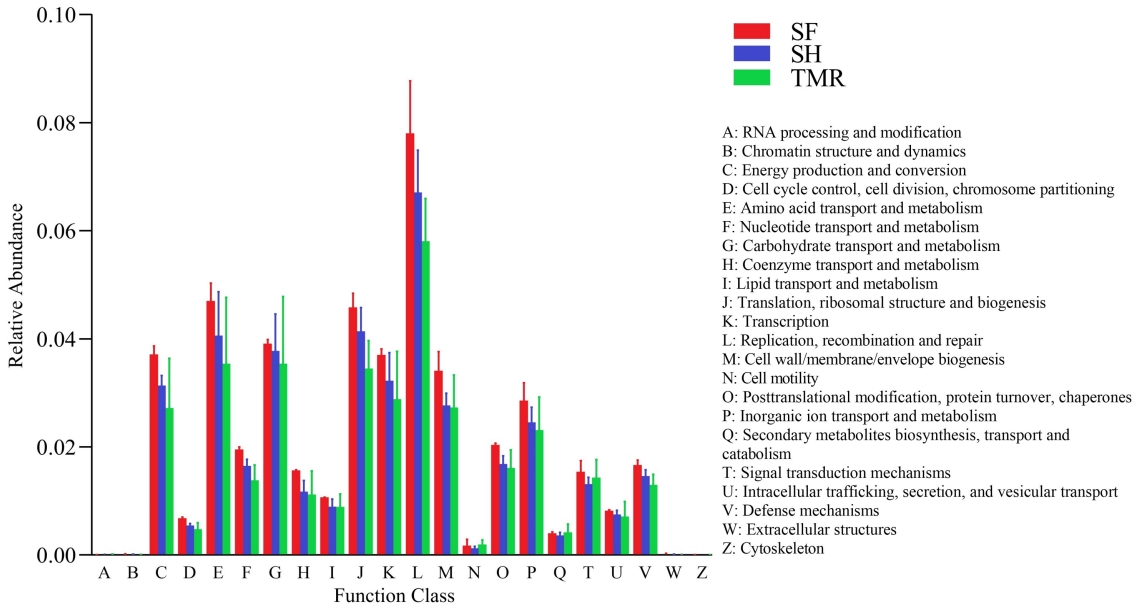

FIGURE 7
EggNOG enrichment analysis.

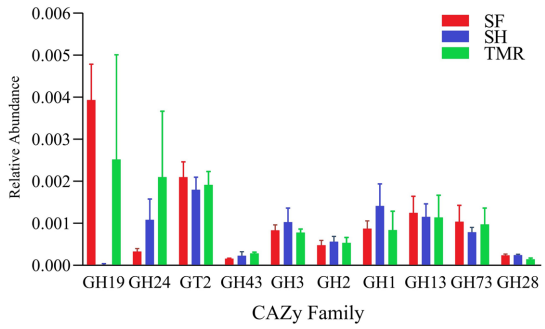

FIGURE 8
CAZy enrichment analysis top 10 between groups.

epithelial cells such as goblet cells, and reduce the risk of inflammatory bowel disease in individuals (43).

The dominant bacteria in the SF group were *Chlamydiae* and *Proteobacteria*. *Chlamydiae* is a pathogen that may cause enteritis (44, 45), but no diarrhea symptoms were found in this individual, and there may be a case of invisible infection of *Chlamydiae* (46). At the same time, other pathogens with high correlation with *Chlamydiae*, such as Campylobacter, were not found. *Chlamydiae*, as a potential pathogen, may be related to the local inflammation observed in the SF group, which may lead to impaired intestinal absorption and induce other diseases (47). Timely treatment is needed. The study of Xia in weaned piglets showed that the addition of plant essential oils containing substances such as tributyrin to the feed can resist Chlamydia, regulate microbial morphology, and improve intestinal villus morphology (48).

Proteobacteria in both TMR group and SF group have high abundance. Previous studies have shown that many intestinal pathogenic bacteria belong to *Proteobacteria*, and the relative abundance of *Proteobacteria* is too high, which may cause inflammation and cause calf diarrhea (49, 50). Shin used the enrichment of *Proteobacteria* as one of the important markers of intestinal microflora imbalance in healthy individuals. In general, *Proteobacteria* will not be significantly enriched in healthy individuals (51). The study of Han on weaned piglets showed that *Proteobacteria* could activate inflammation-related cytokines and cause intestinal inflammation damage. At the same time, the daily weight gain of piglets with enrichment of *Proteobacteria* was significantly reduced, which indicated that the intestinal nutrition absorption function was also affected (52). The study of Varada on newborn buffaloes found that feeding probiotics can regulate the composition of intestinal microbial communities by reducing the abundance of *Proteobacteria* (53). Numerous studies have shown that the enrichment of *Proteobacteria* can lead to inflammatory response and cause intestinal epithelial cell dysfunction (54, 55). This may be one of the reasons why the TMR group had a large number of inflammatory cell infiltration, intestinal epithelial cell shedding, and even diarrhea symptoms. *Sarcina* enriched in TMR is a pathogen that has been reported many times. Katharine found that *Sarcina* was associated with gastric ulcer and fungal infection in calves and goats, which may be the cause of gastritis and enteritis (56). The study of Simpson also found that *Sarcina* may cause necrosis of the intestinal epithelial surface and may be related to parasitic infection (57). Many studies have pointed out that *Sarcina* is associated with diarrhea, inflammation and other symptoms. *Sarcina* is significantly enriched in the intestinal tract of horses parasitized by ascaris, and *Sarcina* is often found in goats, rabbits and other animals with diarrhea symptoms or fungal infection (58, 59). The study of Zhuo on piglets directly pointed out that the enrichment of *Sarcina* is not conducive to the healthy development of the intestinal tract, affects the production performance and feed absorption rate, and increases the probability of intestinal pathogen infection. They also pointed out that *Sarcina* has a great relationship with the reduction of microorganisms that produce short-chain fatty

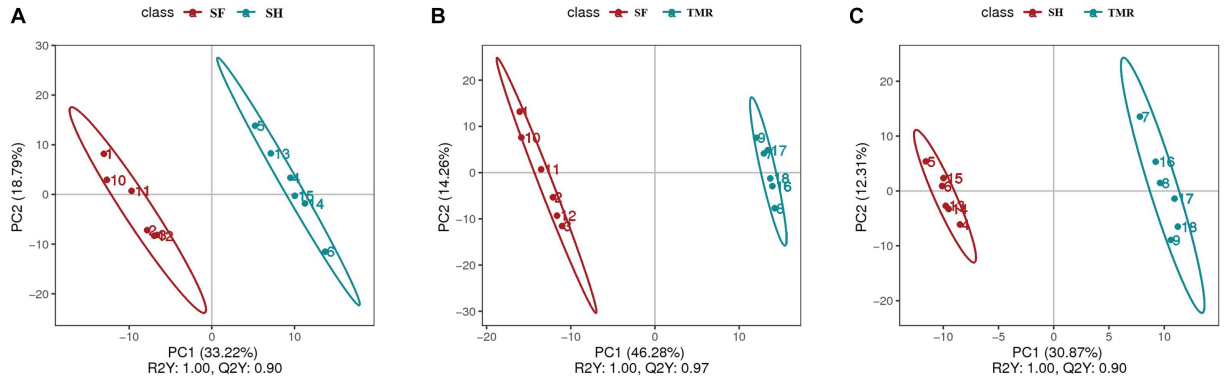

FIGURE 9
PLS-DA analysis of serum metabolites between groups. **(A)** PLS-DA analysis of SF group and SH group; **(B)** PLS-DA analysis of SF group and TMR group; **(C)** PLS-DA analysis of SH group and TMR group.

TABLE 3 Number of differential metabolites in serum.

Compared samples	N total identified	N Signif. different	N Signif. up	N Signif. down
SF vs. SH	403	87	17	90
SF vs. TMR	403	108	26	82
SH vs. TMR	403	152	27	125

N Total Identified, Total number of differential metabolites. N Signif. Different, Number of significant differential metabolites. N Signif. Up, Number of significantly higher metabolite contents. N Signif. Down, Number of significantly lower metabolite.

acids and feeding patterns (60). This is similar to our findings. Therefore, we believe that the inflammatory response, parasitic infection and diarrhea symptoms in the TMR group are closely related to *Sarcina*.

After comparing the enrichment of KEGG signaling pathway, we found that in Cell cycle-Caulobacter (pathway ID: ko04112) and Necroptosis (pathway ID: ko04217) signaling pathways, SH group was less, SF group and TMR group were more. Cell cycle-Caulobacter is a key pathway that can regulate DNA replication, cell cycle and cell topology. Cell cycle response regulator is the core of his network. Cell cycle-Caulobacter regulates the asymmetric division of bacterial cells, resulting in stalked cells and swarmer cells with different fates (61). The stalked type of progeny cell type will enter the S phase, while the swarmer type will remain in the G1 phase (62). We compared the metabolites annotated in the three groups, and found that the unique metabolites in the SF group and the TMR group eventually pointed to polar morphogenesis and pili biogenesis, while the unique metabolites in the SH group pointed to flagellar ejection holdfast biogenesis, stalk formation, that is, the cells in the SF group, TMR group and SH group went to different divisions, and different daughter cells had different cell membrane structures and cell morphology. This leads to differences in subsequent functions (63, 64). Studies have shown that phages with pili and flagella structures are more susceptible to the host through CtrA regulation (65). This molecular network regulation has a direct relationship with bacterial infection. McAdams 's study pointed out that the function within the Cell cycle-Caulobacter network is conserved, but after asymmetric division, due to differences in cell function and protein, downstream specific coupling species will be very different (66). Therefore, we boldly speculate that the Cell cycle-Caulobacter pathway in the SH group is not as active as the other two groups, and the final cell division results are different, which may be one of the important reasons for the differences in bacterial infection and inflammatory response between the SH group and the TMR group and the SF group. The difference in Necroptosis is clearer. SH has no unique metabolites, while the unique metabolites of SH group and TMR group are the same, which are H2AX, VDAC, Drp1, ESCRT-III. These metabolites directly or indirectly point to inflammatory response, mitochondrial damage, etc., and their activity may lead to cell damage, and ultimately necroptosis (67). Yi's study found that plasma pathogens such as *Staphylococcus aureus* can lead to cell necroptosis by affecting mitochondrial membrane potential and membrane permeability. This necroptosis is regulated by calmodulin-dependent protein kinase II (CaMKII) and mixed lineage kinase domain-like (MLKL) (68). The unique metabolites we found in TMR group and SF group were involved in the above reaction process. Huang's study also pointed out that activation of MLKL and CaMKII can lead to mitochondrial dysfunction and pro-inflammatory response (69). We also found that the unique metabolites of TMR group and SF group were concentrated in cytochrome c oxidase \ reductase after comparing the Oxidative phosphorylation (pathway ID: ko00190) of the three groups. We believe that this may be due to bacterial infection that activates pathways such as MLKL, which leads to necroptosis caused by mitochondrial dysfunction and promotes inflammatory response, and ultimately leads to damage to intestinal epithelial structure (70). In addition, intestinal flora may also interact with metabolic intermediates in Oxidative phosphorylation. Existing studies have found that NAD (nicotinamide adenine dinucleotide) can promote the secretion of intestinal mucin 2 (MUC2), enhance the ability of antibacterial infection, and alleviate intestinal inflammation by inhibiting NF-kB. Oxidative phosphorylation abnormalities and mitochondrial damage are likely to affect the levels of metabolites such as NAD, increasing the risk of intestinal inflammation and bacterial infection (71).

In the two signaling pathways of Purine metabolism (pathway ID: ko 00230) and Pyrimidine metabolism (pathway ID: ko00240), the three groups have more enrichment, but their metabolic process is different. As shown in Figure 10, some of the reactions in the TMR and SF groups were more active than SH in these two pathways, including several key

TABLE 4 KEGG enrichment analysis results of differential metabolites.

	Map ID	Map title	Value of *p*	*N*	Meta IDs
SF vs. SH	map04927	Cortisol synthesis and secretion	0.03241	81	Pregnenolone$^{\#\#}$; Cortodoxone**
	map04934	Cushing's syndrome	0.03241	81	Pregnenolone$^{\#\#}$; Cortodoxone**
	map00140	Steroid hormone biosynthesis	0.04173	81	Pregnenolone$^{\#\#}$; Androsterone glucuronide*; Cortodoxone**
SF vs. TMR	map04024	cAMP signaling pathway	0.04283	81	Dopamine$^{\#\#}$; Noradrenaline$^{\#\#}$; Adenosine 5′-monophosphate$^{\#\#}$
	map04728	Dopaminergic synapse	0.04283	81	Dopamine$^{\#\#}$; Levodopa$^{\#\#}$; 3-Methoxytyramine$^{\#\#}$
	map04742	Taste transduction	0.04283	81	Noradrenaline$^{\#\#}$; Saccharin**; Adenosine 5′-monophosphate$^{\#}$
SH vs. TMR	map04024	cAMP signaling pathway	0.02372	81	Dopamine$^{\#\#}$; Noradrenaline$^{\#\#}$; Adenosine 5′-monophosphate$^{\#}$
	map04728	Dopaminergic synapse	0.02372	81	Dopamine$^{\#\#}$; Levodopa$^{\#\#}$; 3-Methoxytyramine$^{\#}$
	map04742	Taste transduction	0.02372	81	Noradrenaline$^{\#\#}$; Saccharin**; Adenosine 5′-monophosphate$^{\#}$
	map00350	Tyrosine metabolism	0.04598	81	Dopamine$^{\#\#}$; Levodopa$^{\#\#}$; Noradrenaline$^{\#\#}$; Tyrosol**; 3-Methoxytyramine$^{\#}$

In the Meta IDs annotation, $^{\#}$ represents a significant down-regulation, $^{\#\#}$ represents a significant down-regulation, * represents a significant up-regulation, ** represents a significant up-regulation.

enzymes that catalyze one-way reactions, such as apyrase (EC: 3.6.1.5) and AMP deaminase (EC: 3.5.4.6), which are unique to the SF and TMR groups. Studies have shown that when the body is in an inflammatory or stress state, the demand for nucleotides will increase to promote intestinal cell proliferation and tissue repair (72, 73). The activity of apyrase (EC: 3.6.1.5) will lead to excessive metabolic intermediates such as GMP and AMP. Their final products all point to uric acid, but the enzyme for the next metabolism of uric acid is not active. Although nucleotide metabolism in the SF and TMR groups may be an autonomous repair of inflammation, it also increases the risk of accumulation of substances such as uric acid. In general, purine catabolism produces metabolites with potential toxicity (74, 75). Excessive accumulation of uric acid may lead to the formation of sodium salt deposition in shutdown, skin and other parts, causing pain. It may also cause kidney damage and lead to the occurrence of uric acidemia, which is common in long-term high protein intake of livestock and poultry (76, 77); in serious cases, it may also lead to atherosclerosis (78). Apyrase is involved in the dephosphorylation of GTP, ATP, UTP, CTP and other substances, and is a one-way reaction. ATP, GTP and ITP were dephosphorylated to produce Uric acid. UTP and CTP were also dephosphorylated to produce Uracil, which was converted into β-Alanine to participate in the next reaction. After dephosphorylation, dTTP is dephosphorylated to generate dTMP, which will further generate Thymine, and then generate (R)-3-Amino-2-methylpropanoate to participate in Valine, leucine and isoleucine degradation pathway. Therefore, we inferred that the Nucleotide metabolism of the three groups was more enriched because the intestine of the calves was in the developmental stage, and the metabolism was vigorous because it responded to stimuli such as feed differences and microbial flora changes. However, the SF group and the SH group showed uniqueness in some metabolic processes due to the need for anti-inflammatory and epithelial cell repair. The activity of enzymes such as apyrase (EC: 3.6.1.5), and the enzymes not annotated to uric acid catabolism may lead to the risk of uric acid accumulation in the SF group and the TMR group.

By comparing the analysis results of the three groups of CAZy databases. The differences are mainly reflected in the GH1 family and the GH19 family. Under level 3 classification, the enrichment of β-glucosidase (EC 3.2.1.21) belonging to GH1 family in SH group was more than that in the other two groups. β-glucosidase mainly plays a role in glycolipid metabolism and is a key enzyme that catalyzes cellulose to produce glucose. We believe that the higher β-glucosidase activity in the SH group is closely related to its microbial community. Studies have shown that adding β-glucosidase to feed or activating β-glucosidase through diet can improve the digestive capacity of the gastrointestinal tract and benefit animal growth (79). β-glucosidase can also increase the activity of intestinal amylase (80). This is also consistent with our found, the amylase content of SH group was the highest. Zhang 's study found that castration changed the cecal digestion environment of dairy cows, significantly reduced the activity of β-glucosidase, reduced the relative abundance of Ruminococcaceae and other bacteria related to β-glucosidase, and reduced the daily gain of dairy cows (81). Therefore, the enrichment of β-glucosidase has a positive effect on calves. The chitinase (EC 3.2.1.14) in GH19 family showed more obvious differences. The enrichment of chitinase in SH group was much less than that in SF group and TMR group. Chitinase has strong specificity and can cleave chitin glycosidic bonds. Chitin is a component of invertebrates, arthropods, fungi, etc., but mammals can hardly synthesize chitin themselves. Therefore, attention should be paid when excessive chitinase is enriched in mammals, and it may be food containing chitin or microbial pathogens invade the body (82). Recent studies have shown that chitin protein is related to gastrointestinal diseases. Chitinase can play an important role in gastrointestinal digestion and diseases due to its specificity, and it is likely to become a new idea for the treatment of gastrointestinal diseases (83, 84). The study of Beier found that microorganisms can also produce chitinase, including Firmicutes, *Actinobacteria*, etc. Chitinase can specifically hydrolyze chitin and make it lose pathogenicity, which is particularly critical for mammals (85). We observed local inflammatory reactions in both the SF group and the TMR group. Parasites also appeared in the TMR group. It is most likely that foreign pathogens produce chitin, which stimulates some of the bacteria in the intestine that can release chitinase to respond, causing differences in intestinal flora.

In addition to this, there is a more critical phenomenon, we found that in the TMR group, a unique enrichment of many neurotransmitters and their precursors: Dopamine, Noradrenaline, Levodopa, etc., which

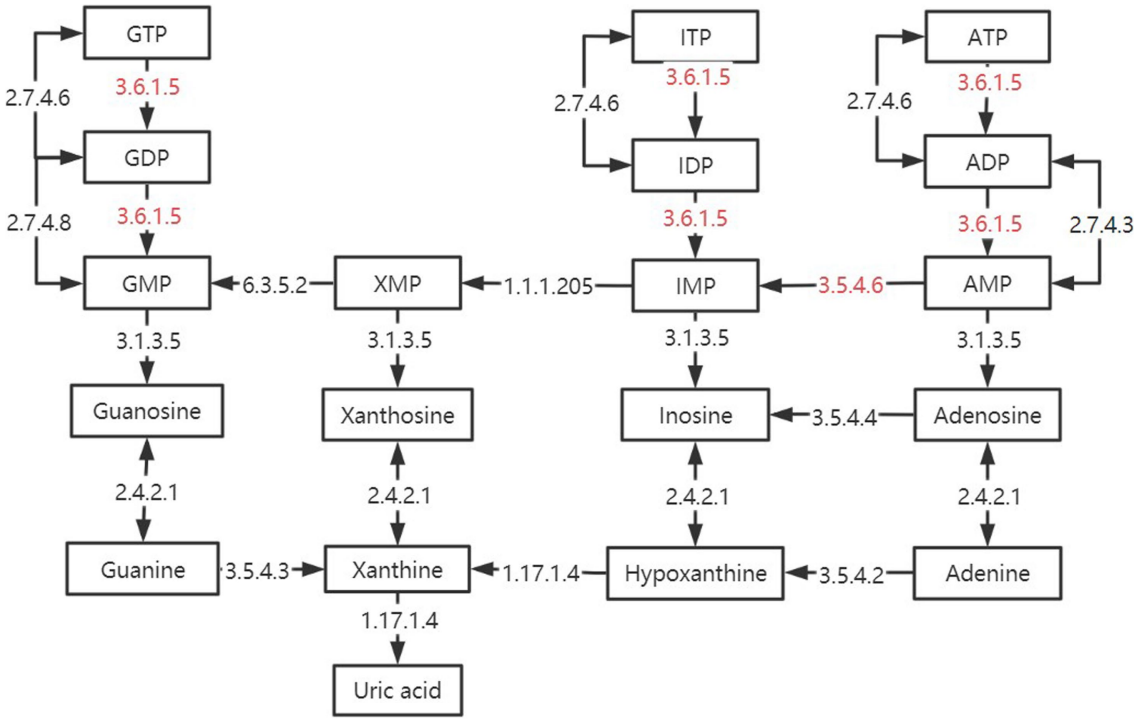

FIGURE 10
Differences in purine metabolic pathways between groups. The black box is marked as the intermediate product in the purine metabolic pathway. The black arrow is marked with enzymes annotated in the kegg enzyme database. The red-labeled enzyme is unique in SF and TMR.

may point to a new research field: neuro microbiology. In recent years, more and more studies have shown that microorganisms not only have the functions of assisting digestion and maintaining barriers, but also participate in the regulation of the enteric nervous system and the central nervous system by producing neurotransmitters or neurotransmitter precursors (86). It is reported that intestinal microorganisms can synthesize neurotransmitters such as dopamine, norepinephrine, 5-hydroxytryptamine and compounds involved in the metabolism of neurotransmitters in the body, which can act on the local intestinal nervous system and regulate the intestinal environment (87, 88). Taking the synthesis mechanism of dopamine (phenylalanine-tyrosine-L-dopa-dopamine) as an example, phenylalanine hydroxylase converts L-phenylalanine into L-tyrosine, which can cross the blood–brain barrier and enter the brain. In the brain, it is converted to (s)-3,4-dihydroxyphenylalanine (L-DOPA) by tyrosine hydroxylase, and then L-DOPA is converted to dopamine by dopa decarboxylase (89, 90). Although the pathway by which these neurotransmitters are synthesized by the gut microbiome is unclear, it is likely that the gut microbiome also has the ability to convert local dopamine to norepinephrine, such as dopamine-beta oxidase (91). Intestinal microorganisms can also synthesize neurotransmitter precursors such as L-tyrosine. Due to the lack of relevant transporters, some neurotransmitters circulating in the blood, such as dopamine and norepinephrine, cannot cross the blood–brain barrier. However, the precursors of these neurotransmitters can cross the blood–brain barrier, synthesize related substances in the brain, and then regulate, which may be the way that microorganisms connect the central nervous system.

Intestinal flora communicates with the central nervous system, sympathetic nerve and vagus nerve through neurotransmitters, regulates the level of neurotransmitters such as auxin, and affects the growth and development, gastrointestinal digestion and immune function of animals (92). For example, slight changes in the vagus nerve will lead to dramatic changes in the release of downstream neurotransmitters, which will affect digestive level, intestinal permeability, intestinal motility and immune response (93, 94). The central nervous system receives signals from these neurotransmitters and regulates the digestive environment and microbial community composition in the gastrointestinal tract by producing neurochemicals such as 5-hydroxytryptamine, cholecystokinin, and dopamine. Microorganisms also stimulate intestinal endocrine cells to produce hormones and complete feedback to the central nervous system through neural afferent fibers. For example, in the presence of dopamine and norepinephrine, *Escherichia coli* will proliferate faster and may also enhance its motility and toxicity (95). Liu 's study found that abnormal casein synthesis aggravated enteritis, disrupted microbial flora and its metabolic function, and led to abnormal immune response (96). The intermediate compound of neurotransmitters: short-chain fatty acids, also participate in the regulation of microorganisms and neurotransmitters. Studies have shown that short-chain fatty acids can regulate the metabolism of many neurotransmitters by acting on the response element binding proteins in the cAMP signaling pathway (97, 98). Taking dopamine as an example, short-chain fatty acids can regulate the production of dopamine and the conversion of dopamine and norepinephrine by regulating the expression of tyrosine carboxylase and dopamine-β-carboxylase (99). These metabolic pathways are reflected in the differential metabolic pathways in the TMR group. Especially for ruminant mammals such as calves, the short-chain fatty acids in the

body will be more abundant, and the regulation mechanism of short-chain fatty acids will be more complicated. In some animal model experiments, it has also been found that metabolic abnormalities caused by intestinal microbial community disorders and the release of monoamine substances may lead to depression (100). This proves the deep relationship between intestinal microbial changes and host mental disorders. In animal husbandry, whether it will affect animal feed intake and estrus cycle needs further study.

In recent years, many studies have shown that intestinal microorganisms can communicate and regulate with the gastrointestinal tract and brain through neurotransmitters, and interact to maintain the dynamic balance of the intestinal environment. One of the difficulties in the field of neurobiology is to identify whether the source of various neuroactive compounds is host or microorganism. Secondly, the influence between intestinal microorganisms and neurotransmitters, and the complex communication between intestinal microorganisms and the brain are difficult. Combining the analysis results of metabolomics and metagenomics will help us understand the operation relationship between the microbial-intestinal-brain axis from the perspective of neuro microbiology. In the future, specific microbial targeted interventions can be performed on the host from the perspective of neuro microbiology, which can be used to adjust the intestinal flora and improve digestion. It is even possible to regulate specific microbial communities by understanding the relationship between gut microbes and brain health, and to treat neurological diseases through microbial regulation of the central nervous system.

5. Conclusion

In summary, our study found that the feeding mode of 'starter + hay + milk 'was more conducive to the development of calves. The calves in the SH group had better intestinal development, intestinal microbial community was more conducive to digestion and absorption, and immunity was stronger. The feeding mode of the SH group was conducive to the healthy development of the calves. Therefore, the calves in the SH group had higher daily weight gain, stronger digestive ability, and no pathological inflammatory reaction was found. For calves, daily weight gain is important, and more importantly, there can be a stronger digestive system and immune system to ensure that calves can have better growth and development after weaning, which is the advantage of SH group compared with SF group. The enrichment of neurotransmitters found in the TMR group also inspired us to study intestinal microorganisms from a new perspective. The research ideas of neuro microbiology will be one of our future focuses. Therefore, the results of this study provide a basis for improving the feeding mode of calves, and inspire us to explore the relationship between microorganisms and hosts from the perspective of neural metabolites.

Data availability statement

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

Ethics statement

The animal study was approved by the Institutional Animal Care and Use Committee of the College of Animal Science and Technology. The study was conducted in accordance with the local legislation and institutional requirements.

Author contributions

JW: Data curation, Writing – review & editing, Writing – original draft. YC: Data curation, Writing – original draft, Writing – review & editing. ML: Data curation, Investigation, Project administration, Writing – review & editing. SX: Investigation, Software, Writing – review & editing. KZ: Investigation, Software, Writing – review & editing. HF: Investigation, Software, Writing – review & editing. JN: Investigation, Software, Writing – review & editing. WS: Conceptualization, Investigation, Software, Writing – review & editing. XJ: Conceptualization, Investigation, Software, Writing – review & editing. SL: Methodology, 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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Effects of *Leymus chinensis* hay and alfalfa hay on growth performance, rumen microbiota, and untargeted metabolomics of meat in lambs

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Objective: The objective of this study was to compare the effects of *Leymus chinensis* hay and alfalfa hay as the roughage on the rumen bacterial and the meat metabolomics in lambs.

Methods: Fourteen male lambs were randomly assigned to two dietary treatments (one group was fed with concentrate and *Leymus chinensis* hay; another was fed with concentrate and alfalfa hay) with seven replicates per treatment. The feeding experiment lasted for 60 days. Lambs were slaughtered at the end of the feeding experiment. Growth performance, carcass performance, and weights of various viscera were determined. The longissimus dorsi and rumen contents were collected for untargeted metabolomics and 16S rDNA amplicon sequencing analysis, respectively.

Results: The lambs fed with alfalfa hay showed a significantly increased in average daily gain, carcass weight, dressing percentage, loin-eye area, and kidney weight. Feeding *Leymus chinensis* hay and alfalfa hay diets resulted in different meat metabolite deposition and rumen bacterial communities in the lambs. The relative abundance of phyla *Fibrobacteres*, *Bacteroidetes*, and *Spirochaetes* were greater in the *Leymus Chinensis* hay group, while, the relative abundance of *Firmicutes*, *Proteobacteria*, *Fusobacteria*, and *Verrucomicrobia* were greater in the alfalfa hay group. Based on untargeted metabolomics, the main altered metabolic pathways included alanine, aspartate and glutamate metabolism, D-glutamine and D-glutamate metabolism, phenylalanine metabolism, nitrogen metabolism, and tyrosine metabolism. Several bacteria genera including *BF31*, *Alistipes*, *Faecalibacterium*, *Eggerthella*, and *Anaeroplasma* were significantly correlated with growth performance and meat metabolites.

Conclusion: Alfalfa hay improved growth performance and carcass characteristics in lambs. *Leymus chinensis* hay and alfalfa hay caused different meat metabolite deposition by modifying the rumen bacterial community. These findings will be beneficial to future forage utilization for sheep growth, carcass performance, and meat quality improvement.

KEYWORDS

alfalfa hay, growth performance, lamb, *Leymus chinensis* hay, metabolomics, rumen bacteria

1. Introduction

Mutton is one of the most widely consumed meats around the world due to its high protein and low cholesterol (1). With the growing requirement for high-quality meat, meat quality has induced more and more attention. Improving animal diets is one of the most effective ways to ameliorate animal growth performance, carcass traits, and meat quality (2, 3). Roughage is a necessary nutrient source for ruminants. In particular, types and quality of forage are key factors affecting ruminant productivity, carcass composition, rumen microbiota, and quality of meat nutrition such as amino acids, fatty acids, and mineral elements (4, 5).

Leymus chinensis, a perennial species of Gramineae, is widely distributed in the Eurasian Steppe including the eastern Inner Mongolian Plateau and the Songnen Plain in China (6). *Leymus chinensis* has been one of the main forages due to its high yield, appropriate nutritional values, and palatability (7). It has been reported that adding *Leymus chinensis* hay in the diet to replace part of the corn silage and alfalfa hay facilitated the improvement of milk yield, milk fat and protein yield, and milk fat concentration (8). Enhancing the ratio of *Leymus chinensis* silage decreased dry matter and neutral detergent fiber degradability but promoted crude protein degradability in the combinations of *Leymus chinensis* silage and corn silage in beef cattle (7). Moreover, the *Leymus chinensis* hay diet increased the C15:0 fatty acid contents in lamb meat compared to the mixed forage diet (5). As the average daily gain (ADG) of ewes was higher in the *Leymus chinensis* hay treatment group, *Leymus chinensis* hay was of better quality for ewes than *Vigna radiata* stalk (9).

Alfalfa hay is famous for its high quality and widely used as an important dietary roughage for ruminants. Feeding with alfalfa hay increased the growth performance in lambs, such as the ADG, compared with wheat straw diets (10). Feeding lactating ewes with alfalfa hay promoted milk production compared with the wheat straw diets (10). A forage diet mixture of alfalfa hay and maize stover in a ratio of 60:40 optimized the growth and carcass traits of lambs (11). Additionally, alfalfa hay as an ingredient to supplement the low-energy diet increased omega-3 fatty acids and lowered the omega-6: omega-3 ratio in lamb meat (12), indicating that alfalfa affects the meat metabolites and nutritional quality.

The rumen is a complex ecosystem containing functional microbiota responsible for the rumen fermentation and an important part in producing nutrient substances and calories in ruminants (13, 14). The ruminal bacteria community is closely correlated with diets (15). Interestingly, altering the diet has a cascading effect on the rumen microbiota, which affects animal growth performance and meat quality (15). Previous reports have revealed that various forages have a great effect on rumen and fecal microbiota composition (16–18). For example, lambs fed with alfalfa had a higher relative abundance of *Akkermansia* and *Asteroleplasma* than the mix of purple prairie clover and alfalfa treatment group (18). The alfalfa hay diet elevated the proportion of *Prevotella* and *Selenomonas* compared with the cornstalk diet, while cornstalk feeding increased the proportion of *Thermoactimoyces*, *Bacillus*, *Papillibacter*, *Anaerotruncus*, and *Streptomyces* compared with the *Leymus chinensis* hay or alfalfa hay feeding in dairy cows (19). It has also been revealed associations between the rumen bacterial community with metabolite deposition. For instance, *Bacteroidales_UCG-001_norank* was negatively related to fatty acids including C18:2 and C20:4 in the longissimus dorsi (LD) of sheep (20). Amino acids including isoleucine and glycine were positively correlated with *Anaeroplasma* and negatively associated with *Parabacteroides* and *Alloprevotella* (21). *Moryella* was positively associated with fatty acids such as C16:0 and C18:1n9c, and negatively related to C20:4 n6, C20:3 n6, and C20:5 in lamb lumborum muscle (22). *Moryella* also exhibited a positive relationship with meat metabolites including L-carnosine, N-acetyl-L-histidine, and negatively related to N-acetylaspartylglutamate, L-carnitine, L-citrulline, and Pro-Glu (22). Therefore, it is important to characterize the relationship between rumen bacteria and meat metabolites.

To our knowledge, the comparison of growth performance, carcass characteristics, and meat metabolites of sheep fed with *Leymus chinensis* hay and alfalfa hay remains poorly defined. The alterations in the rumen bacterial community and interactions between bacteria and metabolites are also lacking. The lack of in-depth evaluation may affect the application of forages in ruminant farming. Therefore, this study focused on investigating the effects of *Leymus chinensis* hay and alfalfa hay on growth and carcass traits, meat metabolite deposition, and rumen bacterial community in lambs. This study will facilitate assessing growth and carcass performance, and meat quality of sheep fed with forages different forage types, and provide an important reference for improving ruminant growth and carcass performance through manipulating diets in the future.

2. Materials and methods

2.1. Animals and experimental design

The animal protocol in this study was approved by the Animal Care and Use Committee of Inner Mongolia University (Approval No. IMU-sheep-2020-041).

Healthy male East Friesian × Small-tail Han lambs were purchased from Inner Mongolia Lark Biotechnology Co., Ltd. Fourteen lambs with an average age of 60 days and with body weight of about 22.03 ± 1.08 kg were selected. The feeding trials were conducted at the Inner Mongolia University Meat Sheep Nutritional Base, where each lamb was housed individually in the same pens. Fourteen lambs were randomly allocated to two dietary treatment groups: one group was feed with concentrate and *Leymus chinensis* hay (Lc group); another was fed with concentrate and alfalfa (*Medicago sativa*) hay (Ms group) with free access to water. The Lc group consumed about 325 g of concentrate and 803 g of (*Leymus chinensis* hay each sheep per day, while the Ms group consumed about 325 g of concentrate and 925 g of alfalfa hay each sheep per day. Ingredient and chemical compositions of diets are shown in Supplementary Table S1. Before the experiment, lambs were acclimatized to the environment for about 1 week. The experiment lasted for 60 days. At the end of the experiment, lambs were selected for slaughter after fasting for about 12 h, and the LD and rumen contents samples were collected. Muscle samples were collected using a disposable scalpel at the same location in the LD of each lamb into 50 mL RNAase-free centrifuge tubes and stored at −80°C for

Abbreviations: Lc, *Leymus chinensis*; Ms, *Medicago sativa*; LD, Longissimus dorsi; OTUs, Operational taxonomic units; ADG, Average daily gain; FBW, Final body weight; IBW, Initial body weight; F/G, Feed to gain ratio; ADFI, Average daily feed intake.

metabolite analysis. After the rumen was opened, the rumen contents were filtered using 4 layers of sterile gauze, and the rumen contents were collected in a 50 mL RNAase-free centrifuge tube and stored at −80°C for 16S rDNA amplicon analysis.

2.2. Chemical analysis of roughage (*Leymus chinensis* hay and alfalfa hay)

Dry matter and crushed ash of *Leymus chinensis* hay and alfalfa hay samples were determined according to Ran et al. (23). Crude protein, acid detergent fiber, neutral detergent fiber, and crude fat in the *Leymus chinensis* hay and alfalfa hay samples were determined using an Automatic Kjeldahl Protein/Nitrogen Analyzer (K1160, Hanon Advanced Technology Group, China), Automatic Fiber Analyser (F2000, Hanon Advanced Technology Group, China) and Automatic Soxhlet Extractor (SOX606, Hanon Advanced Technology Group, China) respectively with reference to Shi et al. (24). The content of mineral elements was determined with using microwave (REVO, LabTech, China) and inductively coupled plasma-optical emission spectrometer (PQ 9000, analytikjena, German) (25).

2.3. Determination of growth and carcass performance and organ index

Body weight and carcass weight were measured following overnight fasting and evisceration, respectively. Loin-eye area between the 12th and 13th ribs was traced on sulphate papers and calculated (26). Dressing percentage = carcass weight/body weight. Organ index = organ weight/body weight (27). Total weight = final body weight (FBW) − initial body weight (IBW). Average daily gain (ADG) = total weight gain/total days. Feed to gain ratio (F/G) = average daily feed intake (ADFI)/ADG.

2.4. 16S rDNA amplicon sequencing and bioinformatics analysis

The rumen contents were collected and stored at −80°C. Microbial DNA was extracted using a TIANGEN kit. The V3–V4 region of the bacterial 16S rDNA gene was amplified by PCR (95°C for 3 min, followed by 30 cycles at 95°C for 30 s, 50°C for 30 s, 72°C for 45 s, and an extension at 72°C for 10 min) using the F3 (ACTCCTACGGG AGGCAGCAG) and R4 (GGACTACHVGGGTWTCTAAT) primer pair (28). DNA Library Prep Kit for Illumina following manufacturer's recommendations and index codes were added. High throughput sequencing was performed utilizing the Illumina MiSeq PE300 platform to detect the 16S rDNA amplicons according to standard protocols.

After sequencing, paired-end reads from sequencing were merged by FLASH, and low-quality reads were filtered by Trimmomatic. UPARSE was used to align operational taxonomic units (OTUs) at 97% identity. Taxonomy was assigned to OTUs by searching against the Greengenes database version 13.8. α- and β- diversities were calculated using QIIME2. The biomarkers with statistical differences were screened by Linear discriminant Effect Size (LEfSe) analysis, and the screening criteria were LDA threshold ≥4.0 and p-value <0.05. The redundancy analysis (RDA) and Spearman's rank correlation analysis were performed using the R packages: vegan and pheatmap, respectively.

2.5. Untargeted metabolomics and bioinformatics analysis

Untargeted metabolomics analysis was referenced to the previous method (29, 30). Briefly, 200 mg LD samples were mixed with 2-chlorophenylalanine (4 ppm) methanol (−20°C) and ground by a high-throughput tissue grinder for 90 s at 60 Hz. The samples were centrifuged at 4°C for 10 min at 12,000 rpm, and the supernatant was filtered through 0.22 μm membrane to obtain the prepared samples for LC-MS. Twenty microlitre from each sample were taken to the quality control samples, and the rest of the samples were used for LC-MS detection.

LC-MS detection was referenced against the previous method (31). Chromatographic separation was accomplished in a Thermo Ultimate 3000 system equipped with an ACQUITY UPLC® HSS T3 column maintained at 40°C. The temperature of the autosampler was 8°C. Gradient elution of analytes was carried out with 0.1% formic acid in water (C) and 0.1% formic acid in acetonitrile (D) or 5 mM ammonium formate in water (A) and acetonitrile (B) at a flow rate of 0.25 mL/min. Injection of 2 μL of each sample was performed after equilibration. An increasing linear gradient of solvent B (v/v) was used as follows: 0–1 min, 2% B/D; 1–9 min, 2%–50% B/D; 9–12 min, 50%–98% B/D; 12–13.5 min, 98% B/D; 13.5–14 min, 98% B/D; 14–20 min, 2% D-positive model (14–17 min, 2% B-negative model) (31).

The ESI-MSn experiments were executed on the Thermo Q Exactive Focus mass spectrometer with the spray voltage of 3.8 kV and −2.5 kV in positive and negative modes, respectively. Sheath gas and auxiliary gas were set at 30 and 10 arbitrary units, respectively. The capillary column temperature was 325°C. The analyzer scanned over a mass range of m/z 81–1,000 for a full scan at a mass resolution of 70,000. Data-dependent acquisition (DDA) MS/MS experiments were performed with an HCD scan. The normalized collision energy was 30 eV. Raw data were converted to the mzXML format by Proteowizard (v3.0.8789), and then processed by the MetaboAnalystR package (32).

2.6. Statistical analyses

GraphPad Prism was used for statistical analysis. The results of growth performance, carcass performance, and organ index are presented as means ± standard error (SE). All data were considered statistically significant at $p < 0.05$. Statistical significance of organ index, growth and carcass performance, and metabolites were determined by t-test. The statistical significance of α-diversity was performed with Wilcoxon tests.

3. Results

3.1. Improvement of growth and carcass traits in lambs fed a diet with alfalfa hay

To investigate the effects of *Leymus chinensis* hay and alfalfa hay on growth and carcass performance, we first compared IBW, FBW, ADG, carcass weight, and loin-eye area between the two groups. IBW was not significantly distinct, while total weight gain, FBW, and ADG were significantly greater in the Ms. group. Carcass weight, dressing percentage, and loin-eye area were also obviously increased in the

TABLE 1 Effects of *Leymus chinensis* hay and alfalfa hay on growth and carcass performance of sheep ($n = 7$).

Items	Lc group[f] (average ± SE)	Ms group[g] (average ± SE)	*p*-value[h]	
IBW[a] (kg)	21.63 ± 1.58	22.43 ± 1.60	0.727	NS
FBW[b] (kg)	30.71 ± 1.38	36.67 ± 1.76	0.020	*
ADG[c] (kg)	0.14 ± 0.01	0.20 ± 0.00	0.009	**
ADFI[d] (kg)	1.13 ± 0.04	1.32 ± 0.07	0.026	*
F/G[e]	10.71 ± 4.19	10.66 ± 2.46	0.639	NS
Carcass weight (kg)	13.29 ± 0.70	16.60 ± 1.18	0.033	*
Dressing percentage, %	43.21 ± 0.76	47.69 ± 0.58	0.0005	***
Loin-eye area (cm^2)	15.39 ± 1.03	20.25 ± 1.35	0.014	*

[a]IBW, Initial body weight.
[b]FBW, Final body weight.
[c]ADG, Average daily gain.
[d]ADFI, Average daily feed intake.
[e]F/G, Feed to gain ratio.
[f]Lc group indicates lambs fed with *Leymus chinensis* hay.
[g]Ms group indicates lambs fed with alfalfa hay.
[h]* $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, NS indicates no significant differences.

TABLE 2 Effects of *Leymus chinensis* hay and alfalfa hay on organ index ($n = 7$).

Items	Lc group[a] (average ± SE)	Ms group[b] (average ± SE)	*p*-value[c]	
Spleen index, %	0.14 ± 0.01	0.15 ± 0.01	0.538	NS
Lung index, %	0.95 ± 0.04	1.07 ± 0.03	0.052	NS
Liver index, %	1.47 ± 0.06	1.63 ± 0.07	0.114	NS
Kidney index, %	0.27 ± 0.01	0.31 ± 0.00	0.001	**
Heart index, %	0.40 ± 0.02	0.43 ± 0.02	0.270	NS

[a]Lc group indicates lambs fed with *Leymus chinensis* hay.
[b]Ms group indicates lambs fed with alfalfa hay.
[c]* $p \leq 0.05$, ** $p \leq 0.01$, NS indicates no significant differences.

Ms. group. However, lambs in the Ms. group had higher ADFI. The F/G was similar between the two groups (Table 1). In addition, we analyzed the organ index in lambs. Kidney index was increased in the Ms. group, while others including spleen, lung, liver, and heart index were not significantly affected (Table 2). These data indicated that compared to *Leymus chinensis* hay, alfalfa hay improved growth and carcass traits in lambs.

3.2. Alterations of rumen bacterial diversity and composition between two groups

To investigate the effects of *Lemus chinensis* hay and alfalfa hay on rumen microbiota, we performed and analyzed bacterial 16S rDNA sequencing of rumen contents between two groups. The observed OTU and Shannon curves reached the saturation phase, suggesting the sufficient and reliable sequence depth captured in this study (Figure 1A). A total of 2,140 OTUs were identified in both groups, among which 3,491 and 3,861 specific OTUs were observed in the Lc and Ms. groups, respectively (Figure 1B). Shannon and Simpson indexes reflected the bacterial community richness and diversity. No significant differences were observed in the Shannon and Simpson index between the two groups (Figure 1C). The β-diversity presenting as Principal Co-ordinates Analysis (PCoA), Non-metric multidimensional scaling (NMDS), Principal Components Analysis (PCA), and Partial Least Squares Discrimination Analysis (PLS-DA) was further applied to analyze the variation of bacterial structure. The NMDS, PCA, and PLS-DA are in Supplementary Figure S3. The Lc group rumen contents (LcRc) formed a different rumen bacterial community clustered separately from the Ms group rumen contents (MsRc) (Figure 1D).

To further define the composition of rumen microbiota in the LcRc and MsRc, the percentages of bacterial community abundance were analyzed at phylum and genus levels. At the phylum level, *Bacteroidetes*, *Firmicutes*, *Spirochaetes*, and *Fibrobacteres* were the dominant phyla (Figure 2A). The relative abundance of *Fibrobacteres*, *Bacteroidetes*, and *Spirochaetes* were obviously greater in the Lc group (Supplementary Table S2); the relative abundance of *Firmicutes*, *Actinobacteria*, *Fusobacteria*, *Verrucomicrobia*, and *Proteobacteria* were obviously greater in the MsRc (Supplementary Table S2). At the genus level, the top 20 genera were present with relatively high abundance (Figure 2B). The 17 genera significantly altered between two groups, among which only 5 genera, i.e., *Clostridiu*, *Fibrobacter*, *Selenomonas*, *BF311*, and *Treponema*, were greater in the LcRc. Most of the altered bacteria in the MsRc, including *Moryella*, *Blautia*, *p_75_a5*, *Faecalibacterium*, *Mogibacteriaceae*, *Anaerostipes*, *Bifidobacterium*, *Eggerthella*, *and Collinsella* belong to *Firmicutes* and *Actinobacteria* (Supplementary Table S3).

The rumen bacterial composition was further compared through LEfSe analysis with 4.0 as the threshold on the LDA score to identify specific species in each group. The constitution of rumen bacteria changed between LcRc and MsRc. A total of 17 taxa sequences were enriched in the LcRc and mainly belonged to *Fibrobacteres*, *Bacteroidetes*, and *Spirochaetes* (Figure 3). A total of 7 rumen bacteria mainly belonging to *Firmicute* were determined as enriched in the MsRc (Figure 3). Taken together, lambs in Lc and Ms. groups had distinct compositions of rumen bacterial community, which may lead to different growth performance, carcass performance, and metabolic profiles.

3.3. Key rumen microbiota associated with ADG, carcass weight, and body weight

To further investigate the potential relationship between rumen microbiota variation and ADG, carcass weight, and body weight, we performed the RDA and Spearman's correlation between differential rumen bacteria and host parameters. According to RDA, the *Lemus chinensis* hay and alfalfa hay treatments were distinct and two different clusters were observed. ADG, carcass weight, and body weight showed a significant correlation with bacterial community (Figures 4A,B). The Spearman's correlation data showed that the relative abundance of *BF311* and *Pseudobutyrivibrio* were negatively related to ADG, carcass weight, and body weight (Figure 4C). Additionally, most genus-level bacteria such as *Enterococcus*, *Lactobacillus*, *Acidaminococcus*, and *Moryella* belonging to *Firmicutes* showed a strongly positive correlation with ADG, carcass weight, and body weight (Figures 4C). *Alistipes* belonging to *Bacteroidetes*, *Eggerthella* and *Bifidobacterium* belonging to *Bacteroidetes*, and

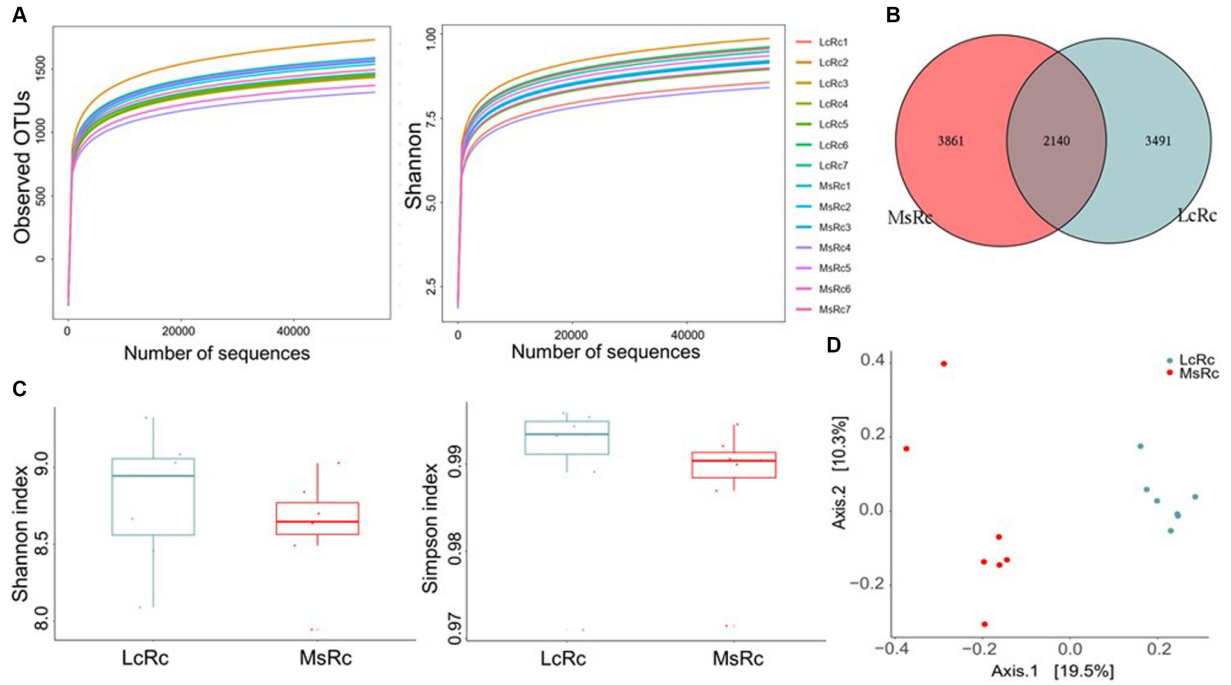

FIGURE 1
The diversity of the rumen microbiota of lambs fed a diet with *Lemus chinensis* hay and alfalfa hay. **(A)** Observed OTUs and Shannon curves of rumen microbiota. **(B)** The Venn diagram illustrates the overlap of microbial OTUs between the two groups. **(C)** The α-diversity includes Shannon and Simpson index. **(D)** The β-diversity presents as PCoA. $n = 7$ in each group. LcRc and MsRc indicate rumen contents from lambs fed with *Leymus chinensis* hay and alfalfa hay, respectively.

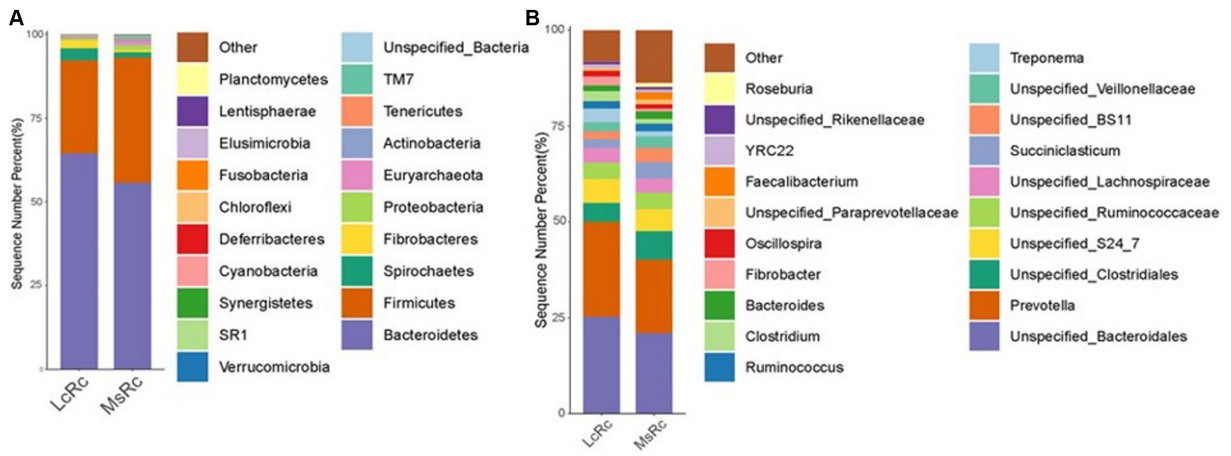

FIGURE 2
The composition of the rumen microbiota of lambs fed a diet with *Lemus chinensis* hay and alfalfa hay. Taxonomic distribution of rumen bacterial communities of different groups at phylum level **(A)** and genus level **(B)**. LcRc and MsRc indicate rumen contents from lambs fed with *Leymus chinensis* hay and alfalfa hay, respectively. "p_" denotes phylum-level classification, "f_" denotes phylum-level classification, and "g_" denotes genus-level classification.

Anaeroplasma belonging to *Tenericutes*, were also positively associated with ADG, carcass weight, and body weight (Figure 4C).

3.4. Metabolome profiles of longissimus dorsi

To define similarities and differences between lamb meat fed with *Leymus chinensis* hay and alfalfa hay, we performed and analyzed the untargeted metabolome profiles of LD samples. PCA score plots with positive and negative ionization modes revealed no significant clustering of the two treatment groups (Figure 5A), but further analysis revealed significant differences in metabolite composition. These metabolites mainly consisted of peptides, lipids, organic acids, steroids, carbohydrates, nucleic acids, vitamins and cofactors, hormones, and transmitters (Figure 5B). These two comparisons identified 96 significant differential metabolites (67 in the positive

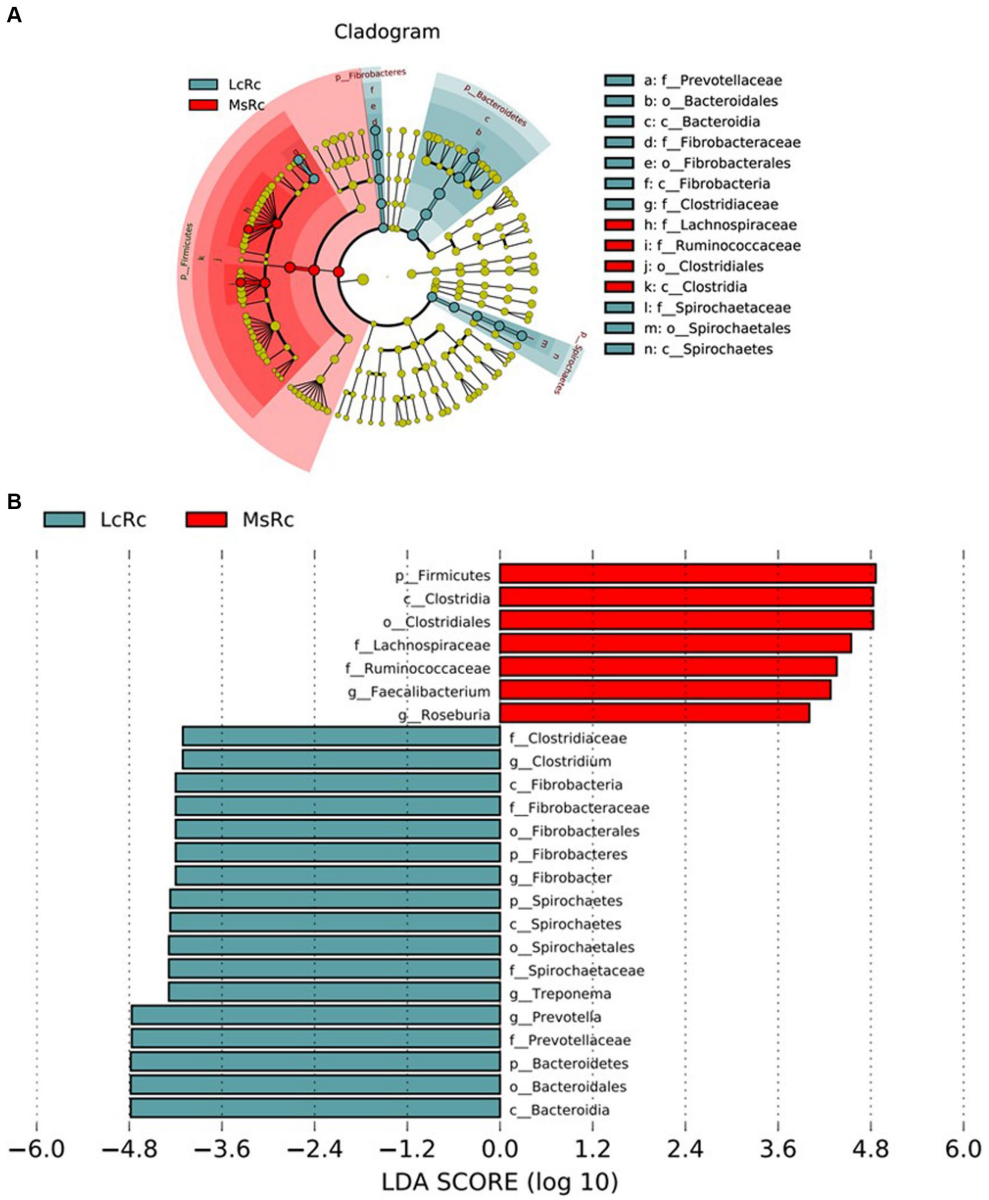

FIGURE 3
The effect of *Lemus chinensis* hay and alfalfa hay on the taxonomic diversity of the rumen bacteria. The cladogram **(A)** and histogram **(B)** show rumen bacterial taxa with a linear discriminant analysis (LDA) score >4.0 by LEfSe analysis. LcRc and MsRc indicate rumen contents from lambs fed with *Leymus chinensis* hay and alfalfa hay, respectively.

mode and 29 in the negative mode), among which 52 and 44 differential metabolites were elevated in the Lc and Ms. groups, respectively. In detail, organic acid and derivatives including N-Acetyl-L-leucine, Pantetheine, Cysteinyl glycine, Alanyl tyrosine, and Ala-Ile were greater in the Ms group longissimus dorsi (MsLD), while Cinnamoyl glycine, L-arginosuccinate, and Glu-Thr were greater in the Lc group longissimus dorsi (LcLD). Lipids and lipid-like molecules such as 2-methylbutyroylcarnitine, Glycoursodeoxycholic acid, and 2-Hydroxymyristic acid were higher in the LcLD, while Hexadecanedioic acid, prostaglandin F2alpha, and tetradecanedioic acid were higher in the MsLD. Interestingly, LcLD contained higher organoheterocyclic compounds including Momocrotaline, (+)-alpha-lipoic acid, Methyl indole-3-acetate, benzenoids (i.e., hippuric acid), phenylpropanoids, and polyketides (i.e., 2-phenylpropionic acid). MsLD had a higher relative abundance of alkaloids and derivates including ecgonine methyl ester (Figures 5C,D). Between *Leymus chinensis* hay and alfalfa hay treatment, peptides including Ala-Ile, Glu-Thr, N-Acetyl-Asp-Glu, gamma-glutamyl-cysteine, and gamma-glutamyl-L-leucine, significantly changed (Figures 5C,D). Furthermore, based on the pathway analysis, we found that these differential metabolites were mainly enriched in alanine, aspartate and glutamate metabolism, D-glutamine and D-glutamate metabolism, phenylalanine metabolism, nitrogen metabolism, and tyrosine metabolism (Figures 6A,B). Changed metabolites in these pathways were shown in Table 3, indicating that different forages affected amino acid composition and contents, and its metabolism, leading to various meat nutritional qualities.

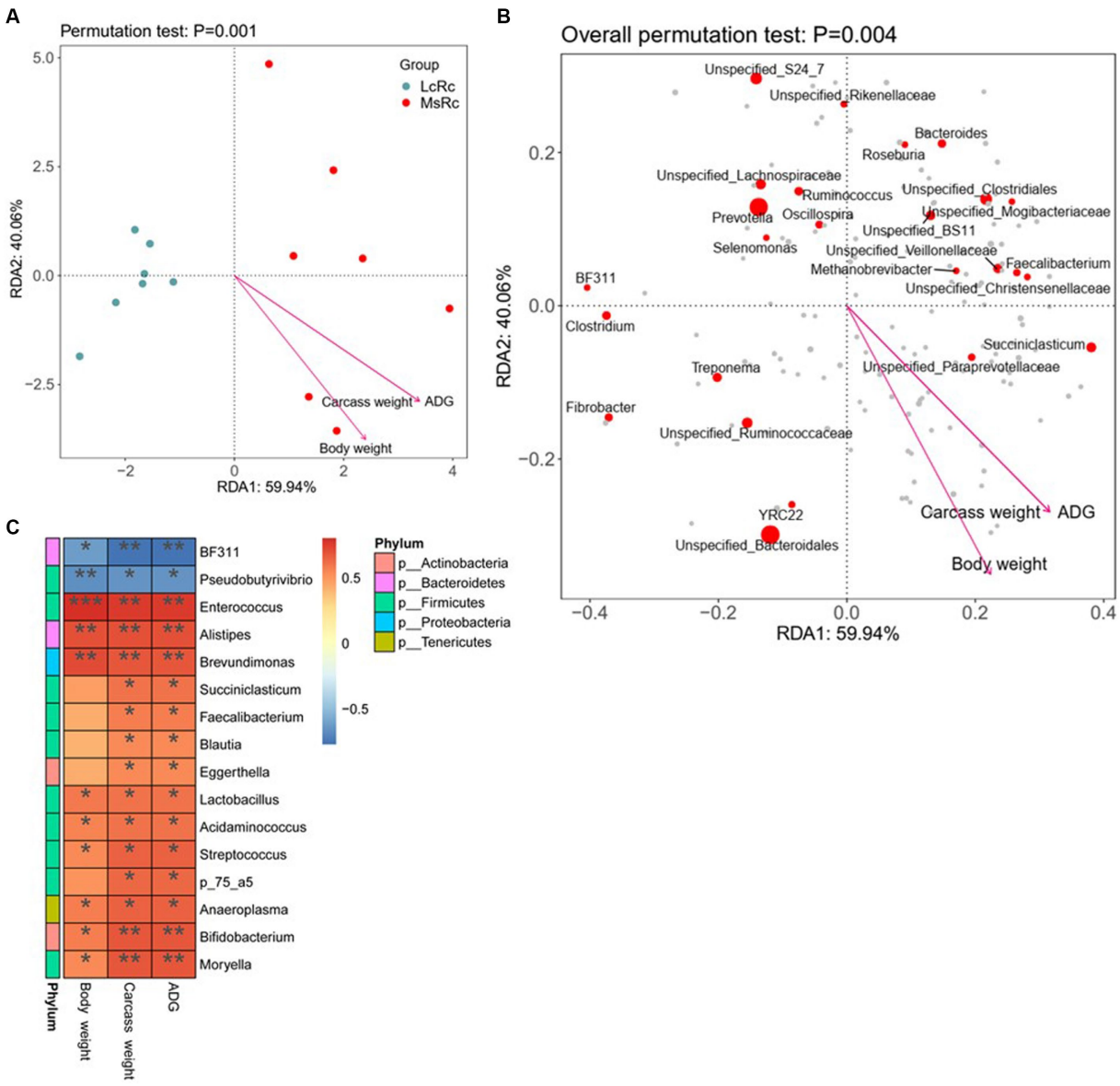

FIGURE 4
Relationship between key rumen bacteria with ADG, carcass weight, and body weight. **(A,B)** RDA was performed on genus-level taxonomic profile and host parameters including ADG, carcass weight, and body weight. Arrows indicate the correlation between community structure and host parameters. **(C)** Correlation analysis of key rumen microbiota with ADG, carcass weight, and body weight. Statistical significance was calculated by Spearman's correlation analysis. $*p \leq 0.05$, $**p \leq 0.01$, $***p \leq 0.001$.

To facilitate the identification of potential biomarkers between the two groups, PLS-DA and OPLS-DA were applied to identify the major differences in metabolites. PLS-DA score plots showed the best separation of LcLD and MsLD samples (Supplementary Figure S2A). Several potential biomarkers with VIP >2 including Monocrotaline, Hippuric acid, 6-Hydroxytestosterone, N-Acetyl-L-leucine, LysoPE 18:0, Ala-Ile, Methadone-d9, Cysteinylglycine, Cinnamoylglycine, Pilocarpine, and 2-Phenylpropionic acid and were highlighted in the volcano plots (Supplementary Figure S2B). To minimize overfitting in the PLS-DA model, we further explored potential markers using OPLS-DA between groups. The analysis presented a cross-validated score plot (R2X = 0.311, R2Y = 0.993, Q2 = 0.737, p-value = 0.01) of the discriminating model between LcLD and MsLD in positive ionization mode. The cross-validated score plot (R2X = 0.454, R2Y = 0.995, Q2 = 0.526, p-value = 0.05) was shown in negative ionization mode. Identified biomarkers were the same as those identified by PLS-DA as having a trend toward being distinct between the two groups (Supplementary Figure S3). Additionally, random forest showed top differential metabolites including N-Acetyl-L-leucine, Hippuric acid, (+)-alpha-lipoic acid, and 2-Phenylpropionic acid (Supplementary Figure S4).

Differential metabolites and potential biomarkers have been clarified as mentioned above. The correlation of potential biomarkers with growth performance was further analyzed. A total of 30 differential metabolites were significantly associated with ADG,

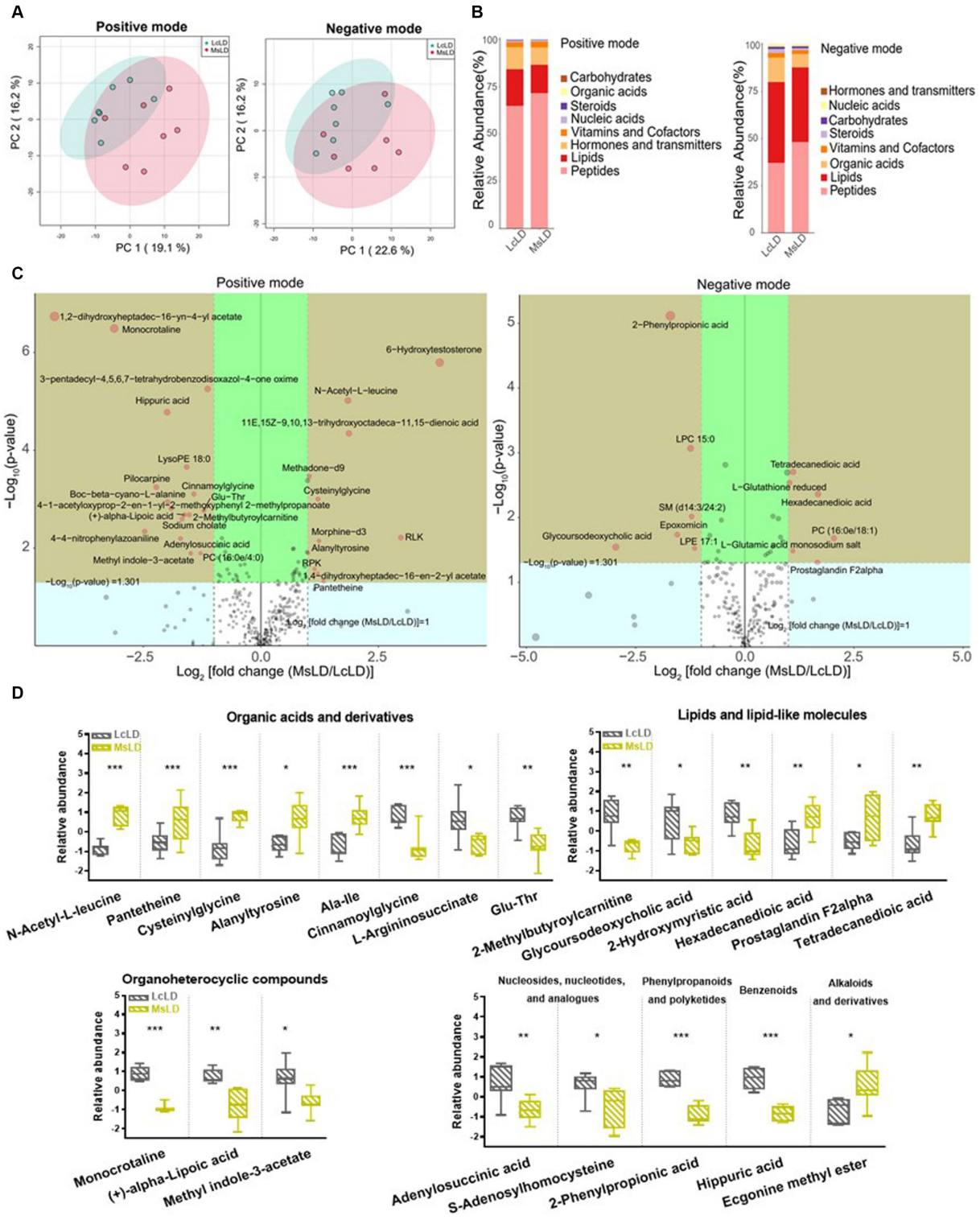

FIGURE 5
Effects of *Lemus chinensis* hay and alfalfa hay on metabolites of LD in lambs. **(A)** Scatter plots of the PCA model based on identified metabolite features. **(B)** Composition of metabolites in LcLD and MsLD. **(C)** Volcano plot of differential metabolites between LcLD and MsLD. **(D)** Boxplot of selected differential metabolites. $n = 7$ in each group. $^{*}p \leq 0.05$, $^{**}p \leq 0.01$, $^{***}p \leq 0.001$. LcLD and MsLD indicate LD from lambs fed with *Leymus chinensis* hay and alfalfa hay, respectively.

carcass weight, and body weight. Among them, 14 metabolites including N-Acetyl-L-leucine, Methadone-d9, Hexadecanedioic acid, and alanyltyrosine were positively related to ADG, carcass weight, and body weight. Sixteen metabolites such as LysoPE 18:0, Cinnamoylglycine, and 2-Methylbutyroylcarnitine exhibited a negative relationship with growth performance (Figure 7).

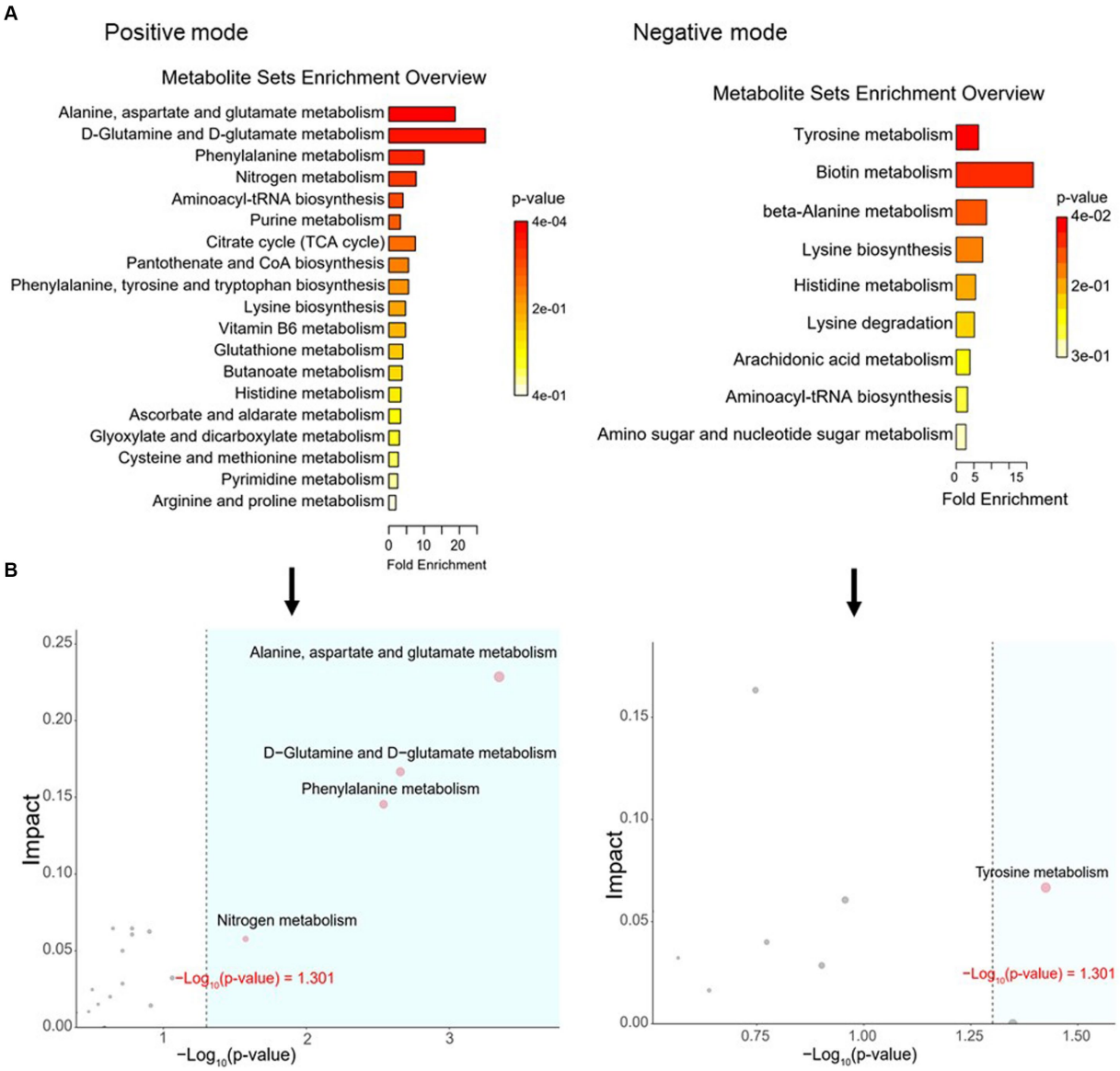

FIGURE 6
Pathway analysis of differential metabolites. **(A)** Enriched KEGG pathways of the comparison between LcLD and MsLD. **(B)** Pathway enrichment and topology analysis.

3.5. Correlation of key rumen microbiota with differential metabolites

The 16S rDNA sequencing revealed that lambs with the consumption of dietary *Leymus chinensis* hay and alfalfa hay exhibited distinct rumen microbiota communities. The metabolomics analysis indicated that exposure to *Leymus chinensis* hay and alfalfa hay resulted in different metabolic profiles of LD. Therefore, we explored the correlations between the rumen microbiota and the altered LD metabolites through Spearman's correlation analysis, revealing high correlations between key rumen microbiota with differential metabolites (Figure 8). In detail, *Fibrobacter*, *Selenomonas*, *BF311*, *Treponema*, and *Clostridium* exhibited a strong positive relationship with organic acid and derivatives such as Glu-Thr, benzenoids including hippuric acid, phenylpropanoids and polyketides including 2-phenylpropionic acid, and organoheterocyclic compounds including Methyl indole-3-acetate, Momocrotaline, and (+)-alpha-lipoic acid. And they were negatively associated with other differential metabolites including Ala-Ile, N-Acetyl-L-leucine, Tetradecanedioic acid, and Methadone-d9. Other genera including *Bifidobacterium*, *Faecalibacterium*, *Blautia*, *Roseburia*, *Moryella*, *Streptococcus*, *[Ruminococcus]*, and *Odoribacter*, showed an obviously opposite correlation trend with the five genera mentioned above (Figure 8). These results indicated that the rumen bacterial compositions obviously and specifically impacted host LD metabolites.

4. Discussion

The forage is one of the main factor affecting ruminant production, rumen microbiota community, and meat quality. Previous studies explored ratios of various forages such as alfalfa hay, *Leymus*

TABLE 3 KEGG pathway enrichment of the changed metabolites for LcLD vs. MsLD.[a]

KEGG pathway	Changed metabolite	*p*-value	Upregulation
Alanine, aspartate, and glutamate metabolism	L-Glutamine	0.0004	LcLD
	2-Oxoglutaric acid		LcLD
	Adenylosuccinic acid		LcLD
D-Glutamine and D-glutamate metabolism	L-Glutamine	0.0022	LcLD
	2-Oxoglutaric acid		LcLD
Phenylalanine metabolism	L-Phenylalanine	0.0029	MsLD
	Phenylacetylglycine		MsLD
	Hippuric acid		LcLD
Nitrogen metabolism	L-Phenylalanine	0.0266	MsLD
	L-Glutamine		LcLD
Tyrosine metabolism	L-Dopa	0.0376	MsLD
	Homovanillic acid		MsLD
Biotin metabolism	L-Lysine	0.0449	MsLD

[a]LcLD and MsLD indicate LD from lambs fed with *Leymus chinensis* hay and alfalfa hay, respectively.

chinensis hay, *Vigna radiata* stalk, and corn stalk, which had a greater effect on the ruminants (5, 9, 12). To our knowledge, few studies have systematically compared the growth and carcass traits of lambs fed *Leymus chinensis* hay and alfalfa hay. In this study, we found that feeding with alfalfa hay increased lamb carcass weight, FBW, ADG, loin-eye area, and dressing percentage compared to those in the Lc group, supporting that those lambs fed with the alfalfa hay exhibited better carcass characteristics and growth performance.

The composition and diversity of rumen bacteria exhibit a close relationship with the host. Diet is one of the key factors that triggers changes in rumen microbial communities, alongside different environmental factors (33). To investigate the impacts of *Leymus chinensis* hay and alfalfa hay on the host rumen bacterial community, 16S rDNA amplicon sequencing was performed and analyzed. Obviously, there was no significant difference in Shannon and Simpson indexes in the LcRc and MsRc. This indicates no significant altered in the diversity of rumen bacteria.

Exploring changes in rumen microbiota, which are closely related to muscle metabolites, can help to investigate the mechanisms for improving lamb meat quality under different roughage conditions. At the phylum level, *Firmicutes* and *Bacteroidetes* were the major bacterial phyla in the rumen as reported (20, 22), whose relative abundances were significantly distinct in the LcRc and MsRc. *Firmicutes* are dominant across rumen bacterial communities and consist of various fibrolytic and cellulolytic bacterial genera (34). *Bacteroidetes* are beneficial for digesting carbohydrates (35). Then, we analyzed differences in key rumen bacteria at the genus level between the two groups and their correlation with metabolite deposition in the LD. *Unspecified bacteroidales* and *Prevotella* were the dominant bacterial genera in both groups. Based on the LEfSe analysis, lambs in the Lc group have a higher relative abundance of *Prevotella*, which plays an important role in polysaccharide and protein metabolism, and utilization of hemicelluloses (20). *Fibrobacter* was also higher in the LcRc, which is a cellulolytic bacteria in the rumen of ruminants (36). This may be related to the higher fiber intake of lambs in the Lc group, while we found that they positively regulate Glu-Thr levels in the LD. Dipeptides are important flavor precursors in lamb meat (37), suggesting that rumen microorganisms may influence lamb meat quality by regulating flavor substances in muscle after feeding *Leymus chinensis* hay. It has been reported that *Fibrobacter* was able to synthesize oligosaccharides and contributed to rapidly adapting to sudden environmental changes (33). In addition, three genera mainly belonging to the fibrolytic bacteria, such as *Selenomonas*, *BF311*, and *Treponema* were obviously elevated in the LcRc. *Treponema* is closely related to pectin treatments due to its ability to degrade pectin, which shares mutual interaction with *Fibrobacter* (33, 38). *Selenomonas* can oxidize lactate and produce propionic acid as a main fermentation product (39). And the correlation between these three fibrolytic bacteria and the metabolite levels of the LD had the same trend as *Fibrobacter*, suggesting that they may have the same mechanism of action. Most of the significant differential bacteria, such as *Bifidobacterium*, *Faecalibacterium*, *Mogibacterium*, *Anaerostipes*, *Alistipes*, and *Blautia*, were elevated in the MsRc. A previous study has shown that *Bifidobacterium* is saccharolytic bacteria can generate acetate and lactate (10). *Faecalibacterium* is one of the butyrate-producing bacteria linked to higher weight gain, and displays anti-inflammatory action (40). *Anaerostipes* is also a butyrate-producing bacterium (41). In the present study also the relative abundance of *Faecalibacterium* was found to be positively correlated with body weight and carcass weight. Changes in *Mogibacterium* are associated with feed efficiency in ruminants (42). Additionally, *Blautia* has been proven to enhance beneficial anti-inflammatory effects of hosts (43). These results suggested that alfalfa hay may have altered feed efficiency, influenced volatile fatty acid production, and also facilitated rumen resistance to inflammation. Taken together, the forage types, i.e., *Leymus chinensis* hay and alfalfa hay, resulted in different rumen bacterial compositions of lambs.

The compositions of nutrient metabolites have a direct impact on meat quality (44). Thus, meat metabolic profiles aroused great public concern. Amino acids are important ingredients for the nutritional value of mutton (45). According to the analysis of untargeted metabolomics, we found significant differences in amino acid-related metabolites in meat between Lc and Ms. groups. Some amino acid metabolism, including alanine, aspartate, and glutamate metabolism, phenylalanine metabolism, D-glutamine, and D-glutamate metabolism, and tyrosine metabolism were changed, suggesting forages regulated amino acid contents and metabolism, and thus affected the nutritional quality of meat. Free amino acids and dipeptides are important flavor precursors in meat (37). Importantly, some peptides have bioactive properties (37). Cysteinylglycine belonging to the dipeptide is a structural component of glutathione (46), which was higher in the MsLD. Between *Leymus chinensis* hay and alfalfa hay treatment, peptides including Ala-Ile, Glu-Thr, N-Acetyl-Asp-Glu, gamma-glutamyl-cysteine and gamma-glutamyl-L-leucine changed significantly. The gamma-glutamyl peptide, gamma-glutamyl-L-leucine, has been recognized as a kokumi-imparting molecule (47). Another gamma-glutamyl peptide, gamma-glutamyl-cysteine, can activate the calcium-sensing receptor and then impart the kokumi peptide-induced responses (48).

Rumen microorganisms provide energy for muscle metabolism to the host mainly in the form of volatile fatty acids (49). Feeding *Leymus chinensis* hay and alfalfa hay not only altered the substrates available for rumen fermentation but may have also altered the distribution of

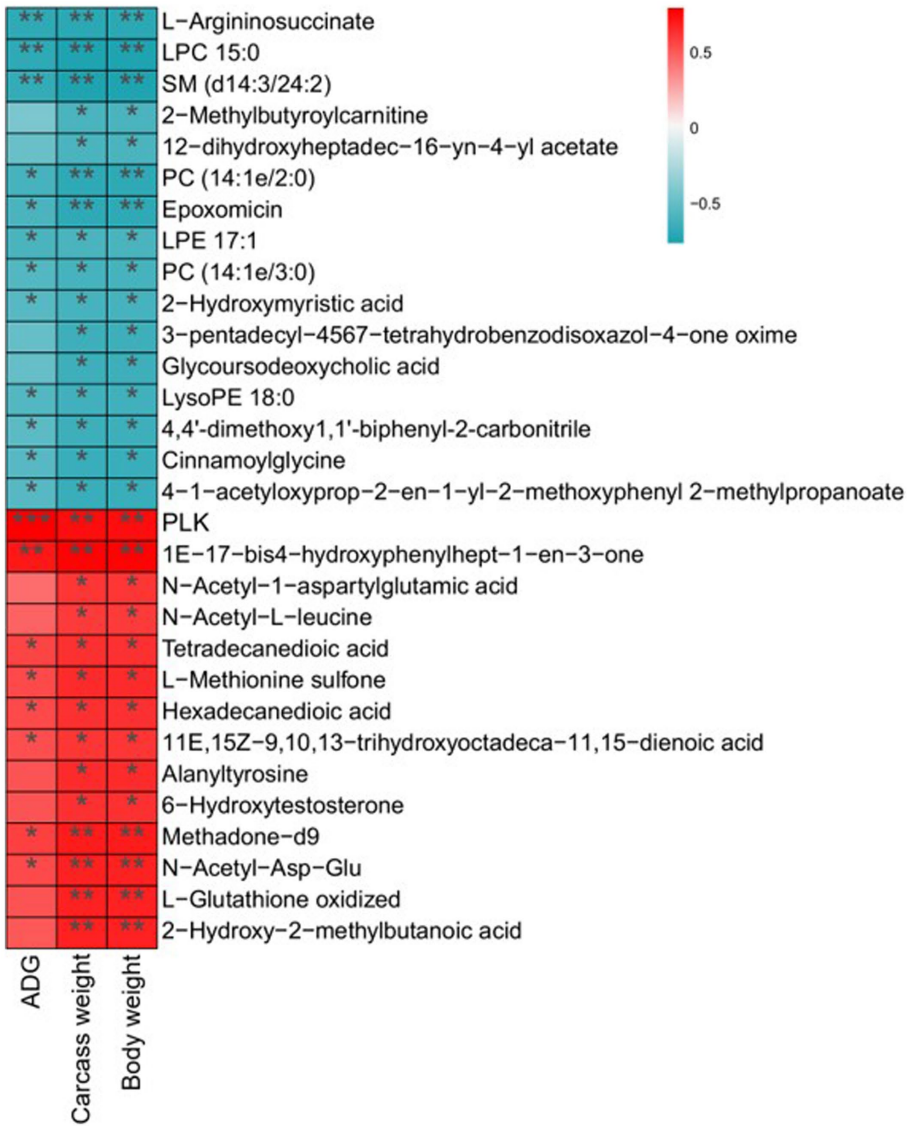

FIGURE 7
Correlation analysis of differential metabolites with ADG, carcass weight, and body weight. Statistical significance was calculated by Spearman's correlation analysis. *$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$.

various fatty acids in the rumen, thereby affecting the composition of the rumen microbiota and the levels of LD muscle metabolites (50). For example, Du et al. (51) found that *Ruminiclostridium_6* and *U29-B03* might be participating in carbohydrate metabolism to produce volatile fatty acids, which promote IMF deposition affecting tenderness in muscles. The significant changes in the relative abundance of the associated bacteria in the Lc and Ms. groups in the present study may similarly cause alterations in the levels of metabolites in muscle via the volatile fatty acid pathway. However, more research would be required to reveal the connection between different roughages and changes in rumen microbiota and muscle metabolites.

5. Conclusion

Compared to lamb with the *Leymus chinensis* hay diet, lambs fed with alfalfa hay exhibited better growth performance and carcass performance. *Leymus chinensis* hay led to the enrichment of the genera *Fibrobacter*, *Treponema*, *Selenomonas*, and *BF311*. Alfalfa hay led to the enrichment of *Blautia*, *Anaerostipes*, *Faecelibacterium*, *Alistipes*, *Bifidobacterium*, and other genera. Ruminal bacteria after feeding different roughages affect the quality of lamb meat by influencing the metabolism of several amino acids and polypeptides in lamb meat. It was also demonstrated that alanine, aspartate and glutamate metabolism, D-glutamine and D-glutamate metabolism, phenylalanine metabolism, nitrogen metabolism, and tyrosine metabolism were the key metabolic pathways involved after feeding *Leymus chinensis* hay and alfalfa hay. In addition, conjoint analysis of rumen microbes and metabolomics indicated a close relationship between rumen microbial composition and muscle metabolites. These results have important significance for the future adequate and rational utilization of pasture to improve the quality of lamb meat.

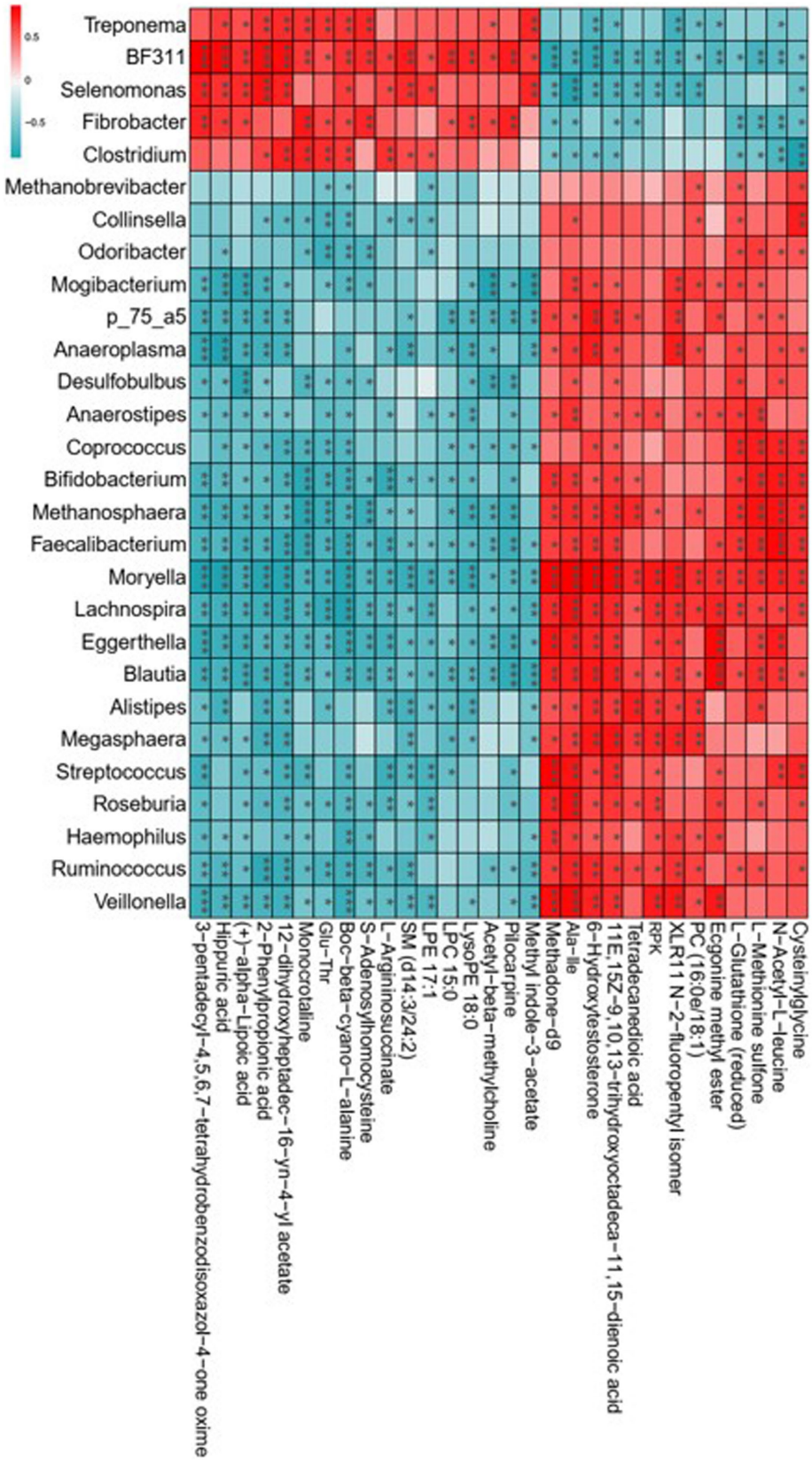

FIGURE 8
Correlation analysis of key rumen microbiota with differential metabolites. Statistical significance was calculated by Spearman's correlation analysis. *$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$.

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 at: https://www.ncbi.nlm.nih.gov/bioproject; PRJNA995904.

Ethics statement

The animal study was approved by Animal Care and Use Committee of Inner Mongolia University (Approval No. IMU-sheep-2020-041). The study was conducted in accordance with the local legislation and institutional requirements.

Author contributions

HW: Writing – original draft. LiM: Data curation, Writing – review & editing. LaM: Writing – review & editing, Data curation.

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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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Dietary supplementation with β-mannanase and probiotics as a strategy to improve laying hen performance and egg quality

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The objective of this study was to assess the impact of β-mannanase and probiotic on the performance, serum biochemistry, gut morphometric traits, and fresh egg quality of laying hens. A total of 120 cages, housing light-weight laying hens (36weeks old), were randomly assigned to four different treatments. These treatments included a control group fed non-supplemented diets; diets supplemented with 300g/ton of beta-mannanase; diets supplemented with 50g/ton of probiotic; or diets containing both 300g/ton of β-mannanase and 50g/ton of probiotics. The trial spanned a duration of 26weeks and was divided into three productive phases, each lasting 28days. The inclusion of β-mannanase resulted in a significant improvement in the laying rate by 11% ($p<0.05$) compared to the control treatment. Similarly, the addition of probiotics also enhanced the laying rate by 7% ($p<0.05$), as well as the supplementation with combined additives (11.5%). Combined additives showed an increase in egg masses, and additive association improved by 13.9% ($p<0.001$) in contrast to the control treatment. Overall, β-mannanase and combined additives used during the supplementation period resulted in improvements in the weight of fresh eggs. These benefits were observed after a period of 14weeks without supplementation (p <0.05). Furthermore, significant differences were observed in the serum biochemistry and egg masses of birds that were fed diets containing both additives (β-mannanase+probiotics) compared to the control group. Parameters such as uric acid, total cholesterol, and triglycerides displayed notable variations. The villi height: crypt depth showed differences with combined additives (β-mannanase + probiotics). The β-mannanase improved specific gravity, yolk height, length, and pH, and yolk color traits compared to the control treatment. The use of probiotics helped to improve yolk height, pH, and color score. Besides, combined additives (β-mannanase+probiotics) improve yolk height, length, weight, pH, and better traits in yolk color. Hence, incorporating β-mannanase and probiotics into laying hen diets proves to be a highly effective strategy for enhancing laying rate and overall health status, while simultaneously elevating certain quality attributes of fresh eggs.

KEYWORDS

additives, biochemical indicators, egg quality, feeding, gut health, nutrition

1 Introduction

In 2019, the World Health Organization (WHO) launched a campaign calling on governments to adopt measures to contain antimicrobial resistance. The inappropriate use of antibiotics both in human medicine and in animal production has become a public health problem, which has been worsening (1). The use of antibiotics is less frequent in laying poultry due to the possibility of residues in the eggs. Still, the use of feed additives is a possible alternative to improve productivity, health status, and even egg quality.

Probiotics were defined by Fuller (2) as a supplement consisting of live microorganisms that benefit the host and improve its intestinal microbial balance. The mechanisms of action of probiotics occur through different processes, which may or may not be associated. Physical effects arise from competitive exclusion or competition for binding sites on the intestinal mucosa. Beneficial bacteria present in probiotics form a protective physical barrier, preventing opportunistic pathogens from occupying the same binding sites. Biological effects occur due to the presence of anaerobic bacteria in probiotics. These bacteria promote a low oxygen tension environment within the gut, which inhibits the growth of pathogens, creating an unfavorable environment for their survival and reproduction. Chemical effects are observed through the production of bacteriocins by probiotic bacteria. Bacteriocins are antimicrobial substances that are effective against various pathogens, further inhibiting their growth and reducing their impact on the body. Additionally, probiotics can have a nutritional effect, providing essential nutrients and promoting a healthy gut environment, which supports the growth and maintenance of beneficial bacteria (3–6).

Enzyme supplementation is a valuable strategy for enhancing gut health by mitigating the effects of anti-nutritional components (7). β-mannanase, in particular, may support nonruminant animals in digesting non-starch polysaccharides, which can otherwise hamper nutrient digestibility (8–11). These polysaccharides, mainly β-mannans, are abundant in plant cell walls and commonly found in animal feed ingredients like soybeans (12). β-mannans are also present on the surface of microorganisms, triggering the animal's innate immune system and leading to the activation of monocytes, macrophages, dendritic cells, and increased cytokine production. Consequently, this incurs unnecessary energy expenditure and heightened inflammatory response (13). By hydrolyzing β-mannans, this enzyme enhances mannans' digestibility, boosts the population of beneficial bacteria, fortifies immunity, improves nutrient digestion and absorption, and restricts the proliferation of potential pathogens in the intestine (7).

Although the advantages mentioned earlier are noteworthy, it is important to note that the majority of the existing data was derived from different poultry categories, specifically broilers. Moreover, both additives function in complementary ways, suggesting the potential for synergistic effects when combined in feed supplementation. There is currently no literature available that describes the combined effects of these additives. Therefore, the objective of this study was to assess whether supplementing commercial laying hens with β-mannanase and probiotics alone or in combination could enhance performance, health status, and egg quality.

2 Materials and methods

2.1 Animals, housing, and experimental design

The experimental protocol described was approved by the Institutional Ethics Committee on the Use of Animals (CEUA/UFRGS) under protocol number 39783. The experimental units consisted of randomly selected hens from a commercial farm located in Salvador do Sul, Rio Grande do Sul, Brazil. The farm housed approximately 28,000 Hyline W 36 lineage light-weight laying hens, 36 weeks old. For the trial, 120 cages, each containing four birds, were utilized as replicates. These replicates were randomly assigned to the four treatments. The treatments consisted of: (1) Control (CON) treatment: This group received a basal diet without any supplementation with additional additives. (2) β-mannanase (BMA) treatment: The birds in this group received the control diet supplemented with 300 g/ton of β-mannanase. (3) Probiotic (PRO) treatment: The birds in this group received the control diet supplemented with 50 g/ton of a multi-strain probiotic additive. (4) β-mannanase + probiotic (BMA + PRO) treatment: The birds in this group received the control diet supplemented with 300 g/ton of β-mannanase and 50 g/ton of a multi-strain probiotic.

The β-mannanase employed in this trial was Hemicell™ HT, sourced from Elanco Animal Health in São Paulo, Brazil. It is an exogenous enzyme derived from the fermentation of the *Paenibacillus lentus* bacteria. The probiotic additive used in this trial was Protexin™ Concentrate, also provided by Elanco Animal Health in São Paulo, Brazil. The probiotic comprises a combination of beneficial bacterial strains, including: *Lactobacillus acidophilus* (2.06×108 UFC/g), *L. bulgaricus* (2.06×108 UFC/g), *L. plantarum* (1.26×108 UFC/g), *L. rhamnosus* (2.06×108 UFC/g), *Bifidobacterium bifidum* (2.0×108 UFC/g), *Enterococcus faecium* (6.46×108 UFC/g), and *Streptococcus thermophilus* (4.10×108 UFC/g).

The experiment spanned a duration of 26 weeks. During the initial 84 days of the trial, the birds received supplementation. To facilitate evaluation, this period was divided into three distinct phases: phase 1 (36–40 weeks), phase 2 (41–44 weeks), and phase 3 (45–48 weeks). Upon completion of the supplementation period, all birds were transitioned to the control diet for a period of 14 weeks. Subsequently, a new evaluation was conducted at week 62 to assess the outcomes.

The basal diet (Table 1) was formulated as a corn-soybean meal-based feed to fulfill the genetic nutritional requirements (14). To account for the absence of β-mannanase and/or probiotic additives, inert material in the form of kaolin was incorporated into the basal feed. During the entire experimental period, the birds had *ad libitum* access to feed and water. Nipple drinkers and gutter feeders were utilized, ensuring unrestricted access for the birds.

The birds were accommodated in conventional sheds that were oriented in an east–west direction. These sheds were constructed with concrete floors and masonry walls, and wire mesh extended up to the ceiling. To ensure optimal thermal comfort, the sheds were equipped with adjustable side curtains, which were managed based on prevailing weather conditions. The lighting regime followed a schedule of 16 h of light starting at 04:00 pm until 08:00 am followed by 8 h of darkness each day, providing a consistent lighting pattern for the birds. Throughout the entire duration of the experiment, the birds were

TABLE 1 Composition of control diet.

	Control treatment
Ingredient composition	
Corn	61.790
Soybean meal 45%	23.556
Limestone	9.283
Soybean oil	1.645
Dicalcium phosphate	1.549
Corn gluten 60%	1.024
Inert (washed sand)	0.262
Salt	0.497
DL-methionine	0.183
Vitamin premix[1]	0.100
Mineral Premix[2]	0.060
Choline chloride 70%	0.050
Calculated composition	
Metabolizable energy (kcal/kg)	2.800
Crude protein (%)	16.50
Calcium (%)	4.020
Available phosphorus (%)	0.380
Digestible methionine (%)	0.431
Digest. methionine+cystine (%)	0.668
Digestible lysine (%)	0.731
Digestible threonine (%)	0.559
Digestible tryptophan (%)	0.174
Digestible arginine (%)	0.984
Digestible valine (%)	0.690
Sodium (%)	0.220
Chlorine (%)	0.339
Potassium (%)	0.621

[1]Composition per kg of product: A vit.—10,000,000 IU; D3 vit.—2,500,000 IU; E vit.—6,000 IU; K vit.—1,600 mg; B12 vit.—11,000 mg; Niacin—25,000 mg; folic acid—400 mg; pantothenic acid—10,000 mg; and Se—300 mg. [2]Composition per kg of product: MN—150,000 mg; zinc—100,000 mg; iron—100,000 mg; copper—16,000 mg; and iodine—1,500 mg.

housed in galvanized-wire cages. These cages measured 100 cm in length, 40 cm in width, and 45 cm in height, offering a floor area of 500 cm^2 per hen.

2.2 Performance analyses

Egg production was evaluated at weeks 4, 8, and 12 in 120 cages with four birds each, corresponding to 30 replicates per treatment. All eggs produced were individually weighed. Laying rate and egg mass were calculated considering all eggs (including non-marketable eggs) for each replicate (cage). The coefficient of variability was calculated for each cage considering the individual weight of all the eggs produced each week. The same procedure was adopted for egg masses [Egg mass = average weight (g) × percentage of egg production %]. Due to management limitations related to the commercial system, feed intake measurement was not possible in this study. For that reason, feed conversion was also not evaluated.

2.3 Dirtiness degree of the eggshells

All eggs produced in each repetition at weeks 4, 8, and 12 were individually inspected for the presence of feces in the shells, which was classified by the same observer through visual analysis as clean eggs (absent; score 0), minor presence (score 1), and major presence (score 3 and 4). During data analysis, scores 3 and 4 were considered together due to the low casuistry of score 3.

2.4 Serum biochemistry

At the end of week 12, blood samples were collected from eight randomly selected birds in each treatment. The samples were obtained from the ulnar vein of the birds, which were chosen from different cages. To obtain serum, the blood was collected in tubes without anticoagulant. The collected blood samples were then subjected to centrifugation at 3,500 rpm for 10 min. This process allowed for the separation of serum from other components of the blood. The resulting serum was carefully collected and frozen at −20°C to preserve its integrity for subsequent biochemical analysis.

For the biochemical analysis, the frozen serum samples were processed and examined using the Bio-Plus 2000® Biochemical Analyzer, manufactured by Bioplus in São Paulo, Brazil. Commercial kits from Wiener Lab Group, São Paulo, Brazil, were utilized to measure various parameters including total protein, albumin, uric acid, total cholesterol, triglycerides, glucose, alkaline phosphatase, alanine aminotransferase, and aspartate aminotransferase.

2.5 Parasitology test

At the conclusion of the experimental period, excreta samples were collected from three birds in each of the 10 cages. The collection was done with care, ensuring that the samples were processed within 2 h of collection to maintain their integrity. The centrifugal-flotation technique (15) was employed to process the excreta samples. To initiate the process, a subsample of 1 g of excreta was diluted in 15 mL of sucrose solution. This diluted sample was then subjected to centrifugation for 5 min. Following centrifugation, an optical microscope was utilized to examine the sample on a glass slide. The microscope was set at magnifications of 10, 40, and 100x to ensure accurate counting of oocysts.

2.6 Gut morphometric analyses

In accordance with the animal welfare and euthanasia standards outlined in the euthanasia practice guidelines of the National Council for Control of Animal Experimentation (16), six birds per treatment were humanely slaughtered using cervical dislocation. After euthanasia, 2-cm samples were collected from the duodenum, jejunum, and cecum of each bird. These tissue samples were carefully

stored in flasks containing a 10% formaldehyde solution to preserve their structural integrity.

Histological slides were prepared from the collected tissue samples and stained with Archived Hematoxylin and Eosin (H&E). To capture the histological images, a microchamber Digital Eyepiece Camera Video, coupled with a biological trinocular microscope model TNB-41 T-PL at a magnification of 40x, was utilized. To determine the crypt depth and villus length, a line was measured from the base of the crypt to the upper portion for crypt depth. For villus length, a straight line was drawn from the tip of the villi to the upper portion of the crypts. ImageJ software bundled with 64-bit Java 1.8.0_172 was employed for accurate measurements.

2.7 Quality of fresh eggs

On the last day of weeks 4, 8, and 12, a total of 15 fresh eggs from each treatment in each phase were randomly collected for quality evaluation. Cracked eggs were excluded from this assessment. To determine the specific gravity, the eggs' weight in both air and water was measured, following Archimedes's principle.

The albumen height was determined by measuring three different points on the albumen, each 10 mm away from the yolk, using a digital caliper (TMX PD-150, China). The average of these measurements was used to calculate the Haugh Unit (HU), employing the equation developed by Haugh (17).

Yolk quality was evaluated by calculating the yolk index, which is the ratio of yolk height to yolk diameter, using the formula: YI = yolk height (mm)/yolk diameter (mm). Yolk height (mm) was measured using an altimeter, while yolk diameter (mm) was measured using a digital caliper.

Yolk width and height measurements (mm) were obtained using a digital caliper (TMX PD-150, China). The yolk index was calculated using the formula: yolk index = yolk weight/yolk width.

To assess yolk color, the Roche colorimetric fan (DSM, São Paulo, Brazil) was employed. This fan utilizes a scoring system ranging from 1 (representing a light yellow color) to 15 (indicating a reddish-orange hue). Additionally, a spectrophotometer device (Delta Vista model 450G, Delta Color, São Leopoldo, Brazil) was employed for this evaluation, providing colorimetric coordinates of luminosity (L*), red intensity (a*), and yellow intensity (b*). Chroma, which represents the actual yolk color for analysis, was estimated using the following equation: $C = \sqrt{(a^2 + b^2)}$.

Once the yolk and albumen were separated, the weights of both components were measured. To ensure uniformity, the dense and fluid portions of the albumen, as well as the yolk, were homogenized for 20 s. To assess the pH levels, a digital pH meter (Kasvi model k39-2014B, Paraná, Brazil) was employed.

The total solid content of the albumen and yolk was assessed individually. For this, 5 g of albumen and yolk were weighed in pre-dried porcelain crucibles. The crucibles containing the samples were then placed in an oven set at 60°C for a duration of 12 h. Following the drying process, the crucibles were re-weighed to determine the weight of the dried albumen and yolk. To obtain the final solid content, the crucibles were subjected to a higher temperature of 105°C for another 12 h. After this drying step, the crucibles were weighed again to calculate the total solid content of the albumen and yolk accurately.

To determine the weight of the shell, a separate procedure was followed. The shells were carefully separated, thoroughly washed, and dried. Subsequently, the dried shells were weighed precisely to obtain their weight.

2.8 Statistical analyses

Data analyses were performed using the SAS statistical program (v 9.3, SAS Institute Inc., Cary, NC). Experimental units varied among the responses, but briefly, it was the cage for performance, the bird for biochemical and gut responses, and each egg for quality assessment. Data were tested for normality and homocedasticity and then submitted to variance analyses using PROC MIXED, except for the coefficient of variance of egg weight, which was analyzed using PROC GLIMMIX. All statistical models included the fixed effect of treatments and the error. Performance data were analyzed considering repeated measures over time. Egg quality was analyzed considering also the random effect of phase in the model, although only pooled means are presented here due to the lack of interaction between treatment and phase. Eventual mean differences were compared by the Tukey test at 5 and 10% probability.

3 Results

The measurements of temperature and humidity were obtained using a datalogger. The average recorded values for the minimum and maximum temperatures were 18 and 36°C, respectively. The average air relative humidity values ranged from 35.8 to 94.7%. This study was conducted in Salvador do Sul (Southern region, Brazil) which largely experiences a subtropical climate. The project implementation started in December (summer) and lasted until March (summer-autumn). Throughout the entire trial, the animals exhibited performance consistent with the expectations for their specific genotype. Furthermore, the animals remained in good health throughout the experimental period, as no severe health problems or illnesses were observed.

3.1 Performance and dirtiness degree of the eggshells

In phase 1, the BMA and BMA + PRO groups presented a 12% higher posture rate compared to the CON ($p < 0.001$, Table 2). In phase 2, all supplemented treatments had higher laying rates (21%) compared to CON ($p < 0.001$). In phase 3, the group fed with BMA + PRO was 5% superior to CON ($p < 0.001$), while the BMA and PRO groups were intermediate in relation to CON and BMA + PRO. In the overall period, all treatments had a higher ($p < 0.001$) laying rate compared to CON, in which BMA + PRO had the highest laying rate followed by BMA and PRO.

Regarding egg weight, the BMA + PRO group differed from the CON, with higher ($p < 0.001$) egg weight in phase 1. In phase 2, all treatments differed ($p < 0.001$) from CON, with BMA and BMA + PRO being similar to each other. In phase 3, the PRO and BMA + PRO treatments differed from CON ($p < 0.001$); however, the PRO group was similar to the control which also occurs in the overall period. At week 62, all treatments differed ($p = 0.013$) from CON.

TABLE 2 Performance of laying hens fed diets supplemented with β-mannanase (BMA) and/or probiotics (PRO).

Responses	Treatments				SEM[1]	*p* value[2]
	CON	BMA	PRO	BMA + PRO		
Laying rate (%)						
Phase 1[4]	85.31^{C}	93.71^{B}	83.25^{C}	97.66^{A}	0.11	<0.001
Phase 2	78.34^{B}	96.69^{A}	96.36^{A}	90.55^{A}	0.11	<0.001
Phase 3	91.05^{B}	92.59^{AB}	92.60^{AB}	95.90^{A}	0.09	<0.001
Overall	84.90^{C}	94.33^{A}	90.74^{B}	94.70^{A}	0.59	<0.001
Weight of fresh eggs (g)						
Phase 1	61.95^{B}	62.36^{B}	61.38^{B}	63.18^{A}	0.14	<0.001
Phase 2	61.28^{C}	63.09^{A}	61.98^{B}	62.79^{A}	0.15	<0.001
Phase 3	64.13^{B}	65.21^{A}	63.76^{B}	65.52^{A}	0.16	<0.001
Overall	62.47^{B}	63.55^{A}	62.37^{B}	63.83^{A}	0.09	<0.001
62 weeks[3]	62.53^{B}	64.40^{A}	64.10^{A}	64.28^{A}	0.13	0.013
Coefficient of variability in egg weight (%)						
Phase 1	5.944^{b}	5.897^{b}	5.193^{a}	5.292^{ab}	0.014	0.072
Phase 2	7.152^{B}	5.625^{A}	5.750^{A}	5.734^{A}	0.018	0.007
Phase 3	7.088^{B}	5.397^{A}	5.608^{A}	5.405^{A}	0.019	0.004
Overall	6.728^{B}	5.640^{A}	5.517^{A}	5.477^{A}	0.01	<0.001
62 weeks[3]	7.94	8.285	8.272	7.891	0.124	0.564
Total solids[5]						
Albumen	10.78	10.59	10.66	11.59	0.21	0.383
Yolk	50.28	49.94	48.73	50.51	0.38	0.339

[1]Standart error of mean. [2]Probability of treatment effect. Means followed by different uppercase letters differ statistically at 5%, while lowercase letters were used to indicate differences at 10%. [3]Treatments were not provided from week 48 to 62. Thus, the last evaluation was performed after 14 weeks without supplementation. [4]Phase 1: 36–40 weeks; phase 2: 41–44 weeks; and phase 3: 45–48 weeks.

TABLE 3 Egg masses of laying hens fed diets supplemented with β-mannanase (BMA) and/or probiotics (PRO).

Responses	Treatments				SEM[1]	*p* value[2]
	CON	BMA	PRO	BMA + PRO		
Egg mass (g/hen/day)						
Phase 1[3]	52.85^{B}	58.44^{A}	51.10^{B}	61.70^{A}	0.67	<0.001
Phase 2	48.01^{C}	61.00^{A}	59.72^{AB}	56.86^{B}	0.39	<0.001
Phase 3	58.30^{B}	60.38^{A}	59.04^{AB}	62.83^{A}	0.63	<0.001
Overall	53.08^{C}	59.94^{A}	56.62^{B}	60.46^{A}	0.39	<0.001

[1]Standart error of mean. [2]Probability of treatment effect. Means followed by different uppercase letters differ statistically at 5%, while lowercase letters were used to indicate differences at 10%. [3]Phase 1: 36–40 weeks; phase 2: 41–44 weeks; phase 3: 45–48 weeks.

All treatments differed ($p < 0.001$) from CON in terms of the overall coefficient of variability of egg weight, with the lowest values observed in BMA + PRO group. A trend effect ($p = 0.072$) was observed in phase 1, with the lowest coefficient of variability attributed to PRO treatment. In phases 2 ($p = 0.007$) and 3 ($p = 0.004$) all treatments differed from CON, with lowest values observed in BMA group. However, at week 62 (after treatment removal), no significant differences were observed among the groups ($p = 0.564$).

Regarding egg masses (Table 3), in phases 1 and 3 ($p < 0.001$), treatments BMA and BMA + PRO were different from the CON group, showing higher egg masses. In phase 2 and overall ($p < 0.001$), all treatments increased egg masses compared to the CON.

The occurrence of clean eggs in treated birds differed from CON in all phases of the experiment ($p < 0.05$, Figure 1). The BMA, PRO, and BMA + PRO groups were superior to CON in all phases. In the 62nd week of production, after 14 weeks without supplementation, the occurrence of clean eggs still differed ($p < 0.001$) in the BMA and BMA + PRO groups compared to CON.

3.2 Serum biochemistry

Uric acid differed from CON ($p < 0.001$) in all treatments, with the lowest values observed in BMA + PRO, BMA, and PRO, respectively (Table 4). Total cholesterol and triglycerides differed from CON

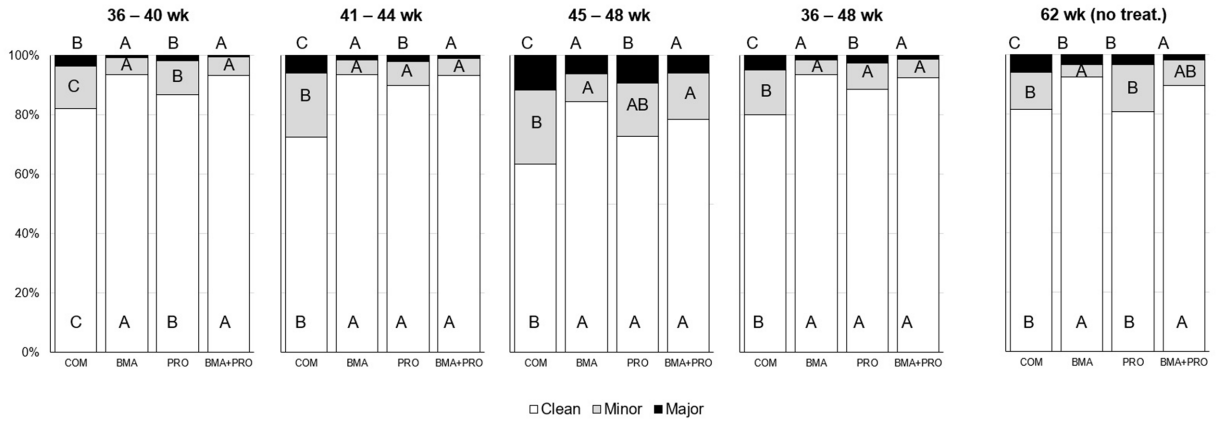

FIGURE 1
Occurrence of clean eggs or minor/major presence of feces (%) in eggs from laying hens fed β-mannanase and/or probiotics. Comparisons were performed among treatments in each period. The probability of treatment effect was $p < 0.001$ for all responses, except for the period from 45 to 48 weeks in which all responses showed $p < 0.05$. Different uppercase letters differ statistically at 5%. Treatments were not provided from week 48 to 62. Thus, the last evaluation was performed after 14 weeks without supplementation.

TABLE 4 Serum biochemistry and intestinal morphometry of laying hens fed β-mannanase and/or probiotics.

Responses	Treatments				SEM[1]	*p* value[2]
	CON	BMA	PRO	BMA + PRO		
Serum biochemistry						
Total protein (g/dL)	5.475	7.113	5.038	6.575	0.361	0.148
Albumin (g/dL)	1.850	2.100	1.875	2.043	0.052	0.237
Uric acid (mg/dL)	5.171 ^{A}	2.400 ^{BC}	3.171 ^{B}	2.062 ^{C}	0.247	<0.001
Total cholesterol (mg/dL)	301.3 ^{A}	149.5 ^{B}	204.1 ^{B}	161.9 ^{B}	14.1	<0.001
Triglycerides (mg/dL)	832.1 ^{A}	929.0 ^{A}	539.0 ^{B}	659.6 ^{B}	45.6	0.013
Glucose (mg/dL)	367.0 ^{A}	293.0 ^{B}	401.5 ^{A}	269.1 ^{B}	11.5	<0.001
FA (U/L)[3]	378.1 ^{B}	624.0 ^{AB}	896.0 ^{A}	831.8 ^{A}	64.0	0.007
ALT (U/L)	8.207 ^{A}	7.020 ^{A}	3.201 ^{B}	1.667 ^{B}	0.811	0.005
AST (U/L)	144.3	149.7	149.0	140.4	2.7	0.579
Gut morphometry						
Villi height (μm)	1,459	1,294	1,375	1,561	30.6	0.428
Villi width (μm)	249.0 ^{ab}	226.7 ^{b}	246.4 ^{ab}	283.1 ^{a}	5.69	0.064
Villi area (μm^2)	367,039	288,838	343,272	450,579	1,798	0.67
Crypt depth (μm)	224.9	186.4	227.6	202.1	4.96	0.397
Villi height: Crypt depth	6.839 ^{B}	7.245 ^{AB}	6.304 ^{B}	8.396 ^{A}	0.171	0.007

[1]Standart error of mean. [2]Probability of treatment effect. Means followed by different uppercase letters differ statistically at 5%, while lowercase letters were used to indicate differences at 10%.
[3]FA, Alkaline phosphatase; ALT, Alanine aminotransferase; AST, Aspartate aminotransferase.

($p < 0.001$), with smaller values shown in supplemented treatments. Serum glucose levels were higher in CON ($p < 0.001$) birds in relation to the BMA and BMA + PRO treatments. The PRO treatment did not differ from the CON. Serum alkaline phosphatase was higher ($p = 0.007$) in PRO and BMA + PRO treatments than in CON. The BMA was similar to CON and other treatments in this study. Alanine aminotransferase was higher in CON ($p = 0.005$) than in PRO and BMA + PRO treatments.

No significant differences were observed in aspartate aminotransferase ($p = 0.579$) and total protein ($p = 0.148$). There were also no significant differences in serum albumin ($p = 0.237$).

3.3 Gut morphometry and parasitological analysis

No difference was observed in villus height, villus area, and crypt depth among the treatments. The villi width tended ($p = 0.064$) to be smaller in the BMA treatment compared to the control, whereas the BMA + CON treatment tended to be superior to CON and the PRO treatment was similar to CON. The relationship between the height of the villus and the depth of the crypt was significant ($p = 0.007$), with the highest relationship observed in the BMA + PRO treatment compared to the CON. The

PRO treatment was similar to CON and BMA similar to CON and BMA + PRO.

No parasites or oocysts were found in the fresh excrete samples, including the control treatment. This condition did not allow the evaluation of the eventual effect of treatments on parasite challenges.

3.4 Quality of fresh eggs

The BMA group showed higher ($p < 0.001$) specific gravity when compared to the CON (Table 5), with higher values. In addition, higher ($p = 0.009$) shell weights were observed in the BMA and BMA + PRO groups compared to CON.

Yolk height showed significant differences in the BMA, PRO, and BMA + PRO groups compared to CON ($p = 0.037$), all with higher values. Yolk width was also higher ($p = 0.002$) in the BMA and BMA + PRO groups compared to CON. Yolk weight was higher in the BMA + PRO group ($p = 0.004$) compared to the CON group. Yolk pH, on the other hand, differed from CON in all groups ($p = 0.002$), showing lower pH values.

The BMA group showed a higher color score in the Roche colorimetric assessment compared to the CON ($p = 0.032$). Regarding luminosity values (L* color), the BMA + PRO group was superior to the CON group ($p = 0.002$). Such findings indicate lower luminosity, that is, they were opaquer as they transmit less light. Higher red intensity (A* color) and chroma values were observed in all supplemented groups than CON ($p < 0.001$; Figure 2).

4 Discussion

4.1 Performance and dirtiness degree of the eggshells

In the current study, an improvement in overall laying rates across all treatments were observed when compared to the control group. This finding aligns with previous research conducted by Zhan et al. (18), who reported a significant increase in laying rates in birds when fed with probiotics, particularly when administered at a dosage of 5×104 cfu/g of *Clostridium butiricum*. Similarly, Ribeiro et al. (19) documented a substantial increase in laying rates with a dosage of 8×105 cfu/g of *Bacillus subtilis*, while Saleh et al. (20) observed improvement when utilizing 0.05% of *Aspergillus awamori*. Probiotics have been recognized for their positive impact on egg production, which is attributed to several beneficial mechanisms. These include enhanced nutrient absorption (19), improved immune function, reduced stress in birds, and promotion of intestinal health (18) which are effects that may help explain the results found in this study. In this context, it is important to mention that the current study was developed under commercial farm conditions. Such

TABLE 5 Quality of fresh eggs from laying hens fed diets supplemented with β-mannanase (BMA) and/or probiotics (PRO).

Responses[3]	Treatments				SEM[1]	*p* value[2]
	CON	BMA	PRO	BMA + PRO		
General traits						
Spec. gravity (g/ml)	1.006 ^{B}	1.007 ^{A}	1.006 ^{B}	1.006 ^{B}	0.001	<0.001
Albumen traits						
Height (mm)	8.04	8.06	8.18	8.17	0.104	0.129
Weight (g)	36.82	37.39	36.30	36.59	0.239	0.424
pH	8.41	8.40	8.38	8.44	0.028	0.178
Yolk traits						
Height (mm)	17.98 ^{B}	18.15 ^{A}	18.27 ^{A}	18.18 ^{A}	0.063	0.037
Length (mm)	40.67 ^{B}	41.62 ^{A}	41.25 ^{AB}	41.82 ^{A}	0.118	0.002
Index	0.443	0.435	0.443	0.435	0.017	0.194
Weight (g)	15.33 ^{B}	15.70 ^{AB}	15.45 ^{B}	16.08 ^{A}	0.096	0.004
Haugh unit	89.40	90.10	89.88	89.55	0.558	0.132
pH	6.04 ^{B}	5.96 ^{A}	5.99 ^{A}	6.00 ^{A}	0.013	0.002
Yolk color						
Color score	5.60 ^{B}	5.98 ^{A}	5.77 ^{AB}	5.87 ^{AB}	0.052	0.032
Lightness (L*)	50.85 ^{B}	50.66 ^{B}	51.33 ^{AB}	52.16 ^{A}	0.161	0.002
Redness (a*)	7.12 ^{B}	7.66 ^{A}	7.67 ^{A}	7.66 ^{A}	0.100	<0.001
Yellowness (b*)	57.41	58.88	58.93	58.75	0.354	0.122
Chroma	57.85 ^{B}	59.67 ^{A}	59.67 ^{A}	59.25 ^{A}	0.357	0.003
Shell traits						
Weight (g)	5.81 ^{B}	6.15 ^{A}	5.96 ^{AB}	6.11 ^{A}	0.041	0.009

[1]Standart error of mean. [2]Probability of treatment effect. Means followed by different uppercase letters differ statistically at 5%, while lowercase letters were used to indicate differences at 10%.
[3]A subsample of 15 eggs from each treatment in each phase.

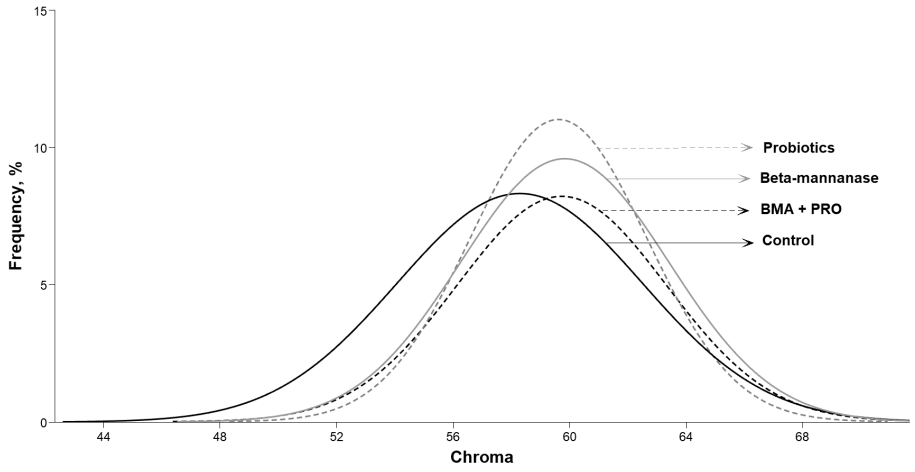

FIGURE 2
Frequency of different chroma indexes in egg yolks from laying hens fed β-mannanase and/or probiotics.

condition generally indicates that animals are exposed to certain challenges (biosecurity, environment, number of animals, among others) that would be limited under small-scale experimental conditions. Both study types are relevant and able to provide useful data. However, this characteristic needs to be considered when interpreting the results.

In reference to β-mannanase, our current findings show an enhanced laying rate when compared to the control treatment. These results are in agreement with the results of Zheng et al. (21), who reported a significant boost in egg production among laying hens that were supplemented with β-mannanase on low-energy diets. Notably, the values were similar to high-energy diets without enzymes. Similar data were also found by Wu et al. (22). These findings may be associated with the fact that β-mannanase, by avoiding the immune response triggered by β-mannanase, redirects energy and nutrients for the bird's performance (23).

The current study noted a significant increase in egg weight, aligning with previous research by Khan et al. (24), Alaquil et al. (25), and Mikulski et al. (26) in diets supplemented with probiotics. Moreover, concerning β-mannanase, our findings agree with the results of Ryu et al. (27), when using 0.8 g β-mannanase/kg, observed an increase in egg weight compared to the control. Regarding the coefficient of variability of egg weight, we observed a stable and predictable production, which facilitates the processes and increases profitability by decreasing the number of declassified eggs.

In regard to egg masses, our study revealed an increase in egg masses across all treatments when compared to the control. These results agree with Ryu et al. (27) that observed higher egg masses using β-mannanase. This effect may be attributed to the improved digestion and utilization of dietary components, leading to increased nutrient absorption and utilization for egg production. As for probiotics, Saleh et al. (20), Alaquil et al. (25), and Ribeiro et al. (19) also observed higher egg masses when compared to the control group. Probiotics are known to foster a balanced gut microbiota, which can positively influence nutrient metabolism and absorption, subsequently contributing to improved egg mass production.

The higher occurrence of clean eggs in treated birds observed in this study differed from the control in all phases of the experiment. The use of β-mannanase can be explained by the probable decrease in feces viscosity. Soluble non-starch polysaccharides increase digesta viscosity by increasing water retention, impairing nutrient diffusion and transport. Daskiran et al. (28) demonstrated that diets that used β-mannanase significantly reduced the water of total fecal production in broilers. Likewise, Mehri et al. (29) demonstrated that the viscosity of digesta from the jejunum of broiler chickens decreased in diets with the enzyme. The cleaner eggs observed in the probiotic treatment can likely be attributed to the enhanced stability of the intestinal microbiota and lower count of opportunistic bacteria. Higgins et al. (30) and Deng et al. (31) observed a decrease in *Salmonella* sp. colonies in birds supplemented with probiotics. Aalaei et al. (32) found that the addition of multi-strain probiotics reduced the presence of *E. coli* in broilers, and with that, it was possible to reduce diarrhea in the birds. Therefore, in the present study, the reduction in diarrhea could be the cause of the decrease in dirty eggs from the PRO treatment. To our knowledge, this is the first study that evaluates the occurrence of dirty eggs in laying hens fed β-mannanase alone or combined with probiotics.

4.2 Serum biochemistry

Uric acid is the main product of nitrogen metabolism in birds, which is synthesized in the liver and kidneys. Disorders in renal function can increase the concentration of uric acid in the serum and plasma of birds (33), as well as elevated temperatures (34). Low uric acid levels also indicate lower protein turnover (35), that is, lower endogenous losses of nitrogen and ammonia. In the present study, which took place in summer, the birds faced high temperatures, and even so the values of uric acid found in the blood were lower in all treatments compared to the control, which may indicate an improvement in the health of the birds and better efficiency in protein utilization due to additives.

Furthermore, in relation to protein metabolism, uric acid may elucidate the observed phenomena of increased albumen weight and egg mass in this study, potentially attributable to enhanced protein deposition within these eggs.

Total cholesterol and triglyceride levels were significantly lower in the groups that received probiotics and the combined additives (BMA + PRO) when compared to the control group. These findings are consistent with previous research (20, 36, 37) in birds that were supplemented with probiotics. Furthermore, birds that were fed β-mannanase exhibited lower total cholesterol levels, which aligns with the results reported by Karimi and Shokrollari (38), who observed a decrease in LDL-cholesterol levels. Serum cholesterol and triglyceride levels reflect lipid metabolism. Saleh et al. (39) reported that one of the possible mechanisms of cholesterol reduction by probiotics occurs through the production of HMG-CoA reductase (3-hydroxy-3-methyl-glutaryl-CoA reductase), which reduces the deposition of abdominal fat by influencing the activity of the hormone-sensitive lipase and malate dehydrogenase enzyme in adipose tissues (40). Also, one of the supposed mechanisms of probiotics occurs through the reduction of hepatic bile acid synthesis (41). Lactic acid bacteria such as those found in the tested product have the ability to reduce cholesterol in the bloodstream (42). The decrease in cholesterol by β-mannanase can be explained by the hypolipidemic effect of the enzyme, which reduces the absorption of lipids (38, 43).

Regarding serum glucose, our findings are in agreement with Tang et al. (44). The decrease in glucose by β-mannanase can be explained by the fact that this enzyme stimulates insulin secretion (45), which may stimulate feed intake behavior and consequently be linked to increasing egg production.

The increase in serum alkaline phosphatase agrees with the findings of Yalcin et al. (37). And the higher alanine aminotransferase also is in agreement with Saleh et al. (39) and Tang et al. (44). The serum concentration of liver enzymes such as alkaline phosphatase and alanine aminotransferase can provide information about tissue and organ damage (46). Alkaline phosphatase is also associated with calcium and phosphorus metabolism and with participation in osteoblastic and chondrogenic activities. Therefore, the increase in this enzyme is associated with bone growth, fracture consolidation, and pre-ovulation and medullary calcification phase in chickens (33). Furthermore, changes in alkaline phosphatase levels may indicate that the medullary bone promotes calcium during the formation of eggshells and stores calcium when there is no egg in the uterus (46). In relation to alanine aminotransferase in birds, it is believed that it may be elevated due to damage to multiple tissues, making its interpretation difficult (47). In the present study, results observed that the birds fed with PRO, and BMA + PRO groups had higher serum alkaline phosphatase values, which indicates better health for these birds. The lower values of alanine aminotransferase observed in this study may indicate a more efficient metabolism of these birds due to less liver damage, which may explain the positive performance results.

4.3 Gut morphometry and parasitological analysis

Crypt height and depth measurements are often used to assess intestinal integrity. The height of the villi indicates a greater area for nutrient absorption and a deeper crypt indicates that there is greater tissue renewal (48, 49). In the present study, the group treated with BMA + PRO showed a greater villus height: crypt depth ratio, demonstrating an improvement in intestinal health.

Previous studies have shown significant differences in the ratio between villus height and crypt depth (36) in the intestine of laying hens fed with probiotics. Even though there are no studies in relation to β-mannanase in laying hens, based on these results, it is believed that this additive can benefit the intestinal health of birds. The higher villus: crypt ratio in these groups, as it is associated with a greater surface area for nutrient absorption, may explain the better performance of these birds, especially in relation to egg weight and egg mass.

4.4 Quality of fresh eggs

It is crucial to recognize that the composition of the eggshell primarily consists of calcium carbonate, along with magnesium carbonate and calcium phosphate, among other components. The balance between calcium and phosphorus ions plays a vital role in the formation of the eggshell (50). Specific gravity is an indicator of the proportion of the shell in relation to other components of the egg. It is closely associated with shell thickness and, consequently, the deposition of calcium carbonate. Evaluating the specific gravity provides insights into the quality of the shell. Additionally, shell weight can be used to support the findings obtained from specific gravity measurements and assess calcium metabolism.

In our study, we observed higher specific gravity and increased eggshell weight in the BMA group compared to the CON. These findings suggest a higher quality of the shell and a reduced likelihood of breakage during handling (51, 52). The improved specific gravity and increased eggshell weight indicate enhanced shell integrity and strength, which are desirable attributes in terms of egg quality and marketability.

Yolk and albumen weight exhibit a positive correlation with egg weight. Eggs with higher weights tend to have greater yolk and albumen masses compared to those with lower weights. Egg weight is influenced by various factors, including heritability, age, and bird weight (53). Additionally, egg weight strongly influences dietary protein requirements (54). In this study, we observed lower uric acid levels in birds fed with the BMA group compared to the CON group, suggesting reduced protein turnover. β-mannans, found in the diet, are known to reduce viscosity and inhibit enzyme action (55). The addition of β-mannanase in the BMA group facilitated enzyme activity by breaking down β-mannans, potentially leading to increased protein absorption. This mechanism may explain the higher yolk weight observed in the BMA group. Another hypothesis is associated with the viscosity-reducing effect of β-mannanase (56). This enzymatic activity alters the structure of micelles (57, 58), which are lipid compounds of significant importance as they are deposited in the yolk. The decrease in viscosity caused by β-mannanase could potentially enhance the formation or function of micelles, contributing to an increased yolk weight.

The results related to yolk color demonstrated lower luminosity in the BMA + PRO group compared to the CON, indicating a more opaque appearance and reduced light transmission. Additionally, the PRO and BMA groups exhibited desirable yellowish and reddish colors, which are considered attractive to consumers (59). The pigmentation of the yolk occurs through the absorption of carotenoid pigments present in the bird's diet (60). Corn, for instance, contains carotenoids such as xanthophyll, lutein, and zeaxanthin (61). These lipophilic and unsaturated compounds accumulate in the yolk, which

has the highest concentration of fat in the egg (62). One hypothesis for the observed color changes is that β-mannanase may enhance nutrient absorption and/or increase the production of micelles, which are responsible for transporting carotenoids and accumulating them in the yolk. However, further studies are needed to validate this hypothesis.

It is worth mentioning that parameters such as yolk pH, height, and width are indicators of egg freshness (63, 64). In this trial, the treatments resulted in decreased pH values and increased yolk height and width, indicating improved egg quality and freshness.

5 Conclusion

The present study provides evidence that the supplementation of β-mannanase, probiotics, and their combination in feed can significantly enhance performance (laying rate and weight of fresh eggs) and gut health (ratio villi height to crypt depth). Furthermore, the supplementation of these additives in the poultry feed leads to the occurrence of eggs more homogeneous in terms of weight, cleaner, and with an better quality.

Data availability statement

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

Ethics statement

The animal study was approved by the experimental protocol described was approved by the Institutional Ethics Committee on the Use of Animals (CEUA/UFRGS) under protocol number 39783. The study was conducted in accordance with the local legislation and institutional requirements.

Author contributions

CC, IA, and MK: conceptualization, methodology, validation, and formal analysis. CC, GG, NC, TB, RiM, and GP: investigation. IA and MK: resources, visualization, project administration, and funding acquisition. CC and IA: data curation. CC: writing—original draft preparation. CC, IA, GG, BB, and MK: writing—review and editing. IA, RaM, and MK: supervision. All authors contributed to the article and approved the submitted version.

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Conflict of interest

MK was employed by Elanco Animal Health, São Paulo, Brazil.

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.

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Fecal microbiota characterization of an Italian local horse breed

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The Bardigiano horse is a traditional native Italian breed with a rich history and peculiar characteristics. Local breeds are proven to have unique genetic traits developed over generations to adapt to defined geographical regions and/or conditions. The specific microbial communities that coexist within these animals are unraveled by studying their microbiota, which permits a further step in the characterization of local heritage. This work aimed to characterize Bardigiano horse fecal microbiota composition. The data obtained were then compared with published data of a mix of athlete breeds to evaluate potential differences among local and specialized breeds. The study involved 11 Bardigiano mares between 3 and 4 years of age, from which stool was sampled for the study. Samples were processed for 16S rRNA sequencing. Data obtained were analyzed and plotted using R, RStudio, and FastTree software. The samples analyzed were similar to what literature has reported on horses of other breeds and attitudes at higher taxonomic levels (from phylum to genera). While at lower taxonomic levels, the difference was more marked highlighting specific families found in the Bardigiano breed only. Weight, province of origin, and breeding sites significantly affected microbiota composition (p-value $\leq$0.02, p-value $\leq$0.04, and p-value $\leq$0.05, respectively). The comparison with athlete breed showed a significant difference confirming that animal and environmental factors are crucial in determining fecal microbiota composition (p-value <0.001). Understanding the microbiota composition in local breeds like the Bardigiano horse is crucial for preserving biodiversity, managing animal health, and promoting sustainable farming practices.

KEYWORDS

horse, local breed, Bardigiano, microbiota, biodiversity

1 Introduction

The Bardigiano horse, as represented in Figure 1, is a traditional native Italian breed with a rich history and peculiar characteristics. Its name is related to Bardi, a small town on the hill in the province of Parma, Italy. The Bardigiano breed played a crucial role in human society during the 19th century, serving as a means of transportation in agriculture as well as for meat production (1). Unfortunately, after the Second World War, the breed faced a major threat to its survival since only five stallions and 150 mares survived. To overcome this severe bottleneck, the Bardigiano studbook was founded in 1977 to preserve the breed's unique features while improving its use for riding and draft purposes. Furthermore, recent strategies have been implemented in the breed both using pedigree and genotype data to reduce the loss of genetic diversity (2, 3). Nowadays, the Bardigiano breed counts roughly 3,000 live horses, mainly used for riding and light draft purposes. This breed is an example of a successful project to preserve local heritage through adaptation to the current market demand. This is possible through the

FIGURE 1
Example of Bardigiano horse. Picture by Francesca Rossi.

implementation of breeding values as well as optimal contribution selection tools (2, 4).

To meet the market demand, the selection of this breed is leading it towards a meso-endomorph morphological type, characterized by moderate dimensions and a calm temperament. The distinctive traits of the breed can be summarized as follow: (a) the height ranges between 140 and 149 cm for males and 135 and 147 cm for females; (b) chest circumference higher than 165 cm; (c) the coat color is bay, with a preference for dark bay; and (d) limited white markings on the legs and face are allowed, although not preferred. Typical conformation includes a small head with a straight or concave profile, low withers, a slightly straight back, deep girth, and an overall muscular appearance. Bardigiano horses also show unique traits ranging from excellent resilience to harsh climates, roughage diet, pasture conditions, gentle temperament, and willingness to work (5).

Therefore, Bardigiano horses represent a unique breed with a deep history, cultural significance, and a current position in the market thanks to their peculiar characteristics. The genetic identity of the Bardigiano breed was likewise corroborated by a recent study where the Italian equine gene pool was analyzed by genotype data (6). Several research activities have been performed to fully characterize this breed from a genetic point of view; however, nothing is yet known about the microbiota of Bardigiano horses. By studying those horses' microbiota, we might better understand their unique adaptations and potential environmental influences that have shaped the breed over time.

The microbiota is the microbial community that inhabits a certain environment or organ. Fecal microbiota composition has been widely described in human and major animal species (7–10). The characterization of the microbiota in local breeds is important since they are proven to have unique genetic traits developed over generations to adapt to specific geographical regions and/or conditions. By characterizing the microbiota, we better understand the specific microbial communities that coexist within these animals (11, 12). Therefore, studying the microbiota of native breeds might lead to a further step to unravel peculiar within-breed characteristics. Several works have used the characterization of microbiota as a tool to evaluate animals' good health and resilience because of its tight links to the immune system (13, 14). It is proven that the more diverse the microbiome the more the probability of the individual being in good health. Moreover, knowing the breed microbiota composition allows for identifying any changes (dysbiosis) that could be caused by or lead to pathological states (15). Since the intestinal microbial community is involved in nutrient absorption, digestion, and building a strong immune response, its description has also a functional role. Understanding the microbiota can help target measures to promote animal wellness and prevent diseases. Studying microbiological diversity might help to enhance the repopulation of endangered breeds: since the microbiota is transmitted from the mother to the offspring, it allows to detect the healthiest individuals and potentially more resistant to adverse conditions (16). Therefore, that knowledge can help to preserve the biodiversity of local breeds and to ensure their long-term survival. The characterization of the microbiota in local breeds can also provide valuable insights into the genetic and phenotypic characteristics associated with specific microbial communities (17). By studying the interactions between host genetics, microbiota, and environmental factors, researchers can identify potential genetic markers or traits that are correlated with desirable microbial profiles. This information can be useful in selective breeding programs to improve animal health, productivity, and resilience (18). Characterization of local breeds and species has been encouraged in several international projects. The United Nations, as part of its Sustainable Development Goals outlined in the 2030 Agenda, has set ambitious targets aimed at preserving Life on Earth. These objectives encompass halting biodiversity loss, fostering genetic diversity, and championing the appreciation and valorization of local breeds (19).

Thus, the identification of the microbiota composition in local breeds is essential for preserving biodiversity, managing animal health and disease, promoting sustainable farming practices, and unlocking the potential for genetic and phenotypic improvements. Finally, it helps to understand the intricate relationship between animals and their microbial partners, leading to better strategies for animal welfare, conservation, and agricultural sustainability.

This work aimed to study the fecal microbial community in the Bardigiano horse breed, an Italian local breed originating in the hills of the province of Parma, to add a further step in its characterization. The study evaluated various aspects that potentially influence microbiota composition, considering both intrasubject characteristics as well as environmental factors acting in the development age or adulthood. In addition, the data obtained from Bardigiano horses were compared to the athletic breeds' from Plancade et al. (20) to further disentangle potential differences between local breeds and cosmopolitan ones.

2 Materials and methods

2.1 Inclusion criteria

The 11 female horses, subscribed to the Bardigiano studbook, were selected by ANAREAI (National Breeders Association for Italian Equine and Asinine breeds), based on their age, breed standards, and biometrical measurements. All breeders were aware of this experiment as they agreed through ANAREAI to subject their animals to experiments for the characterization and valorization of the breed itself. A maximum of two animals for each breeder were allowed for a total of 10 breeders (6 at Site 1 and 4 at Site 2). Before starting the protocol, all horses underwent examinations by judges and veterinarians to ensure they did not have any pathologies or lameness. The areas where the 11 horses were born were classified into plains and hills, based on altitude following the guidelines from the Italian National Statistical Institute (ISTAT, 2018), where hills are defined as lands from 300 to 700 m and lowlands lower than 300 m from sea level. The horses were reared in two different riding centers (Site 1 and Site 2); feeding, training, and environmental conditions were identical within each riding center; however, there were differences comparing the conditions between the two riding centers. Site 1 is situated in a flatland in the province of Parma (Italy), at coordinates 44.93452935855489° N, 10.359899154478137° E, and hosts a total population of 50 horses with several other breeds. At this site, the horses have been subjected to a daily training session lasting 20 min, with 1 day of rest per week. Site 2 is situated in a hilly area in the province of Parma (Italy) at coordinates 44.5042826805862° N, 9.658427278379634° E; these stable counts more than 40 horses, and most of them belong to the Bardigiano breed. The tested horses at this site are housed all in the same stable, while the remaining ones have access to a large pasture. The training routine for the tested horses at Site 2 was a one-hour session every 2 days. A 70 days training protocol started, during which the eleven horses were trained by one single trainer in a conditioning stable. The diet of the horses was standardized among horses belonging to the study consisting of 12 kg of hay and 1.3 kg of concentrate for Site 1 and 15 kg of hay and 500 g of concentrate in Site 2. The quantity of the hay is similar between Site 1 and Site 2, although there are differences in their quality and origin due to different altitudes and therefore availability at the two sites. At Site 1, the hay originates from flatland locations, while, at Site 2, the hay is sourced from the hills surrounding the riding center, resulting in less variability in the diet of the horses. Both sites use concentrate feedstuff, although the specific type of feed differs between them. At Site 1, a supplementary feed indicated for the rehydration of horses during the summer months is used. Also, the bedding differs, in Site 1 wood flakes were used while in Site 2 straw.

2.2 Weight of horse

The weight of the horses was estimated according to Marcenac et al. (21), where weight expressed in kilograms = (chest circumference expressed in meters)^3× 80. Three weight classes were established based on percentile divisions of the estimated weight within the samples. These classes were then assessed and analyzed in the study, as indicated in Supplementary Table S1.

2.3 Fecal sampling and 16S rRNA sequencing

The stool samples were taken in July 2022 after 70 days of conditioning protocol which can be considered as an "acclimation period" where the environmental conditions where set in the two sites as previously described in the "inclusion criteria paragraph." Approximately 30 g of feces were collected and transferred in a sterile plastic flask. The samples were then transported in a cooler to the lab, where three aliquots of each sample were made in sterile cryotubes and stored at – 80°C until extraction.

The DNA of the samples was extracted as indicated by QIAamp UCP Pathogen Mini Kit purchased from QIAGEN (Hilden, Germany), including a blank control. The quantification of the DNA extracted was performed through qPCR of the 16S rRNA sequences (regions V3 and V4) which included a negative control consisting of a MOC Community and filtered sterile water after UV treatment. The library was set up using a Quick-16S NGS Library Prep Kit (Zymo 145 Research, Irvine, CA, United States) and analyzed using a MiSeq Reagent Kit v3 (600 cycles, 2 × 300 bp paired-end reads).

2.4 Data and statistical analysis

Using DADA2 pipeline v1.16 (22) in R v4.2.3 we processed reads obtained from sequencing. R packages used at this phase were dada2 and DECIPHER. Samples obtained were split into individual fastq files containing both forward and reverse reads. Firstly, using filterAndTrim function reads were filtered based on standard filtering parameters suggested by Edgar et al. (23), then the learnErrors function was used on filtered reads and errors were plotted through plotErrors function to visualize estimated error rates. The sequence data underwent filtering and trimming according to the inference algorithm from Callahan et al. (24), afterwards by means of mergePairs function forward and reverse reads were merged. We built an amplicon sequence variant (ASV) table using makeSequenceTable. Chimeras were then removed using the removeBimeraDenovo function. The sequences were classified according to the SILVA

database version 138.1 (Small Subunit rRNA database) with the assignTaxonomy function (20). The quality control (QC) of the data was filtered based on ASV abundance (0.01%) per sample. Taxonomic features, beta diversity, and PairwiseAdonis test were performed using the following R packages: phyloseq, readxl, tibble, ape, MicrobiotaProcess, ggplot2, ggtree, plyr, vegan. Principal Coordinates Analysis (PCoA) allows for plotting ecological dissimilarity distances among microbial communities. Beta diversity was plotted using PCoA based on the Bray-Curtis distance index (Hellinger method) from the OTUs and taxa relative abundance table. A phylogenetic tree was created based on taxa identified using the open-source software FastTree and the following r Packages: DECIPHER, Biostrings, ape, adegenet, ggtree, ggtreeExtra, phyloseq, dplyr. The microbiota difference between horses kept in Site 1 and Site 2 was evaluated based on Welch's *t*-test when two groups were present as in the case of place of origin, their altitude (flatland and hills), and when comparing the two sampling locations. Whereas, in the case of multiple levels within a factor (as in the case of estimated weight) a ANOVA was performed.

2.5 Comparison between Bardigiano and athletic breed

To further describe and distinguish the breed's unique characteristics, the data found in this study was compared to published microbiota data from a pool of athlete breeds subjects. A random sample of 21 horses was downloaded from the open-source project BioProject PRJNA438436 by Plancade et al. (20). The horses, although from different breeds, ranging from Anglo-Arabian to Arabian, all performed endurance racing over 90 km. Therefore, they can be considered as a suited comparison to local breeds as they can be categorized as athlete horses. The data obtained in fastq format were processed using DADA2 pipeline v1.16 in R v4.2.3 and as described above. The relative abundance was compared between the two types of horses (Bardigiano and athletic one) and the ANOVA test was performed to detect potential breed differences in terms of taxa abundance and composition. The principal coordinate analysis was conducted based on the Bray-Curtis distance index.

3 Results and discussion

3.1 Taxonomic description

A total of 1,633,013 reads were obtained from the sequencing. After filtering we had 1,305,837 reads. After the assignments of the taxonomy in R, 14 phyla, 18 classes, 32 orders, 59 families, 118 genes and 132 species were found.

The data showed that at the phylum level, the sample group was made on average mainly of Firmicutes (over 50%) and secondly of Bacteroidota (33.52%) and Spirochaeotota (6.27%), confirming published findings on horses' microbiota (16, 25). Other phyla encountered were Fibrobacterota (3.36%) as shown in Figure 2A. These findings are in line with what has been reported by several authors in terms of taxa encountered in horse fecal microbiota studies (16, 18, 20, 25–27).

Taxa with the highest mean abundance regarding the class data were Clostridia (39.30%) and Bacteroida (33.52%), followed in smaller percentages by Bacilli (6.52%), and Spirochaetia (6.27%) as previously described by Mach et al. (16).

At the order level, as shown in Figure 2B, Bacterioidales covered 33.48% of relative abundance on average, followed by Oscillospirales (21.19%) and Lachnospirales (13.71%). A minor role is played by other orders, whose relative abundances, all under 6.5%, are reported in Supplementary Table S4.

Lachnospiraceae (13.63%), Oscillospiraceae (10.68%), Rikenellaceae (8.39%), Prevotellaceae (8.22%), F082 unclassified (6.47%), and Spirochaetaceae (6.27%) families, in respective decreasing order, composed, on average over 50% of the microbiota. Other families identified in the sample with an average relative abundance lower than 5% are reported in Supplementary Table S5.

Only Rikenellaceae RC9 gut group, F082 unclassified genus, and Treponema had abundance over 5% (respectively 7.52, 6.47, 6.25%). These three genera made up the 52.33% of relative abundance together with Streptococcus (4.63%), Lachnospiraceae AC2044 group (4.57%), NK4A214 group (4.38%), p-251-o5 unclassified genus (4.33%), Phascolarctobacterium (3.83%), UCG-010 unclassified genus (3.49%), Lachnospiraceae unclassified genus (3.49%), Fibrobacter (3.36%).

The list of all taxa is provided with relative abundance in Supplementary Table S7.

3.2 Factors of influence

The potential influence of estimated weight was considered. The animals were categorized into three groups based on their estimated weight percentile classes as indicated in Supplementary Table S1. Bacteroidales were significantly enriched in group 2 based on weight estimate (p-value = 0.02), whereas the Lachnospiraceae were more abundant in groups 1 and 3 (Figure 3A).

Bacteroidetes phyla are known for their vital roles in fiber digestion and organic acid synthesis. On the other hand, the Lachnospiraceae family contributes to the digestion and fermentation of complex plant polysaccharides (28), such as cellulose and hemicellulose, and are essential to produce volatile fatty acids (VFAs) that serve as an energy source (29, 30). Horses with the highest and lowest estimated weights exhibited a lower abundance of Bacteroidetes compared to the intermediate group, indicating that there might be a link between bacterial composition and energy utilization from the diet.

Previous studies have suggested that a decrease in Bacteroidetes abundance (31) and an increased ratio of Firmicutes to Bacteroides could potentially contribute to the promotion of fat deposition as an adaptive response (32). Furthermore, another research has indicated a positive association between high body mass index (BMI) and increased levels of Firmicutes (including Lachnospiraceae) and decreased levels of Bacteroidetes (33). In the context of the breed object of the study, the findings suggest that alterations in the relative abundance of these bacterial groups, with the changes in the proportions in group 3, may impact energy metabolism and contribute to the development of obesity. On the other hand, Firmicutes bacteria produce higher amounts of butyrate (34), which is recognized as a health-promoting molecule due to its ability to enhance insulin sensitivity (35). In specific circumstances, this increased production of butyrate by Firmicutes

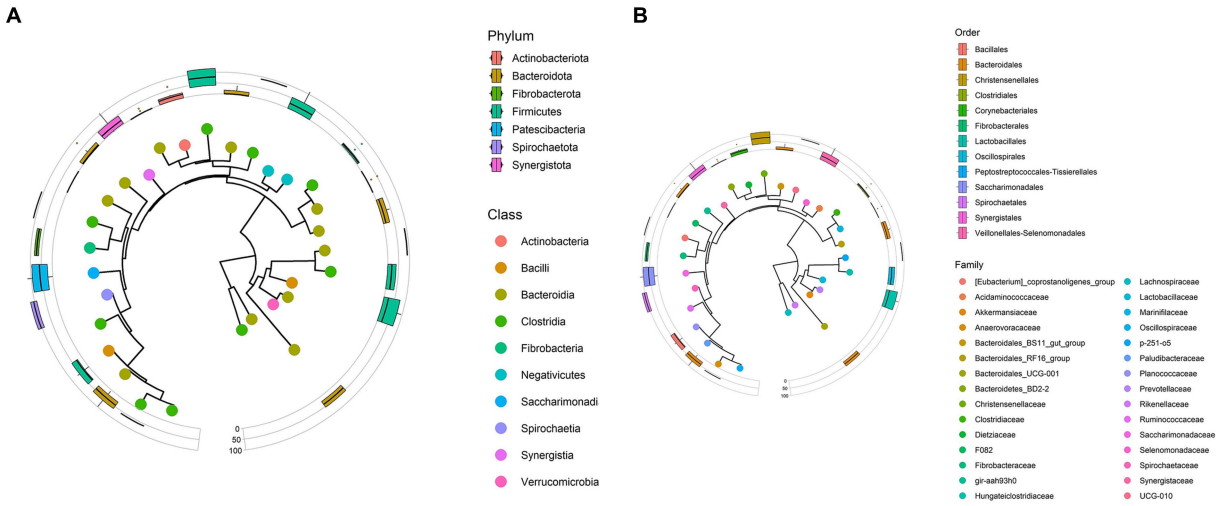

FIGURE 2
(A) Phylogenetic tree representing phyla and classes data observed from Bardigiano horses' fecal samples. **(B)** Phylogenetic tree representing orders and families data observed from Bardigiano horses' fecal samples.

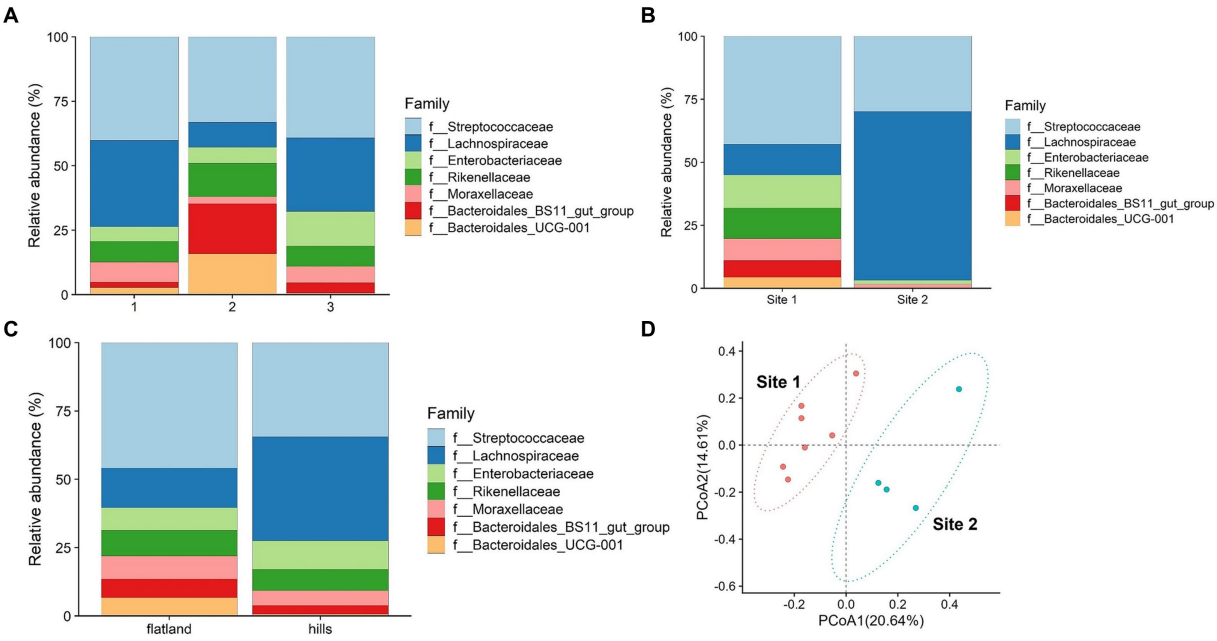

FIGURE 3
(A) Relative abundance of microbiota's family divided into three groups of weight. Group 1 consisted of animals with an estimated weight below 400 kg, group 2 between 401 and 450 kg and group 3 above 451 kg. **(B)** Relative abundance of microbiota's family divided into flatland and hills based on place of origin. **(C)** Relative abundance of microbiota's family divided into the two conditioning sites. **(D)** Principal coordinate analysis of intestinal microbiota families comparing data from two conditioning sites.

could potentially result in elevated energy expenditure and subsequent weight loss. Conversely, Lachnospiraceae were found to be less prevalent in group 2. Studies have shown higher levels of Lachnospiraceae in anorexic patients, and this bacterial presence remains unchanged even after short-term weight recovery (36). These findings underscore the importance of weight-related factors in influencing the composition of fecal microbiota, particularly the distinct roles played by Bacteroidetes and Lachnospiraceae-Firmicutes. Given the relatively limited number of animals involved, further investigation is necessary to gain a clearer understanding of the specific impact of the predominance of Firmicutes compared to Bacteroidetes on the body weight of horses.

The comparison between the place of origin where the horses were kept when they were foals, and their altitude (flatland and hills) showed statistically significant differences for the Lachnospiraceae

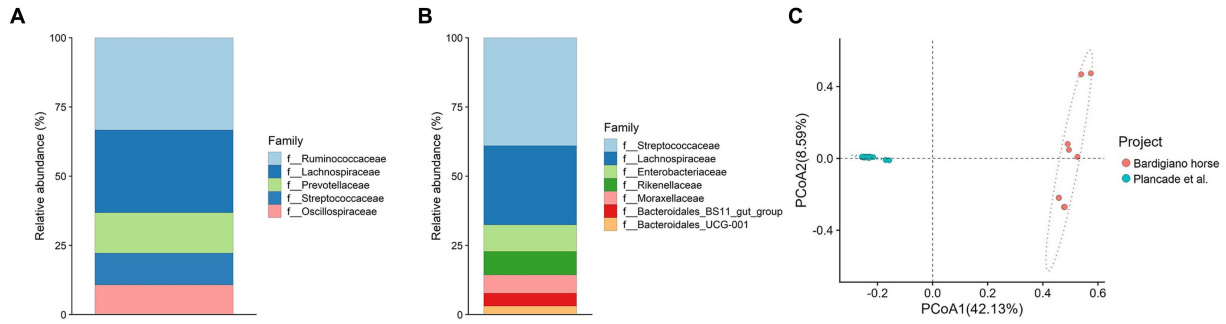

FIGURE 4
(A,B) Relative abundance of families comparing Bardigiano horses **(B)** with athletic breeds **(A)** from Plancade et al. (20). **(C)** Principal coordinate analysis of intestinal microbiota families comparing data from Bardigiano and athlete horses.

family (p-value = 0.04), which could be attributed to the feeding strategies employed during the early years of the horses (Figure 3B). The type of breeding and feeding practices are known to leave a footprint on the animal's microbiome (37, 38). Most of the flatland horses had limited access to pastures during their early years, while all the horses bred in the hills spent at least 6 months in a pasture. This difference in breeding practices may possibly explain the prevalence of Lachnospiraceae observed in the hills horse group. It is plausible to think that maybe the distinct composition of grass and hay in the pasture environment, which contains higher fiber content and is more resistant to degradation, requires a greater abundance of Lachnospiraceae to facilitate lignin degradation. Statistically significant differences were found comparing the two sampling locations (Site 1 and Site 2). Based on the Welch statistics, three families seem to be enriched in Site 1 and not in the other as shown in Supplementary Table S8. Enterobacteriaceae and Rikenellaceae were over-presented in Site 1, whereas Lachnospiraceae in Site 2 (Supplementary Table S8).

Dietary changes, stress, and environmental factors, such as training (25), have been shown to influence the composition of the microbiota in horses (39). As mentioned before, the two riding centers had different management practices. Figure 3C illustrates the abundance and types of bacterial families identified in the microbiota of the horses from the two riding centers. The observations belonging to each riding center cluster together and differ between the two centers, as shown in Figure 3D. At Site 1, a greater diversity of bacteria was observed, with a total of seven different families present. In contrast, Site 2 exhibited a reduced population with only four families, predominantly comprising Lachnospiraceae and Streptococcaceae. As previously mentioned, the horses at Site 2 were primarily fed hay with small amounts of concentrate feed, while at Site 1, the quantity of concentrate feed provided was three times higher. This disparity in diet composition likely contributed to the prevalence of Lachnospiraceae observed at Site 2, as reported by Zhu et al. (40). Thus, this could be an effect of feeding with a high percentage of hay, which contains fewer nutrients and degrades at a slower rate compared to a diet consisting of both hay and concentrate feed. This result shows that probably 70 days of conditioning protocol significantly impacted the horses' fecal microbiota, due to the different feeding, training, and bedding type. Beta diversity performed with 999 permutations showed a significant difference (p-value = 0.05) among the two groups from different sites, confirming that environmental factors affect deeply microbiota composition (Figure 4A).

3.3 Comparison with athlete breeds

The comparison with athlete breeds showed that most abundant families differed between local and athletic horses, having in common Lachnospiraceae only as shown in Figures 4A,B.

Among the two populations, a statistically significant difference (p-value < 0.001) was found through the ANOVA test, which results are shown in Supplementary Table S9, suggesting that indeed breeds' objectives play an important role in microbiota determination.

The Bray–Curtis dissimilarity index was calculated and used to perform a Pairwise Adonis test showing a significant difference (p-value < 0.001) in beta diversity among the two groups. Conversely from what has been suggested by Massacci et al. (18), the Bardigiano horse local breed clustered differently from the athletic breed mix in the PCoA as shown in Figure 4C.

3.4 Limits of the study

The limits of the study were the small number of mares due to the small size of the Bardigiano population. Experimenting with horses requires high expenses in handling and maintaining the subjects. No further metadata was available to deepen the comparison with the athlete breed. In addition, to fully comprehend the effect of the tested factors on the microbiota, the work would have benefit from a microbiota characterization at time 0 (before the conditioning period). However, due to limited budget this was not possible.

3.5 Further developments

Further developments of this study could concern the prevalence of antimicrobial resistance genes in Bardigiano horses compared to more common athlete breeds.

4 Conclusion

This is the first work aimed to describe the fecal microbiota of Bardigiano, an Italian local horse breed. Based on our findings, it seems that environmental changes in horse management impact the microbiota composition. At a broader taxonomic level, the analyzed

samples resembled what has been seen in different horse breeds and attitudes in existing studies. However, at lower taxonomic levels, the distinction became clearer, suggesting distinguished characteristics of the Bardigiano breed. Comparison with the mix of athlete horse breeds reinforced these results. Despite the differences among the two breeding sites, Bardigiano horses clustered together when compare through PCoA against the athlete breeds: thus confirming these suggestions.

Understanding the microbiota composition in local breeds like the Bardigiano horse is important for preserving biodiversity, managing animal health, and promoting sustainable farming practices. It also helps to understand the complex relationship between animals and their microbial partners, leading to better strategies for animal welfare, conservation, and agricultural sustainability. Researches as this one are meaningful for the preservation of biodiversity, in alignment with the guidelines outlined by European directives and authorities involved in the safeguarding of biodiversity.

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: BioProject, PRJNA1001795.

Ethics statement

The animal studies were approved by responsible for the welfare of animals at the University of Parma (protocol 14/CESA/2023). 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

AMCH, VA, and MA performed conceptualization, data curation, and writing – original draft preparation. AMCH performed data collection. ASa, ASu, and SB performed writing – review and editing. All authors contributed to the article and approved the submitted version.

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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.

Publisher's note

Supplementary material

The Supplementary material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fvets.2024.1236476/full#supplementary-material

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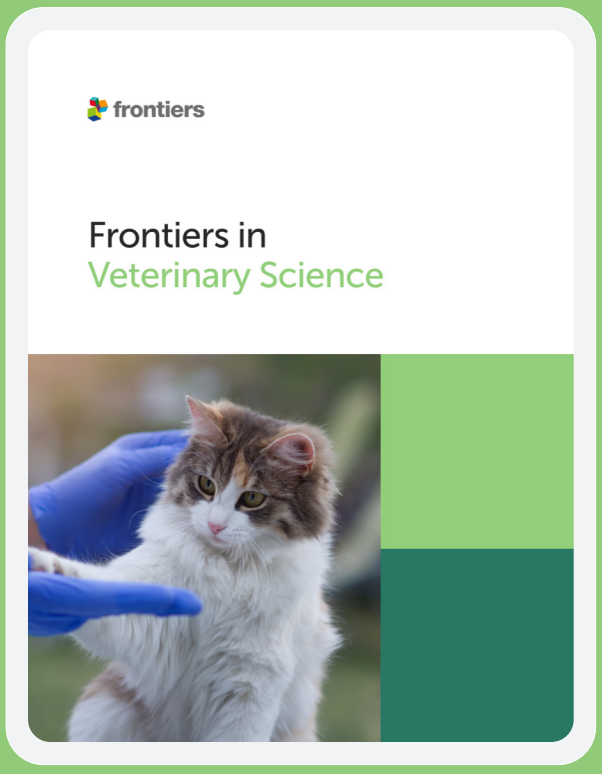

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