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# Gastrointestinal nutrients-microbiome-host interactions in livestock

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# Gastrointestinal nutrients-microbiome-host interactions in livestock

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# Effect of dietary resveratrol supplementation on growth performance, antioxidant capacity, intestinal immunity and gut microbiota in yellow-feathered broilers challenged with lipopolysaccharide

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Resveratrol (RES) displays strong antioxidant and anti-inflammatory properties in protecting the animals from various stressors and inflammatory injuries, but its interrelationship with the gut microbiota remained largely unclear. This study was carried out to investigate the effects of dietary RES supplementation on growth performance, antioxidant capacity, intestinal immunity and gut microbiota in yellow-feathered broilers challenged by lipopolysaccharide (LPS). A total of 240 yellow-feathered broilers were randomly assigned to four treatment groups in a 2 × 2 factorial design. The broilers were fed with the control diet or control diet supplemented with 400 mg/kg RES, followed by challenge with LPS or the same amount of saline. Dietary RES supplementation significantly alleviated the decreases in the final body weight (BW), average daily gain (ADG), and ADFI induced by LPS ($P < 0.05$). LPS challenge significantly increased plasma concentrations of triglyceride, high-density lipoprotein cholesterol (HDL-C), aspartate aminotransferase (AST), and cortisol levels, but decreased triiodothyronine (T3) and insulin levels ($P < 0.05$). Dietary supplementation with RES significantly reversed the elevated creatinine concentrations and the decreased concentrations of T3 and insulin caused by LPS ($P < 0.05$). Moreover, dietary RES supplementation significantly increased plasma total antioxidant capacity (T-AOC) and catalase (CAT) activities and superoxide dismutase (SOD) and T-AOC activities in jejunal mucosa and reduced malondialdehyde (MDA) concentration in the plasma ($P < 0.05$). The reduction in the villus height to crypt depth ratio in duodenum, jejunum and ileum and the shortening of villus height in jejunum and ileum caused by LPS were also alleviated by RES treatment ($P < 0.05$). Furthermore, the increased concentrations of intestinal tumor

necrosis factor-α (TNF-α), interleukin-6 (IL-6), and IL-1β caused by LPS were significantly decreased by RES treatment ($P < 0.05$). Dietary RES treatment increased the mRNA expression of *claudin-1*, *claudin-5*, *occludin*, and *zonula occludens-1* (*ZO-1*), and decreased mRNA expression of *IL-1β*, *IL-8*, *IL-17*, and *TNF-α* after LPS challenge ($P < 0.05$). Dietary RES treatments significantly decreased the dominance of cecal microbiota, and increased the Pieiou-e and Simpson index. Moreover, dietary RES supplementation increased relative abundance of *UCG_ 009*, *Erysipelotrichaceae*, *Christensenellaceae_R-7_group*, *Anaerotruncus*, *RF39*, and *Ruminococcus* while decreasing the abundance of *Alistipes* at genus level. Spearman correlation analysis revealed that the microbes at the order and genus levels significantly correlated with indicators of growth performance, antioxidant capacity, and intestinal health. Collectively, dietary supplementation with 400 mg/kg RES could improve growth performance and antioxidant capacity, and modulate intestinal immunity in yellow-feathered broilers challenged by LPS at early stage, which might be closely associated with the regulation of gut microbiota community composition.

KEYWORDS

**lipopolysaccharide, resveratrol, yellow-feathered broilers, growth performance, gut microbiota**

## Introduction

With the increasing demands for animal products, the intensive systems has greatly developed over the last few decades. However, the improvement of intensive production makes poultry more vulnerable to various stressors including pathogenic and toxin infections (Kumar et al., 2012). Immune stress represents a great threat to the poultry farming industry. Under immune stress, the overproduction of inflammatory cytokines would lead to impaired intestinal structure and barrier function, thereby affecting the nutrient digestion and absorption and increasing the risk of intestinal inflammation and diseases (Chen et al., 2018; Sun et al., 2021). Moreover, prolonged immune stress can cause suppression of immune function, especially in the early growth stage when the immune system of broiler has not yet well developed (Song et al., 2021). Lipopolysaccharide (LPS) is a major component of the outer membrane of Gram-negative bacteria and has been widely used as a non-pathogenic immune activator to induce immune stress in broilers. Therefore, finding the effective nutrient regulation strategies to protect the intestinal health and maintain immune function are important for improving the production efficiency and disease resistance of poultry.

Resveratrol (RES) (*trans*-3,5,4′-trihydroxystilbene) is a naturally polyphenol found in a variety of plants, and displays strong antioxidant and anti-inflammatory properties (Liang et al., 2019; Meng et al., 2021), which has great application prospects in animal production. Previous study has shown that RES can effectively alleviate the damages of intestinal morphology and barrier integrity induced by heat stress in broilers (He et al., 2019a, 2020). Moreover, dietary RES treatment can reduce diarrhea and intestinal inflammation in piglets by altering intestinal gene expression and gut microbiota (Meng et al., 2019). Indeed, gut microbiota play an important role in the digestion and absorption of nutrients and in the immune response of animals, thus affecting the growth development and health of the host animal (Yao et al., 2021). Previous study has shown that the improvement of growth performance and immunity might be associated with the balanced cecum microbiota in broilers challenged by *Escherichia coli* (Mohebodini et al., 2019). However, it remained unclear whether the protective effect of RES on the growth performance and intestinal health was involved with the changes of gut microbiota composition and diversity in yellow-feathered broilers challenged with LPS.

Therefore, the purpose of this experiment was to investigate the effects of dietary supplementation with RES on growth performance, plasma biochemical parameters, hormones levels, antioxidant capacity and intestinal health, and tempted to identify their potential relationships with the alterations of gut microbiota in yellow-feathered broilers challenged with LPS.

# Materials and methods

## Experimental design, diets and management

The experimental protocol (FOSU2022004) was approved by Animal Care and Use Committee of Foshan University (Foshan, China). A total of 240 healthy 1-day-old yellow-feathered broiler male chicks with initial body weight (BW) of 34.43 ± 0.24 g were randomly divided into four treatment groups in a 2 × 2 factorial design, which included the control group (CON), RES group, LPS group, and RES + LPS group. The broilers in CON group and LPS group were fed the basal diet, while those in RES group or RES + LPS group were fed with the basal diet supplemented with 400 mg/kg RES. Each treatment group had five replicates of 12 birds each replicate. The basal diet (**Table 1**) was formulated according to the nutritional levels recommended by Ministry of Agriculture of the People's Republic of China. (2020). RES (purity ≥ 98%) was purchased from Shaanxi Sciphar Natural Products Co., Ltd., while LPS (*E. coli* O55:B5) was provided from Sigma-Aldrich (Saint Louis, MO, United States).

The experiment lasted 21 days. The chickens of each replicate were kept in individual cage and had free access to feed and drinking water during the whole experiment. At days 16, 18, and 20 of the experiment, the broilers in LPS group and RES + LPS group were injected intraperitoneally with LPS solution (1 mg/kg BW, LPS dissolved in 0.9% saline at a concentration of 1 mg/mL), while the broilers in CON and RES groups were received the same amount of sterilized saline. The challenge dosage and treatment of LPS was chosen accordingly to the previous study (Han et al., 2020).

TABLE 1 The composition and nutrient levels of the basal diet for yellow-feathered broilers.

| Ingredients | % | Calculated nutrient levels[1] | % |
|---|---|---|---|
| Corn | 39.07 | MJ (kcal/kg) | 12.35 |
| Wheat | 25.00 | CP | 21.50 |
| Soybean meal (43% CP) | 22.00 | EE | 3.40 |
| Peanut meal | 3.00 | CF | 2.50 |
| Corn gluten meal (58% CP) | 5.00 | Ca | 0.90 |
| Limestone | 1.45 | Total P | 0.60 |
| $CaHPO_4$ | 1.10 | L-Lysine | 1.38 |
| L-lysine sulfate | 0.64 | DL-Met | 0.61 |
| DL-Met | 0.32 | L-Thr | 0.82 |
| NaCl | 0.28 | | |
| L-Thr | 0.19 | | |
| Lard oil | 1.45 | | |
| Vitamin-mineral premix[2] | 0.50 | | |
| Total | 100.00 | | |

[1]Values were calculated from data provided by China Nutrient Requirements for yellow-feathered broilers (2020).
[2]The premix provided the following per kg of diet: VA, 6,000 IU; $VD_3$, 2,000 IU; VE, 30 mg; $VK_3$, 2 mg; $VB_1$, 3 mg; $VB_2$, 5 mg; pantothenic acid, 800 mg; choline chloride 1,500 mg; nicotinic acid, 30 mg; pyridoxine, 3 mg; folic acid, 500 mg; biotin, 0.2 mg; $VB_{12}$, 1 mg; Fe, 100 mg; Cu, 8 mg; Mn, 100 mg; Zn, 100 mg; I, 0.42 mg; Se, 0.3 mg.

## Sample collection

The boilers in each group were weighed on days 1, 16, 18, 20, and 21 of the experiment, and one chicken with average BW of each replicate was selected for sample collections on day 21. After fasting for 12 h, the blood (10 mL) was collected from the wing veins of the chickens, and centrifuged at 3,000 rpm for 10 min to get the plasma samples for the determination of plasma biochemical parameters, hormone levels and antioxidant indicators. After blood collection, the chickens were subjected for slaughter. Briefly, the middle sections (about 2 cm) of the duodenum, jejunum, and ileum were collected and placed in 10% formalin fixation to determine the intestinal morphology. Then, the mucosal samples were gently scraped from another portion of middle jejunum (about 10 cm) after rinsing with ice-cold saline, and placed into 1.5 mL EP tubes for the determinations of antioxidant indicators and intestinal cytokines. Moreover, the samples of cecum contents were collected and immediately snap-frozen in liquid nitrogen and stored at −80°C to determine the changes of gut microbiota in yellow-feathered broilers.

## Determinations

### Growth performance

Feed consumption of each replicate was recorded before each weighing or in case of mortality. The average daily gain (ADG), average daily feed intake (ADFI), and feed-to-gain ratio (F/G) were calculated accordingly.

### Plasma biochemical parameters and hormones concentrations

The plasma biochemical parameters, including total protein, uric acid, glucose, cholesterol, triglyceride, high-density lipoprotein cholesterol (HDL-C), low-density lipoprotein cholesterol (LDL-C), none-esterified fatty acid (NEFA), total bile acid, aspartate aminotransferase (AST), alanine aminotransferase (ALT), creatine kinase, creatine kinase-MB, and creatinine concentrations were determined according to the methods described previously (Zhu et al., 2020).

The plasma concentrations of hormones including triiodothyronine (T3), thyroxine (T4), insulin, and cortisol

concentrations were determined by commercial Elisa kits according to the instructions of the manufacturer (Beijing North Institute of Biotechnology Co., Ltd., Beijing, China).

### Analyses of antioxidant capacity in the plasma and jejunal mucosa

The antioxidant indexes of plasma and jejunal mucosa samples including glutathione peroxidase (GSH-Px), superoxide dismutase (SOD), catalase (CAT), total antioxidant capacity (T-AOC), and malondialdehyde (MDA) were measured using the commercial kits from Nanjing Jiancheng Institute of Biological Engineering (Nanjing, China) according to the manufacturer's instructions.

### Measurement of intestinal morphology

The mid-sections of duodenum, jejunum, and ileum were fixed in 10% formalin for 24 h. The fixed samples were embedded in paraffin, and cross sections of each sample were fixed on slides coated with polylysine, deparaffinized, rehydrated, and then stained with hematoxylin-eosin (HE). Sections were observed using an Olympus MD50-T microscope. The villus height and crypt depth of at least 25 villus samples were measured by MShot Image Analysis System (Guangzhou, China), and the ratio of villus height to crypt depth was calculated accordingly.

### Measurement of intestinal cytokine secretions

Intestinal cytokines including intestinal tumor necrosis factor-α (TNF-α), interleukin-6 (IL-6), IL-1β, IL-17, and transforming growth factor-β (TGF-β) were quantified using the commercial Elisa kits according to manufacturer protocols (Cusabio, Barksdale, DE, United States).

### RNA extraction, cDNA synthesis and real time quantitative PCR

Total RNA was isolated from jejunal mucosa samples using TRIzol reagent (Invitrogen, Carlsbad, CA, United States) according to the manufacturer's instructions. The purity and concentration of RNA were determined using a model DS-11 spectrophotometer (DeNovix, Wilmington, DE, United States). The total RNA (1 μg) was used to synthesize cDNA using a T100 Thermal Cycler (Bio-Rad, Hercules, CA, United States). The real time quantitative PCR (RT-qPCR) analysis was performed in triplicate using a final volume of 10 μL of SYBR Green (Bio-Rad, Hercules, CA, United States) by a 10-fold dilution of cDNA. The targeted gene-specific primers (**Table 2**) were designed by Primer 5.0 and synthesized by Tsingke Biotechnology Co., Ltd. (Beijing, China). The RT-qPCR conditions were 95°C for 5 min, followed by 40 amplification cycles (95°C for 15 s, 60°C for 30 s, and 72°C for 30 s). The β-actin was used as an internal control and the fold change of targeted genes was calculated with β-actin for each sample using the $2^{-\Delta\Delta Ct}$ method.

### Analysis of gut microbial composition and diversity

The 16S RNA sequencing technique was used to determine the potential changes in the composition and diversity of the gut microbiota in yellow-feathered broilers fed with RES-supplemented diets followed by challenge with LPS. Microbial DNA was extracted from cecum contents using a DNA kit (Omega Bio-tek, Norcross, GA, United States) according to the manufacturer's instructions. The qualified extracted DNA samples were then diluted to 1 ng/μL using sterile water and the V3–V4 variable region of the 16S rRNA gene was amplified using specific primers (341 F: 5′-CCTAYGGGRBGCASCAG-3′; 806 R: 5′-GGACTACNNGGGTATCTAAT-3′). PCR amplicons were purified using the Qiagen Gel Extraction Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. The PCR products that meet the library construction requirements were used for library construction using TruSeq DNA PCR-Free Sample preparation library construction kit (Illumina, San Diego, CA, United States) and then sequenced by HiSeq 2500 PE 250 platform (Novogene Bioinformatics Technology Co., Ltd., Tianjin, China). The 16S rRNA sequencing data was performed using the QIIME software package. The high-quality sequences of operational taxonomic units (OTUs) at a 97% identity were aligned against the SILVA database (Ribocon GmbH, Bremen, Germany) after removing chimeric sequences *via* UCHIME software (Tiburon, CA, United States). The Venn diagram with shared and unique OTUs was used to identify the similarity and difference among treatments. The alpha-diversity parameters included Chao 1, Dominance Observed-OTUs, Pieiou-e, Shannon index, and Simpson index. The principal coordinate analysis (PCoA) and non-metric multidimensional scaling (NMDS) were accessed to calculate the β-diversity between groups. The differences in the relative abundances of microbiota among treatments were compared using the linear discriminant analysis effect size (LEfSe) and Metastat analysis. The Spearman correlations between the representative bacteria at phylum, order, and genus levels and the significant phenotype parameters concerning growth performance, antioxidant capacity, intestinal health were plotted as heatmaps using the R package (version 2.15.3).

## Statistical analysis

The data were subjected to two-way ANOVA by SPSS 23.0 (SPSS, Inc., Chicago, IL, United States) to analyze the main effects of LPS or RES and their interactions. Significant differences between means were compared using Duncan's multiple comparison. The replicates ($n$ = 5) were considered as experimental units. The results were expressed as mean and combined standard error (SEM). $P < 0.05$ was considered as significantly different while $P < 0.10$ indicated a trend.

TABLE 2 Sequence, product size and accession number of primers for quantitative real-time PCR.

| Item | Nucleotide sequence of primers (5′→3′) | Product size (bp) | GenBank accession |
|---|---|---|---|
| *Claudin-1* | F: CATACTCCTGGGTCTGGTTGGT | 100 | NM 001013611.2 |
| | R: GACAGCCATCCGCATCTTCT | | |
| *Claudin-5* | F: CATCACTTCTCCTTCGTCAGC | 111 | NM_204201 |
| | R: GCACAAAGATCTCCCAGGTC | | |
| *Occludin* | F: ACGGCAGCACCTACCTCAA | 123 | XM 025144247.2 |
| | R: GGGCGAAGAAGCAGATGAG | | |
| *ZO-1* | F: CTTCAGGTGTTTCTCTTCCTCCTC | 131 | XM 030016322.2 |
| | R: CTGTGGTTTCATGGCTGGATC | | |
| *IL-1*β | F: ACTGGGCATCAAGGGCTACA | 142 | Y15006.1 |
| | R: GCTGTCCAGGCGGTAGAAGA | | |
| *IL-6* | F: CTCCTCGCCAATCTGAAGTC | 99 | NM_204628 |
| | R: CCTCACGGTCTTCTCCATAAAC | | |
| *IL-8* | F: GGCTTGCTAGGGGAAATGA | 200 | DQ393272.2 |
| | R: AGCTGACTCTGACTAGGAAACTGT | | |
| *IL-17* | F: CTCCTCTGTTCAGACCACTGC | 126 | AJ493595.1 |
| | R: ATCCAGCATCTGCTTTCTTGA | | |
| *TGF*-β | F: AGGATCTGCAGTGGAAGTGGAT | 138 | M31160 |
| | R: CCCCGGGTTGTGTTGGT | | |
| *TNF*-α | F: GAGCGTTGACTTGGCTGTC | 64 | GU230788.1 |
| | R: AAGCAACAACCAGCTATGCAC | | |
| β-*actin* | F: ATGATATTGCTGCGCTCGTT | 145 | L08165.1 |
| | R: TCTTTCTGGCCCATACCAACC | | |

ZO-1, zonula occludens-1; IL-1β, interleukin-1β; IL-6, interleukin-6; IL-8, interleukin-8; IL-17, interleukin-17; TGF-β, transforming growth factor-β; TNF-α, tumor necrosis factor-α.

# Results

## Growth performance

As shown in Table 3, LPS challenge caused significant decreases in final BW at days 21, ADG and ADFI during days 16–21 (post-challenge with LPS) and the whole period (days 1–21) in yellow-feathered broilers ($P < 0.01$), but dietary supplementation with 400 mg/kg RES significantly alleviated the decreases in these growth performance parameters during this period caused by LPS ($P < 0.05$). Moreover, no significant RES × LPS interaction for final BW, ADG, ADFI, or F/G during days 16–21 was observed after LPS challenge ($P > 0.05$). Additionally, dietary supplementation with 400 mg/kg RES significantly increased the ADFI during days 1–16 (pre-challenge) in yellow-feathered broilers compared to those fed with the basal diets ($P < 0.05$) (data not shown).

## Plasma biochemical parameters and hormones concentrations

Lipopolysaccharide challenge resulted in significant increase of plasma concentrations of triglyceride, HDL-C, AST, total bile acid, creatine kinase, creatine kinase-MB and creatinine in yellow-feathered broilers ($P < 0.05$) (Table 4). In contrast, dietary supplementation with 400 mg/kg RES significantly alleviated the elevated creatinine concentrations and decreased the concentration of creatine kinase-MB caused by LPS ($P < 0.05$). Moreover, there was a significant RES × LPS interaction in plasma NEFA concentrations of yellow-feathered broilers ($P = 0.038$). However, the plasma concentrations of total protein, glucose, urea acid, cholesterol, ALT, and LDL-C in yellow-feathered broilers were not significantly changed among different treatments ($P > 0.05$).

According to Table 4, the plasma hormone concentrations of T3, T4, and insulin was significantly decreased and plasma cortisol concentrations was significantly increased in yellow-feathered broilers ($P < 0.05$). In contrast, dietary supplementation with 400 mg/kg RES significantly reversed the decrease in plasma T3 and insulin concentrations and the increase in cortisol concentrations induced by LPS challenge ($P < 0.05$).

## Antioxidant capacity

The results of antioxidant capacity of plasma and jejunal mucosa are shown in Table 5. LPS challenge caused a

TABLE 3 Effect of dietary resveratrol supplementation on growth performance in yellow-feathered broilers with lipopolysaccharide[1].

| Item | Treatment | | | | SEM | *P*-value | | |
|---|---|---|---|---|---|---|---|---|
| | CON | RES | LPS | RES + LPS | | LPS | RES | LPS × RES |
| BW 16 days (g) | 353.97 | 360.46 | 353.59 | 354.13 | 7.38 | 0.529 | 0.510 | 0.575 |
| BW 21 days (g) | 468.12$^{b}$ | 485.77$^{a}$ | 447.19$^{c}$ | 466.67$^{b}$ | 6.75 | 0.001* | 0.001* | 0.847 |
| **Day 16–21** | | | | | | | | |
| ADG (g/d) | 19.03$^{b}$ | 20.88$^{a}$ | 15.59$^{c}$ | 18.76$^{b}$ | 0.54 | <0.001* | <0.001* | 0.108 |
| ADFI (g/d) | 47.17$^{b}$ | 48.39$^{a}$ | 45.34$^{c}$ | 47.70$^{ab}$ | 0.41 | 0.001* | <0.001* | 0.067 |
| F/G | 2.48$^{bc}$ | 2.32$^{c}$ | 2.92$^{a}$ | 2.54$^{b}$ | 0.09 | <0.001* | 0.001* | 0.110 |
| **Day 1–21** | | | | | | | | |
| ADG (g/d) | 19.62$^{b}$ | 20.43$^{a}$ | 18.71$^{c}$ | 19.68$^{b}$ | 0.32 | 0.002* | 0.001* | 0.738 |
| ADFI (g/d) | 33.34$^{bc}$ | 34.32$^{a}$ | 32.70$^{c}$ | 33.62$^{b}$ | 0.32 | 0.008* | 0.001* | 0.878 |
| F/G | 1.70 | 1.68 | 1.75 | 1.71 | 0.03 | 0.061 | 0.111 | 0.631 |

[1]Values are means and standard error of the means ($n = 5$).
BW, body weight; ADG, average daily gain; ADFI, average daily feed intake; F/G, feed to gain ratio; CON, control; RES, resveratrol; LPS, lipopolysaccharide; RES + LPS, dietary resveratrol treatment followed by LPS challenge.
$^{abc}$Means in the same row with different superscripts differ ($P < 0.05$).
The * indicates statistically significant difference ($P < 0.05$).

TABLE 4 Effect of dietary resveratrol supplementation on plasma biochemical parameters and hormones concentrations in yellow-feathered broilers with lipopolysaccharide[1].

| Item | Treatment | | | | SEM | *P*-value | | |
|---|---|---|---|---|---|---|---|---|
| | CON | RES | LPS | RES + LPS | | LPS | RES | LPS × RES |
| Total protein (g/L) | 31.83 | 32.53 | 29.17 | 32.13 | 1.98 | 0.305 | 0.226 | 0.441 |
| Urea acid (μmol/L) | 156.33 | 139.67 | 215.33 | 286.00 | 34.40 | 0.062 | 0.372 | 0.801 |
| Glucose (mmol/L) | 13.10 | 13.17 | 12.06 | 12.27 | 1.12 | 0.258 | 0.868 | 0.935 |
| Triglyceride (mmol/L) | 0.35$^{b}$ | 0.34$^{b}$ | 0.44$^{a}$ | 0.40$^{ab}$ | 0.03 | 0.003* | 0.246 | 0.480 |
| Cholesterol (mmol/L) | 4.22 | 3.92 | 4.63 | 4.22 | 0.33 | 0.072 | 0.382 | 0.738 |
| HDL-C (mmol/L) | 2.70$^{b}$ | 2.66$^{b}$ | 3.25$^{a}$ | 2.83$^{b}$ | 0.16 | 0.015* | 0.076 | 0.147 |
| LDL-C (mmol/L) | 0.86 | 0.73 | 0.91 | 0.91 | 0.13 | 0.240 | 0.493 | 0.472 |
| NEFA (mmol/L) | 0.76 | 0.96 | 1.01 | 0.77 | 0.14 | 0.777 | 0.808 | 0.038* |
| AST (U/L) | 171.0$^{ab}$ | 162.2$^{b}$ | 185.2$^{a}$ | 177.2$^{ab}$ | 7.15 | 0.011* | 0.116 | 0.938 |
| ALT (U/L) | 1.25 | 1.00 | 1.50 | 1.25 | 0.32 | 0.295 | 0.295 | 1.000 |
| Total bile acids (umol/L) | 4.16$^{b}$ | 3.64$^{b}$ | 7.28$^{a}$ | 5.74$^{ab}$ | 1.60 | 0.015* | 0.301 | 0.604 |
| Creatine kinase (U/L) | 1073.33$^{ab}$ | 906.33$^{b}$ | 1299.67$^{a}$ | 1130.67$^{ab}$ | 114.96 | 0.024* | 0.073 | 0.990 |
| Creatine kinase-MB (U/L) | 1285.27$^{a}$ | 962.30$^{b}$ | 1426.00$^{a}$ | 1321.33$^{a}$ | 70.81 | 0.001* | 0.003* | 0.061 |
| Creatinine (μmol/L) | 11.20$^{b}$ | 9.00$^{b}$ | 17.20$^{a}$ | 11.60$^{b}$ | 2.57 | 0.031* | 0.048* | 0.364 |
| T3 (ng/mL) | 1.55$^{a}$ | 1.82$^{a}$ | 0.96$^{b}$ | 1.41$^{ab}$ | 0.20 | 0.008* | 0.040* | 0.577 |
| T4 (ng/mL) | 46.79$^{ab}$ | 51.70$^{a}$ | 39.86$^{b}$ | 43.94$^{ab}$ | 3.47 | 0.017* | 0.104 | 0.869 |
| Insulin (μIU/mL) | 6.35$^{ab}$ | 7.86$^{a}$ | 5.00$^{b}$ | 6.16$^{ab}$ | 0.69 | 0.014* | 0.025* | 0.731 |
| Cortisol (ng/mL) | 3.87$^{ab}$ | 1.57$^{a}$ | 7.35$^{b}$ | 3.89$^{ab}$ | 1.66 | 0.038* | 0.039* | 0.631 |

[1]Values are means and standard error of the means ($n = 5$).
$^{ab}$Means in the same row with different superscripts differ ($P < 0.05$).
HDL-C, high-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol; NEFA, none-esterified fatty acid; AST, aspartate aminotransferase; ALT, alanine aminotransferase; CON, control; RES, resveratrol; LPS, lipopolysaccharide; RES + LPS, dietary resveratrol treatment followed by LPS challenge.
The * indicates statistically significant difference ($P < 0.05$).

significant decrease in plasma GSH-Px, SOD, CAT, and T-AOC activities and a significant increase in plasma MDA concentrations in yellow-feathered broilers ($P < 0.05$). Dietary supplementation with 400 mg/kg RES significantly the increase in plasma CAT and T-AOC activities caused by LPS ($P < 0.05$). Moreover, a significant increase in SOD and T-AOC activities was found in the jejunal mucosa of yellow-feathered broilers by LPS challenge ($P < 0.05$).

TABLE 5 Effect of dietary resveratrol supplementation on antioxidant capacity in yellow-feathered broilers with lipopolysaccharide[1].

| Item | Treatment | | | | SEM | *P*-value | | |
|---|---|---|---|---|---|---|---|---|
| | CON | RES | LPS | RES + LPS | | LPS | RES | LPS × RES |
| **Plasma** | | | | | | | | |
| GSH-Px (U/mL) | 872.65$^{a}$ | 896.98$^{a}$ | 713.50$^{b}$ | 836.60$^{ab}$ | 69.24 | 0.039* | 0.152 | 0.328 |
| SOD (U/mg) | 175.96$^{a}$ | 179.11$^{a}$ | 140.71$^{b}$ | 174.47$^{a}$ | 12.73 | 0.042* | 0.057 | 0.109 |
| CAT (U/mL) | 3.37$^{b}$ | 4.23$^{a}$ | 2.57$^{b}$ | 3.15$^{b}$ | 0.40 | 0.004* | 0.021* | 0.632 |
| T-AOC (mmol/L) | 0.58$^{b}$ | 0.72$^{a}$ | 0.43$^{c}$ | 0.56$^{b}$ | 0.05 | <0.001* | 0.001* | 0.732 |
| MDA (nmol/mL) | 4.46$^{ab}$ | 4.01$^{b}$ | 5.18$^{a}$ | 4.74$^{ab}$ | 0.44 | 0.048* | 0.189 | 0.979 |
| **Jejunal mucosa** | | | | | | | | |
| GSH-Px (U/mg prot) | 197.79 | 209.22 | 181.74 | 193.13 | 18.11 | 0.228 | 0.386 | 0.999 |
| SOD (U/mg prot) | 29.57$^{ab}$ | 33.84$^{a}$ | 25.20$^{b}$ | 30.64$^{ab}$ | 3.25 | 0.054 | 0.022* | 0.858 |
| CAT (U/mg prot) | 1.10 | 1.12 | 0.91 | 0.98 | 0.12 | 0.065 | 0.586 | 0.757 |
| T-AOC (mmol/mg prot) | 58.25$^{ab}$ | 69.32$^{a}$ | 46.89$^{b}$ | 59.96$^{ab}$ | 6.54 | 0.040* | 0.019* | 0.832 |
| MDA (nmol/mg prot) | 0.70 | 0.62 | 0.78 | 0.72 | 0.062 | 0.076 | 0.154 | 0.892 |

[1]Values are means and standard error of the means, $n = 5$ per treatment.
$^{ab}$Means in the same row with different superscripts differ ($P < 0.05$).
CON, control; RES, resveratrol; LPS, lipopolysaccharide; RES + LPS, dietary resveratrol treatment followed by LPS challenge; GSH-Px, glutathione peroxidase; SOD, superoxide dismutase; CAT, catalase; T-AOC, total antioxidant capacity; MDA, malondialdehyde.
The * indicates statistically significant difference ($P < 0.05$).

Dietary supplementation with 400 mg/kg RES significantly increased jejunal SOD and T-AOC activities ($P < 0.05$). However, no significant LPS × RES interaction for these variables was observed. And there were no significant changes in plasma SOD activity as well as the CAT and MDA activities in jejunal mucosa among different treatments ($P > 0.05$).

## Intestinal morphology

The analysis of intestinal morphology (Figure 1) showed that LPS challenge resulted in intestinal damages in the duodenum, jejunum, and ileum of yellow-feathered broilers, as evidenced by a significant shortening of villus height and a highly significant decrease in villus height to crypt depth ratio of duodenum, jejunum, and ileum ($P < 0.01$) and an increase in crypt depth of duodenum and ileum ($P < 0.05$). However, the addition of RES at 400 mg/kg significantly alleviated the reduction in the ratio of villus height to crypt depth in the duodenum, jejunum and ileum and prevented the shortening of villus height in jejunum and ileum caused by LPS ($P < 0.05$), and significantly reduced the crypt depth in the ileum in yellow-feathered broilers ($P < 0.05$).

## The mRNA expression and secretion of cytokines in the jejunal mucosa

The real-time PCR analysis (Figure 2A) showed that LPS challenge upregulated ($P < 0.01$) the mRNA expression levels of *IL-1*β, *IL-8*, *IL-17*, and *TNF*-α and downregulated ($P < 0.01$) the mRNA expression levels of *TGF*-β in the jejunum mucosa of yellow-feathered broilers. However, the addition of RES significantly alleviated the changes in the above inflammatory factors caused by LPS ($P < 0.01$). Specially, dietary supplementation with RES significantly decreased the mRNA expression levels of *IL-6*, *IL-8*, and *IL-17* in the jejunal mucosa of yellow-feathered broilers ($P < 0.05$).

The Elisa assays (Table 6) also showed that LPS challenge caused a significant increase in TNF-α level and a significant decrease in TGF-β level in the jejunal mucosa of yellow-feathered broilers ($P < 0.05$). In contrast, the addition of RES at 400 mg/kg significantly alleviated the significant elevation of jejunal TNF-α, IL-6, and IL-1β concentrations compared to LPS group ($P < 0.05$). However, there is no significant difference in IL-17 level at jejunal mucosa and no significant LPS × RES interaction for these variables observed in present study ($P > 0.05$).

## The mRNA expression of intestinal tight junctions

As shown in Figure 2B, LPS challenge significantly decreased ($P < 0.01$) the mRNA expression levels of *claudin-1*, *claudin-5*, *occludin*, and *ZO-1* in jejunal mucosa. Furthermore, dietary supplementation with RES at 400 mg/kg significantly increased the mRNA expression levels of *claudin-5*, *occludin*, and *ZO-1* in jejunal mucosa of yellow-feathered broilers ($P < 0.01$).

10.3389/fmicb.2022.977087

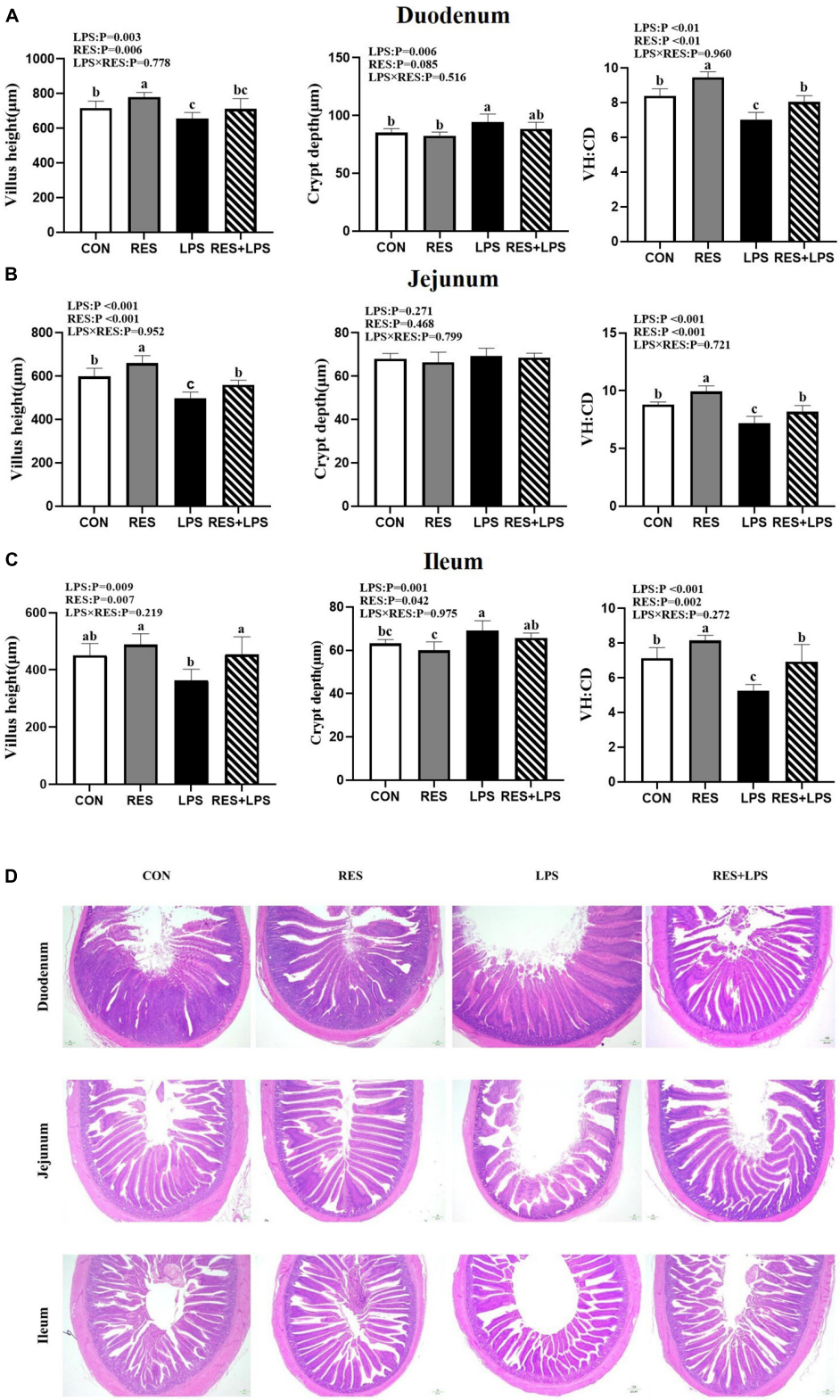


FIGURE 1
Effect of dietary resveratrol supplementation on intestinal morphology in yellow-feathered broilers with lipopolysaccharide. **(A)** Duodenum. **(B)** Jejunum. **(C)** Ileum. **(D)** The represent images of HE staining. Original magnification 40×, scale bar 20 μm. Values are means and standard error of the means, $n = 5$ per treatment. $^{abc}$ Means in the columns with different superscripts differ ($P < 0.05$). CON, control; RES, resveratrol; LPS, lipopolysaccharide; RES + LPS, dietary resveratrol treatment followed by LPS challenge.

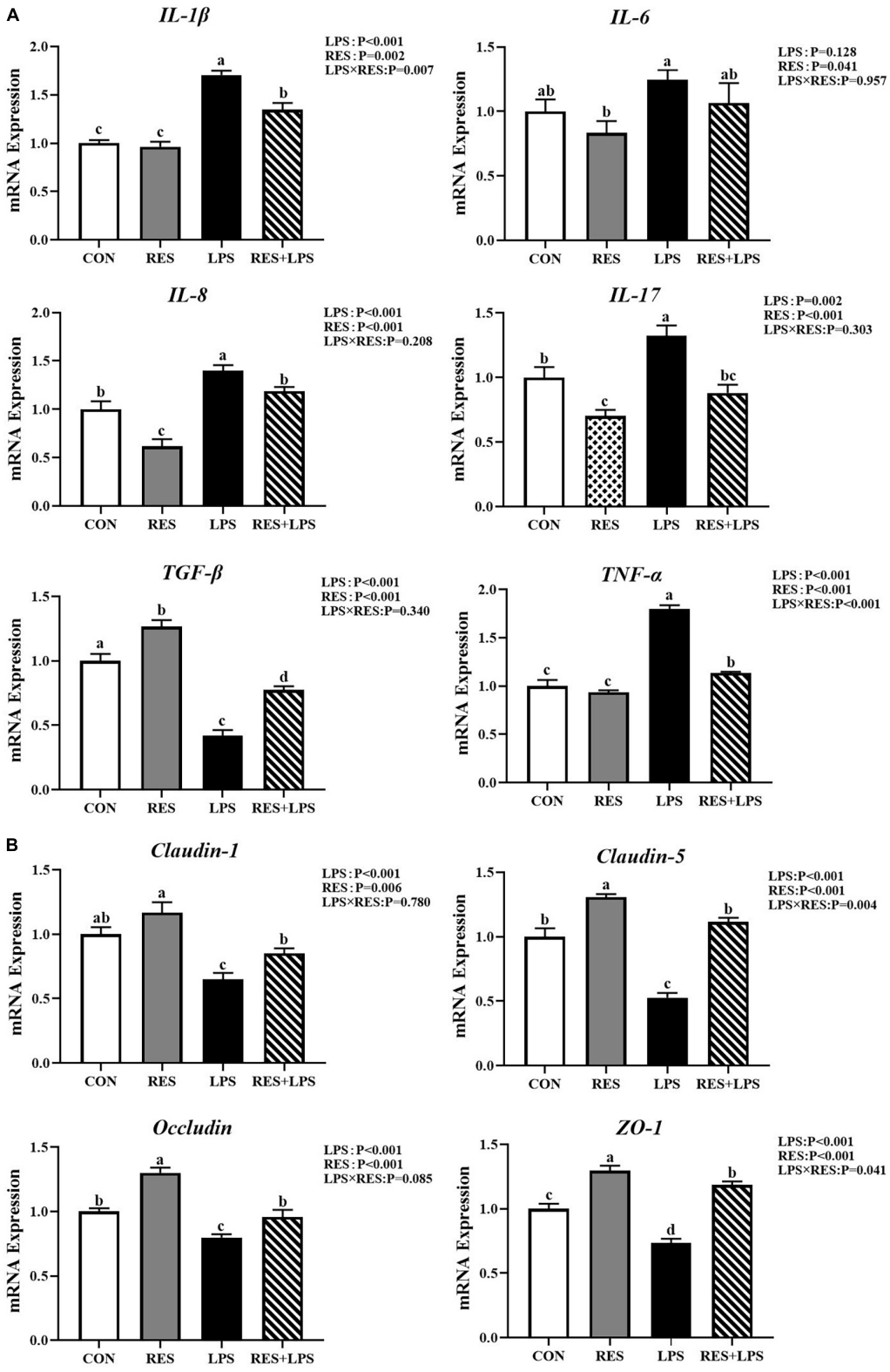


FIGURE 2
Effect of dietary resveratrol supplementation on intestinal gene expression in yellow-feathered broilers with lipopolysaccharide. **(A)** The mRNA expression of intestinal cytokines. **(B)** The mRNA expression of tight junctions. Values are means and standard error of the means, $n = 5$ per treatment. $^{abc}$ Means in the columns with different superscripts differ ($P < 0.05$). CON, control; RES, resveratrol; LPS, lipopolysaccharide; RES + LPS, dietary resveratrol treatment followed by LPS challenge.

TABLE 6 Effect of dietary resveratrol supplementation on cytokine secretion in jejunal mucosa of yellow-feathered broilers with lipopolysaccharide[1].

| Item | Treatment | | | | SEM | *P*-value | | |
|---|---|---|---|---|---|---|---|---|
| | CON | RES | LPS | RES + LPS | | LPS | RES | LPS × RES |
| TNF-α (pg/mL) | 1.71[b] | 1.22[b] | 2.52[a] | 1.71[b] | 0.30 | 0.007* | 0.007* | 0.479 |
| IL-6 (pg/mL) | 4.86[b] | 4.18[b] | 6.76[a] | 4.70[b] | 0.87 | 0.066 | 0.041* | 0.276 |
| IL-1β (pg/mL) | 2.11[ab] | 1.72[b] | 3.22[a] | 1.82[b] | 0.57 | 0.157 | 0.043* | 0.227 |
| IL-17 (pg/mL) | 6.66 | 5.23 | 7.94 | 6.21 | 1.15 | 0.186 | 0.71 | 0.853 |
| TGF-β (ng/mL) | 5.85[a] | 6.86[a] | 2.79[b] | 5.56[ab] | 1.21 | 0.034* | 0.059 | 0.331 |

[1]Values are means and standard error of the means, $n = 5$ per treatment.
[ab]Means in the same row with different superscripts differ ($P < 0.05$).
CON, control; RES, resveratrol; LPS, lipopolysaccharide; RES + LPS, dietary resveratrol treatment followed by LPS challenge; TNF-α, tumor necrosis factor-α; IL-6, interleukin-6; IL-1β, interleukin-1β; IL-17, interleukin-17; TGF-β, transforming growth factor-β.
The * indicates statistically significant difference ($P < 0.05$).

## Gut microbiota composition and diversity

There were 1139, 846, 932, and 345 unique OTUs in the CON group, LPS group, RES group, and RES + LPS group, respectively. A total of 578 common OTUs were found among four treatment groups (Figure 3A). At the phylum level (Figure 3B), the top 10 phyla bacteria were Firmicutes, Proteobacteria, Bacteroidota, Acidobacteriota, Spirochaetota, Campylobacterota, Cyanobacteria, Verrucomicrobiota, Desulfobacterota, and others. At the order level (Figure 3C), the top 10 bacteria were *Oscillospirales*, *Lachnospirales*, *Lactobacillales*, *Clostridia_UCG-014*, *Enterobacterales*, *Bacteroidales*, *Pseudomonadales*, *Clostridia_vadinBB60_group*, *RF39*, *Erysipelotrichales*, and others. At the genus level (Figure 3D), the top 10 genera bacteria were *Faecalibacterium*, *Clostridia_UCG-014*, *Lactobacillus*, *Escherichia-Shigella*, *CHKC1001*, *Ruminococcus_torques_group*, *Streptococcus*, *Enterococcus*, *PseuDomonas*, *Clostridia_vadinBB60_group*, and others.

As shown in Figure 4, the LPS challenge did not affect the alpha diversity of cecal microbiota in yellow-feathered broilers, but the RES treatments significantly decreased the dominance ($P = 0.047$) of cecal microbiota, and significantly increased the Pieiou-e ($P = 0.015$) and Simpson index ($P = 0.047$). There was significant interaction effect of LPS × RES in Pieiou-e of cecal microbiota of yellow-feathered broilers ($P = 0.027$).

The PCoA analysis (Figure 5A) and NMDS analysis (Figure5B) showed that the distances between CON group and LPS groups or between Control and RES groups were significantly distributed separately. The LEfSe analysis (Figure 5C) also indicated that three bacteria including *Lachnospiraceae* (Family), *Lachnospirales* (order), and *Ruminococcus_torques_group* (genus) were enriched in LPS when comparing to the CON group which enriched *Faecalibacterium* (genus). In contrast to control, LPS group enriched *Lachnospiraceae* (family) and *Lachnospirales* (order), while the CON group enriched *Deltia* (genus), *Ruminococcaceae* (family), *Faecalibacterium* (genus), and *Oscillospirales* (order). However, the RES + LPS group enriched four bacteria including Firmicutes (phylum), and *Clostridia_UCG-014* (order, family, and genus), while the LPS challenge enriched the *Enterobacteriaceae* (family), *Enterobacterales* (order), *Escherichia_Shigella* (genus), and Proteobacteria (phylum).

The Metastat analysis (Figure 6) showed that compared to the CON group, the relative abundances of *PeH15*, *UCG-009*, *Succinivibrio*, *Acetitomacuium*, *Christensenellaceae_R-7_group*, *Fibrobacter*, *Lachnospiraceae_NK3A20_group*, and *Rikenellaceae_RC9_gut_group* were significantly decreased while those of *CHKC1002, IS-44*, *Leptonema*, and *Alkaliphilus* were significantly increased in LPS group. Compared to the CON group, the relative abundances of *PeH15*, *C39*, *Fibrobacter*, *Lachnospiraceae_NK3A20_group*, *Alistipes*, *Pseudobutyrivibrio*, *Saccarofermentans*, and *Eubacterium_ruminantium_group* were significantly decreased while those of *Enterococcus*, *Weissella*, *Nesterenkonia*, and *Bacteriap25* were significantly increased in RES group. However, compared to the LPS group, the relative abundances of *IS-44*, *Leptonema*, *Alkaliphilus*, *Clostridium_innocuum_group*, *Catenibacillus*, and *Castellaniella* were significantly decreased while those of *UCG_009*, *Erysipelotrichaceae*, *Christensenellaceae_R-7_group*, *Anaerotruncus*, *RF39*, and *Ruminococcus* were significantly increased in RES + LPS group.

The Spearman correlation analysis showed that there was no significant correlation between top 10 phyla bacteria with the representative significant phenotypes of the yellow-feathered broilers challenged with LPS (Figure 7A) ($P > 0.05$). However, at the order level, the relative abundance of *Lachnospirales* was negatively associated with jejunal SOD and T-AOC activities as well the villus height in the jejunum (Figure 7B) ($P < 0.05$). Moreover, the *Clostridia_vadinBB60_group* at both order and genus level was positively correlated with the plasma T-AOC

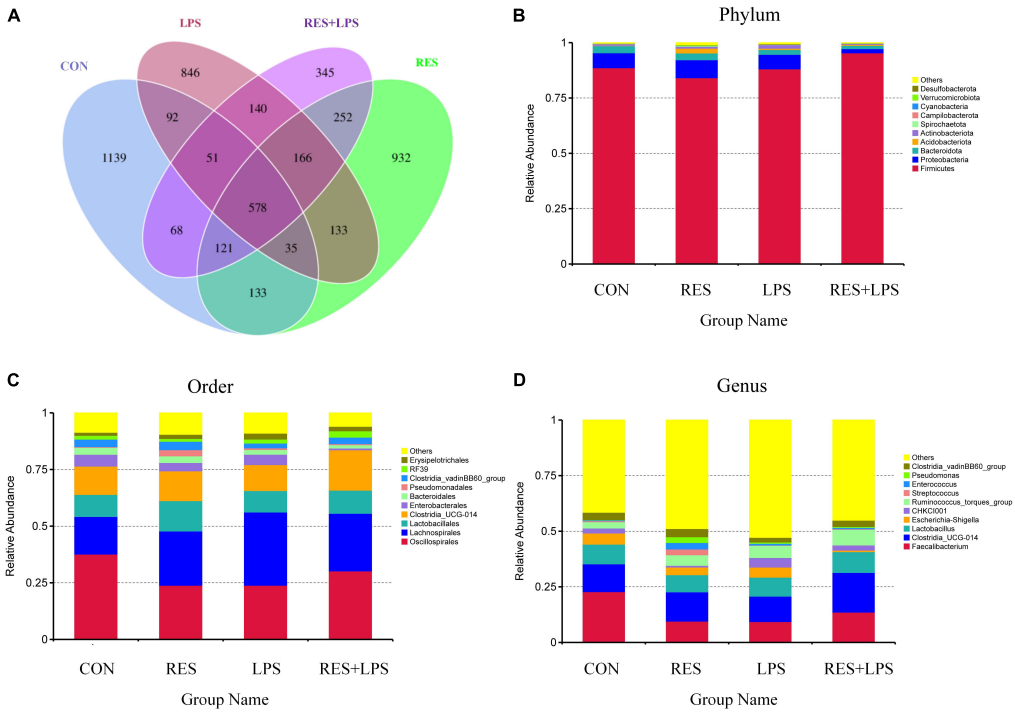


FIGURE 3
The Venn diagram and relative abundances of bacteria at the phylum, order, and genus levels. **(A)** Venn diagram. **(B)** Top 10 bacteria at phylum level. **(C)** Top 10 bacteria at order level. **(D)** Top 10 bacteria at genus order. CON, control; RES, resveratrol; LPS, lipopolysaccharide; RES + LPS, dietary resveratrol treatment followed by LPS challenge.

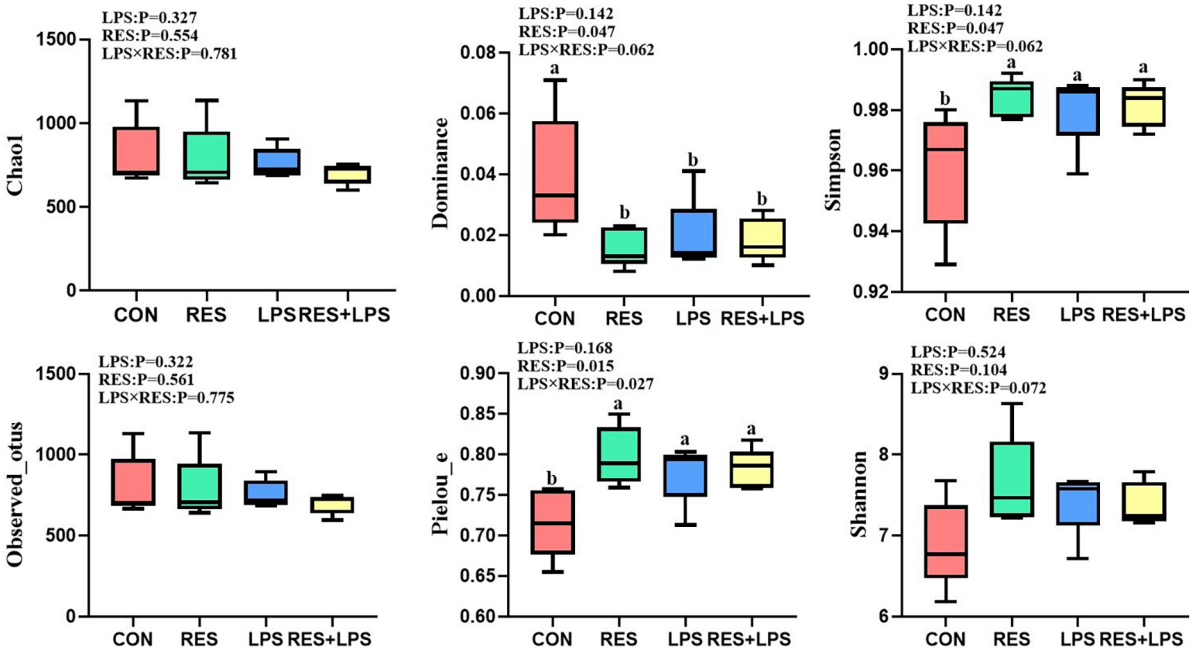


FIGURE 4
The effect of dietary resveratrol supplementation on the alpha diversity of cecal microbiota in yellow-feathered broilers. $^{ab}$ Means in the columns with different superscripts differ ($P < 0.05$). CON, control; RES, resveratrol; LPS, lipopolysaccharide; RES + LPS, dietary resveratrol treatment followed by LPS challenge.

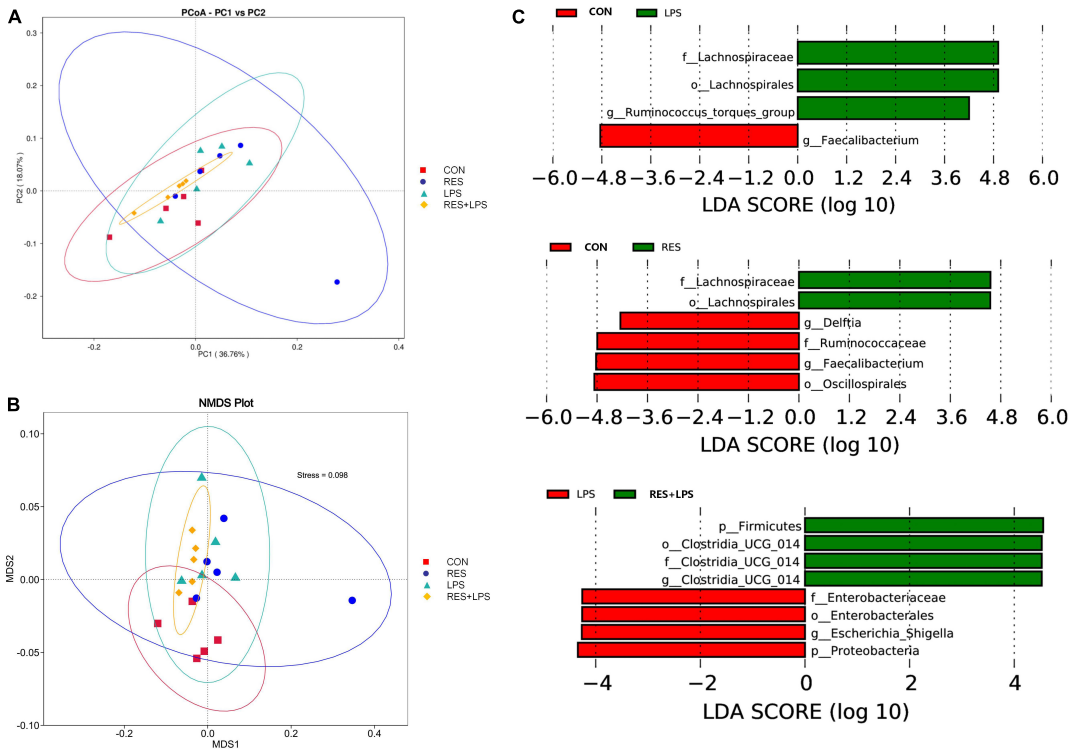


FIGURE 5
The effect of dietary resveratrol supplementation on the beta-diversity of cecal microbiota in yellow-feathered broilers. **(A)** The principal coordinate analysis (PCoA) plot; **(B)** Non-metric multidimensional scaling (NMDS) plot. **(C)** The LEfSe analysis (LDA score > 4). CON, control; RES, resveratrol; LPS, lipopolysaccharide; RES + LPS, dietary resveratrol treatment followed by LPS challenge.

activity and ileal villus height (Figures 7B,C) ($P < 0.05$). In addition, the intestinal IL-1β concentration was positively correlated with the relative abundance of *Lactobacillus* at both order ($P < 0.05$) and genus levels ($P < 0.01$) (Figures 7B,C), whereas the plasma CAT activity was negatively correlated with the *Lactobacillus* abundance at genus level (Figure 7C) ($P < 0.01$). Furthermore, the relative abundance of *Streptococcus* at the genus level was positively correlated with ADG at 1–21 days ($P < 0.05$), plasma T-AOC activity ($P < 0.05$) and jenunal villus height ($P < 0.01$), but was negatively correlated with intestinal TNF-α ($P < 0.01$) and IL-1β concentrations (Figure 7C) ($P < 0.05$). Additionally, the genera *Blautia* abundance was negatively associated with ileal villus height while plasma MDA concentration was negatively correlated with that of *UCG_005* and bacteria *p.251.05* (Figure 7C) ($P < 0.05$). Notably, there was a negative correlation between the relative abundance of genera *Alistipes* and ADFI at 1–21 days and ileal villus height in yellow-feathered broilers (Figure 7C) ($P < 0.05$).

## Discussion

Resveratrol is a natural polyphenolic compound with strong antioxidant and anti-inflammatory activities, which have been widely used for the prevention and treatments of many human diseases and disorders (Wang et al., 2016; Mayangsari and Suzuki, 2018). Increasing evidence also suggested that RES can effectively improve intestinal barrier function (Wang et al., 2022), alleviate the oxidative stress and intestinal inflammation for the enhancement of gut health and performance in piglets (Cao et al., 2019; Qiu et al., 2021), ducks (Yang C. et al., 2021), rats (Wang et al., 2022), and mice (Hao et al., 2022) under different experimental models. It has been well demonstrated that LPS could be used to mimick pathogen infection in broilers for studying immune inflammatory responses (Zhang et al., 2020, 2021; Erinle et al., 2022a). However, studies on the effects of RES on the growth performance, antioxidant capacity, intestinal health and gut microbiota of yellow-feathered broilers at early stage under LPS challenge conditions remained limited. Moreover, whether the protective of RES on broiler performance and health involved with the alterations of gut microbiota remained largely unknown.

Therefore, we investigated the effects of RES on the growth performance, antioxidant capacity and intestinal barrier function and gut microbiota in yellow-feathered broilers challenged with LPS. As expected, the present results showed that LPS significantly impaired the growth performance of yellow-feathered broilers by reducing ADG and ADFI and

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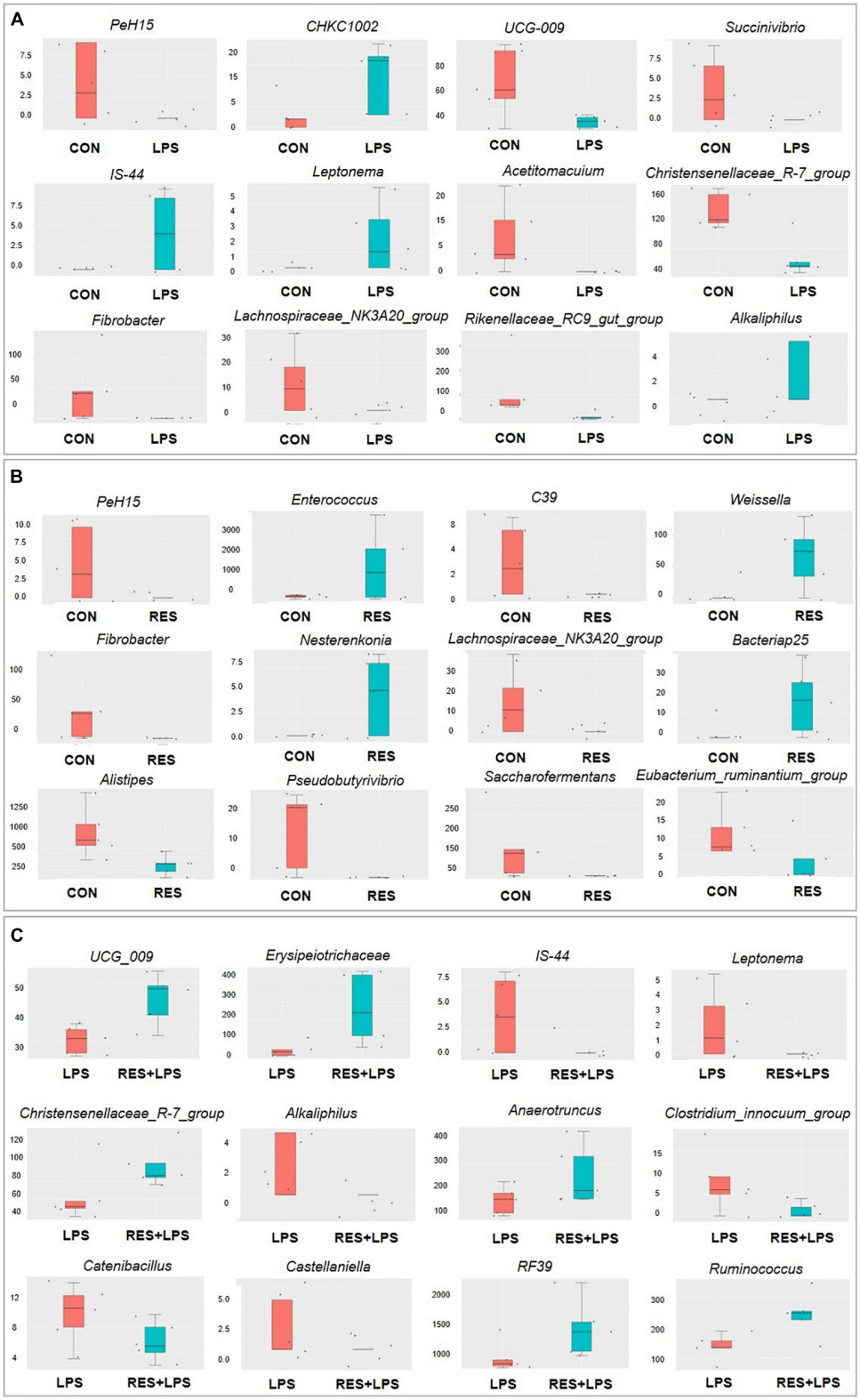


FIGURE 6
The Metastat analysis of cecal microbiota between treatments of cecal microbiota changes in yellow-feathered broilers. **(A)** Control group vs. LPS group. **(B)** Control group vs. RES group. **(C)** LPS group vs. RES + LPS group. CON, control; RES, resveratrol; LPS, lipopolysaccharide; RES + LPS, dietary resveratrol treatment followed by LPS challenge.

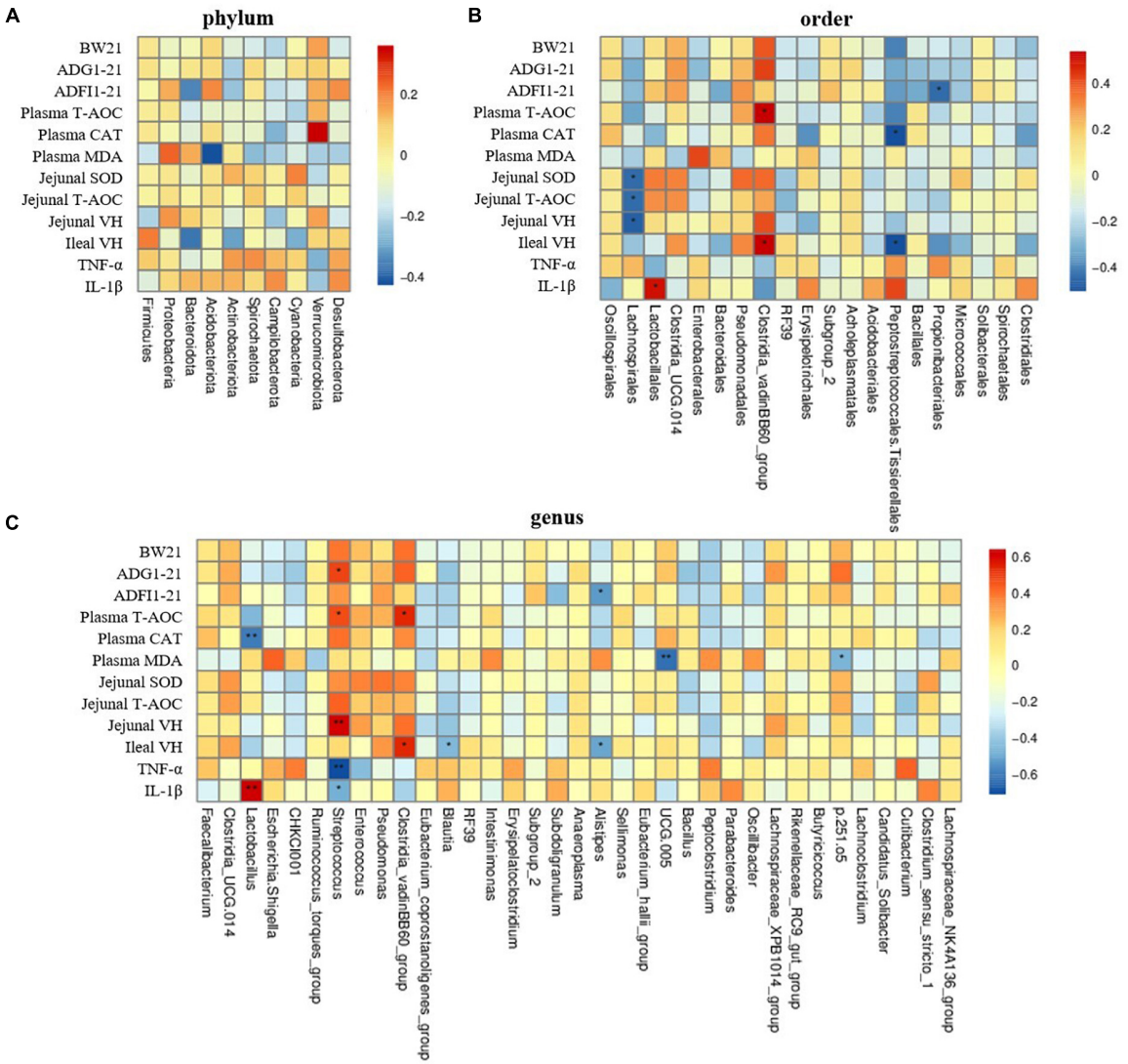


FIGURE 7
The Spearman correlation analysis of gut microbial composition at phylum, order, and genus levels with growth performance, antioxidant capacity, and intestinal health in yellow-feathered broilers. Spearman correlation coefficients of BW at 21 days, ADG and ADFI during 1–21 days, plasma and jenual antioxidant capacity, villus height at jejunum and ileum, and cytokine concentrations with representative cecal microbiota at phylum **(A)**, order **(B)**, and genus **(C)** level, are represented by color ranging from red (positive correlation) to blue (negative correlation), respectively. * and ** indicates statistically significant difference ($P < 0.05$) and ($P < 0.01$), respectively. BW, body weight; ADG, average daily gain; ADFI, average daily feed intake; T-AOC, total antioxidant capacity; CAT, catalase; SOD, superoxide dismutase; MDA, malondialdehyde; VH, villus height; TNF-α, tumor necrosis factor-α; IL-1β, interleukin-1β.

increasing the F/G during days 16–21. Consistently, previous studies have clearly demonstrated that immune stress induced by LPS excitation severely affected ADG and ADFI in broiler chickens; Yang S. et al., 2021; Zhang et al., 2021 (Kamboh et al., 2016; Li X. et al., 2021). Moreover, the induction of immune stress by LPS injection after 14 days significantly affected the growth performance in weaned piglets (Li Y. S. et al., 2021). Similarly, treatment with LPS for 3–10 times significantly reduced the ADG of rats (Zhong et al., 2018). However, we found that dietary supplementation with RES significantly improved the final BW, as well as the ADG and ADFI during days 16–21 and days 1–21, and significantly reduced the F/G during days 16–21 of yellow-feathered broilers, which indicated that dietary RES supplementation was able to mitigate the LPS-induced decline in growth performance of yellow-feathered broilers at early stage. Our results was consistent with the previous report on the protective effect of RES on the growth performance of broilers under heat stress (He et al., 2019b; Wang et al., 2021) or when receiving conventional vaccinations (Zhang et al., 2014). Additionally,

these above results may strengthen the rationale for applying RES to broiler production to improve the growth performance of broilers under LPS challenge as observed in current study.

Plasma biochemical biomarkers are generally used to reflect the physiological and health status of animals. The concentrations of plasma AST and ALT indirectly indicate the health status of the liver and their abnormal activities imply potential hepatic tissue damage (Nyblom, 2004; Zhang et al., 2017a). The present results showed that the injection of LPS could increase the plasma AST level in yellow-feathered broilers. Our results were in accordance with previous study demonstrating an increase in plasma AST activity in response to stress in broilers injected with Salmonella-derived LPS (Erinle et al., 2022a). Previous study also showed that the activity of AST in yellow-feathered broilers was increased by heat stress (He et al., 2019b). Besides, triglyceride mainly participates in body fat and energy metabolism, and infection and inflammation can be leading to multiple alterations in lipid and lipoprotein metabolism (Khovidhunkit et al., 2004). Moreover, HDL-C and LDL-C possess a core structure composed of total cholesterol and triglyceride that associates with the hydrophobic portion of LPS (Feingold and Grunfeld, 2011). The present results showed that LPS challenge resulted in significant increases of plasma creatine kinase, HDL-C and triglyceride concentrations in yellow-feathered broilers, but dietary RES supplementation had no significant mitigating effect on the elevated plasma triglyceride levels, which is consistent with the previous study (Mohebodini et al., 2019). However, the current study showed that RES had a significant alleviating effect on the elevated creatine kinase-MB and creatinine concentrations induced by LPS stress. This is consistent with previous findings that mice pre-treated with RES almost completely counteracted the deleterious effects of LPS-induced creatinine elevation in the liver and kidney (Sebai et al., 2010).

Stressors often cause dynamic changes in the hormones in the hypothalamic-pituitary-adrenal (HPA) axis (Khilji et al., 2018). The activation of the HPA axis in broilers under heat stress conditions leads to elevated plasma glucocorticoid corticosterone (Quinteiro-Filho et al., 2012). Besides, thyroid hormones (T3 and T4) are major metabolic hormones of the body to regulate liver, skeletal muscles, and kidney energy metabolism, as well as maintenance of physiological body temperature (Decuypere et al., 2005). The current results showed that LPS significantly reduced the plasma concentrations of T3, T4 and insulin and significantly increased cortisol levels in yellow-feathered broilers. Our results were in consistent with previous study that heat stress increased the cortisol levels and decreased the T3 and T4 levels in broilers (He et al., 2019b). However, our study shows that dietary supplementation with RES could alleviate the increase of cortisol concentration and the decrease of T3 and insulin caused by LPS stimulation, which might partially explain the improved growth performance of broilers after RES supplementation. Similarly, previous study has shown that supplementation with RES can reduce serum cortisol, T3 and T4, which is beneficial in reducing stress in broilers (Zhang et al., 2017b). This suggested that dietary supplementation with RES might positively regulated the abnormal levels of plasma hormones under LPS challenge to support the enhancements in growth performance.

The broilers are prone to oxidative stress when invaded by pathogenic microorganisms or stimulated by the environment which lead to the imbalance of antioxidant system. In the present study, the plasma GSH-Px, SOD, CAT, and T-AOC activities were significantly reduced and MDA concentration was significantly increased by LPS challenge in yellow-feathered broilers, which is consistent with previous studies that significantly increased MDA levels and reduced GSH-Px and SOD activities in broilers under LPS stimulation (Li X. et al., 2021; Xing et al., 2021). However, RES has a strong antioxidant capacity to capture and scavenge free radicals, which helps to alleviate oxidative stress-induced intestinal damage. Previous study has confirmed that RES can promote the expression of antioxidant genes, regulate the expression of inflammatory cytokines, and can effectively inhibit the apoptosis of piglet intestinal epithelial cells and alleviate the oxidative stress caused by deoxynivalenol (Yang et al., 2019). In our study, dietary addition of RES alleviated the decrease in plasma CAT and T-AOC activities caused by LPS, while increasing the activities of SOD and T-AOC in jejunal mucosa and decrease plasma MDA concentration, which suggested the potential of RES in alleviating the LPS-induced oxidative damages in yellow-feathered broilers. These results might be partially attributed to the hepatoprotective effects and antioxidant capacity of RES (Yang C. et al., 2021). Similarly, dietary RES supplementation has been demonstrated to effectively alleviate the decrease of SOD and CAT activities induced by heat stress with reducing the concentration of MDA at the same time (Liu et al., 2014). These results confirm that RES could effectively exert its antioxidant function to ensure the intestinal health and growth of animals (Kim et al., 2011).

The gastrointestinal tract is not only the main site to digest and absorb nutrients, but also represents the biggest immune organ and endocrine organ in the body (Cannon et al., 2011). Villus height, crypt depth, and their ratio are often used as indicators of gut integrity and maintenance of normal intestinal function (Erinle and Adewole, 2022). Indeed, the increase in the villus height is closely related to enhanced digestive ability, thus promoting the growth and development of animals (Erinle et al., 2022b). In this study, LPS significantly decreased the villus height and the ratio of villus height to crypt depth in the duodenum, jejunum and ileum of broilers. We found that the addition of RES could increase the villus height, and alleviate the intestinal morphological damages induced by LPS, thus improving the intestinal mucosa integrity in yellow-feathered broilers. These results are consistent with previous studies on the protective effect of RES on intestinal morphology and

structure (Liu et al., 2016; Zhuang et al., 2021; Hong et al., 2022). Moreover, the intestine mucosal barrier represents the first line of the host defence against invading enteric pathogens and toxins, which plays key roles in nutrient digestion and absorption (Turner, 2009). LPS stimulation can cause intestinal barrier damage, oxidation and immune stress, and then affect the intestinal development and growth performance of livestock and poultry (Gu et al., 2022). Tight junctions (TJs) are protein complexes in intestinal epithelial cells that can regulate intestinal epithelial permeability and maintain the intestinal barrier integrity (Suzuki, 2020). The most commonly recognized TJs included both the intracellular proteins zona occludens (*ZO-1*, *ZO-2*, and *ZO-3*) and transmembrane proteins (*claudins* and *occludin*) (Suzuki, 2013). In our study, LPS caused a decrease in the relative mRNA expression of *ZO-1*, *claudin-1*, and *claudin-5* in jejunal mucosa of yellow-feathered broilers. The previous results showed that LPS-induced duck ileitis accompanied with the destruction of intestinal structure and permeability alteration, severe oxidative stress, mitochondrial dysfunction, and inflammatory response (Wang et al., 2021; Yang H. et al., 2021). Dietary RES supplementation has been demonstrated to effectively alleviated LPS-induced intestinal dysfunction and the increase of intestinal permeability by linearly increasing mRNA levels of several tight junction protein genes (*claudin-1*, *occludin-1*, and *ZO-1*) as well as the protein expression of claudin-1 (Xun et al., 2021). In our study, the *claudin-1*, *occludin*, *ZO-1* mRNA expression was also significantly increased by RES treatment, which was consistent with a previous study in pigs (Chen et al., 2020). These above results suggested that RES might facilitate the maintenance of intestinal physical barrier function by increasing the expression of tight junction proteins.

The specificity and efficiency of the defense mechanism of intestinal immune response depend on the intestinal mucosa integrity (Ziegler et al., 2003). When the body is stimulated by microbial antigens such as LPS, it will stimulate macrophages, monocytes and endothelial cells to release a series of inflammatory mediators such as cytokines, oxygen free radicals and histamine to cause inflammatory reaction and reduce barrier function (Wu et al., 2015). The classical LPS cytokine cascade system includes IL-1β, IL-6, and TNF-α (Reisinger et al., 2021). In broilers, LPS could result in elevated inflammatory cytokines and reduced immune function, which lead to the damage to the gut integrity and thus causes intestinal immune dysfunction and impaired performance (Kamboh and Zhu, 2014; Wu et al., 2015). In this study, compared with the control group, LPS increased the concentration of TNF-α, IL-6, and IL-1β in jejunal mucosa of broilers, and decreased the concentration of TGF-β. Similarly, LPS significantly increased the mRNA expression of *IL-1*β, *IL-8*, *IL-17*, and *TNF*-α in jejunal mucosa. In addition, pro-inflammatory cytokines are effective stimulators of the HPA axis. It has been shown that excess of these cytokines may increase energy consumption and reduce food intake (Dec et al., 2020), which might account for the decline in growth performance of broilers by LPS observed in this experiment. However, we found that dietary RES inhibited the increase of the concentration of TNF-α, IL-6, and IL-1β, and increased the TGF-β concentration, as well as inhibiting the mRNA expression of *IL-1*β, *IL-8*, *IL-17*, and *TNF*-α in jenual mucosa. These results suggested that dietary RES supplementation might effectively reduce the intestinal inflammation to alleviate intestinal injury induced by LPS. Similarly, Qian et al. (2021) also showed that RES could attenuate the prostatic inflammation and reduce the expression of *IL-6*, *IL-8*, and *TNF*-α in rat with oestradiol-induced chronic prostatitis.

The maintenance of the dynamic balance of gut microbiota plays an important role in alleviating inflammation and improving animal intestinal health. The change of cecal microbial community is an important index of intestinal non-specific immune response (Jiang et al., 2021). Firmicutes, Bacteroidetes, Proteobacteria, Actinobacteria, and Cyanobacteria are the main phyla of broiler (Chen et al., 2020). Moreover, previous studies have shown that the addition of RES can regulate the balance of intestinal microflora (Sung et al., 2017; Cai et al., 2020). Consistently, our results showed that RES + LPS group increased the abundance of Firmicutes and decreased the abundance of the Proteobacteria compared to the LPS group. Similarly, previous study found that RES (200 mg per kg/day) attenuated the intestinal microflora dysbiosis in mice fed with a high-fat diet especially by increasing the ratio of Bacteroides to Firmicutes ratio, and enhancing the abundance of *Lactobacillus* (Qiao et al., 2014). Moreover, at the genus level, RES treatments significantly increased the relative abundances of *Erysipelotrichaceae* and *Ruminococcus* in the cecal digesta of yellow-feathered broilers. Previous study has demonstrated that the abundance of intestinal *Erysipelotrichaceae* are positively correlated with carbohydrate consumption (Cox et al., 2012), while the abundance of *Erysipelotrichaceae* and *Ruminococcus* has a positive association with short-chain fatty acids (SCFAs) levels (Kong et al., 2019; Li L. et al., 2020). Moreover, we found that the relative abundance of *Lachnospirales* negatively associated with jejunal SOD and T-AOC activities as well the villus height in the jejunum by Spearman correlation analysis. Importantly, *Lachnospiraceae* (family) and *Lachnospirales* (order) was found to be enriched in LPS-induced immune stress group, coupled with reduced antioxidant capacity and impaired intestinal morphology found in current study. Accordingly, a recent study also revealed close correlations between bacterial communities belonging to *Bacteroidales* and *Lachnospirales* orders and putative reactive aldehydes covariated with oxidative stress and leaky gut phenotype in mice (Mazenc et al., 2022). Furthermore, another study also showed that the abundance of butyrate-producing bacteria *Lachnospiraceae* was significantly reduced in rats with stress-induced visceral hypersensitivity (Zhang et al., 2019). However, due to the fact that SCFAs play

an important role as an energy substrate to influence the host energy metabolism (Besten et al., 2013), it requires further investigations to clarify whether RES could regulate the SCFAs production and energy metabolism of broilers by modulating the gut microbiota community.

In addition, dietary supplementation with RES significantly reduced the abundance of genera *Alistipes* in the cecal digesta of yellow-feathered broilers when compared to the control group. *Alistipes* is highly associated with dysbiosis and inflammation and has been identified as a potential opportunistic pathogen for many diseases, such as liver fibrosis, colorectal cancer, cardiovascular disease, and mood disorders (Rodríguez-Nogales et al., 2017; Kong et al., 2019). Here, the significant negative correlations were found between the relative abundance of cecal *Alistipes* and ADFI during days 1–21 and villus height in the ileum. So, the reduced abundances of *Alistipes* by dietary supplementation with RES might be associated with the restore of intestinal morphology and improvement of performance in yellow-feathered broilers. Our results was consistent with previous study in layer pullets demonstrating a significant negative correlation between growth parameters (including ADFI and feed conversion ratio) and Alistipes (Liu et al., 2021). Furthermore, the microbial alpha diversity analysis showed that RES treatments significantly decreased the dominance of cecal microbiota, but increased the Simpson index and Pieiou-e in yellow-feathered broilers, indicating RES impact on regulation of microbial diversity. This was in accordance with previous study that dietary RES altered microbial composition and restored microbial community diversity in dextran sulfate sodium-treated mice (Li F. et al., 2020). In summary, dietary supplementation with RES could cause dynamic changes in the bacterial community as well as microbial diversity in the cecal digesta in yellow-feathered broilers under LPS challenge at early stage, which might be associated with the enhancement of the broiler growth performance and antioxidant capacity, and restore intestinal health and immunity disturbed by LPS.

## Conclusion

Collectively, the present results suggest that dietary supplementation with 400 mg/kg of RES could improve the growth performance, antioxidant capacity and immunity, which might be closely linked with the alterations in the gut microbiota community composition in yellow-feathered broilers. The results of this study may provide scientific basis for the application of RES as a feed additive for protecting intestinal health and performance in yellow-feathered broilers. However, further investigations are necessary to elucidate the potential mechanism of gut microbiota as biomarker targets interacting with animal performance and wellbeing through nutritional intervention strategies.

## Data availability statement

The datasets presented in this study can be found in online repositories. The data can be found below: Submission ID: SUB11567250, NCBI BioProject number: PRJNA846164, available at: http://www.ncbi.nlm.nih.gov/bioproject/846164/.

## Ethics statement

This animal study was reviewed and approved by Animal Care and Use Committee of Foshan University.

## Author contributions

ZH, YL, TX, and XN performed the methodological research. ZH, YL, and TX data analysis and wrote the original manuscript. ZH, YL, TX, CZ, and HZ wrote, reviewed, and edited the manuscript. CZ and HZ contributed to conceptualization and obtaining the funding. 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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# References


Besten, G. D., Eunen, K. V., Groen, A. K., Venema, K., Reijngoud, D., and Bakker, B. M. (2013). The role of short-chain fatty acids in the interplay between diet, gut microbiota, and host energy metabolism. *J. Lipid Res.* 54, 2325–2340. doi: 10.1194/jlr.R036012

Cai, T., Ye, X., Li, R., Chen, H., Wang, Y., Yong, H., et al. (2020). Resveratrol modulates the gut microbiota and inflammation to protect against diabetic nephropathy in mice. *Front. Pharmaco* 11:1249. doi: 10.3389/fphar.2020.01249

Cannon, M. J., Goyne, H., Stone, P. J. B., and Chiriva-Internati, M. (2011). Dendritic cell vaccination against ovarian cancer - tipping the Treg/TH17 balance to therapeutic advantage? *Expert Opin. Biol. Ther.* 11, 441–445. doi: 10.1517/14712598.2011.554812

Cao, S., Shen, Z., Wang, C., Zhang, Q., Hong, Q., He, Y., et al. (2019). Resveratrol improves intestinal barrier function, alleviates mitochondrial dysfunction and induces mitophagy in diquat challenged piglets1. *Food Funct.* 10, 344–354. doi: 10.1039/C8FO02091D

Chen, K., Zhao, H., Shu, L., Xing, H., Wang, C., Lu, C., et al. (2020). Effect of resveratrol on intestinal tight junction proteins and the gut microbiome in high-fat diet-fed insulin resistant mice. *Int. J. Food Sci. Nutr.* 71, 965–978. doi: 10.1080/09637486.2020.1754351

Chen, Y., Zhang, H., Cheng, Y., Li, Y., Wen, C., and Zhou, Y. (2018). Dietary L-threonine supplementation attenuates lipopolysaccharide-induced inflammatory responses and intestinal barrier damage of broiler chickens at an early age. *Br. J. Nutr.* 119, 1254–1262. doi: 10.1017/S0007114518000740

Cox, L. M., Cho, I., Young, S. A., Anderson, W. H. K., Waters, B. J., Hung, S. C., et al. (2012). The nonfermentable dietary fiber hydroxypropyl methylcellulose modulates intestinal microbiota. *FASEB J.* 27, 692–702. doi: 10.1096/fj.12-219477

Dec, M., Puchalski, A., Stępień-Pyśniak, D., Marek, A., Urban-Chmiel, R. (2020). Susceptibility of chicken Lactobacillus bacteria to coccidiostats. *J. Vet. Med. Sci.* 82, 333–336. doi: 10.1292/jvms.19-0533

Decuypere, E., Van As, P., Van der Geyten, S., and Darras, V. M. (2005). Thyroid hormone availability and activity in avian species: A review. *Domest. Anim. Endocrin.* 29, 63–77. doi: 10.1016/j.domaniend.2005.02.028

Erinle, T. J., and Adewole, D. I. (2022). Fruit pomaces—their nutrient and bioactive components, effects on growth and health of poultry species, and possible optimization techniques. *Anim Nutr.* 9, 357–377. doi: 10.1016/j.aninu.2021.11.011

Erinle, T. J., MacIsaac, J., Yang, C., and Adewole, D. I. (2022a). Effect of red osier dogwood extract on growth performance, blood biochemical parameters, and gut functionality of broiler chickens challenged or unchallenged intraperitoneally with *Salmonella* Enteritidis lipopolysaccharide. *Poult. Sci.* 101:101861. doi: 10.1016/j.psj.2022.101861

Erinle, T. J., Oladokun, S., MacIsaac, J., Rathgeber, B., and Adewole, D. (2022b). Dietary grape pomace - effects on growth performance, intestinal health, blood parameters, and breast muscle myopathies of broiler chickens. *Poult. Sci.* 101:101519. doi: 10.1016/j.psj.2021.101519

Feingold, K. R., and Grunfeld, C. (2011). The role of HDL in innate immunity. *J. Lipid Res.* 52, 1–3. doi: 10.1194/jlr.E012138

Gu, Y. F., Chen, Y. P., Jin, R., Wang, C., Wen, C., and Zhou, Y. M. (2022). Dietary chitooligosaccharide supplementation alleviates intestinal barrier damage, and oxidative and immunological stress in lipopolysaccharide-challenged laying hens. *Poult. Sci.* 101:101701. doi: 10.1016/j.psj.2022.101701

Han, H., Zhang, J., Chen, Y., Shen, M., Yan, E., Wei, C., et al. (2020). Dietary taurine supplementation attenuates lipopolysaccharide-induced inflammatory responses and oxidative stress of broiler chickens at an early age. *J. Anim. Sci.* 98:skaa311. doi: 10.1093/jas/skaa311

Hao, W., Zhu, X., Liu, Z., Song, Y., Wu, S., Lu, X., et al. (2022). Resveratrol alleviates aluminum-induced intestinal barrier dysfunction in mice. *Environ. Toxicol.* 37, 1373–1381. doi: 10.1002/tox.23490

He, S., Chen, L., He, Y., Chen, F., Ma, Y., Xiao, D., et al. (2020). Resveratrol alleviates heat stress-induced impairment of intestinal morphology, barrier integrity and inflammation in yellow-feather broilers. *Anim. Prod. Sci.* 60:1547. doi: 10.1071/AN19218

He, S., Yu, Q., He, Y., Hu, R., Xia, S., and He, J. (2019a). Dietary resveratrol supplementation inhibits heat stress-induced high-activated innate immunity and inflammatory response in spleen of yellow-feather broilers. *Poult. Sci.* 98, 6378–6387. doi: 10.3382/ps/pez471

He, S., Li, S., Arowolo, M. A., Yu, Q., Chen, F., Hu, R., et al. (2019b). Effect of resveratrol on growth performance, rectal temperature and serum parameters of yellow-feather broilers under heat stress. *Anim. Sci. J.* 90, 401–411. doi: 10.1111/asj.13161

Hong, Q., Li, X., Lin, Q., Shen, Z., Feng, J., and Hu, C. (2022). Resveratrol improves intestinal morphology and anti-oxidation ability in deoxynivalenol-challenged piglets. *Animals* 12:311. doi: 10.3390/ani12030311

Jiang, J., Qi, L., Wei, Q., and Shi, F. (2021). Maternal stevioside supplementation ameliorates intestinal mucosal damage and modulates gut microbiota in chicken offspring challenged with lipopolysaccharide. *Food Funct.* 12, 614–628. doi: 10.1039/d0fo02871a

Kamboh, A. A., Hang, S. Q., Khan, M. A., and Zhu, W. Y. (2016). *In vivo* immunomodulatory effects of plant flavonoids in lipopolysaccharide-challenged broilers. *Animals* 10, 1619–1625. doi: 10.1017/S1751731116000562

Kamboh, A. A., and Zhu, W. (2014). Individual and combined effects of genistein and hesperidin on immunity and intestinal morphometry in lipopolysacharide-challenged broiler chickens. *Poult. Sci.* 93, 2175–2183. doi: 10.3382/ps.2014-03971

Khilji, M. S., Sandhu, M. A., Yousaf, M. S., Saeed, A. A., Rehman, H. U., Zaneb, H., et al. (2018). Differential effects of experimental hyperthyroidism on declined immunity of broiler chicken. *J. Anim. Physiol. An. N.* 102:e948–e956. doi: 10.1111/jpn.12861

Khovidhunkit, W., Kim, M., Memon, R. A., Shigenaga, J. K., Moser, A. H., Feingold, K. R., et al. (2004). Thematic review series: The Pathogenesis of Atherosclerosis. Effects of infection and inflammation on lipid and lipoprotein metabolism mechanisms and consequences to the host. *J. Lipid Res.* 45, 1169–1196. doi: 10.1194/jlr.R300019-JLR200

Kim, S., Jin, Y., Choi, Y., and Park, T. (2011). Resveratrol exerts anti-obesity effects via mechanisms involving down-regulation of adipogenic and inflammatory processes in mice. *Biochem. Pharmacol.* 81, 1343–1351. doi: 10.1016/j.bcp.2011.03.012

Kong, C., Gao, R., Yan, X., Huang, L., and Qin, H. (2019). Probiotics improve gut microbiota dysbiosis in obese mice fed a high-fat or high-sucrose diet. *Nutrition.* 60, 175–184. doi: 10.1016/j.nut.2018.10.002

Kumar, B., Manuja, A., and Aich, P. (2012). Stress and its impact on farm animals. *Front. Biosci.* 4:1759. doi: 10.2741/496

Li, F., Han, Y., Cai, X., Gu, M., Sun, J., Qi, C., et al. (2020). Dietary resveratrol attenuated colitis and modulated gut microbiota in dextran sulfate sodium-treated mice. *Food Funct.* 11, 1063–1073. doi: 10.1039/c9fo01519a

Li, L., Wang, Y., Zhu, L., Liu, Z., Ye, C., and Qin, S. (2020). Inulin with different degrees of polymerization protects against diet-induced endotoxemia and inflammation in association with gut microbiota regulation in mice. *Sci. Rep.* 10:978. doi: 10.1038/s41598-020-58048-w

Li, X., Liu, S., Wang, J., Yi, J., Yuan, Z., Wu, J., et al. (2021). Effects of ND vaccination combined LPS on growth performance, antioxidant performance and lipid metabolism of broiler. *Res. Vet. Sci.* 135, 317–323. doi: 10.1016/j.rvsc.2020.10.007

Li, Y. S., San Andres, J. V., Trenhaile-Grannemann, M. D., van Sambeek, D. M., Moore, K. C., Winkel, S. M., et al. (2021). Effects of mannan oligosaccharides and Lactobacillus mucosae on growth performance, immune response, and gut health of weanling pigs challenged with *Escherichia coli* lipopolysaccharides. *J. Anim. Sci.* 99:skab286. doi: 10.1093/jas/skab286

Liang, Y., Zhou, J., Ji, K., Liu, H., Degen, A., Zhai, M., et al. (2019). Protective effect of resveratrol improves systemic inflammation responses in LPS-Injected lambs. *Animales* 9:872. doi: 10.3390/ani9110872

Liu, L., Fu, C., Yan, M., Xie, H., Li, S., Yu, Q., et al. (2016). Resveratrol modulates intestinal morphology and HSP70/90, NF-κB and EGF expression in the jejunal mucosa of black-boned chickens on exposure to circular heat stress. *Food Funct.* 7, 1329–1338. doi: 10.1039/C5FO01338K

Liu, L. L., He, J. H., Xie, H. B., Yang, Y. S., Li, J. C., and Zou, Y. (2014). Resveratrol induces antioxidant and heat shock protein mRNA expression in response to heat stress in black-boned chickens. *Poult Sci.* 93, 54–62. doi: 10.3382/ps.2013-03423

Liu, Y., Yan, T., Ren, Z., and Yang, X. (2021). Age-associated changes in caecal microbiome and their apparent correlations with growth performances of layer pullets. *Anim. Nutr.* 7, 841–848. doi: 10.1016/j.aninu.2020.11.019

Mayangsari, Y., and Suzuki, T. (2018). Resveratrol enhances intestinal barrier function by ameliorating barrier disruption in Caco-2 cell monolayers. *J. Funct. Foods* 51, 39–46. doi: 10.1016/j.jff.2018.10.009

Mazenc, A., Mervant, L., Maslo, C., Lencina, C., Bézirard, V., Levêque, M., et al. (2022). Maternal heme-enriched diet promotes a gut pro-oxidative status associated with microbiota alteration, gut leakiness and glucose intolerance in mice offspring. *Redox Biol.* 53:102333. doi: 10.1016/j.redox.2022.102333

Meng, Q., Sun, S., Luo, Z., Shi, B., Shan, A., and Cheng, B. (2019). Maternal dietary resveratrol alleviates weaning-associated diarrhea and intestinal inflammation in pig offspring by changing intestinal gene expression and microbiota. *Food Funct.* 1, 5626–5643. doi: 10.1039/c9fo00637k

Meng, T., Xiao, D., Muhammed, A., Deng, J., Chen, L., and He, J. (2021). Anti-Inflammatory action and mechanisms of resveratrol. *Molecules* 26:229. doi: 10.3390/molecules26010229

Ministry of Agriculture of the People's Republic of China. (2020). *Nutrient requirements of yellow-feathered chickens*. Beijing: China Agricultural Press.

Mohebodini, H., Jazi, V., Bakhshalinejad, R., Shabani, A., and Ashayerizadeh, A. (2019). Effect of dietary resveratrol supplementation on growth performance, immune response, serum biochemical indices, cecal microflora, and intestinal morphology of broiler chickens challenged with *Escherichia coli*. *Livest. Sci.* 229, 13–21. doi: 10.1016/j.livsci.2019.09.008

Nyblom, H. B. U. B. J. (2004). High AST/ALT ratio may indicate advanced alcoholic liver disease rather than heavy drinking. *Alcohol Alcoholism* 39, 336–339. doi: 10.1093/alcalc/agh074

Qian, X., Gu, Z., Guan, W., Qi, J., and Xu, D. (2021). Resveratrol could attenuate prostatic inflammation in rats with Oestradiol-induced chronic prostatitis. *Andrologia* 53:e14004. doi: 10.1111/and.14004

Qiao, Y., Sun, J., Xia, S., Tang, X., Shi, Y., and Le, G. (2014). Effects of resveratrol on gut microbiota and fat storage in a mouse model with high-fat-induced obesity. *Food Funct.* 5, 1241–1249. doi: 10.1039/c3fo60630a

Qiu, Y., Yang, J., Wang, L., Yang, X., Gao, K., Zhu, C., et al. (2021). Dietary resveratrol attenuation of intestinal inflammation and oxidative damage is linked to the alteration of gut microbiota and butyrate in piglets challenged with deoxynivalenol. *J. Anim. Sci. Biotechnol.* 12:71. doi: 10.1186/s40104-021-00596-w

Quinteiro-Filho, W. M., Gomes, A. V. S., Pinheiro, M. L., Ribeiro, A., Ferraz-de-Paula, V., Astolfi-Ferreira, C. S., et al. (2012). Heat stress impairs performance and induces intestinal inflammation in broiler chickens infected with *Salmonella* Enteritidis. *Avian Pathol.* 41, 421–427. doi: 10.1080/03079457.2012.709315

Reisinger, N., Wendner, D., Schauerhuber, N., and Mayer, E. (2021). Effect of lipopolysaccharides (LPS) and lipoteichoic acid (LTA) on the inflammatory response in rumen epithelial cells (REC) and the impact of LPS on claw explants. *Anim.* 11:2058. doi: 10.3390/ani11072058

Rodríguez-Nogales, A., Algieri, F., Garrido-Mesa, J., Vezza, T., Utrilla, M. P., Chueca, N., et al. (2017). Differential intestinal anti-inflammatory effects of Lactobacillus fermentum and Lactobacillus salivarius in DSS mouse colitis: Impact on microRNAs expression and microbiota composition. *Mol. Nutr. Food Res.* 61:1700144. doi: 10.1002/mnfr.201700144

Sebai, H., Sani, M., Ghanem-Boughanmi, N., and Aouani, E. (2010). Prevention of lipopolysaccharide-induced mouse lethality by resveratrol. *Food Chem. Toxicol.* 48, 1543–1549. doi: 10.1016/j.fct.2010.03.022

Song, B., Tang, D., Yan, S., Fan, H., Li, G., Shahid, M. S., et al. (2021). Effects of age on immune function in broiler chickens. *J. Anim. Sci. Biotechnol.* 12:42. doi: 10.1186/s40104-021-00559-1

Sun, X., Cui, Y., Su, Y., Gao, Z., Diao, X., Li, J., et al. (2021). Dietary fiber ameliorates Lipopolysaccharide-Induced intestinal barrier function damage in piglets by modulation of intestinal microbiome. *mSystems* 6:e1374–e1320. doi: 10.1128/mSystems.01374-20

Sung, M. M., Byrne, N. J., Robertson, I. M., Kim, T. T., Samokhvalov, V., Levasseur, J., et al. (2017). Resveratrol improves exercise performance and skeletal muscle oxidative capacity in heart failure. *Am. J. Physiol.-Heart C.* 312:H842–H853. doi: 10.1152/ajpheart.00455.2016

Suzuki, T. (2013). Regulation of intestinal epithelial permeability by tight junctions. *Cell. Mol. Life Sci.* 70, 631–659. doi: 10.1007/s00018-012-1070-x

Suzuki, T. (2020). Regulation of the intestinal barrier by nutrients: The role of tight junctions. *Anim. Sci. J.* 91:e13357. doi: 10.1111/asj.13357

Turner, J. R. (2009). Intestinal mucosal barrier function in health and disease. *Nat. Rev. Immunol.* 9, 799–809. doi: 10.1038/nri2653

Wang, C., Zhao, F., Li, Z., Jin, X., Chen, X., Geng, Z., et al. (2021). Effects of Resveratrol on Growth Performance, Intestinal Development, and Antioxidant Status of Broilers under Heat Stress. *Animales* 11:1427. doi: 10.3390/ani11051427

Wang, N., Han, Q., Wang, G., Ma, W., Wang, J., Wu, W., et al. (2016). Resveratrol protects oxidative Stress-Induced intestinal epithelial barrier dysfunction by upregulating heme oxygenase-1 expression. *Digest. Dis. Sci.* 61, 2522–2534. doi: 10.1007/s10620-016-4184-4

Wang, Y., Hong, C., Wu, Z., Li, S., Xia, Y., Liang, Y., et al. (2022). Resveratrol in Intestinal Health and Disease: Focusing on Intestinal Barrier. *Front. Nutr.* 9:848400. doi: 10.3389/fnut.2022.848400

Wu, Q. J., Wang, Q. Y., Wang, T., and Zhou, Y. M. (2015). Effects of clinoptilolite (zeolite) on attenuation of lipopolysaccharide-induced stress, growth and immune response in broiler chickens. *Ann. Anim. Sci.* 15, 681–697. doi: 10.1515/aoas-2015-0010

Xing, Y. Y., Zheng, Y. K., Yang, S., Zhang, L. H., Guo, S. W., Shi, L. L., et al. (2021). Artemisia ordosica Polysaccharide Alleviated Lipopolysaccharide-induced Oxidative Stress of Broilers via Nrf2/Keap1 and TLR4/NF-κB Pathway. *Ecotox. Environ. Safe* 223:112566. doi: 10.1016/j.ecoenv.2021.112566

Xun, W., Fu, Q., Shi, L., Cao, T., Jiang, H., and Ma, Z. (2021). Resveratrol protects intestinal integrity, alleviates intestinal inflammation and oxidative stress by modulating AhR/Nrf2 pathways in weaned piglets challenged with diquat. *Int. Immunopharmacol.* 99:107989. doi: 10.1016/j.intimp.2021.107989

Yang, C., Luo, P., Chen, S., Deng, Z., Fu, X., Xu, D., et al. (2021). Resveratrol sustains intestinal barrier integrity, improves antioxidant capacity, and alleviates inflammation in the jejunum of ducks exposed to acute heat stress. *Poult. Sci.* 100:101459. doi: 10.1016/j.psj.2021.101459

Yang, H., Wang, Y., Jin, S., Pang, Q., Shan, A., and Feng, X. (2021). Dietary resveratrol alleviated lipopolysaccharide-induced ileitis through Nrf2 and NF-κB signalling pathways in ducks (*Anas platyrhynchos*). *J. Anim. Physiol. An. N* [Epub ahead of print]. doi: 10.1111/jpn.13657

Yang, J., Zhu, C., Ye, J., Lv, Y., Wang, L., Chen, Z., et al. (2019). Protection of porcine intestinal-epithelial cells from deoxynivalenol-induced damage by resveratrol via the Nrf2 signaling pathway. *J. Agr. Food Chem.* 67, 1726–1735. doi: 10.1021/acs.jafc.8b03662

Yang, S., Zhang, J., Jiang, Y., Xu, Y. Q., Jin, X., Yan, S. M., et al. (2021). Effects of Artemisia argyi flavonoids on growth performance and immune function in broilers challenged with lipopolysaccharide. *Anim Biosci.* 34, 1169–1180. doi: 10.5713/ab.20.0656

Yao, Q., Li, H., Fan, L., Zhang, Y., Zhao, S., Zheng, N., et al. (2021). Dietary regulation of the crosstalk between gut microbiome and immune response in inflammatory bowel disease. *Foods* 10:368. doi: 10.3390/foods10020368

Zhang, C., Tian, Y., Yan, F., Kang, X., Han, R., Sun, G., et al. (2014). Modulation of growth and immunity by dietary supplementation with resveratrol in young chickens receiving conventional vaccinations. *Am. J. Vet. Res.* 75:752. doi: 10.2460/ajvr.75.8.752

Zhang, C., Wang, L., Zhao, X. H., Chen, X. Y., Yang, L., and Geng, Z. Y. (2017a). Dietary resveratrol supplementation prevents transport-stress-impaired meat quality of broilers through maintaining muscle energy metabolism and antioxidant status. *Poult. Sci.* 96, 2219–2225. doi: 10.3382/ps/pex004

Zhang, C., Zhao, X., Wang, L., Yang, L., Chen, X., and Geng, Z. (2017b). Resveratrol beneficially affects meat quality of heat-stressed broilers which is associated with changes in muscle antioxidant status. *Anim. Sci. J.* 88, 1569–1574. doi: 10.1111/asj.12812

Zhang, H., Chen, Y., Chen, Y., Li, Y., Jia, P., Ji, S., et al. (2020). Dietary pterostilbene supplementation attenuates intestinal damage and immunological stress of broiler chickens challenged with lipopolysaccharide. *J. Anim. Sci.* 98:skz373. doi: 10.1093/jas/skz373

Zhang, H., Yu, X., Li, Q., Cao, G., Feng, J., Shen, Y., et al. (2021). Effects of rhamnolipids on growth performance, immune function, and cecal microflora in linnan yellow broilers challenged with lipopolysaccharides. *Antibiotics* 10:905. doi: 10.3390/antibiotics10080905

Zhang, J., Song, L., Wang, Y., Liu, C., Zhang, L., Zhu, S., et al. (2019). Beneficial effect of butyrate-producing Lachnospiraceae on stress-induced visceral hypersensitivity in rats. *J Gastroenterol. Hepatol.* 34, 1368–1376. doi: 10.1111/jgh.14536

Zhong, Y., Zhang, X., Hu, X., and Li, Y. (2018). Effects of repeated lipopolysaccharide treatment on growth performance, immune organ index, and blood parameters of Sprague-Dawley rats. *J. Vet. Res.* 62, 341–346. doi: 10.2478/jvetres-2018-0048

Zhu, C., Gong, L., Huang, K., Li, F., Tong, D., and Zhang, H. (2020). Effect of Heat-Inactivated compound probiotics on growth performance, plasma biochemical indices, and cecal microbiome in Yellow-Feathered broilers. *Front. Microbiol.* 11:585623. doi: 10.3389/fmicb.2020.585623

Zhuang, Y., Huang, H., Liu, S., Liu, F., Tu, Q., Yin, Y., et al. (2021). Resveratrol improves growth performance, intestinal morphology, and microbiota composition and metabolism in mice. *Front. Microbiol.* 12:726878. doi: 10.3389/fmicb.2021.726878

Ziegler, T. R., Evans, M. E., Fernández-Estívariz, C., and Jones, D. P. (2003). Trophic and cytoprotective nutrition for intestinal adaptation, mucosal repair, and barrier function. *Annu. Rev. Nutr.* 23, 229–261. doi: 10.1146/annurev.nutr.23.011702.073036

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# Mixed silage with Chinese cabbage waste enhances antioxidant ability by increasing ascorbate and aldarate metabolism through rumen *Prevotellaceae UCG-004* in Hu sheep

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Silage is rich in nutrients, which can make up for the lack of seasonal roughage, and has a certain promotion effect on the intensive feeding of ruminants. In addition, silage can maintain the rumen function of ruminants to a certain extent and reduce the risk of rumen acidosis and abomasum translocation. The purpose of this study was to investigate the effects of the mixed silage of Chinese cabbage waste and rice straw (mixed silage) on antioxidant performance, rumen microbial population, and fermentation metabolism of Hu sheep. The 16 healthy Hu sheep (eight rams and eight ewes, 39.11±1.16kg, 5.5months) were randomly divided into two groups (the control group and the mixed silage group) with eight animals (four rams and four ewes) in each group. The control group was fed with farm roughage (peanut seedlings, corn husk, and high grain shell) as forage, and the mixed silage group was fed with the mixed silage as forage. The results showed that the mixed silage had no effect on the growth performance of Hu sheep ($p$ >0.05). Ruminal butyric acid, total volatile fatty acids (TVFA), and ammonia nitrogen ($NH_3$-N) concentration in the mixed silage group were increased, whereas the pH was decreased ($p$ <0.05). The blood and rumen total antioxidants capacity (T-AOC) concentration in the mixed silage group was higher, and the malondialdehyde (MDA) content in rumen, serum, liver, and kidney was lower than that in the control group ($p$ <0.05). PCoA and ANOSIM results of Illumina sequencing indicated that the mixed silage affected the bacterial composition of the rumen microbes. The mixed silage increased the proportion of *Prevotellaceae UCG-004* which was in a positive correlation with Vitamin C (Vc). In addition, PICRUSt functional

prediction analysis showed that ascorbate and aldarate metabolism were up-regulated in the mixed silage group ($p$ <0.05). In conclusion, higher contents of $V_C$ and acid detergent fiber (ADF) in the mixed silage were beneficial to the growth and reproduction of *Prevotellaceae UCG-004*, resulting in increased production of the butyric acid significantly upregulated the metabolism of ascorbate and aldarate metabolism, thereby improving the antioxidant properties of Hu sheep.

KEYWORDS

**mixed silage, Hu sheep, antioxidant properties, high-throughput sequencing, bacterial community**

## Introduction

As the global population increases, so does the demand for animal products. The growing demand for animal products has also created a huge requirement for animal feed, and the efficient use of existing feed is essential for efficient livestock farming and food safety (Makkar and Ankers, 2014). Chinese vegetable production accounts for 50% of the world's total production (FAOSTAT, 2016), but it is also accompanied by a large amount of vegetable waste. Vegetable waste, such as cabbage tails, accounts for the majority of food waste, especially in supermarkets, fresh food markets and households (Ma et al., 2017). The rational use of vegetable waste can not only reduce environmental pollution (water and soil pollution, bacteria reproduction, etc.) but also reduce the harm to consumers' health (Qiao et al., 2020). In addition, vegetable tails contains minerals, vitamins and other nutrients, which can promote the antioxidant properties of ruminants (Seong et al., 2016; Sahoo et al., 2021). The annual production of rice straw in China is about 21 million t/year, accounting for about 47% of the straw production (Wang et al., 2010; Chen, 2016). Fresh crop stalks dry and wilt rapidly, resulting in reduced nutrients such as moisture and soluble substances (Liu et al., 2019). Furthermore, The lower nutritional value and higher silica content of straw results in lower feed utilization and digestibility of rice straw (Cherdthong et al., 2020; Suntara et al., 2020).

There is a certain complementary effect between Chinese cabbage waste and rice straw in terms of physical structure, nutrients, and water content. Air-dried straw stalks and Chinese cabbage waste are available to complete common silage (Ren et al., 2018). Previous studies showed that vegetable waste can be mixed with straw for silage, leading to the higher rate of utilization, for example, broccoli by-products were mixed with wheat straw (Partovi et al., 2020); corn stover were mixed with cabbage (Ren et al., 2018), co-ensiling of sugar beet waste, and wheat straw (Hillion et al., 2018).

Silage treatment can be used for feed utilization of unconventional feed, and has certain positive impacts on the health status, physiology and rumen microorganisms of ruminants (Bampidis and Robinson, 2006; Abd El Tawab et al., 2020). Broccoli by-product and wheat straw (ratio 69:31) mixed feed did not affect rumen fermentation parameters in Fasander lambs (Partovi et al., 2020). In addition, adding 10% silage mulberry leaves to the total mixed diet of Hanwoo cattle can improve the activities of antioxidant enzymes such as total superoxide dismutase (T-SOD) and glutathione peroxidase (GSH-Px; Cheong et al., 2012). Hassanat et al. (2013) found that by increasing the proportion of maize silage in the diet, the richness and diversity of bacterial communities was decreased, but the number of total bacteria and *Prevotella* spp., was increased which favored the propionate production.

The rumen environment and microflora structure are crucial to the digestion and absorption of nutrients, production performance, and health. The rumen is home to a large number of microorganisms that grow in an anaerobic environment and can adhere to the feed surface and ferment (Palma-Hidalgo et al., 2021). Diet level, geographic distribution, feeding management, and health status are several factors that affect the rumen environment and microbial community structure while the diet level is the biggest factor affecting rumen fermentation and microbial population changes (Henderson et al., 2015; van Lingen et al., 2016).

We hypothesized that the mixed silage could improve the rumen environment and microbial flora structure, thereby enhancing the antioxidant properties of Hu sheep. Therefore, in the present study, we fed Hu sheep mixed silage to explore its effects on the antioxidant properties, rumen fermentation and microorganisms, so as to provide some reference for the application of mixed silage in Hu sheep.

## Materials and methods

### Mixed silage source and production process

The rice straw was collected from the experimental farms of Yangzhou University in Jiangsu Province. The Chinese cabbage

waste was collected from the East Garden Farmers Market in Yangzhou City, Jiangsu Province, China. Lactobacillus plantarum and cellulase used in the experiment were purchased from Guangzhou Lvhui Biotechnology Co., Ltd.

The rice straw and Chinese cabbage tails were cut to 2–3 cm, and then were mixed thoroughly with *Lactobacillus plantarum* and *cellulase* in a ratio of 4:6. *Lactobacillus plantarum* 0.035 g/kg and *cellulase* 0.25 g/kg were added to each kilogram of mixed silage. After mixing, it was put into silage bags, vacuum-treated with a Meggis vacuum machine, sealed in a cool place for 45 days, and then silaged and sampled after anaerobic fermentation.

## Determination of vitamin content

After the mixed silage was completed, the contents of vitamin A ($V_A$), vitamin $B_2$ ($V_{B2}$), $V_C$, and vitamin E ($V_E$) were determined by commercial kits (Shanghai Enzyme Link Biotechnology Co., Ltd.).

## Experimental animals and feeding

We selected 16 healthy Hu sheep with an initial weight of 39.97 ± 1.22 kg and an age of about 5.5 months, and randomly divided them into two groups ($n = 8$, four rams and four ewes). We took the sheep farm roughage (peanut seedlings, corn husks and high grain husks) as the control group, and the mixed silage group took the mixed silage as the roughage. Both the control group and the mixed silage group were fed 50% roughage and 50% concentrate (based on dry matter (DM), Table 1). Feed formulations were designed according to the nutritional requirements of mutton sheep (NRC, 2007). The feeding experiment was carried out for a total of 5 weeks. The first week was the pre-feeding period, and the remaining period from the second to the fifth week was the experimental period. The experimental Hu sheep were raised in single pens, and the sheep house was cleaned and disinfected before the experiment. All sheep were uniformly dewormed and immunized before entering into the sheep house. In the pre-feeding period and the formal experimental period, each Hu sheep was fed twice a day (8:00 and 18:00). During the experiment, each Hu sheep was free to get clean drinking water.

## Determination of growth performance

We recorded the fasting weight of each sheep before the formal start and end of morning feeding, that is, the initial weight (IW) and final weight (FW), to calculate the average daily gain (ADG). The amount of feed and leftovers fed to each sheep was recorded during the experiment to calculate the mean daily feed intake (DMI). Finally, the feed-to-weight ratio (F/W) was calculated according to ADG and DMI.

TABLE 1 Experimental diet formula and nutrition level (DM basis).

| Item | Groups[1] | |
|---|---|---|
| | Control | Mixed silage |
| Ingredients (% of DM) | | |
| Peanut seedling | 30.00 | – |
| Corn husk | 15.00 | – |
| Sorghum shell | 5.00 | – |
| The Mixed Silage | 0.00 | 50.00 |
| Corn | 34.00 | 34.00 |
| Soybean meal | 7.00 | 5.50 |
| Bran | 7.50 | 8.00 |
| Corn gluten meal | – | 1.00 |
| $NaHCO_3$ | 0.50 | 0.50 |
| Premix contained[2] | 0.50 | 0.50 |
| Salt | 0.50 | 0.50 |
| Total | 100.00 | 100 |
| Nutrient composition (% of DM) | | |
| Digestive energy/DE (MJ/Kg)[3] | 13.52 | 14.73 |
| CP (%) | 15.08 | 15.11 |
| Ash (%) | 4.36 | 12.33 |
| NDF (%) | 47.64 | 48.23 |
| ADF (%) | 23.71 | 27.17 |
| Ca (%) | 0.48 | 0.45 |
| P (%) | 0.38 | 0.39 |

[1]Control: Based on peanut seedling, corn husk and sorghum shell for roughage; Mixed Silage: Based on the mixed silage for roughage.
[2]Premix contained (per kg): VA 80 kIU, VD 25 kIU, VE 130 kIU, Fe 0.6 g, Mn 0.7 g, Zn 2.3 g, Cu 0.2 g, Se 8 mg, Ca 10%, P 1%, NaCl 10%.
[3]DE was estimated according to NRC (2007). The others were measured values.

$$ADG = (FW - IW) / 28$$

$$DMI = (\text{feeding amount} \times \text{DM content}) - (\text{residual feed amount} \times \text{residual feed DM content})$$

$$F / W = DMI / ADG$$

## Sampling

Before morning feed, 5 Hu sheep were randomly selected from the experimental group and the control group for slaughter after fasting for 24 h and having no access to water for 2 h. The sheep were euthanized by injecting thiopental sodium (0.125 mg/kg BW) and potassium chloride (5–10 ml), and then immediately bloodletting. After slaughtering, the rumen fluid was collected. About 50 ml of rumen fluid from each sheep was collected and filtered with four layers of gauze, and the pH value was measured by a pH meter (Mettler Toledo, Switzerland) immediately. Then, it was sub-packed into four 15 ml centrifuge tubes and stored at −80°C for analysis. Rumen fluid for VFA determination requires the addition of deproteinized solution (100 g metaphosphoric acid and 0.6 g crotonic

acid per L) in a 1:1 ratio. During slaughter, we immediately collected blood, muscle (longissimus dorsi), rumen, kidney, spleen, liver, and small intestine (duodenum, jejunum, ileum) samples of Hu sheep.

## Determination of rumen fermentation parameters

The content of NH3-N in rumen fluid was determined by phenol sodium hypochlorite colorimetry (Broderick and Kang, 1980). Microprotein (MCP) content was determined by the trichloroacetic acid method (Cotta and Russell, 1982). The content of VFA in rumen fluid was determined by gas chromatography (GC-14B, Kyoto, Japan) meteorological chromatography internal standard method (Pan et al., 2016), and the TVFA and the ratio of acetate to propionate were calculated. The instruments and conditions of gas chromatography were the same as Zhang's method (Zhang et al., 2021).

## Determination of antioxidant enzymes

The levels of superoxide dismutase (SOD), total antioxidant capacity (T-AOC), GSH-Px, malondialdehyde (MDA) and Catalase (CAT) in blood, muscle (longissimus dorsi), rumen, kidney, spleen, liver, and small intestine (duodenum, jejunum and ileum) samples were measured. The kit for index determination was purchased from Nanjing Jiancheng Biotechnology Co., Ltd., Nanjing, China.

## DNA extraction, PCR amplification, and computer

After the rumen fluid samples were thawed, the total microbial DNA of the rumen fluid was extracted using the HiPure Soil DNA Kit (purchased from D3142B, Magen Biotechnology Co., Ltd., GuangZhou, China). The extracted DNA was tested for purity and concentration (OD260/280 and OD260/230) with an ultra-micro spectrophotometer (NzanoDrop-1000, Thermo Fisher Scientific Co., Ltd., United States), and then 1% agarose gel electrophoresis was used.

Amplification was performed using bacterial V3-V4 variable region universal primers 341F (5′-CCTACGGGNGGCWG CAG-3′), 806R (5′-GGACTACHVGGGTWTCTAAT-3′). PCR used 2× Phanta Master Mix, 30 μl reaction system: 10 ng Genomic DNA, 15 μl 2 × Phanta Master Mix, 1 μl Bar-PCR primer F (10 μM), 1 μl Primer R (10 μM), dd$H_2O$ supplemented to 30 μl. Reaction program: initial denaturation at 95°C for 5 min; 27 cycles of denaturation at 95°C for 30 s, annealing at 55°C for 30 s, and elongation at 72°C for 45 s; stretch for 10 min at 72°C. After PCR amplification, the obtained products were detected by 2% gel electrophoresis. The PCR products with correct band size and appropriate concentration were sequenced on the computer. The sequencing company was Genepioneer Biotechnologies Co., Ltd (Nanjing, China) and the platform was Illumina miseq.

## Data analysis

We used SPSS Statistics V20.0 Software to test the normal distribution and homogeneity of the data. Vitamin content ($V_A$, $V_{B2}$, $V_C$ and $V_E$) and rumen fermentation parameters (pH, VFA, MCP and $NH_3$-N) were tested in SPSS using independent samples T-test.

Species richness (Chao1 index) and diversity (Simpson and Shannon index) were calculated with the R language picante package and the difference test was performed with Mann–Whitney U nonparametric rank-sum test using IBM SPSS Statistics 20.0 software. Venn diagrams and Principal coordinate analysis (PCoA) diagrams were drawn through the ggplot2 package of the R language. At the same time, the similarity analysis (ANOSIM) calculated by the vegan package of R language shows the similarity between groups, where 0 = indistinguishable, 1 = identical. Determination of ANOSIM's $p$-value based on 999 permutation tests. Canonical correlation analysis (CCA) was used to analyze the influence of environmental factors on microorganisms, which was performed through the vegan and ggrepel packages and visualized with ggplot2. Only those bacterial taxa with an abundance >0.1% in at least one sample were analyzed. KEGG microbial gene function prediction analysis was performed using PICRUSt according to previous protocol (Zhang et al., 2022). The Pearson correlations between differential flora and rumen fermentation parameters, as well as differential flora and differential metabolic pathways, were completed by the complot package in R language. When the correlation coefficient (R) was $>|0.65|$ and the $p$-value was <0.05, the bacterial community and rumen fermentation parameters were considered to be significantly correlated (Wang et al., 2016).

# Results

## Vitamin content of roughage

In Table 2, the contents of $V_{B2}$ and $V_C$ in the mixed silage group were higher than those in the control group ($p < 0.05$), and there was no difference in the contents of $V_A$ and $V_E$ ($p > 0.05$).

TABLE 2 Comparison of vitamin contents between mixed silage and control.

| | Groups | | | |
|---|---|---|---|---|
| Vitamin, μg/L | Control | Mixed silage | SEM | Value of $p$ |
| $V_A$ | 72.54 | 75.95 | 1.01 | 0.089 |
| $V_{B2}$ | 3.69$^b$ | 4.10$^a$ | 0.10 | 0.028 |
| $V_C$ | 23.23$^b$ | 27.85$^a$ | 0.94 | 0.003 |
| $V_E$ | 8.16 | 7.70 | 0.15 | 0.114 |

In the same row, values with no letter or the same letter superscripts mean no significant difference ($p \geq 0.05$), while with different small letter superscripts mean significant difference ($p < 0.05$).

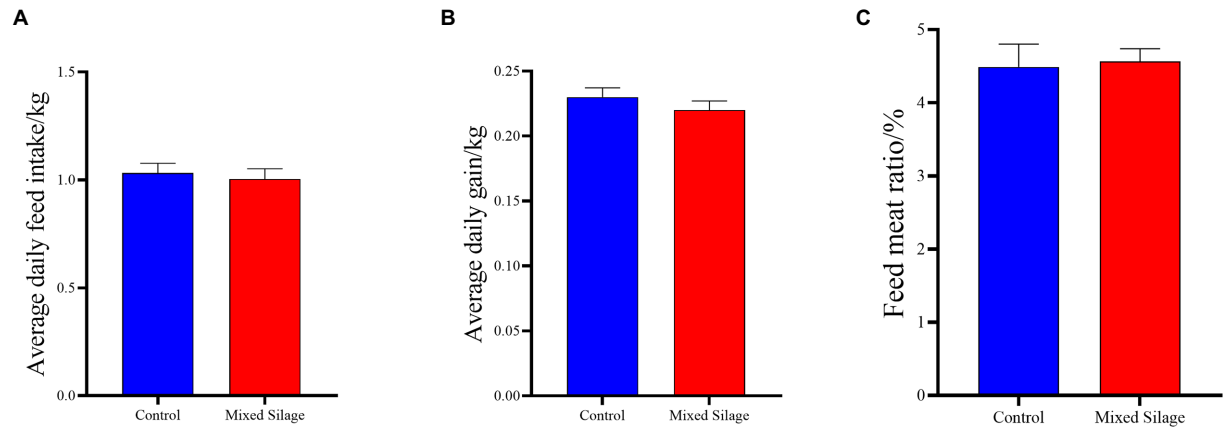


FIGURE 1
Effects of the mixed silage on growth performance of Hu sheep. **(A)** Average daily feed intake; **(B)** Average daily gain; **(C)** Feed meat ratio. Control: Based on peanut seedling, corn husk and sorghum shell for roughage in the diet; Mixed Silage: Based on the mixed silage for roughage in the diet.

## Effects of mixed silage on growth performance of Hu sheep

It can be seen from Figure 1 that the mixed silage did not affect the DMI (Figure 1A), ADG (Figure 1B) and F/W (Figure 1C) of Hu sheep ($p > 0.05$).

## Effects of mixed silage on antioxidant properties of Hu sheep

Figure 2 shows the comparison of antioxidant properties of blood and organs between mixed silage group and control group. Compared with the control group, the mixed silage increased the concentration of T-AOC in serum and SOD in kidney ($p < 0.05$). In addition, the content of MDA in serum, liver and kidney decreased ($p < 0.05$). The effects of the mixed silage on intestinal antioxidant performance of Hu sheep is shown in Figure 3. The mixed silage could increase the content of T-AOC and CAT in rumen and duodenum. In addition, it could reduce the content of MDA in rumen ($p < 0.05$). The results showed (Table 3) that the content of MDA in the mixed silage group was lower ($p < 0.05$), while the antioxidant indexes such as T-AOC, SOD, GSH-Px, and CAT were higher than those in the control groups with no differences ($p > 0.05$).

## Rumen fermentation parameters

The results in the Table 4 showed that the mixed silage could reduce the pH and the concentrations of isovalerate and isobutyrate ($p < 0.05$). In addition, it increased the concentrations of TVFA, $NH_3$-N, butyrate, and valerate in rumen fluid ($p < 0.05$).

## Rumen microbial diversity, richness, and structure

The results in the Supplementary Table 1 showed that the OTU, Chao1 index, Shannon index and Simpson index of the silage group were higher than those of the control group ($p < 0.05$). It can be seen from the species accumulation curve (Supplementary Figure 1A) that when the number of samples taken reaches 8, the detection rate of new species is further slowed down, and the species accumulation curve becomes flat (close to the horizontal state), indicating that adding new sequencing samples was important for the discovery of rumen. The contribution of new microbial species was getting smaller and smaller, so the sequencing samples in this experiment can completely cover most of the microorganisms in the rumen environment of Hu sheep. Clustering of sequences with 97% similarity revealed that the two groups shared 1,187 OTUs, 348 were shared by the silage group, and 172 were exclusively shared by the control group (Supplementary Figure 1B). PCoA based on Bray–Curtis distance was drawn for comparison of the two treatments. As it can be seen from Figure 4A that the samples of the mixed silage group were completely separated from the samples of the control group. The results of ANOSIM analysis showed that mixed silage could affect the rumen microflora structure of the fattening Hu sheep ($R = 0.536$, $p = 0.011$).

At the phyla level, Bacteroidetes and Firmicumtes were the dominant phyla in both the mixed silage group and the control group (Figure 4B). Furthermore, the ratio of Firmicutes to Bacteroidetes was not different between the two groups ($p > 0.05$; Figure 4C). Compared with the control group, the mixed silage group increased the proportion of Synergistetes and Kiritimatiellaeota in rumen fluid ($p < 0.05$; Figure 4D). At the genera level, 21 genera (relative abundance of more than 0.1%) were different between the two groups ($p < 0.05$; Figure 4E). Compared with the control group, the mixed silage group increased the proportion of *Olsenella*, *Prevotellaceae NK3B31 group*, *Prevotellaceae UCG-001*, *Prevotellaceae UCG-004*,

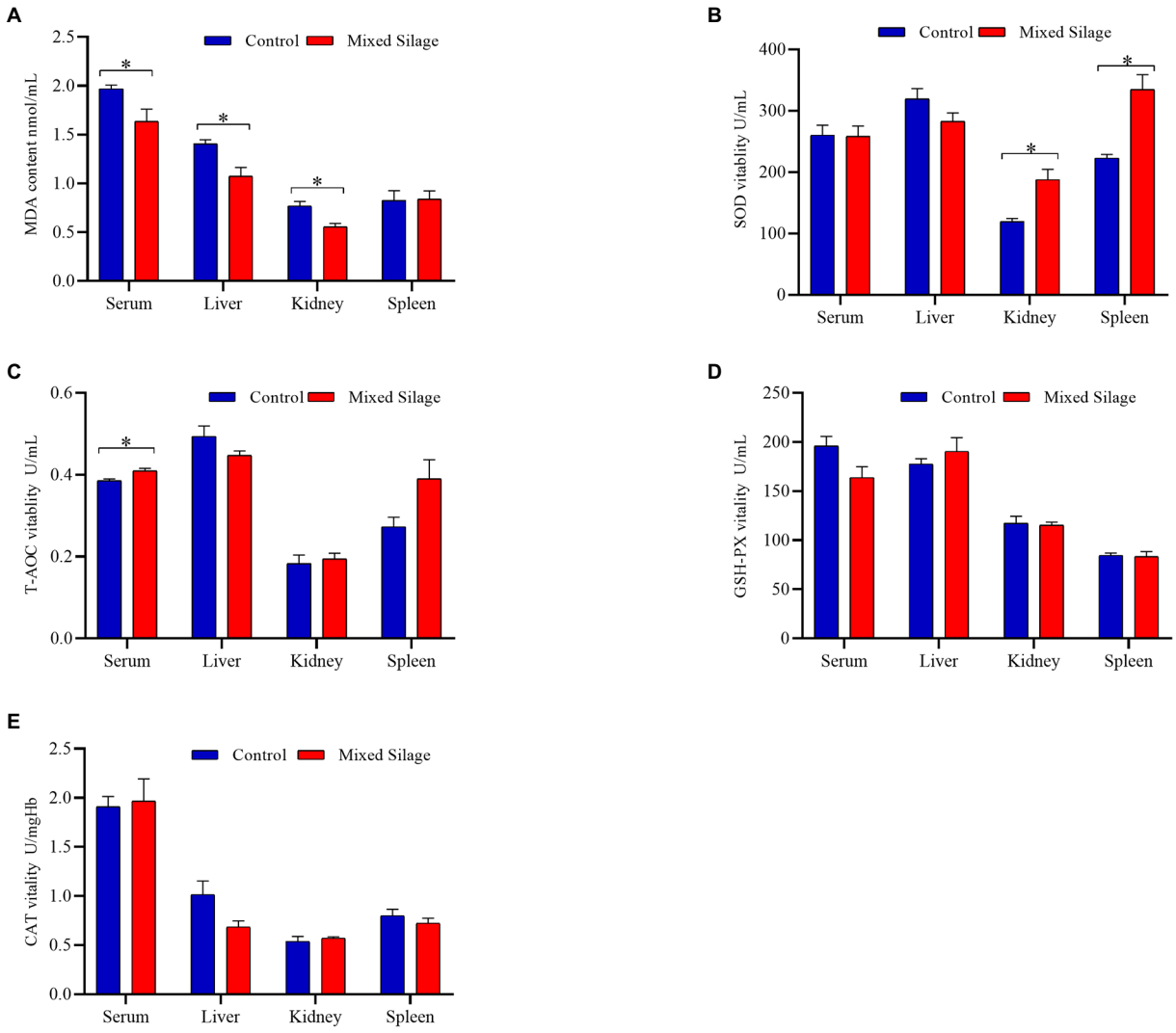


FIGURE 2
Effects of the mixed silage on antioxidant capacity of Hu sheep. **(A)** MDA content. **(B)** SOD vitality. **(C)** T-AOC vitality. **(D)** GSH-PX vitality. **(E)** CAT vitality. Control: Based on peanut seedling, corn husk and sorghum shell for roughage in the diet; Mixed Silage: Based on the mixed silage for roughage in the diet. "*" means indicates a significant difference ($p$<0.05). No "*" indicates that the difference is not significant ($p$>0.05). The same as below.

*Elusimicrobium*, *Anaerovorax*, *Lachnoclostridium 10*, *probable genus 10*, *Papillibacter*, *Syntrophococcus*, *Erysipelotrichaceae*, *Ruminiclostridium 1*, *Ruminococcaceae UCG-005*, *Quinella*, *Schwartzia*, *Veillonellaceae UCG 001* and *Psychrobacter* ($p < 0.05$), but decreased the proportion of *Howardella*, *Lachnospiraceae AC2044 group*, *Veillonellaceae UCG 001*, *CPla 4 termite group* and *M2PT2 76 termite group* relative abundance ($p < 0.05$).

## CCA of rumen microflora and differential rumen fermentation parameters

The relationship between the rumen bacterial community and rumen fermentation parameters were analyzed by Canonical correspondence analysis (CCA). As shown in Figure 5A, the relationship between the rumen microflora and isovalerate ($p = 0.002$), $NH_3$-N ($p = 0.003$), butyrate ($p = 0.021$), pH ($p = 0.022$), valerate ($p = 0.048$) and TVFA ($p = 0.064$) contributed to the flora.

## Correlation analysis of microorganisms, vitamins and fermentation parameters

As shown in Figure 5B, there was a negative correlation between pH and *Anaerovorax*, and a positive correlation with *Erysipelotrichaceae UCG-004* ($p < 0.05$). $NH_3$-N showed a positive correlation with *Prevotellaceae UCG-001*, *Lachnoclostridium 10* and *probable genus 10*, but a negative correlation with *Schwartzia* and *Howardella* ($p < 0.05$). There

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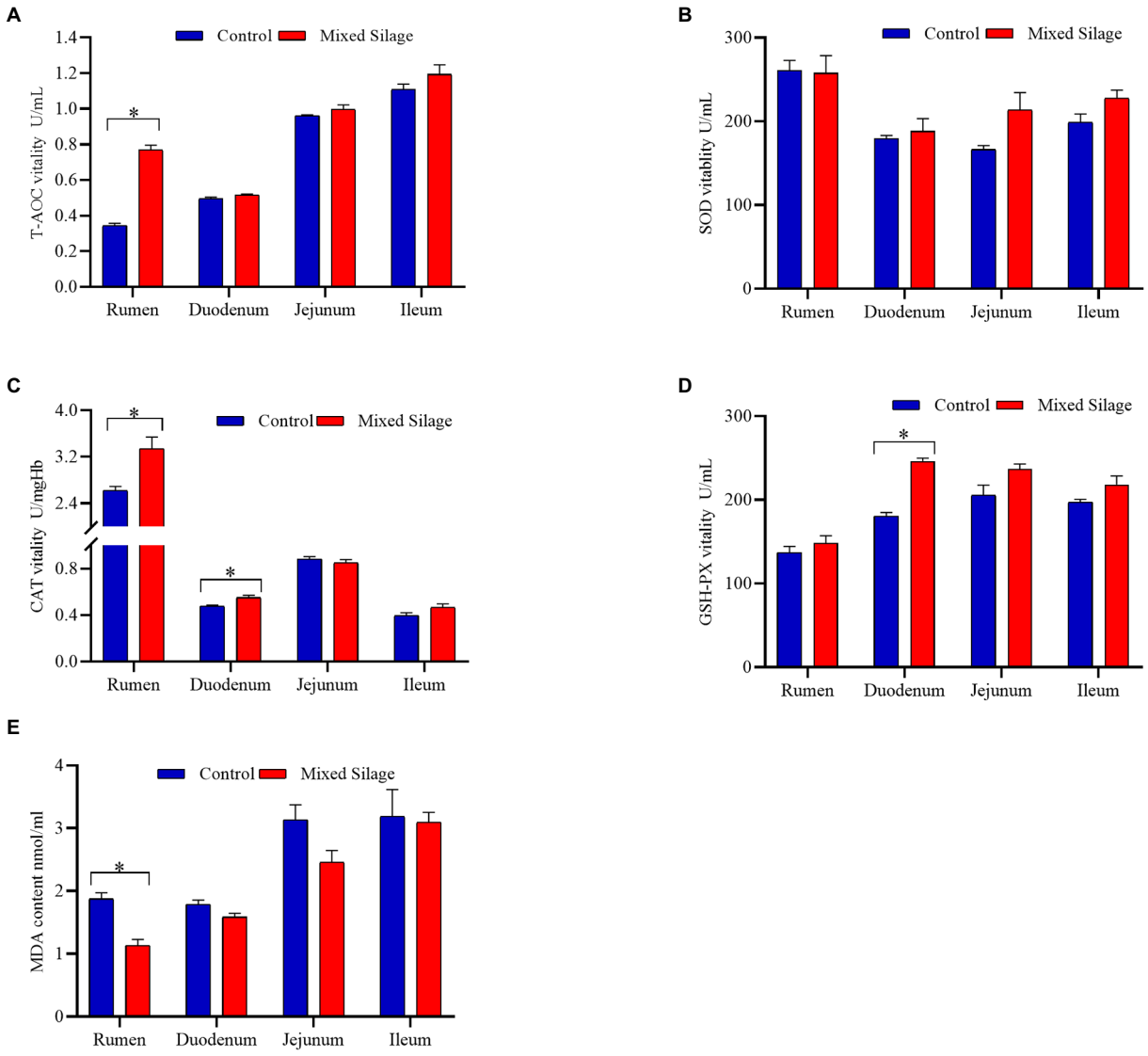


FIGURE 3
Effects of the mixed silage on antioxidant capacity in the digestive tract of fattening Hu sheep. **(A)** T-AOC vitality. **(B)** SOD vitality. **(C)** CAT vitality. **(D)** GSH-PX vitality. **(E)** MDA content. Control: Based on peanut seedling, corn husk and sorghum shell for roughage in the diet; Mixed Silage: Based on the mixed silage for roughage in the diet.

**TABLE 3** Effects of the mixed silage on muscle anti-oxidation of Hu sheep.

| Item | Groups[1] | | SEM[2] | Value of *p* |
|---|---|---|---|---|
| | Control | Mixed silage | | |
| T-AOC, U/mg | 1.39 | 1.50 | 0.07 | 0.477 |
| SOD, U/mg | 200.73 | 206.58 | 5.02 | 0.591 |
| GSH-Px, U/mg | 138.90 | 140.24 | 3.48 | 0.861 |
| CAT, U/mg | 1.32 | 1.48 | 0.05 | 0.095 |
| MDA, mmol/mg | 5.01[a] | 3.83[b] | 0.28 | 0.022 |

[1]Control: Based on peanut seedling, corn husk and sorghum shell for roughage; Mixed Silage: Based on the mixed silage for roughage.
[2]SEM, standard error of the mean.
[a,b]In the same row, values with no letter or the same letter superscripts mean no significant difference ($p \geq 0.05$), while with different small letter superscripts mean significant difference ($p < 0.05$).

was a positive correlation between TVFA and *Prevotellaceae NK3B31 group*, *Papillibacter* and *probable genus 10* ($p < 0.05$). Propionate showed a positive correlation with *Psychrobacter*, *Papillibacte* and *CPla-4 termite group* ($p < 0.05$). Isobutyrate showed a negative correlation with *Anaerovorax* and *Papillibacter*. Butyrate showed a positive correlation with *Prevotellaceae NK3B31* and *Prevotellaceae UCG 004* ($p < 0.05$). Valerate showed a positive correlation with *Quinella*, *Elusimicrobium*, *Lachnoclostridium 10* and *probable genus 10* ($p < 0.05$). $V_{B2}$ was positively correlated with *Elusimicrobium*, *Quinella*, and *Lachnoclostridium 10* ($p < 0.05$). $V_C$ was positively correlated with *Anaerovorax*, *Prevotellaceae UCG-004* and *Prevotellaceae UCG-001*, but negatively correlated with *Erysipelotrichaceae UCG-004* ($p < 0.05$).

TABLE 4 Effects of the mixed silage on rumen fermentation parameters in Hu sheep.

| Item | Groups[1] | | SEM[2] | Value of *p* |
|---|---|---|---|---|
| | Control | Mixed silage | | |
| Ruminal pH | 7.09[a] | 6.91[b] | 0.04 | 0.019 |
| $NH_3$-N (mg/dL) | 7.38[b] | 11.73[a] | 0.86 | 0.002 |
| MCP (mg/dL)[3] | 19.24 | 18.71 | 0.35 | 0.479 |
| VFAs[4] | | | | |
| TVFA (mmol/L)[5] | 68.77[b] | 78.23[a] | 1.93 | 0.004 |
| Acetate (mmol/L) | 43.31 | 45.08 | 0.80 | 0.293 |
| Propionate (mmol/L) | 17.39 | 18.82 | 0.88 | 0.448 |
| Isobutyrate (mmol/L) | 1.13[a] | 0.88[b] | 0.07 | 0.049 |
| Butyrate (mmol/L) | 4.43[b] | 8.62[a] | 0.83 | 0.002 |
| Isovalerate (mmol/L) | 1.98[a] | 1.14[b] | 0.19 | 0.012 |
| Valerate (mmol/L) | 0.53[b] | 1.11[a] | 0.12 | 0.005 |
| A:P[6] | 2.51 | 2.57 | 0.14 | 0.850 |

[1]Control: Based on peanut seedling, corn husk and sorghum shell for roughage; Mixed Silage: Based on the mixed silage for roughage.
[2]SEM, standard error of the mean.
[3]MCP, Microbial protein.
[4]VFAs, volatile fatty acids.
[5]TVFA, total volatile fatty acids.
[6]A:P, Ratio of acetic acid to propionic acid.
[a,b]In the same row, values with no letter or the same letter superscripts mean no significant difference ($p \geq 0.05$), while with different small letter superscripts mean significant difference ($p < 0.05$).

## Prediction of rumen bacterial function

There were 10 significantly different metabolic pathways in the microbiota prediction function between the mixed silage group and the control group (Figure 6A). The mixed silage up-regulated epithelial cell signaling in helicobacter pylori infection, phosphotransferase system (PTS), primary immunodeficiency and ascorbate and aldarate metabolism ($p < 0.05$), while downregulated aminobenzoate degradation, ethylbenzene degradation, glycolysis/gluconeogenesis, lysine degradation, propanoate metabolism and spliceosome metabolic pathways ($p < 0.05$).

## Correlation analysis between microorganisms and pathways

As shown in Figure 6B, aminobenzoate degradation had a positive correlation with *Erysipelotrichaceae UCG-004*, *Howardella* and *Schwartzia*, and a negative correlation with *Prevotellaceae UCG-001* ($p < 0.05$). Ascorbate and aldarate metabolism were positively correlated with *Veillonellaceae UCG-001*, *Prevotellaceae UCG-004*, *Elusimicrobium* and *Quinella* ($p < 0.05$). Epithelial cell signaling in helicobacter pylori infection was positively correlated with *Anaerovorax*, *Prevotellaceae NK3B31 group*, *Papillibacter* and *CPla-4 termite group*, while negatively correlated with *Erysipelotrichaceae UCG-004* and *Schwartzia* ($p < 0.05$). Ethylbenzene degradation showed a positive correlation with *Schwartzia* and a negative correlation with *Anaerovorax* and *Prevotellaceae UCG-004* ($p < 0.05$). Glycolysis/gluconeogenesis showed a positive correlation with *Schwartzia*, and a negative correlation with *Elusimicrobium*, *Ruminiclostridium 1*, *Quinella*, *Papillibacter*, *M2PT2–76 termite group*, *Lachnoclostridium 10*, and *probable genus 10* ($p < 0.05$). Lysine degradation had a positive correlation with *Erysipelotrichaceae UCG-004* and *Howardella*, while a negative correlation with *Anaerovorax* ($p < 0.05$). Phosphotransferase system (PTS) had a positive correlation with *Prevotellaceae UCG-004* ($p < 0.05$). Primary immunodeficiency was positively correlated with *Prevotellaceae UCG-001*, *Prevotellaceae UCG-004* and *Elusimicrobium*, but negatively correlated with *Quinella* ($p < 0.05$). Spliceosome had a positive correlation with *Erysipelotrichaceae UCG-004* and *Howardella* ($p < 0.05$). In addition, propanoate metabolism was found negatively correlated with *Ruminiclostridium 1* ($p < 0.05$).

# Discussion

The production, utilization and elimination of free radicals in the body are in a dynamic balance. When this balance is broken, the body produces oxidative stress, the reactive oxygen species cause lipid peroxidation, giving rise MDA as a final product (Honaramooz et al., 2011). The complex antioxidant system in the body is influenced by dietary intake of antioxidants (taurine, etc.), vitamins ($V_C$ and $V_E$), and minerals (Zn etc.; Agarwal and Gupta, 2005). SOD is an intracellular antioxidant enzyme that protects against oxidative stress (Bhatia et al., 2003). CAT exists in red blood cells and peroxides in certain tissues, and can make $H_2O_2$ react to generate water and oxygen molecules (Zhao et al., 2017). Serum T-AOC is primarily considered to be a proxy for the balance between oxidative and antioxidant compounds in the body, and it provides more biologically relevant information than any other individual antioxidants (Ghiselli et al., 2000). The results showed that T-AOC in serum and rumen of the mixed

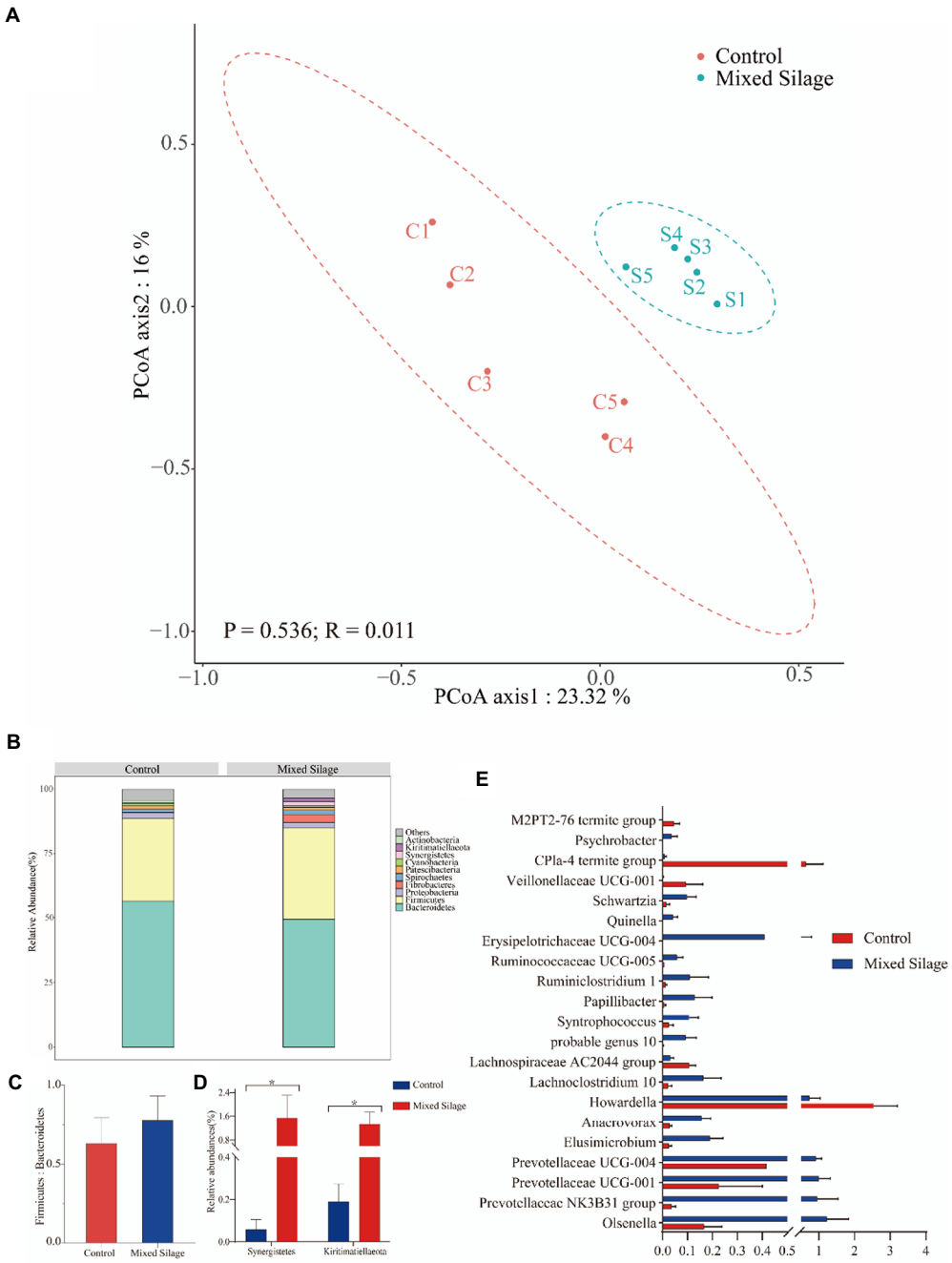


FIGURE 4
Structure of rumen bacterial flora in Hu Sheep. **(A)** Principal coordinate analysis (PCoA) of the bacterial community structure of ruminal microbiota in the control and mixed silage groups. **(B)** The composition of the top 10 phyla in rumen fluid. **(C)** Ratio of the proportion of phyla Firmicutes to the proportion of Bacteroidetes. **(D)** The phyla with the difference in the top 10 phyla. **(E)** The proportion of the bacterial genus with significant differences among the two groups. Generas with a proportion >0.1% in any one sample will be analyzed. Control: Based on peanut seedling, corn husk and sorghum shell for roughage in the diet; Mixed Silage: Based on the mixed silage for roughage in the diet. PCoA plots were constructed using Bray–Curits distance.

silage group were significantly higher than those in the control group. SOD activity in the kidney was observed significantly higher, while CAT activity in the rumen and duodenum was seen higher significantly. In addition, MDA content in the muscle, serum, liver, kidney and rumen of the mixed silage group was significantly lower than that of the control group. This may be caused by the higher content of $V_C$ in the mixed silage. Sahoo et al. (2021) results showed that the addition of fresh fruit and vegetable tails to the diet increased the ability of T-AOC in the blood of ewes. $V_C$ can be used to maintain the oxidation state of

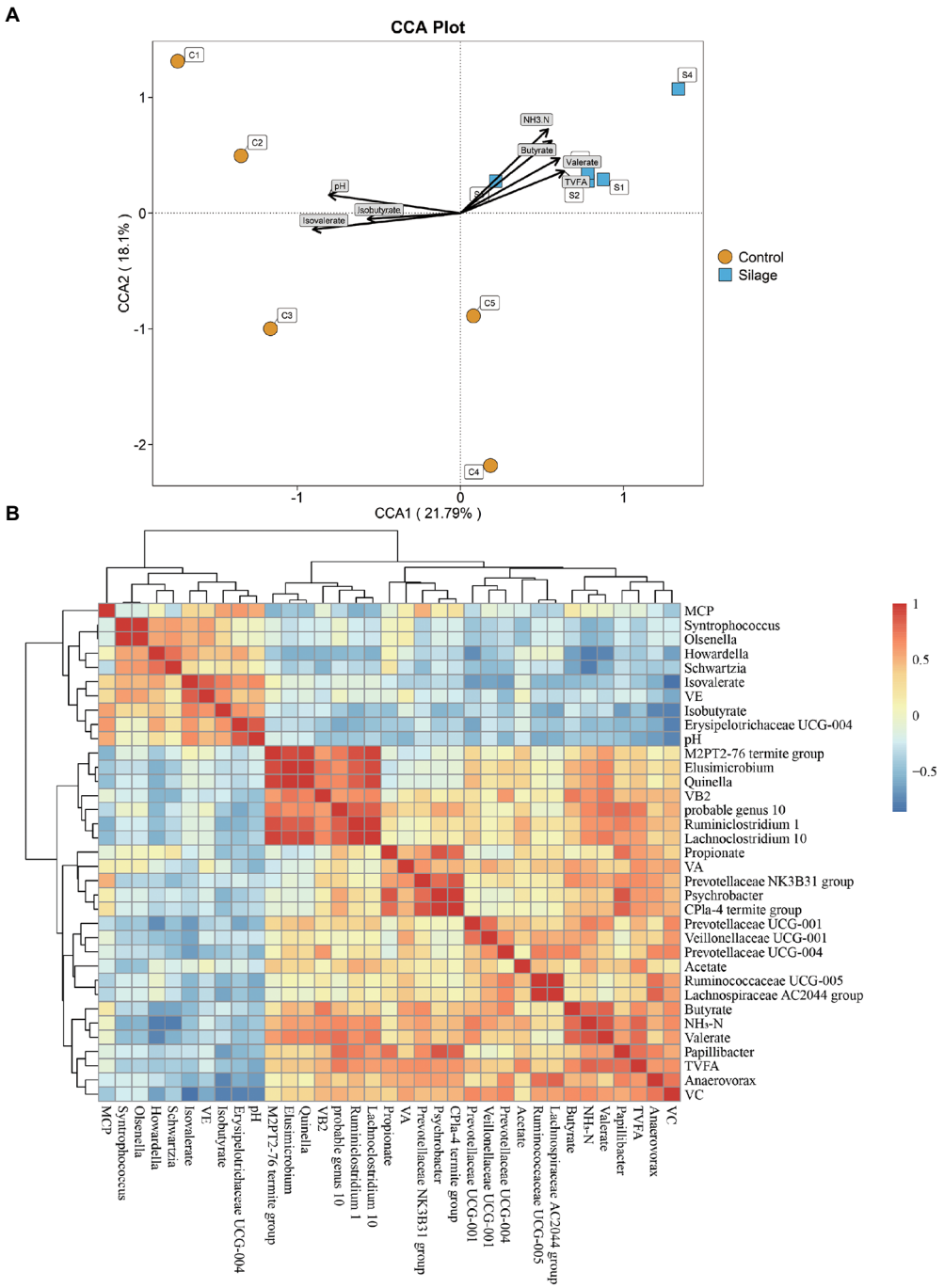


FIGURE 5
Correlation results between rumen microbiota structure and fermentation parameters or vitamins. **(A)** CCA to prove the associations between the rumen fermentation parameters and the bacterial community structure. The length of an arrow expresses the relative influence of its corresponding rumen fermentation parameters on the distribution of the bacterial community analyzed. Centroid is displayed for each treatment. The samples are represented by circles and square symbols, respectively. **(B)** Correlation analyses between proportion of differential bacteria genera and ruminal fermentation parameters and vitamins.

cells and remove other oxidizing substances, so as to improve the T-AOC of cells (Sordillo and Aitken, 2009). A study by Seifzadeh et al. (2022) also showed that by adding 20 mg of $V_C$ to prenatal dairy cow diets significantly reduced blood MDA concentration. By adding 50 mg/ml $V_C$ to drinking water can increase the level of T-AOC in the myocardium and serum of chickens, and improves its antioxidant capacity (Yin et al., 2020). In addition, by adding $V_C$ to the diet can also increase the activity of MnSOD (a kind of SOD) in chicken liver and kidney (Ozturk-Urek et al., 2001).

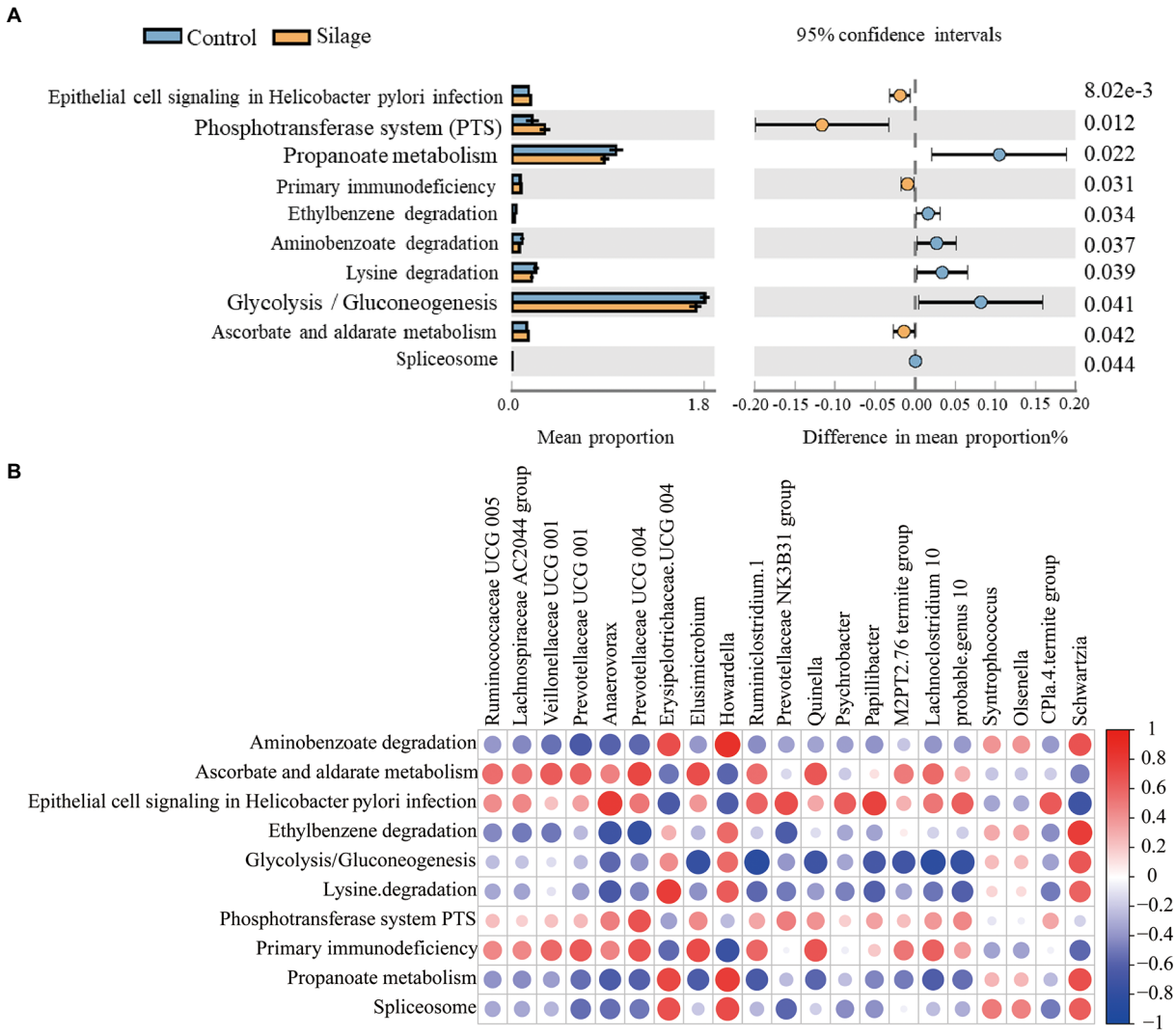


FIGURE 6
Prediction of metabolic pathways and their correlation with rumen microbes. **(A)** Analysis of differences in functional prediction pathways of rumen flora between the control group and the mixed silage group. **(B)** Correlation analysis between differential microorganisms and differential pathways. In this graph, each row represents differential metabolic pathways and each column represents differential microorganisms at the genera level. Red circles indicate positive correlations, and blue circles indicate negative correlations.

Partovi et al. (2020) fed Fasander lambs with a mixed diet of broccoli by-products and wheat straw (ratio 69:31) and found that the mixed diet did not affect the rumen fermentation of lambs. In our experiment, the mixed silage increased the concentrations of TVFA, $NH_3$-N and butyric acid in the rumen fluid, while the pH of the rumen fluid decreased. The normal range of rumen pH is 5.5–7.0, but it may also reach to 7.5 (Kim et al., 2012; Anantasook et al., 2013). The pH of the rumen in the two groups of diets was within the normal range, whereas the pH of the mixed silage group was lower than that of the control group, which may be caused by the increase of TVFA. The $NH_3$-N in the rumen originates from the degradation of endogenous nitrogenous substances or exogenous nitrogenous substances, and is also the main substance for the synthesis of MCP, which can reflect the utilization of nitrogenous substances by rumen microorganisms to a certain extent. All animals in both groups had $NH_3$-N concentrations in the rumen above 5 mg/dl, which is the minimum concentration required to ensure optimal growth of rumen microorganisms (Satter and Slyter, 1974). The differences in $NH_3$-N in the two groups of diets indicated that the mixed silage could affect the release of N in the rumen and the efficiency of MCP synthesis after being absorbed by microorganisms. The increase of VFA content in the mixed silage group may be caused by the increase of rumen microbial reproduction and activity. VFAs which account for 75% of the total energy requirements provided by ruminants, are the main product of rumen microbial fermentation, and are critical to the overall metabolism of ruminants (Bergman, 1990; Dijkstra, 1994). Butyrate can effectively stimulate the proliferation and growth of the rumen epithelium (Gorka et al., 2018). The increase of butyric acid in the

mixed silage group in this experiment may be due to the increase of butyric acid-producing bacteria in the rumen.

The rumen microbes are complex and diverse, and play an important role in the digestion of nutrients and the functioning of the rumen. Diet type, structure and feeding method can significantly affect the species and quantity of ruminant microorganisms in ruminants (van Lingen et al., 2016). The mixed silage increased the species diversity and richness of rumen microorganisms, which may be due to the higher metabolizable and digestible energies of the silage group, promoted the growth and reproduction of microorganisms, and improved the richness and diversity of microorganisms. The results of PCoA and ANOSIM clearly showed that the mixed silage significantly improved the rumen microbial composition. Additionally, CCA analysis showed that among the different rumen fermentation parameters, isovalerate, $NH_3$-N, butyrate, pH, valerate and TVFA had the greatest influence on rumen microorganisms.

The ecosystem of rumen microorganisms is dominated by its core flora which is not affected by the nutrients from feed sources (Petri et al., 2012; Henderson et al., 2015). Bacteroidea and Firmicutes are two dominant microflora in the structure of rumen microbial flora (Singh et al., 2012; Pitta et al., 2014). Previous studies have shown that the main function of Bacteroidea members is to hydrolyze proteins and degrade carbohydrates, while Firmicutes members are mainly involved in energy utilization (Wu et al., 2011; Chen et al., 2015). The ratio of Firmicutes to Bacteroidetes is generally considered to be related to the metabolic potential of the intestinal microbiota and can regulate the metabolism, physiology and health of the host (Tilg and Kaser, 2011; Clemente et al., 2012). Our results showed that the mixed silage did not have a significant effect on Firmicutes, Bacteroidetes or their ratios, which is consistent with previous studies (Zhang et al., 2014). Notably, at the level of the top 10 phyla in relative abundance, the mixed silage increased the proportion of Synergistetes and Kiritimatiellaeota. Synergistetes is part of the gut microbiota of healthy animals and can degrade amino acids (Leser et al., 2002; Menes and Muxi, 2002; Godon et al., 2005). Studies have pointed out that Synergistetes isolated from sheep rumen can not only utilize arginine and histidine to improve nitrogen utilization, but also degrade toxins present in feed (McSweeny et al., 1993; Jumas-Bilak et al., 2009). Kiritimatiellaeota is a supergroup of the planktonic Chlamydia verrucobacterium (PVC) whose primary function is to break down complex polysaccharides and glycoproteins (Spring et al., 2016; Mu et al., 2020).

The experimental results showed that the proportions of *Prevotellaceae NK3B31 group*, *Prevotellaceae UCG-001* and *Prevotellaceae UCG-004* were increased in the mixed silage group. *Prevotellaceae UCG-001*, *Prevotellaceae UCG-004* and *Prevotellaceae NK3B31 group,* all belong to the Prevotellaceae family. The results of association analysis showed that *Prevotellaceae UCG-004* was positively correlated with Vc, ascorbate and aldarate metabolism. In mammals, $V_C$ is abundant and an effective water-soluble antioxidant for reducing oxidative stress, but its impact on the rumen ecology is unknown (Ghanem et al., 2008; Matsui, 2012). The use of antioxidants to improve animal health and product quality has received increasing attention (Schelling et al., 1995). The basic physiological needs of the organism are thought to be met by L-ascorbic acid (or $V_C$) generated in the liver of ruminants (Ranjan et al., 2012). Compared to other animals, ruminants may require vitamin supplementation during feeding because microbes in the rumen completely destroy $V_C$ in the feed (Kim et al., 2012; Ranjan et al., 2012). Another *in vitro* study has shown that high doses of $V_C$ have a certain positive effect on rumen microorganisms (Tagliapietra et al., 2013). Therefore, it is speculated that the higher $V_C$ content in the mixed silage group could increase the proportion of *Prevotellaceae UCG-004*, but it remains to be studied. Prevotellaceae is positively correlated with the content of ADF and NDF in the diet (An et al., 2020). In this experiment, the ADF content in the mixed silage group was higher, which may be another reason for the increase in the relative abundance of *Prevotellaceae UCG-004*. *Prevotellaceae UCG-004* can ferment carbohydrates and produce SCFAs including acetate and butyrate. The process of butyrate metabolism is linked to the interconversion of "pentose and glucuronic acid," and the "ascorbate and aldarate metabolism" through dehydrogenases together (Heinritz et al., 2016; Shi et al., 2020).

## Conclusion

In summary, the mixed silage could improve rumen fermentation and enhance the antioxidant capacity of Hu sheep. In this experiment, the mixed silage also increased the concentrations of TVFA, $NH_3$-N and butyric acid in the rumen, whereas the pH of the rumen fluid was reduced. In addition, the mixed silage increased the activity of T-AOC in blood, SOD in liver and kidney, and decreased the content of MDA in blood. The improvement of the antioxidant capacity of Hu sheep may be directly caused by the higher $V_C$ content in the mixed silage, or it may be caused by the up-regulation of ascorbate and aldarate metabolism in Hu sheep. The upregulation of ascorbate and aldarate metabolism may be related to the increased proportion of *Prevotellaceae UCG-004* caused by higher $V_C$ or ADF content in the mixed silage.

## Data availability statement

The datasets provided in this study can be found in the online repository. The name and login number of the repository are as follows: NCBI SRA Bioproject, login number: PRJNA795847.

## Ethics statement

The animal study was reviewed and approved by Yangzhou Veterinary Animal Welfare Committee of the

Ministry of agriculture of China [Yangzhou, China, license No. syxk (Su) 2019-0029]. Written informed consent was obtained from the owners for the participation of their animals in this study.

## Author contributions

MW and CL designed this study and wrote the manuscript of this study. CL, XZ, RQ, ZZ, ZL, YL, MZ, and XY conducted experiments and collected samples. Animal data were analyzed by CL, MZ, XZ, ZZ, YL, NC, and ZL. CL, KS, NC, WL, and MW performed bioinformatics analysis and visualization of data and draft manuscripts. 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/fmicb.2022.978940/full#supplementary-material

## References

Abd El Tawab, A. M., Kholif, A. E., Hassan, A. M., Matloup, O. H., El-Nor, S. A. A., Olafadehan, O. A., et al. (2020). Feed utilization and lactational performance of Friesian cows fed beet tops silage treated with lactic acid bacteria as a replacement for corn silage. *Anim. Biotechnol.* 31, 473–482. doi: 10.1080/10495398.2019.1622556

Agarwal, A., and Gupta, S. (2005). Role of reactive oxygen species in female reproduction. Part I. oxidative stress: a general overview. *Agro Food Ind. Hi Tech* 16, 21–25.

An, X. J., Zhang, L. Y., Luo, J., Zhao, S. G., and Jiao, T. (2020). Effects of oat Hay content in diets on nutrient metabolism and the rumen microflora in sheep. *Animals* 10:2341. doi: 10.3390/ani10122341

Anantasook, N., Wanapat, M., Cherdthong, A., and Gunun, P. (2013). Effect of plants containing secondary compounds with palm oil on feed intake, digestibility, microbial protein synthesis and microbial population in dairy cows. *Asian-Australas J. Anim. Sci.* 26, 820–826. doi: 10.5713/ajas.2012.12689

Bampidis, V. A., and Robinson, P. H. (2006). Citrus by-products as ruminant feeds: a review. *Anim. Feed Sci. Tech.* 128, 175–217. doi: 10.1016/j.anifeedsci.2005.12.002

Bergman, E. (1990). Energy contributions of volatile fatty acids from the gastrointestinal tract in various species. *Physiol. Rev.* 70, 567–590. doi: 10.1152/physrev.1990.70.2.567

Bhatia, S., Shukla, R., Madhu, S. V., Gambhir, J. K., and Prabhu, K. M. (2003). Antioxidant status, lipid peroxidation and nitric oxide end products in patients of type 2 diabetes mellitus with nephropathy. *Clin. Biochem.* 36, 557–562. doi: 10.1016/S0009-9120(03)00094-8

Broderick, G. A., and Kang, J. H. (1980). Automated simultaneous determination of ammonia and total amino acids in ruminal fluid and in vitro media. *J. Dairy Sci.* 63, 64–75. doi: 10.3168/jds.S0022-0302(80)82888-8

Chen, X. G. (2016). Economic potential of biomass supply from crop residues in China. *Appl. Energy* 166, 141–149. doi: 10.1016/j.apenergy.2016.01.034

Chen, Y.-B., Lan, D.-L., Tang, C., Yang, X.-N., and Li, J. (2015). Effect of DNA extraction methods on the apparent structure of yak rumen microbial communities as revealed by 16S rDNA sequencing. *Pol. J. Microbiol.* 64, 29–36. doi: 10.33073/pjm-2015-004

Cheong, S. H., Kim, K. H., Jeon, B. T., Park, P. J., Hwang, I. H., Choi, N. J., et al. (2012). Effect of mulberry silage supplementation during late fattening stage of Hanwoo (Bos taurus coreanae) steer on antioxidative enzyme activity within the longissimus muscle. *Anim. Prod. Sci.* 52, 240–247. doi: 10.1071/AN11087

Cherdthong, A., Suntara, C., and Khota, W. (2020). Lactobacillus casei TH14 and additives could modulate the quality, gas kinetics and the in vitro digestibility of ensilaged rice straw. *J. Anim. Physiol. Anim. Nutr. (Berl)* 104, 1690–1703. doi: 10.1111/jpn.13426

Clemente, J. C., Ursell, L. K., Parfrey, L. W., and Knight, R. (2012). The impact of the gut microbiota on human health: an integrative view. *Cell* 148, 1258–1270. doi: 10.1016/j.cell.2012.01.035

Cotta, M. A., and Russell, J. B. (1982). Effect of peptides and amino acids on efficiency of rumen bacterial protein synthesis in continuous culture. *J. Dairy Sci.* 65, 226–234. doi: 10.3168/jds.S0022-0302(82)82181-4

Dijkstra, J. (1994). Production and absorption of volatile fatty acids in the rumen. *Livest. Sci.* 39, 61–69. doi: 10.1016/0301-6226(94)90154-6

FAOSTAT (2016). *Food and Agriculture Data (FAO, 2016)*. Available at: http://www.fao.org/faostat/en/#home

Ghanem, A. M., Jaber, L. S., Said, M. A., Barbour, E. K., and Hamadeh, S. K. (2008). Physiological and chemical responses in water-deprived Awassi ewes treated with vitamin C. *J. Arid Environ.* 72, 141–149. doi: 10.1016/j.jaridenv.2007.06.005

Ghiselli, A., Serafini, M., Natella, F., and Scaccini, C. (2000). Total antioxidant capacity as a tool to assess redox status: critical view and experimental data. *Free Radic. Biol. Med.* 29, 1106–1114. doi: 10.1016/S0891-5849(00)00394-4

Godon, J. J., Moriniere, J., Moletta, M., Gaillac, M., Bru, V., and Delgenes, J. P. (2005). Rarity associated with specific ecological niches in the bacterial world:

the 'Synergistes' example. *Environ. Microbiol.* 7, 213–224. doi: 10.1111/j.1462-2920.2004.00693.x

Gorka, P., Kowalski, Z. M., Zabielski, R., and Guilloteau, P. (2018). Invited review: use of butyrate to promote gastrointestinal tract development in calves. *J. Dairy Sci.* 101, 4785–4800. doi: 10.3168/jds.2017-14086

Hassanat, F., Gervais, R., Julien, C., Masse, D. I., Lettat, A., Chouinard, P. Y., et al. (2013). Replacing alfalfa silage with corn silage in dairy cow diets: effects on enteric methane production, ruminal fermentation, digestion, N balance, and milk production. *J. Dairy Sci.* 96, 4553–4567. doi: 10.3168/jds.2012-6480

Heinritz, S. N., Weiss, E., Eklund, M., Aumiller, T., Louis, S., Rings, A., et al. (2016). Intestinal microbiota and microbial metabolites are changed in a pig model fed a high-fat/low-fiber or a low-fat/high-fiber diet. *PLoS One* 11:e0154329. doi: 10.1371/journal.pone.0154329

Henderson, G., Cox, F., Ganesh, S., Jonker, A., Young, W., Janssen, P. H., et al. (2015). Rumen microbial community composition varies with diet and host, but a core microbiome is found across a wide geographical range. *Sci. Rep.-UK* 5, 1–15. doi: 10.1038/srep14567

Hillion, M. L., Moscoviz, R., Trably, E., Leblanc, Y., Bernet, N., Torrijos, M., et al. (2018). Co-ensiling as a new technique for long-term storage of agro-industrial waste with low sugar content prior to anaerobic digestion. *Waste Manag.* 71, 147–155. doi: 10.1016/j.wasman.2017.10.024

Honaramooz, A., Schlatt, S., Orwig, K., and Kim, N. H. (2011). Recent advances in reproductive technologies. *Vet. Med. Int.* 2011:915031. doi: 10.4061/2011/915031

Jumas-Bilak, E., Roudiere, L., and Marchandin, H. (2009). Description of 'Synergistetes' phyl. Nov and emended description of the phylum 'Deferribacteres' and of the family Syntrophomonadaceae, phylum 'Firmicutes'. *Int. J. Syst. Evol. Microbiol.* 59, 1028–1035. doi: 10.1099/ijs.0.006718-0

Kim, J. H., Mamuad, L. L., Yang, C. J., Kim, S. H., Ha, J. K., Lee, W. S., et al. (2012). Hemato-biochemical and cortisol profile of Holstein growing-calves supplemented with vitamin C during summer season. *Asian-Australas J. Anim. Sci.* 25, 361–368. doi: 10.5713/ajas.2011.11438

Leser, T. D., Amenuvor, J. Z., Jensen, T. K., Lindecrona, R. H., Boye, M., and Moller, K. (2002). Culture-independent analysis of gut bacteria: the pig gastrointestinal tract microbiota revisited. *Appl. Environ. Microbiol.* 68, 673–690. doi: 10.1128/AEM.68.2.673-690.2002

Liu, B. Y., Huan, H. L., Gu, H. R., Xu, N. X., Shen, Q., and Ding, C. L. (2019). Dynamics of a microbial community during ensiling and upon aerobic exposure in lactic acid bacteria inoculation-treated and untreated barley silages. *Bioresour. Technol.* 273, 212–219. doi: 10.1016/j.biortech.2018.10.041

Ma, Y., Yin, Y., and Liu, Y. (2017). A holistic approach for food waste management towards zero-solid disposal and energy/resource recovery. *Bioresour. Technol.* 228, 56–61. doi: 10.1016/j.biortech.2016.12.090

Makkar, H. P., and Ankers, P. (2014). A need for generating sound quantitative data at national levels for feed-efficient animal production1. *Anim. Prod. Sci.* 54, 1569–1574. doi: 10.1071/AN14377

Matsui, T. (2012). Vitamin C nutrition in cattle. *Asian-Australas J. Anim. Sci.* 25, 597–605. doi: 10.5713/ajas.2012.r.01

McSweeny, C. S., Allison, M. J., and Mackie, R. I. (1993). Amino acid utilization by the ruminal bacterium Synergistes jonesii strain 78-1. *Arch. Microbiol.* 159, 131–135. doi: 10.1007/BF00250272

Menes, R. J., and Muxi, L. (2002). Anaerobaculum mobile sp nov., a novel anaerobic, moderately thermophilic, peptide-fermenting bacterium that uses crotonate as an electron acceptor, and emended description of the genus Anaerobaculum. *Int. J. Syst. Evol. Microbiol.* 52, 157–164. doi: 10.1099/00207713-52-1-157

Mu, D. S., Zhou, L. Y., Liang, Q. Y., Chen, G. J., and Du, Z. J. (2020). Tichowtungia aerotolerans gen. Nov., sp. nov., a novel representative of the phylum Kiritimatiellaeota and proposal of Tichowtungiaceae fam. Nov., Tichowtungiales Ord. Nov. and Tichowtungiia class. Nov. *Int. J. Syst. Evol. Microbiol.* 70, 5001–5011. doi: 10.1099/ijsem.0.004370

National Research Council (NRC). (2007). *Nutrient Requirements of Small Ruminants, Sheep, Goats, Cervids, and New World Camelids*. Washington, DC: National Academy Press.

Ozturk-Urek, R., Bozkaya, L. A., and Tarhan, L. (2001). The effects of some antioxidant vitamin- and trace element-supplemented diets on activities of SOD, CAT, GSH-Px and LPO levels in chicken tissues. *Cell Biochem. Funct.* 19, 125–132. doi: 10.1002/cbf.905

Palma-Hidalgo, J. M., Jiménez, E., Popova, M., Morgavi, D. P., Martín-García, A. I., Yáñez-Ruiz, D. R., et al. (2021). Inoculation with rumen fluid in early life accelerates the rumen microbial development and favours the weaning process in goats. *Anim. Microbiome.* 3, 1–21. doi: 10.1186/s42523-021-00073-9

Pan, X., Yang, L., Xue, F., Xin, H., Jiang, L., Xiong, B., et al. (2016). Relationship between thiamine and subacute ruminal acidosis induced by a high-grain diet in dairy cows. *J. Dairy Sci.* 99, 8790–8801. doi: 10.3168/jds.2016-10865

Partovi, E., Rouzbehan, Y., Fazaeli, H., and Rezaei, J. (2020). Broccoli byproduct-wheat straw silage as a feed resource for fattening lambs. *Transl. Anim. Sci.* 4:txaa078. doi: 10.1093/tas/txaa078

Petri, R. M., Forster, R. J., Yang, W., McKinnon, J. J., and McAllister, T. A. (2012). Characterization of rumen bacterial diversity and fermentation parameters in concentrate fed cattle with and without forage. *J. Appl. Microbiol.* 112, 1152–1162. doi: 10.1111/j.1365-2672.2012.05295.x

Pitta, D. W., Kumar, S., Vecchiarelli, B., Shirley, D. J., Bittinger, K., Baker, L. D., et al. (2014). Temporal dynamics in the ruminal microbiome of dairy cows during the transition period. *J. Anim. Sci.* 92, 4014–4022. doi: 10.2527/jas.2014-7621

Qiao, Y. Y., Xu, F. F., Ming, X., Feng, S. W., Ji, Y. Y., Jiang, Y., et al. (2020). Valorization of vegetable waste *via* pyrolysis: thermal behavior, volatiles release, and products analysis from its extractives. *Energy Fuel* 34, 1896–1907. doi: 10.1021/acs.energyfuels.9b03970

Ranjan, R., Ranjan, A., Dhaliwal, G. S., and Patra, R. C. (2012). L-ascorbic acid (vitamin C) supplementation to optimize health and reproduction in cattle. *Vet. Q.* 32, 145–150. doi: 10.1080/01652176.2012.734640

Ren, H., Wang, C., Fan, W., Zhang, B., Li, Z., and Li, D. (2018). Effects of formic acid or acetic acid on the mixed-storage quality of air-dried maize Stover and cabbage wastes and microbial community analysis. *Food Technol. Biotechnol.* 56, 71–82. doi: 10.17113/ftb.56.01.18.5455

Sahoo, A., Sarkar, S., Lal, B., Kumawat, P., Sharma, S., and De, K. (2021). Utilization of fruit and vegetable waste as an alternative feed resource for sustainable and eco-friendly sheep farming. *Waste Manag.* 128, 232–242. doi: 10.1016/j.wasman.2021.04.050

Satter, L. D., and Slyter, L. L. (1974). Effect of ammonia concentration of rumen microbial protein production in vitro. *Br. J. Nutr.* 32, 199–208. doi: 10.1079/bjn19740073

Schelling, G. T., Roeder, R. A., Garber, M. J., and Pumfrey, W. M. (1995). Bioavailability and interaction of vitamin A and vitamin E in ruminants. *J. Nutr.* 125, 1799S–1803S. doi: 10.1093/jn/125.suppl_6.1799S

Seifzadeh, S., Seifdavati, J., Abdi-Benemar, H., Salem, A. Z. M., Sharifi, R. S., and Elghandour, M. M. M. Y. (2022). Dietary vitamin C in pre-parturient dairy cows and their calves: blood metabolites, copper, zinc, iron, and vitamin C concentrations, and calves growth performance. *Trop. Anim. Health Pro.* 54:54. doi: 10.1007/s11250-022-03061-6

Seong, G. U., Hwang, I. W., and Chung, S. K. (2016). Antioxidant capacities and polyphenolics of Chinese cabbage (*Brassica rapa* L. ssp Pekinensis) leaves. *Food Chem.* 199, 612–618. doi: 10.1016/j.foodchem.2015.12.066

Shi, B. C., Lux, R., Klokkevold, P., Chang, M., Barnard, E., Haake, S., et al. (2020). The subgingival microbiome associated with periodontitis in type 2 diabetes mellitus. *ISME J.* 14, 519–530. doi: 10.1038/s41396-019-0544-3

Singh, K. M., Jakhesara, S. J., Koringa, P. G., Rank, D. N., and Joshi, C. G. (2012). Metagenomic analysis of virulence-associated and antibiotic resistance genes of microbes in rumen of Indian buffalo (*Bubalus bubalis*). *Gene* 507, 146–151. doi: 10.1016/j.gene.2012.07.037

Sordillo, L. M., and Aitken, S. L. (2009). Impact of oxidative stress on the health and immune function of dairy cattle. *Vet. Immunol. Immunop.* 128, 104–109. doi: 10.1016/j.vetimm.2008.10.305

Spring, S., Bunk, B., Sproer, C., Schumann, P., Rohde, M., Tindall, B. J., et al. (2016). Characterization of the first cultured representative of Verrucomicrobia subdivision 5 indicates the proposal of a novel phylum. *ISME J.* 10, 2801–2816. doi: 10.1038/ismej.2016.84

Suntara, C., Cherdthong, A., Uriyapongson, S., Wanapat, M., and Chanjula, P. (2020). Comparison effects of Ruminal Crabtree-negative yeasts and Crabtree-positive yeasts for improving ensiled Rice straw quality and Ruminal digestion using In vitro gas production. *J. Fungi.* 6:109. doi: 10.3390/jof6030109

Tagliapietra, F., Cattani, M., Hansen, H. H., Bittante, G., and Schiavon, S. (2013). High doses of vitamin E and vitamin C influence in vitro rumen microbial activity. *Anim. Feed Sci. Tech.* 183, 210–214. doi: 10.1016/j.anifeedsci.2013.05.010

Tilg, H., and Kaser, A. (2011). Gut microbiome, obesity, and metabolic dysfunction. *J. Clin. Investigation* 121, 2126–2132. doi: 10.1172/JCI58109

van Lingen, H. J., Plugge, C. M., Fadel, J. G., Kebreab, E., Bannink, A., and Dijkstra, J. (2016). Thermodynamic driving force of hydrogen on rumen microbial metabolism: a theoretical Investig. *PLoS One* 11:e0161362. doi: 10.1371/journal.pone.0161362

Wang, Y. J., Bi, Y. Y., and Gao, C. Y. (2010). The assessment and utilization of straw resources in China. *Agric. Sci. China* 9, 1807–1815. doi: 10.1016/S1671-2927(09)60279-0

Wang, W. M., Li, C., Li, F., Wang, X. J., Zhang, X. X., Liu, T., et al. (2016). Effects of early feeding on the host rumen transcriptome and bacterial diversity in lambs. *Sci. Rep.-UK* 6, 1–14. doi: 10.1038/srep32479

Wu, G. D., Chen, J., Hoffmann, C., Bittinger, K., Chen, Y.-Y., Keilbaugh, S. A., et al. (2011). Linking long-term dietary patterns with gut microbial enterotypes. *Science* 334, 105–108. doi: 10.1126/science.1208344

Yin, B., Di, L., Tang, S., and Bao, E. (2020). Vitamin CNa enhances the antioxidant ability of chicken myocardium cells and induces heat shock proteins to relieve heat stress injury. *Res. Vet. Sci.* 133, 124–130. doi: 10.1016/j.rvsc.2020.09.008

Zhang, Z., Shahzad, K., Shen, S., Dai, R., Lu, Y., Lu, Z., et al. (2021). Altering dietary soluble protein levels With decreasing crude protein may be a potential strategy to improve nitrogen efficiency in Hu sheep based on rumen microbiome and metabolomics. *Front. Nutr.*, 8:815358. doi: 10.3389/fnut.2021.815358

Zhang, R. Y., Zhu, W. Y., Zhu, W., Liu, J. X., and Mao, S. Y. (2014). Effect of dietary forage sources on rumen microbiota, rumen fermentation and biogenic amines in dairy cows. *J. Sci. Food Agric.* 94, 1886–1895. doi: 10.1002/jsfa.6508

Zhang, Z., Wei, W., Yang, S., Huang, Z., Li, C., Yu, X., et al. (2022). Regulation of dietary protein solubility improves ruminal nitrogen metabolism in vitro: role of bacteria–protozoa interactions. *Nutrients* 14:2972. doi: 10.3390/nu14142972

Zhao, J. X., Jin, Y. Q., Du, M., Liu, W. Z., Ren, Y. S., Zhang, C. X., et al. (2017). The effect of dietary grape pomace supplementation on epididymal sperm quality and testicular antioxidant ability in ram lambs. *Theriogenology* 97, 50–56. doi: 10.1016/j.theriogenology.2017.04.010

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# *Rhodotorula benthica* culture as an alternative to antibiotics improves growth performance by improving nutrients digestibility and intestinal morphology, and modulating gut microbiota of weaned piglets

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The effects of *Rhodotorula benthica* culture (RBC) and antibiotics (AB) on the growth performance, nutrients digestibility, morphological indicators, and colonic microbiota of weaning piglets were explored. Ninety-six (Duroc × Landrace × Large) weaned piglets (21-day-old) weighing 7.7 ± 0.83 kg, were randomly allocated to 4 dietary treatments. They were fed with basal diet (CON), basal diet + 25 mg/kg bacitracin zinc + 5 mg/kg colistin sulfate (AB), 5 g/kg reduction in soybean meal of basal diet + 5 g/kg RBC (RBC1), or 10 g/kg reduction in soybean meal of basal diet + 10 g/kg RBC (RBC2). The results showed that dietary RBC1 improved the body gain/feed intake (G/F) of weaned piglets than the CON diet, and the RBC2 diet improved the average daily gain and G/F than CON and AB diets from days 15 to 28 ($P < 0.05$). Supplementation of RBC2 improved the apparent total tract digestibility of dry matter, nitrogen, and gross energy in weaned piglets compared to controls from days 15 to 28 ($P < 0.05$). Dietary AB, RBC1, and RBC2 enhanced the ileal villus height (VH) and VH/crypt depth (CD), and these two indicators were greater in the RBC2-treated piglets than in the AB- and RBC1-treated piglets ($P < 0.05$). The activity of serum superoxide dismutase (SOD) was enhanced by dietary AB, RBC1, and RBC2 ($P < 0.05$). Serum glutathione (GSH) concentration was elevated by dietary RBC1 and RBC2 ($P < 0.05$). According to 16S rRNA sequence analysis, AB- and RBC2-treated piglets had a higher relative abundance of *Firmicutes* and *Lachnospiraceae* in the colon digesta, and more abundant *Lactobacillus* was found in RBC1-treated piglets, as compared to the CON group. Additionally, RBC2 supplementation increased the α diversity

[Chao1, PD-whole-tree, and observed operational taxonomic units (OTUs)] compared to the CON group. Taken together, the dietary RBC improved the growth performance of weaned piglets. In addition, 10 g/kg of RBC2 in the diet achieved better effects on higher ADG, ileal villi morphology, and stronger antioxidant capacity than dietary AB and RBC1 in weaning piglets.

KEYWORDS

**weaning piglets, growth performance, nutrients digestibility, antioxidant property, intestinal morphology, microbiota community**

## Introduction

From the onset of weaning, piglets face many stressors including changed diet (from breast nursing to pelleted feed), separation from sows, and living with unacquainted piglets (Montagne et al., 2004; Campbell et al., 2013). Upon weaning, changes in diet have a profound influence on newly weaned piglets since they are equipped with an immature digestive system that cannot digest and absorb feed adequately, leading to the proliferation of *Escherichia coli* easily in the gut (Upadhaya et al., 2018; Shuai et al., 2019). Meanwhile, adverse effects of weaning are also followed by villous atrophy, crypt hyperplasia, and descendent brush border enzyme activity (Pluske et al., 1997). These issues affect the normal growth speed of piglets.

Reportedly, the presence of antibiotics (AB) alleviates diarrhea by killing or preventing the proliferation of pathogenic bacteria (Neuman et al., 2018), prompting animal growth from weaning stress (Ma et al., 2021). In 2006, the European Union banned AB, and China followed suit in 2020 because AB residues in meat products affect the food chain and its resistance genes can be altered by microbiota in the gut and soil (Lin et al., 2017; Ma et al., 2021). Therefore, healthy alternatives are urgently needed to replace AB.

As a fermented product, yeast culture (YC) fermented by live yeast, like *Saccharomyces cerevisiae* (Saied et al., 2011; Dávila-Ramírez et al., 2020), is commonly used as a feed additive, and it contains yeast cell wall polysaccharides, vitamins, minerals, proteins, and enzymes (Song et al., 2021). One of them, marine *Rhodotorula benthica* culture (RBC) is obtained by fermentation of *Rhodotorula benthica* and contains astaxanthin compared to YC but no live *Rhodotorula benthica*. Probiotic *Rhodotorula benthica* unusually secretes astaxanthin which is the source of vitamin A in animals and effectively scavenges free radicals more than vitamin E in the body (Elwan et al., 2018; Wang et al., 2018). According to research, YC has advantages on livestock embodying improved feed intake, average daily gain (ADG), feed conversion ratio (G: F) of finishing pigs, and ADG of fattening lambs (Haddad and Goussous, 2005; Lei and Kim, 2014; Dávila-Ramírez et al., 2020; Song et al., 2021). These benefits from increased nutrient digestibility by YC and mannan-oligosaccharide (yeast component) enhance the intestinal morphology of the small intestine by inducing more goblet cells and higher ileal villous height (De Los Santos et al., 2007; Ayiku et al., 2020). Since *Rhodotorula benthica* is frequently used in the feed of aquatic animals, few studies have been conducted on livestock (Wang et al., 2015). In 2020, a study reported that dietary fermentation product of *Rhodotorula* improved the egg quality and modulated the intestinal microbiota of hens (Sun et al., 2020). After that, Ge et al. (2021) found that mice drinking water with *Rhodotorula mucilaginosa* for half-month exhibited higher immunoglobulin G (IgG) and immunoglobulin A (IgA) in the serum, and a high abundance of *Firmicutes* and *Lactobacillus* in the feces. However, there has been no reported data regarding the effect of RBC on the weaned piglets. Therefore, this trial aimed to explore the effects of RBC supplementation in diets on the growth performance, nutrient digestibility, and intestinal health of weaned piglets.

## Materials and methods

This experiment was conducted at the experiment base in Ya'an (Sichuan, China). All experimental and animal management procedures conformed to the Animal Care and Use Committee of Sichuan Agricultural University (Sichuan, China) and followed animal protection law (approval number: 20160125).

### RBC preparation

Marine *Rhodotorula benthica strains* are detached, selected, and purified after culturing on a slant medium containing seawater. The strains undergo liquid fermentation expansion and transformation for 36 h on a solid fermentation medium under anaerobic conditions. With a temperature of 50–60°C, marine *Rhododendron benthica* break their wall after fermentation for 6–10 h and then are dried under 50–60°C. The product is in powder form and not in pellet form, and

its ingredients have been assayed using the Food Standards of China (GB/T 15673-2009 for crude protein, GB/T 23745-2009 for astaxanthin, DB22/T 2462-2016 for methionine, and GB/T 18868-2002 for lysine). The RBC contained crude protein ≥45% (w/w), yeast cell wall polysaccharides (β- glucan and mannan oligosaccharide) ≥100 mg/kg, small peptide ≥5% (w/w), lysine ≥2.8% (w/w), astaxanthin ≥0.01% (w/w), and methionine ≥0.65% (w/w). The product was provided by Prosyn Biological Technology Feed CO., LTD. (Guangzhou, China).

## Experimental design and animal care

The experiment consisted of 96 Duroc × Landrace × Yorkshire weaning piglets (21-day-old) that were split based on body weight and sex into 4 treatment groups. Each treatment group included 6 replicate pens of 4 pigs per pen (2 males: 2 females) with a similar piglet weight of 7.7 ± 0.83 kg. Pigs were supplemented with one of four diets, namely basal diet without any additives (CON), basal diet + 25 mg/kg bacitracin zinc + 5 mg/kg colistin sulfate (antibiotics group, AB), and 5 g/kg reduction in soybean meal of basal diet + 5 g/kg RBC (RBC1 group) or 10 g/kg reduction in soybean meal of basal diet +10 g/kg RBC (RBC2 group). Since the RBC product had more than 45% protein content, we replaced the soybean meal with the same percentage of RBC in the diet. The basal diets [phase 1 (days 1–14) and phase 2 (days 15–28)], shown in Table 1, were formulated to meet the recommendations of the National Research Council (NRC, 2012; USA) for piglets weighing 11 to 25 kg. The period of the feeding trial was 28 days. Pigs were reared in the house where chamber temperature (24–28°C) and humidity of 55-70% were controlled. Additionally, they can drink and consume feed *ad libitum via* a nipple drinker and feeder. Bacitracin zinc (purity of 15% w/w) was purchased from Shenzhen Tongde Veterinary Medicine Co., Ltd (Shenzhen, China), and colistin sulfate (purity of 10% w/w, effectiveness ≥19,000 IU/mg) was bought from Hebei Baipin Biological Technology Co., Ltd. (Heibei, China).

## Growth performance and diarrhea score

Before checking individual body weight (BW) on the morning of days 15 and 29, the feed was withdrawn 8 h in advance. The given feed and the residual feed by individual pen were recorded daily. Growth performance parameters included ADG, average daily feed intake (ADFI), and G/F which was calculated by dividing the ADG by ADFI. The dead piglets were recorded daily to revise performance indices.

Diarrhea scores for each piglet were checked twice per day at 09:00 h and 17:00 h, respectively. The calculation for diarrhea rate was done using the formula of Giang et al. (2012). Feces consistency scores criterion was as follows: 0, hard bar or granular; 1, soft stools but shapeable; 2, unshaped; 3, watery stool. According to the criterion, the piglet was in diarrhea when the diarrhea score was not <2 scores. The diarrhea rate = total number of piglets with diarrhea/ (number of all piglets × days of this experiment) × 100%.

## Sample collection

Immediately after BW measurement on days 15 and 29, one female piglet/pen with BW closest to the average BW of treatment was selected. Blood samples taken from the anterior vena cava were put in two 5 mL vacuum tubes (with /without heparin sodium). Then, all heparin sodium tubes were immediately sent to Sichuan Agricultural University Pet Hospital (Ya'an, China) to determine the number of white blood cells (WBCs) and lymphocytes (LYMs) through a BC-2600 automatic blood cell analyzer (Mindray, China) for quantitative analysis. The unit of WBC was $10^9$/L, and the LYM was %. The remaining tubes without heparin sodium were centrifuged (3,500 × *g*, 4°C for 15 min) to obtain serum that was analyzed for immunoglobulin and antioxidant indicators and stored (−20°C).

The digestion trial was implemented twice from days 8 to 14 and from days 22 to 28. Chromic oxide ($Cr_2O_3$) as an analytical marker, was mixed in diets (0.25% w/w). After 4-day adaptation, fresh fecal samples were obtained by stimulating the anal sphincters from 2 piglets in each pen from days 12 to 14 and from days 26 to 28; then 3-day feces per pen were mixed in equal proportions and stored (−20°C).

At the termination of this trial, 7 piglets from each treatment with BW closest to the average BW of treatment were chosen and euthanized by injecting sodium pentobarbital according to the manual. Their intestine was stripped from the mesentery and immediately placed on ice. Sections (about 2 cm) from the middle of the individual duodenum, jejunum, and ileum were cut and placed into 4% (v/v) paraformaldehyde solution for histomorphometry measurement. Colonic chyme was aseptically collected into sterile Eppendorf tubes and stored in liquid nitrogen, then removed to the fridge at −80°C for analyzing microbiota.

## Serum IgG, IgM, and antioxidant index determination

Concentrations of serum IgG and IgM were determined with porcine IgG and IgM ELISA kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) with a microplate reader (SpectraMax® 190, Molecular Devices, USA) at 450 nm. The logistic curves of IgG and IgM were built according to the manufacturer's instructions, and used the analysis software of ELISA calc. The limits for IgG and IgM concentrations were 0.3–90 mg/mL and 0.1–30 mg/mL, respectively. Coefficients of intra-

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TABLE 1 Ingredients and composition of basal diet.

| Items (as-fed, %) | Day 1–14 | | | | Day 15–28 | | | |
|---|---|---|---|---|---|---|---|---|
| | CON | AB | RBC1 | RBC2 | CON | AB | RBC1 | RBC2 |
| Corn | 18 | 18 | 18 | 18 | 38.16 | 38.16 | 38.16 | 38.16 |
| Extruded corn | 17.99 | 17.99 | 17.99 | 17.99 | 10 | 10 | 10 | 10 |
| Soybean meal | 10.40 | 10.40 | 9.90 | 9.40 | 18.04 | 18.04 | 17.54 | 17.04 |
| Extruded soybean | 13 | 13 | 13 | 13 | 10 | 10 | 10 | 10 |
| Extruded wheat | 5 | 5 | 5 | 5 | 5 | 5 | 5 | 5 |
| Extruded rice | 10 | 10 | 10 | 10 | 3 | 3 | 3 | 3 |
| Fish meal | 5 | 5 | 5 | 5 | 4 | 4 | 4 | 4 |
| SDPP | 3 | 3 | 3 | 3 | – | – | – | – |
| Whey powder | 13 | 13 | 13 | 13 | 7 | 7 | 7 | 7 |
| Soy oil | 1.86 | 1.86 | 1.86 | 1.86 | 2.07 | 2.07 | 2.07 | 2.07 |
| $CaHPO_3$ | 0.45 | 0.45 | 0.45 | 0.45 | 0.54 | 0.54 | 0.54 | 0.54 |
| Limestone | 0.95 | 0.95 | 0.95 | 0.95 | 0.83 | 0.83 | 0.83 | 0.83 |
| Bacitracin Zinc | 0 | 0.0025 | 0 | 0 | 0 | 0.0025 | 0 | 0 |
| Colistin sulfate | 0 | 0.0005 | 0 | 0 | 0 | 0.0005 | 0 | 0 |
| RBC | 0 | 0 | 0.5 | 1 | 0 | 0 | 0.5 | 1 |
| Salt | 0.3 | 0.3 | 0.3 | 0.3 | 0.3 | 0.3 | 0.3 | 0.3 |
| L-lysine HCl | 0.35 | 0.35 | 0.35 | 0.35 | 0.36 | 0.36 | 0.36 | 0.36 |
| DL-Methionine | 0.1 | 0.1 | 0.1 | 0.1 | 0.07 | 0.07 | 0.07 | 0.07 |
| L-Threonine | 0.1 | 0.1 | 0.1 | 0.1 | 0.11 | 0.11 | 0.11 | 0.11 |
| L-Tryptophan | – | – | – | – | 0.02 | 0.02 | 0.02 | 0.02 |
| Vitamin/trace element Premix[a] | 0.5 | 0.5 | 0.5 | 0.5 | – | – | – | – |
| Vitamin/trace element Premix[b] | – | – | – | – | 0.5 | 0.5 | 0.5 | 0.5 |
| Calculated nutrient composition, % | | | | | | | | |
| NE, kcal/kg | 2,596 | 2,596 | 2,594 | 2,591 | 2,570 | 2,570 | 2,567 | 2,565 |
| CP | 20.03 | 20.03 | 20.05 | 20.07 | 19.78 | 19.78 | 19.80 | 19.82 |
| Ca | 0.7 | 0.7 | 0.7 | 0.7 | 0.7 | 0.7 | 0.7 | 0.7 |
| Available phosphorus | 0.4 | 0.4 | 0.4 | 0.4 | 0.4 | 0.4 | 0.4 | 0.4 |
| Calculated standardized ideal digestible value, % | | | | | | | | |
| d-Lys | 1.20 | 1.20 | 1.20 | 1.20 | 1.18 | 1.18 | 1.18 | 1.18 |
| d-Met | 0.39 | 3.9 | 3.9 | 3.9 | 3.6 | 3.6 | 3.6 | 3.6 |
| d-Thr | 0.79 | 0.79 | 0.79 | 0.79 | 0.73 | 0.73 | 0.73 | 0.73 |
| d-Trp | 0.23 | 0.23 | 0.23 | 0.23 | 0.2 | 0.2 | 0.2 | 0.2 |
| Analyzed nutrient values, % | | | | | | | | |
| DE, MJ/kg | 14.91 | 14.91 | 14.94 | 14.96 | 14.73 | 14.72 | 14.80 | 14.81 |
| CP | 20.11 | 20.10 | 20.13 | 20.15 | 19.82 | 19.82 | 19.88 | 19.87 |
| Lys | 1.32 | 1.32 | 1.35 | 1.37 | 1.30 | 1.30 | 1.34 | 1.35 |
| Met+Cys | 0.59 | 0.59 | 0.61 | 0.62 | 0.61 | 0.60 | 0.61 | 0.63 |

[a]Provided per kg of complete diet: vitamin A, 2,200 IU; vitamin D3, 220 IU; vitamin E, 16 IU; vitamin K3, 0.5 mg; vitamin B12, 0.0175 mg; riboflavin, 3.5 mg; niacin, 30 mg; thiamine, 10 mg; choline, 500 mg; folic acid, 0.3 mg; vitamin B1, 1 mg; vitamin B6, 7 mg; biotin, 0.05 mg; Zn (as $ZnSO_4$), 100 mg; Mn (as $MnO_2$), 4 mg; Fe (as $FeSO_4 \cdot 7H_2O$), 100 mg; Cu (as $CuSO_4 \cdot 5H_2O$), 6 mg; I (as KI), 0.14 mg; Se (as $Na_2SeO_3 \cdot 5H_2O$), 0.3 mg.

[b]Provided per kg of complete diet: vitamin A, 1,750 IU; vitamin D3, 220 IU; vitamin E, 11 IU; vitamin K3, 0.5 mg; riboflavin, 3 mg; niacin, 30 mg; thiamine, 1 mg; d-pantothenic, 9 mg; choline, 400 mg; vitamin $B_{12}$, 15 $\mu$g; folic acid, 0.3 mg; vitamin B6, 3 mg; biotin, 0.05 mg; Zn (as $ZnSO_4$), 80 mg; Mn (as $MnO_2$), 3 mg; Fe (as $FeSO_4 \cdot 7H_2O$), 100 mg; Cu (as $CuSO_4 \cdot 5H_2O$), 5 mg; I (as KI), 0.14 mg; Se (as $Na_2SeO_3 \cdot 5H_2O$), 0.25 mg.

and inter-sample variations were all <10 and 12% for IgG and IgM, respectively.

The content of reduced glutathione (GSH) and malondialdehyde (MDA) and the activity of superoxide dismutase (SOD) were assayed using specific assay kits (Product code: A006-2, A003-1, A001-3, Nanjing Institute of Jiancheng Biological Engineering, Nanjing, China) according to the manufacturer's instruction. A microplate

reader (SpectraMax® 190, Molecular Devices, USA) with the absorbance of 405, 532, and 450 nm for GSH, MDA, and SOD, respectively was recommended to read the numbers. Parallel determination was conducted for each sample.

## Detection of intestinal morphology

The fixed jejunal segments were rinsed with running water for 30 min and subsequently dehydrated with absolute ethanol at varying concentrations. These tissues were cleared with xylene, embedded in wax, and sliced into 5 μm-thick slices using a Leica RM2235 microtome (Leica, Germany). Finally, these slices were dewaxed and stained with hematoxylin-eosin. For each well-oriented villus, 10 measurements were recorded for both villus height (VH) and crypt depth (CD) using Image Pro Plus 6.0. The average of these 10 measurements was taken to represent the VH and CD for each tissue. The V/C ratio was obtained by dividing the VH by the CD value.

## 16S ribosomal RNA (rRNA) sequencing

The frozen colonic digesta at −80°C were thawed and extracted for total DNA using QIAamp PowerFecal Pro DNA kit (Qiagen, Hilden, Germany) following the manufacturer's instructions. The DNA samples for purity and integrity examination were analyzed using a NanoDrop 2000 spectrophotometer (Thermo, Waltham, USA) and electrophoresis (2% w/v gel). DNA concentration was quantified by Equalbit1 × dsDNA HS Assay Kit (Vazyme Biotech Co., Ltd., Nanjing, China) and diluted to 1 ng/μL, and purified by Qiagen Gel extraction kit (Qiagen, Germany). Library construction using Ion Plus Fragment Library Kit 48 rxns (Thermos Scientific, USA) and Illumina MiSeq sequencing on IonS5™ was performed in Novogene Bioinformatics Technology (Beijing, China). The hypervariable region primers of V3-V4 and amplicons library of 16S rRNA kept consistent with our previous description (Zhang Q. et al., 2020). Raw reads were obtained from the spliced sequences by removing the primer sequences and barcode, and primers and adapters were removed from the raw reads using the Cutadapt (V1.9.1), then clean reads were obtained. Operational taxonomic units (OTUs) were clustered by clean reads using Uparse software (http://www.drive5.com/uparse/) with 97% identity. The feature classifiers were trained according to SILVA 132 database (http://www.arb-silva.de/). Alpha and beta diversities were analyzed using QIIME (version 1.9.1; Caporaso et al., 2010). Differential taxonomic markers for each group were determined using linear discriminant analysis effect size (LEfSe; Segata et al., 2011).

## Statistical analysis

The data were analyzed by mixed procedure with PDIFF (SAS 9.4 Inst., Inc., Cary, NC). The results were expressed as means ± pooled standard error. The *P*-value was used for the comparison of every 2 treatment groups from the least square means. $P < 0.05$ was considered significant. Replicate pen was the statistics for growth performance, diarrhea, and ATTD. Intestinal morphology, serum indices, and grouped microbial were analyzed using each euthanized piglet as an experiment unit. The relative abundance of microorganisms was used as the result of Metastat analysis. The Principal Co-ordinates Analysis (PCoA) was visualized by the "vegan" package of R (2.15.3).

# Results

## Growth performance and diarrhea score

Supplementary RBC1 in the diet notably improved ($P < 0.05$) the G/F compared with the CON diet (Table 2) from days 15 to 28 and from days 1 to 28. Higher ADG and G/F ($P < 0.05$) were found in the RBC2-treated piglets than in the controls from days 15 to 28 and from days 1 to 28 ($P < 0.05$). Compared with AB-treated piglets, the piglets fed with RBC2 diet had higher ADG and G/F from days 15 to 28 and greater G/F from days 1 to 28 ($P < 0.05$). However, the differences were not observed between CON vs. AB, AB vs. RBC1, or RBC1 vs. RBC2 groups. As shown in Table 3, diarrhea scores of weaning piglets were similar among groups.

## Apparent total tract digestibility

Compared with the CON group, the piglets in the AB group had higher ATTD of DM ($P < 0.05$) from days 1 to 14 and from days 15 to 28 (Table 4), and this index was also higher in the RBC1 group from days 15 to 28 ($P = 0.004$). The ATTD of GE from days 1 to14, DM, N, and GE from days 15 to 28 were higher ($P < 0.05$) in the RBC2 group than those in the CON group.

## Intestinal morphology

Compared to piglets in the CON group, the basal diet supplemented with AB or RBC1 was conducive to higher VH and greater V/C ratio of piglets in the ileum ($P < 0.01$, Table 5); while dietary RBC2 improved the jejunal VH, ileal VH, and V/C ($P < 0.05$). Additionally, AB-treated piglets exhibited a higher ($P < 0.05$) duodenal VH and V/C ratio, as compared to RBC1-treated piglets. However, a greater ileal VH and V/C ratio of piglets were found in the RBC2 group, as compared to the AB group ($P < 0.001$). The RBC2 dose exhibited a better effect

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TABLE 2 Effect of dietary RBC and AB supplementation on the growth performance of weaning piglets.

| Items | CON | AB | RBC1 | RBC2 | SEM[a] | *P*-value | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | CON vs. AB | CON vs. RBC1 | CON vs. RBC2 | AB vs. RBC1 | AB vs. RBC2 | RBC1 vs. RBC2 |
| BW, kg | | | | | | | | | | | |
| Day 0 | 7.75 | 7.69 | 7.69 | 7.70 | 0.37 | 0.910 | 0.915 | 0.930 | 0.995 | 0.980 | 0.985 |
| Day 14 | 10.41 | 10.76 | 10.60 | 10.72 | 0.46 | 0.595 | 0.775 | 0.639 | 0.804 | 0.950 | 0.853 |
| Day 28 | 16.83 | 17.40 | 17.78 | 18.30 | 0.63 | 0.534 | 0.304 | 0.117 | 0.678 | 0.326 | 0.565 |
| Day1–14 | | | | | | | | | | | |
| ADG, g/d | 190 | 220 | 208 | 216 | 21 | 0.327 | 0.556 | 0.394 | 0.688 | 0.894 | 0.788 |
| ADFI, g/d | 265 | 292 | 290 | 295 | 24 | 0.447 | 0.476 | 0.393 | 0.962 | 0.923 | 0.886 |
| G/F | 0.728 | 0.741 | 0.723 | 0.734 | 0.040 | 0.814 | 0.928 | 0.914 | 0.745 | 0.898 | 0.843 |
| Day 15–28 | | | | | | | | | | | |
| ADG, g/d | 458 | 474 | 513 | 541 | 23 | 0.638 | 0.108 | 0.018 | 0.243 | 0.049 | 0.381 |
| ADFI, g/d | 721 | 747 | 714 | 727 | 50 | 0.709 | 0.937 | 0.931 | 0.652 | 0.774 | 0.869 |
| G/F | 0.638 | 0.649 | 0.724 | 0.750 | 0.028 | 0.772 | 0.039 | 0.010 | 0.070 | 0.019 | 0.525 |
| Day 1–28 | | | | | | | | | | | |
| ADG, g/d | 324 | 347 | 360 | 379 | 17 | 0.362 | 0.152 | 0.035 | 0.583 | 0.200 | 0.452 |
| ADFI, g/d | 493 | 519 | 502 | 511 | 32 | 0.559 | 0.832 | 0.691 | 0.709 | 0.850 | 0.853 |
| G/F | 0.661 | 0.674 | 0.724 | 0.743 | 0.019 | 0.623 | 0.032 | 0.007 | 0.086 | 0.020 | 0.481 |

[a]Pooled standard error of means, n = 6.

TABLE 3 Effect of dietary RBC and AB supplementation on the diarrhea score of weaning piglets (%).

| Items | CON | AB | RBC1 | RBC2 | SEM[a] | *P*-value | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | CON vs. AB | CON vs. RBC1 | CON vs. RBC2 | AB vs. RBC1 | AB vs. RBC2 | RBC1 vs. RBC2 |
| Day 0–14 | 0.222 | 0.344 | 0.249 | 0.189 | 0.155 | 0.584 | 0.903 | 0.881 | 0.670 | 0.487 | 0.786 |
| Day 15–28 | 0.753 | 0.523 | 0.524 | 0.429 | 0.135 | 0.243 | 0.243 | 0.104 | 0.999 | 0.624 | 0.624 |
| Day 0–28 | 0.488 | 0.434 | 0.386 | 0.309 | 0.113 | 0.739 | 0.532 | 0.276 | 0.769 | 0.443 | 0.634 |

[a]Pooled standard error of means, n = 6.

TABLE 4 Effect of dietary RBC and AB supplementation on nutrients digestibility of weaning piglets (%).

| Items | CON | AB | RBC1 | RBC2 | SEM[a] | *P*-value | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | CON vs. AB | CON vs. RBC1 | CON vs. RBC2 | AB vs. RBC1 | AB vs. RBC2 | RBC1 vs. RBC2 |
| Day 1–14 | | | | | | | | | | | |
| DM | 81.40 | 83.18 | 82.28 | 82.45 | 0.60 | 0.047 | 0.307 | 0.226 | 0.300 | 0.398 | 0.843 |
| N | 81.79 | 83.05 | 83.37 | 83.18 | 0.76 | 0.255 | 0.158 | 0.210 | 0.771 | 0.905 | 0.864 |
| GE | 79.80 | 81.80 | 81.06 | 83.29 | 0.98 | 0.162 | 0.373 | 0.020 | 0.596 | 0.295 | 0.122 |
| Day 15–28 | | | | | | | | | | | |
| DM | 77.36 | 80.67 | 81.50 | 82.22 | 0.90 | 0.017 | 0.004 | 0.001 | 0.526 | 0.237 | 0.573 |
| N | 78.19 | 79.39 | 80.27 | 82.36 | 1.12 | 0.458 | 0.206 | 0.016 | 0.587 | 0.076 | 0.202 |
| GE | 77.51 | 78.50 | 80.38 | 81.90 | 1.17 | 0.559 | 0.101 | 0.016 | 0.274 | 0.055 | 0.370 |

[a]Pooled standard error of means, n = 6.

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TABLE 5 Effect of dietary RBC and AB supplementation on the intestinal morphology of weaning piglets.

| Items | CON | AB | RBC1 | RBC2 | SEM[a] | *P*-value | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | CON vs. AB | CON vs. RBC1 | CON vs. RBC2 | AB vs. RBC1 | AB vs. RBC2 | RBC1 vs. RBC2 |
| Duodenum | | | | | | | | | | | |
| VH, μm | 373.17 | 381.00 | 367.17 | 378.83 | 4.47 | 0.229 | 0.353 | 0.380 | 0.041 | 0.735 | 0.078 |
| CD, μm | 242.00 | 236.50 | 243.33 | 239.33 | 3.41 | 0.267 | 0.785 | 0.586 | 0.172 | 0.563 | 0.416 |
| V/C ratio | 1.54 | 1.61 | 1.51 | 1.58 | 0.02 | 0.051 | 0.369 | 0.252 | 0.007 | 0.382 | 0.049 |
| Jejunum | | | | | | | | | | | |
| VH, μm | 320.17 | 330.33 | 320.83 | 327.67 | 5.63 | 0.216 | 0.934 | 0.358 | 0.247 | 0.741 | 0.401 |
| CD, μm | 172.67 | 171.17 | 166.17 | 163.67 | 3.43 | 0.721 | 0.196 | 0.079 | 0.316 | 0.138 | 0.613 |
| V/C ratio | 1.86 | 1.93 | 1.93 | 2.01 | 0.05 | 0.263 | 0.261 | 0.029 | 0.996 | 0.243 | 0.245 |
| Ileum | | | | | | | | | | | |
| VH, μm | 279.33 | 318.83 | 321.67 | 351.00 | 3.58 | <0.001 | <0.001 | <0.001 | 0.582 | <0.001 | <0.001 |
| CD, μm | 153.00 | 149.67 | 156.33 | 146.83 | 2.84 | 0.417 | 0.417 | 0.141 | 0.113 | 0.489 | 0.028 |
| V/C ratio | 1.83 | 2.14 | 2.06 | 2.42 | 0.05 | 0.013 | <0.001 | 0.002 | 0.246 | <0.001 | <0.001 |

[a]Pooled standard error of means, n = 7.

TABLE 6 Effect of RBC and AB supplementation on the blood profiles of weaning piglets.

| Items | CON | AB | RBC1 | RBC2 | SEM[a] | *P*-value | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | CON vs. AB | CON vs. RBC1 | CON vs. RBC2 | AB vs. RBC1 | AB vs. RBC2 | RBC1 vs. RBC2 |
| Day 14 | | | | | | | | | | | |
| IgG, μg/L | 2.62 | 2.85 | 2.80 | 3.24 | 0.23 | 0.475 | 0.581 | 0.069 | 0.869 | 0.249 | 0.191 |
| IgM, μg/L | 0.198 | 0.298 | 0.272 | 0.338 | 0.07 | 0.301 | 0.446 | 0.154 | 0.780 | 0.676 | 0.488 |
| WBC, $10^9$ /L | 23.02 | 21.67 | 22.48 | 21.56 | 0.74 | 0.210 | 0.612 | 0.178 | 0.444 | 0.921 | 0.389 |
| LYM, % | 73.82 | 74.28 | 74.18 | 76.15 | 2.09 | 0.876 | 0.903 | 0.439 | 0.973 | 0.535 | 0.513 |
| Day 28 | | | | | | | | | | | |
| IgG, μg/L | 4.20 | 4.23 | 4.67 | 4.40 | 0.42 | 0.960 | 0.435 | 0.687 | 0.464 | 0.720 | 0.702 |
| IgM, μg/L | 0.22 | 0.25 | 0.28 | 0.37 | 0.02 | 0.380 | 0.114 | 0.172 | 0.459 | 0.609 | 0.816 |
| WBC, $10^9$ /L | 23.85 | 24.25 | 23.17 | 22.47 | 1.94 | 0.886 | 0.808 | 0.621 | 0.699 | 0.525 | 0.801 |
| LYM, % | 15.22 | 15.95 | 15.07 | 15.69 | 1.66 | 0.757 | 0.949 | 0.843 | 0.709 | 0.911 | 0.793 |

[a]Pooled standard error of means, n = 6.

than the RBC1 dose on piglets, showing a higher VH in the duodenum and ileum, shallower ileal CD, and greater ileal V/C ratio ($P < 0.05$).

## Blood characteristics and antioxidant properties

Serum IgG, IgM, WBC, and LYM were not different among groups on days 14 and 28 (Table 6). Serum SOD activity on day 14 from piglets fed with the AB diet was elevated ($P = 0.001$), as shown in Table 7 when compared with the controls. The RBC1-treated piglets had higher concentration of ($P < 0.01$) serum GSH on days 14 and 28 and the SOD activity on day 28, as compared to the piglets in the CON group, while piglets fed with the RBC2 diet exhibited a higher GSH concentration on days 14 and 28 and the SOD activity on day 14 compared with the controls ($P < 0.001$). The GSH concentration on days 14 and 28 was increased ($P < 0.001$), while the SOD activity was reduced on day 14 but elevated on day 28 in piglets fed with RBC1 diet ($P < 0.05$), as compared to that in the AB group; additionally, RBC2-treated piglets had a higher GSH concentration on days 14 and 28 than AB-treated piglets ($P < 0.001$). Moreover, when compared to RBC1-treated piglets, the dietary RBC2 induced higher SOD activity on days 14 and 28 and GSH activity on day 14 ($P < 0.05$).

TABLE 7 Effect of dietary RBC and AB supplementation on serum antioxidant properties of weaning piglets.

| Items | CON | AB | RBC1 | RBC2 | SEM[a] | *P*-value | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | CON vs. AB | CON vs. RBC1 | CON vs. RBC2 | AB vs. RBC1 | AB vs. RBC2 | RBC1 vs. RBC2 |
| Day 14 | | | | | | | | | | | |
| GSH, ug/mL | 613.11 | 598.41 | 653.35 | 711.85 | 6.48 | 0.124 | < 0.001 | < 0.001 | < 0.001 | < 0.001 | < 0.001 |
| MDA, nmol/mL | 6.78 | 6.25 | 6.26 | 6.35 | 0.31 | 0.239 | 0.247 | 0.332 | 0.985 | 0.829 | 0.844 |
| SOD, U/mL | 98.77 | 108.79 | 101.84 | 110.21 | 1.78 | 0.001 | 0.236 | < 0.001 | 0.012 | 0.577 | 0.003 |
| Day 28 | | | | | | | | | | | |
| GSH, ug/mL | 595.50 | 602.19 | 673.85 | 663.03 | 3.67 | 0.158 | <0.001 | <0.001 | <0.001 | <0.001 | 0.051 |
| MDA, nmol/mL | 6.55 | 6.05 | 6.41 | 6.46 | 0.17 | 0.055 | 0.570 | 0.735 | 0.159 | 0.105 | 0.818 |
| SOD, U/mL | 104.82 | 106.55 | 113.70 | 106.40 | 1.55 | 0.440 | 0.001 | 0.480 | 0.004 | 0.946 | 0.003 |

[a]Pooled standard error of means, n = 6.

## Microbial composition in colonic digesta

In the present study, an average of 77,665 raw reads and 72,560 clean reads were obtained for each group with the length of the sequences ranging between 409 and 420 bp. The sequence number at the OTU level was 440,028 (Supplementary Figure S1), and it tended to reach a plateau, suggesting that the majority of OTU samples were captured. The α diversity indices in Figures 1A–F, 2A are represented by richness (observed-OTUs, ACE, and Chao1), diversity (Simpson and Shannon), goods-coverage (sequencing depth index), and PD_ whole_ tree (index of phylogenetic diversity). A sharp contrast between PRC2 and CON groups showed an increase in Chao1, PD-whole-tree, and observed-OTUs ($P < 0.05$) in the RBC2 group. Shannon and Simpson indicators were similar among groups ($P > 0.05$). The unweighted Unifrac PCoA plot (Figure 2B) visually indicated no separation of microbial communities among groups ($P > 0.05$). The ANOSIM R-value of unweighted Unifrac distance ($R = 0.026$, $P = 0.027$; Figure 2C) was close to zero, which represented the weak separation of the microbiota community between CON and RBC2 groups.

## Relative abundance of microbiota at phylum and genus levels

The top 10 predominant microorganisms at the phylum level depicted in Figure 2D (Supplementary Table S1) showed that bacteria belonging to *Firmicutes* (51.99%), *Bacteroidetes* (31.04%), and *Proteobacteria* (5.27%) occupied more than 88% of the total sequences. Supplementation of RBC and AB seemed to change the microbiota abundance as *Firmicutes* (44.48%) *and Proteobacteria* (9.95%) in the CON group, *Firmicutes* (55.28%) *and Proteobacteria* (3.97%) in the AB group, and *Firmicutes* (50.86%) *and Proteobacteria* (4.95%) in the RBC1 group, *Firmicutes* (57.32%) *and Proteobacteria* (2.18%) in the RBC2 group. The relative abundance of *Firmicutes* in the AB and RBC2 groups was higher ($P = 0.015$) when compared with the CON group. The relative abundance of *Euryarchaeota* was higher ($P < 0.05$) in the RBC2 group than in the RBC1 and CON groups, and in the AB group than in the RBC1 group, while that of *Proteobacteria* was lower in the RBC2 group than in the CON group ($P = 0.019$).

At the genus level (Figure 2E, Supplementary Table S2), a heatmap showed the top 30 microorganisms in the genus level among the total number of 185 genera. *Lactobacillus* (6.73%), *Prevotella_9* (9.69%), *Mitsuokella* (4.86%), *Succinivibrio* (4.65%), and *Dialister* (3.94%) had high relative abundance in all colon digesta samples. Supplementation of RBC1 and RBC2 diets both lowered the relative abundance of *Acidaminococcus*, as compared to the CON and AB diets ($P < 0.05$). The *Succinivibrio* population was lowered in the RBC2 group compared to the CON group ($P = 0.023$). The relative abundance of *Prevotella*, *Prevotellaceae_UCG-003*, and *Ruminococcus* were enriched in the RBC1-treated piglets than in the piglets of the AB group ($P < 0.05$). *Rikenellaceae_RC9_gut*_group and *g__UCG-002* population were enriched in the RBC2 group compared with the RBC1group ($P < 0.05$).

## LEfSe analysis in the OUT level

LEfSe analysis is used to assess the represented biomarker for comparison among groups. Linear discriminant analysis (score set 4) depicted in Figure 3A shows that the AB group had a higher relative abundance of *p_Firmicutes*, *f_Lachnospiraceae*, and *o_ Lachnospirales* in comparison to the CON group. While in Figure 3B, the RBC1 group exhibited enriched *f_Lactobacillaceae*, *o_Lactobacillales*, *g_Lactobacillus*, and *s_Lactobacillus_ johnsonii*, as compared to the CON

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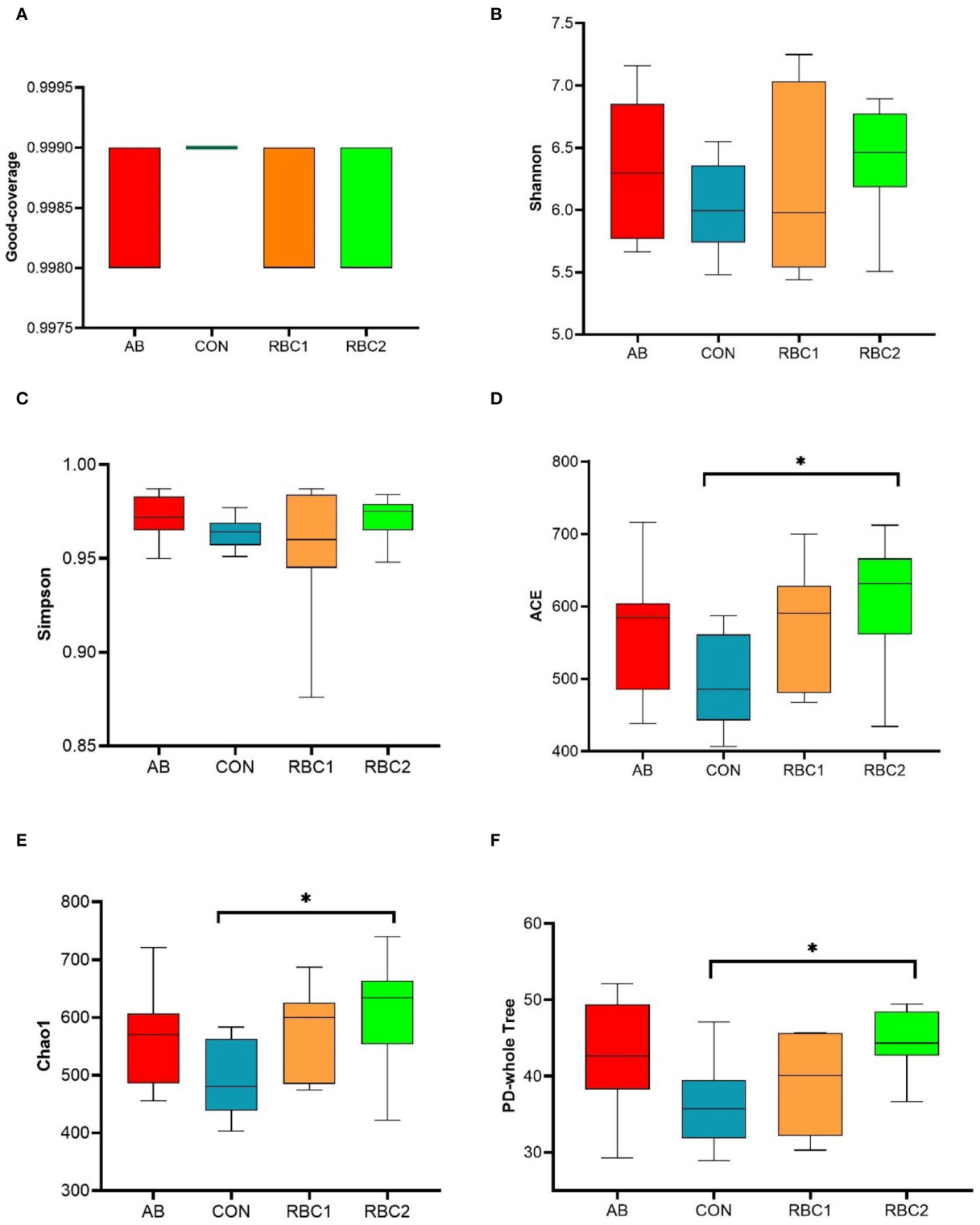


FIGURE 1
Effect of dietary RBC and antibiotics (AB) supplementation on the α and β diversity in the colonic digesta of weaning piglets. **(A–F)** Goods-coverage, Shannon, Simpson, ACE, Chao1, and Ph-whole tree indices of α diversity. $n = 7$ for each treatment. CON, basal diet; AB, basal diet + 25 mg/kg bacitracin zinc+5 mg/kg colistin sulfate; RBC1, 5 g/kg reduction in soybean meal of basal diet +5 g/kg RBC; RBC2, 10 g/kg reduction in soybean meal of basal diet +10 g/kg RBC. *Means $P < 0.05$.

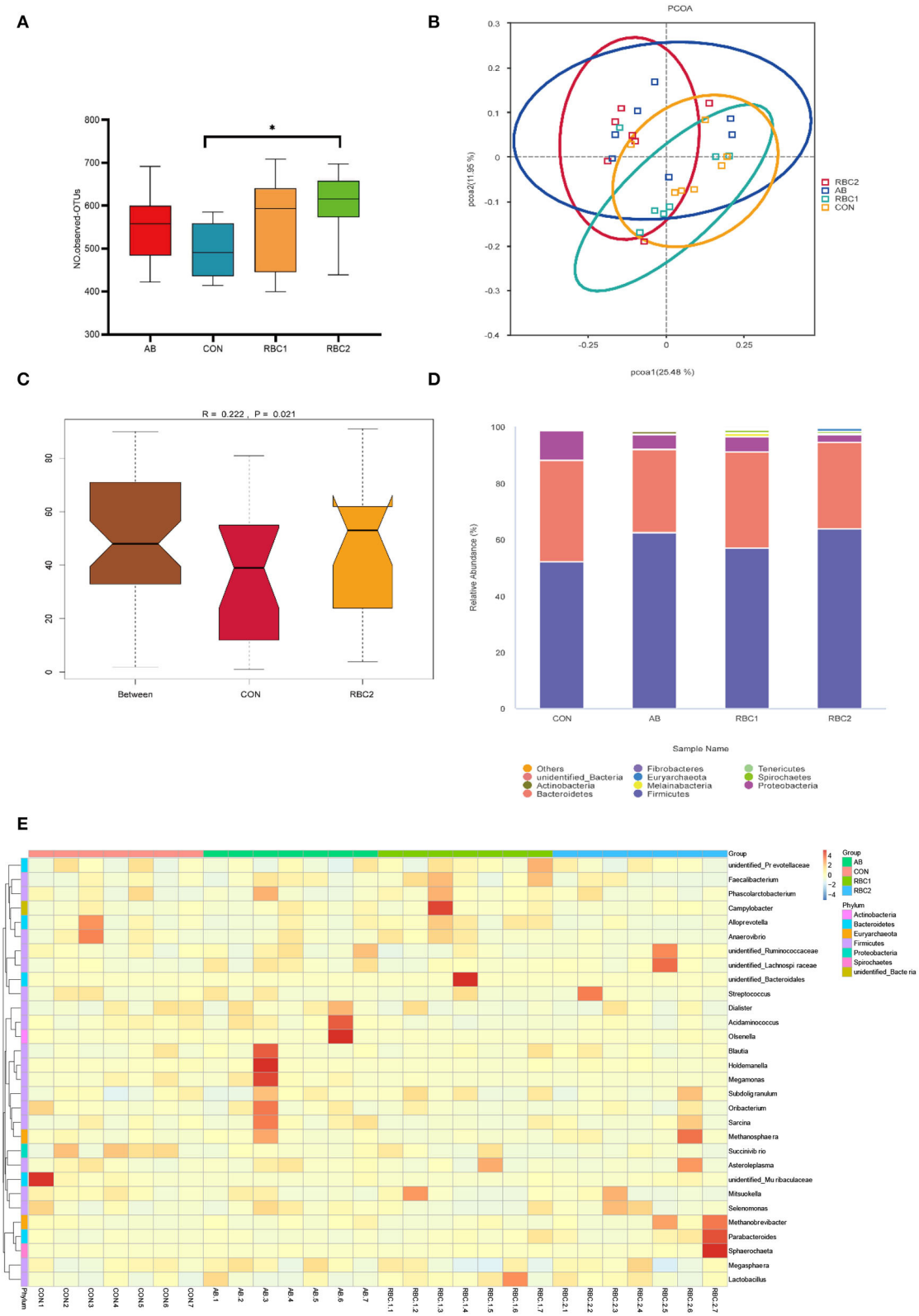


FIGURE 2
Effect of dietary AB and RBC on the relative abundance of microbiota at the phylum and genus level, and PCoA and Adonism of weaned piglets. **(A)** Operational taxonomic units (OTUs) number in each treatment group. **(B)** Principal coordinates analysis (PCoA). **(C)** Analysis of similarities (ANOSIM) between CON and RBC2 group. **(D)** Microbiota abundance at the phyla level. **(E)** Microbiota abundance at the genus level. $n = 7$ for each treatment. CON, basal diet; AB, basal diet + 25 mg/kg bacitracin zinc+5 mg/kg colistin sulfate; RBC1, 5 g/kg reduction in soybean meal of basal diet + 5 g/kg RBC; RBC2, 10 g/kg reduction in soybean meal of basal diet + 10 g/kg RBC. *Means $P < 0.05$.

group whereas g_*Provotella*_7 was abundant. In Figure 3C, *p_Firmicutes*, *c_Clostridia*, *s_Lactobacillus_ johnsonii, f_Lachnospiraceae*, *o_Lachnospirales,* and *f_Paludibacteraceae* were increased in the RBC2-treated piglets when compared to the CON group where *g_Prevotella*_7, *o_Veillonellales_ Selenomonadales,* and *c_ Negativicutes* were enriched. Compared to the microbiota abundance of AB-treated piglets (Figure 3D), piglets fed with RBC1 diet had more *g_Prevotella* populations in the colon, while *o_Erysipelotrichales* and *g_ Acidminococcus* were enriched in the AB-treated piglets; meanwhile, *s_Lactobacillus_johnsonii* and *g_UCG*_002 were enriched in the colon digesta from piglets fed withRBC2 diet (Figure 3E). When compared to RBC1-treated piglets (Figure 3F); more abundant *g_UCG*_002 was found in piglets fed with RBC2 diet.

## Discussion

Natural marine red yeast strains are rich in protein, astaxanthin, and enzyme and characterize high salt tolerance; therefore, they are commonly used as feed additives in aquaculture (Matrosova and Politaeva, 2021; Yun et al., 2021). RBC without live yeast also has the above features but it has fewer applications on animals. For a long time, overcoming weaning syndrome in piglets has always been a research hotspot; thus, we look forward to the influences of RBC on weaning piglets.

In this study, RBC1 and RBC2 supplementation as the alternative to soybean meal in the basal diet improved the ADG and G/F of piglets except in the phase of days 1–14. This result was partially in line with the findings of Dávila-Ramírez et al. (2020), that dietary *Saccharomyces cerevisiae* culture at the dose of 0.2% and 0.3% both improved the final BW, ADG, and ADFI of growing pigs, and the results of Lee et al. (2018), showed that 0.5% fermented YC improved the ADG and feed intake of weaned piglets. Moreover, supplementation of RBC at the dose of 10 g/kg improved the ADG and G/F of piglets more than the effect of diet-containing AB. To our knowledge, the feed intake is a vital factor for growth speed (Bruininx et al., 2001), but there was a similar ADFI among groups. Indeed, supplementary RBC and AB in the diet did not change the dietary nutritional value or physical character; consequently, there was no improvement in the feed intake, and it was normal. Meanwhile, the immunity response of piglets was not affected by RBC although RBC could promote serum protein and albumin synthesis (Saied et al., 2011). Of note, Cui et al. (2019) and Song et al. (2021) reported that the elevated growth may not be related to the feed intake but to digestibility.

The nutrient digestibility and absorption and piglet diarrhea indicators are also decisive factors for growth performance (Haddad and Goussous, 2005; Hu et al., 2015). In this study, although the diarrhea scores were not affected by dietary additives, the RBC2 supplementation improved DM, GE, and N digestibility, and greater DM digestibility was also found in AB and RBC1-treated piglets. These agreed with the effects of fermented YC products on elevating the nutritional digestibility and growth performance of lambs without affecting intakes (Lei and Kim, 2014; Zhang et al., 2019). RBC product contains biologically active substances, such as bioactive peptide, which can be absorbed by villous epithelial cells of the small intestine and stimulates the activity of chorionic brush border enzymes (Wang et al., 2017), as well as modulates the nutrient digestibility (Bao and Wu, 2021). Additionally, the protein from fermented RBC is the most water-soluble protein with easy absorption, which may increase the ATTD of N (Hu et al., 2008). Moreover, RBC containing lysing also prompts nutrient absorption (Zeng et al., 2013). Of course, it was no doubt that improvements in nutritional absorption and growth performance were closely related to the improved gut environment induced by ingredients of RBC.

Intestinal morphology indices that are common standards to estimate the ability of the intestine for nutrient digestion and absorption are widely reported. The potential of yeast derivative that induces morphology on higher VH and greater V/C ratio of weaned piglets or growing-finishing pigs (Zhang et al., 2005; Gang et al., 2017; He et al., 2021), and goblet cell density of broilers (Reisinger et al., 2012) have been reported. In the current study, the RBC induced a higher ileal V/H and V/C ratio, and the dietary RBC2 greatly affected the ileal morphology more than the AB and RBC1 diet. Earlier studies prove that mannan-oligosaccharides, a component of yeast cells, prevent the villi from contacting pathogens (Sims et al., 2004; Castillo et al., 2008), like *Clostridium perfringens* and *Salmonella,* is beneficial to maintain the normal function of mucosa and reduce diarrhea during the weaning period (Gao et al., 2008). Admittedly, AB had the capability of improving the VH and V/C in the small intestine (Oliver and Wells, 2013; Long et al., 2018) due to inhibiting harmful bacteria. This result exhibited that the supplementation of RBC at the dose of 10 g/kg improved the ileal VH and V/C compared to AB-treated piglets. On the one hand, a higher villus manifests faster cell renewal and more mature epithelia and increases the absorptive area of the villus (Gao et al., 2008). On the other hand, taller VH improves the secretion of digestive enzyme activity from the tips of the villi (Hampson, 1986), leading to elevated ATTD. Additionally, a higher magnitude of the V/C ratio directly indicates the strength of absorption (Pluske et al., 1996). Hence, in this research, the larger V/C ratio in RBC treatments may contribute to the higher digestibility.

Oxidant stress results in weaker growth performance and lower nutrient digestibility of weaned pigs (Yuan et al., 2007). In the current study, the dietary inclusion of RBC improved

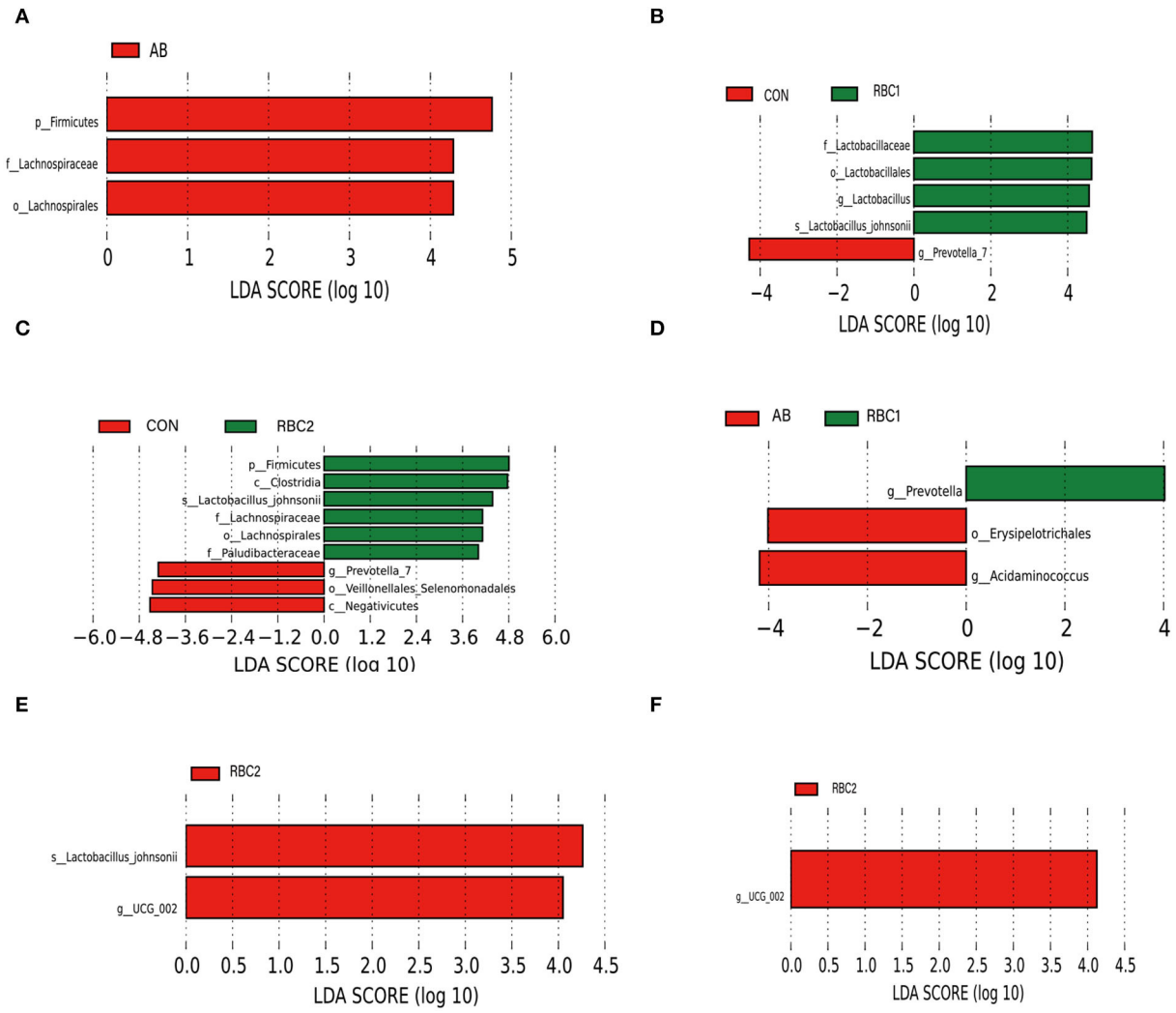


FIGURE 3
LEfSe analysis of digesta microbiota. **(A)** CON vs. AB group, **(B)** CON vs. RBC1 group, **(C)** CON vs. RBC2 group, **(D)** AB vs. RBC1group, **(E)** AB vs. RBC2 group, **(F)** RBC1 vs. RBC2 group. $n = 7$ for each treatment. CON, basal diet; AB, basal diet + 25 mg/kg bacitracin zinc+5 mg/kg colistin sulfate; RBC1, 5 g/kg reduction in soybean meal of basal diet +5 g/kg RBC; RBC2, 10 g/kg reduction in soybean meal of basal diet +10 g/kg RBC.

the GSH content more than CON and AB diets on days 14 and 28, with RBC2 achieving the optimal effect. The GSH can be an index to evaluate the antioxidant capacity of the body because it possesses vital physiological functions, such as free radical scavenging, detoxication, and maintaining cellular immunity (Tang et al., 2008). Supplementation of AB, RBC1, and RBC2 enhanced the SOD activity in the serum. SOD is a type of antioxidant enzyme that removes harmful free radicals from the cellular environment (Fattman et al., 2003). According to researchers, the astaxanthin that is present in the RBC with the structure of a hydroxyl and a keto group can neutralize free radicals, and scavenge singlet oxygen, peroxyl, and hydroxyl radicals (Hardo et al., 2020), and this function has been proved in finishing pigs and fishes (Lei and Kim, 2014; Li et al., 2017). Subsequently, methionine was reported to eliminate reactive oxygen species by methionine residues or through GSH synthesis (Zeitz et al., 2017). The RBC containing methionine is attributed to its higher GSH; this was not observed in the AB group, which was in response to stronger antioxidant capacities in the RBC groups than in the AB group.

Reportedly, gut microbial populations in pigs are influenced by dietary ingredients, such as trace elements, AB, and beneficial bacteria (Zhang et al., 2016). Meanwhile, they also modulate nutrient digestibility and serve as an important barrier pathogen (Fouhse et al., 2016).

The increased PD_ whole_ tree, observed-OTUs, and Chao1 in the RBC2 group indicated that the dietary RBC at

the dose of 10 g/kg increased the richness of colonic flora compared to the controls. At the phylum level, *Firmicutes*, more abundant in AB and RBC2 group, are positively interrelated in energy absorption and produce acetate and lactate (Hu et al., 2020; Oh et al., 2021). Also, *Euryarchaeota* enriched in the RBC2 is well-known for working at metabolizing nutrients and metabolites of other bacteria, resulting in elevated short-chain fatty acid concentration, such as acetate (Primec et al., 2019). The abundant 2 phyla may be connected to improved GE digestibility of RBC piglets. The increased *Acidaminococcus* in the AB group, *Lachnospiraceae,* and *Paludibacteraceae* in the RBC2 group, involve fermenting fibers, polysaccharides, and glutamate (Ren et al., 2019; Tian et al., 2019; Zhang L. et al., 2020; Ryazanov et al., 2021). As we know, RBC contains abundant yeast cell polysaccharides, which may stimulate the proliferation of *Euryarchaeota*, *Lachnospiraceae*, and *Paludibacteraceae.* Importantly, *Lachnospiraceae* and *Paludibacteraceae* protect intestinal cells from injury (Berger et al., 2021). In addition, the abundant genus, *Lactobacillus* in the RBC1 group is well-known to improve nutrient digestibility and against intestinal *Escherichia coli* in weaned piglets (Dowarah et al., 2017; Wang et al., 2019). Moreover, *Lactobacillus_ johnsonii* belonging to the genus, *Lactobacillus* is useful for inhibiting *Clostridium perfringens* in the chicks (La Ragione et al., 2004). *Prevotella* with the characteristic of decomposing starch and plant polysaccharides has strong catabolism of mucin (Gao et al., 2022). This changed abundance of microbiota reflected the dietary supplementation of AB and RBC at the dose of 5 and 10 g/kg, respectively, played a function in modulating the microbiota.

## Conclusion

In this study, the comparative effect of AB and RBC doses on weaning piglets was explored. The RBC1 supplementation at the dose of 5 g/kg improved the G/F and ATTD of DM of weaned piglets than the CON diet, while 10 g/kg of RBC improved the G/F and ADG than CON and AB diet; it also improved the ATTD of DM, N, and GE than the AB diet. These improved indicators may result in elevating the intestinal morphology of the jejunum and ileum and antioxidant properties in the serum (GSH and SOD) of weaning piglets by RBC. Meanwhile, AB supplementation also elevated the digestibility of DM, N, and GE, and the ileum intestinal morphology of weaning piglets than the controls. Additionally, the supplementation of AB, RBC1, and RBC2 modulated the microbiota in the colon but the RBC2 had a more profound influence on improving the α diversity than the controls. Taken together, the dietary RBC at the dose of 10 g/kg achieved a better effect on the weaning piglets than AB; thus, 10 g/kg of RBC could be an alternative to AB.

## Data availability statement

All standard sequence format (.fastq) files generated by Illumina Miseq containing all raw sequence reads have been deposited at the National Center for Biotechnology Information (NCBI) Sequence Read Archive (SRA) (BioProject accession number: PRJNA864102).

## Ethics statement

This experiment was conducted at the experiment base in Ya'an (Sichuan, China). All experimental and animal management procedures conformed to the Animal Care and Use Committee of Sichuan Agricultural University (Sichuan, China), and followed animal protection law (approval number: 20160125).

## Author contributions

JL designed the experiment, provided the funds, and revised the manuscript. QZ analyzed the data and wrote the manuscript. ZL performed the animal trial and determination of indices. XY helped to analyze indices. YL, SX, and YZ helped to design this experiment. ZF, BF, LC, and DW helped to revise the final manuscript. SL provided guide in animal trial. All authors approved the final manuscript.

## Conflict of interest

Author SL was employed by Guangzhou Prosyn Biological Technology Feed 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/fmicb.2022.964531/full#supplementary-material

# References


Ayiku, S., Shen, J. F., Tan, B. P., Dong, X. H., and Liu, H. Y. (2020). Effects of dietary yeast culture on shrimp growth, immune response, intestinal health and disease resistance against Vibrio harveyi. *Fish Shellfish Immun.* 102, 286–295. doi: 10.1016/j.fsi.2020.04.036

Bao, X., and Wu, J. (2021). Impact of food-derived bioactive peptides on gut function and health. *Food Res. Int.* 147, 110485. doi: 10.1016/j.foodres.2021.110485

Berger, K., Burleigh, S., Lindahl, M., Bhattacharya, A., Patil, P., St?lbrand, H., et al. (2021). Xylooligosaccharides increase bifidobacteria and lachnospiraceae in mice on a high-fat diet, with a concomitant increase in short-chain fatty acids, especially butyric acid. *J. Agr. Food Chem.* 69, 3617–3625. doi: 10.1021/acs.jafc.0c0627910.1021/acs.jafc.0c06279

Bruininx, E. M. A. M., Van Der Peet-Schwering, C. M. C., Schrama, J. W., Vereijken, P. F. G., Vesseur, P. C., Everts, H., et al. (2001). Individually measured feed intake characteristics and growth performance of group-housed weanling pigs: effects of sex, initial body weight, and body weight distribution within groups. *J. Anim. Sci.*79, 301–308. doi: 10.2527/2001.792301x

Campbell, J. M., Crenshaw, J. D., and Polo, J. (2013). The biological stress of early weaned piglets. *J. Anim. Sci. Biotechno.* 4, 1–4. doi: 10.1186/2049-1891-4-19

Caporaso, J. G., Kuczynski, J., Stombaugh, J., Bittinger, K., Bushman, F. D., Costello, E. K., et al. (2010). QIIME allows analysis of high-throughput community sequencing data. *Nat. Methods* 7, 335–336. doi: 10.1038/nmeth.f.303

Castillo, M., Martínorúe, S. M., Taylorpickard, J. A., Pérez, J. F., and Gasa, J. (2008). Use of mannanoligosaccharides and zinc chelate as growth promoters and diarrhea preventative in weaning pigs: effects on microbiota and gut function. *J. Anim. Sci.* 86, 94–101. doi: 10.2527/jas.2005-686

Cui, K., Diao, Q., and Zhang, N. (2019). Effects of dietary supplementation with Bacillus subtilis and yeast culture on growth performance, nutrient digestibility, serum indices and faeces microbiota of weaned piglets. *J. Anim. Feed. Sci.* 28, 328–336. doi: 10.22358/jafs/114238/2019

Dávila-Ramírez, J. L., Carvajal-Nolazco, M. R., López-Millanes, M. J., González-Ríos, H., Celaya-Michel, H., Sosa-Castañeda, J., et al. (2020). Effect of yeast culture (*Saccharomyces cerevisiae*) supplementation on growth performance, blood metabolites, carcass traits, quality, and sensorial traits of meat from pigs under heat stress. *J. Anim. Feed. Sci.* 267, 114573. doi: 10.1016/j.anifeedsci.2020.114573

De Los Santos, F. S., Donoghue, A. M., Farnell, M. B., Huff, G. R., Huff, W. E., and Donoghue, D. J. (2007). Gastrointestinal maturation is accelerated in turkey poults supplemented with a mannan-oligosaccharide yeast extract (Alphamune). *Poult. Sci.* 86, 921–930. doi: 10.1093/ps/86.5.921

Dowarah, R., Verma, A. K., and Agarwal, N. (2017). The use of Lactobacillus as an alternative of antibiotic growth promoters in pigs: a review. *Anim. Nutr.* 3, 1–6. doi: 10.1016/j.aninu.2016.11.002

Elwan, H. A. M., Elnesr, S. S., Abdallah, Y., Hamdy, A., and El-Bogdady, A. H. (2018). Red yeast (*Phaffia rhodozyma*) as a source of astaxanthin and its impacts on productive performance and physiological responses of poultry. *World Poultry Sci. J.* 75, 273–284. doi: 10.1017/S0043933919000187

Fattman, C. L., Schaefer, L. M., and Oury, T. D. (2003). Extracellular superoxide dismutase in biology and medicine. *Free Radical Bio. Med.* 35, 236–256. doi: 10.1016/S0891-5849(03)00275-2

Fouhse, J. M., Zijlstra, R. T., and Willing, B. P. (2016). The role of gut microbiota in the health and disease of pigs. *Anim. Front.* 6, 30–36. doi: 10.2527/af.2016-0031

Gang, L., Lei, Y., Martínez, Y., Wenkai, R., Hengjia, N., Naif, A. D., et al. (2017). Dietary saccharomyces cerevisiae cell wall extract supplementation alleviates oxidative stress and modulates serum amino acids profiles in weaned piglets. *Oxid. Med. Cell. Longev.* 3, 1–7. doi: 10.1155/2017/3967439

Gao, J., Zhang, H. J., Yu, S. H., Wu, S. G., Yoon, I., Quigley, J., et al. (2008). Effects of yeast culture in broiler diets on performance and immunomodulatory functions. *Poult. Sci.* 87, 1377–1384. doi: 10.3382/ps.2007-00418

Gao, X., Yu, B., Yu, J., Mao, X., Huang, Z., Luo, Y., et al. (2022). Developmental profiling of dietary carbohydrate digestion in piglets. *Front. Microbiol.* 13, 896660. doi: 10.3389/fmicb.2022.896660

Ge, Y., Huang, K., Xie, W., Xu, C., Yao, Q., and Liu, Y. (2021). Effects of *Rhodotorula mucilaginosa* on the immune function and gut microbiota of mice. *Fronti. Fungal Biol.* 2, 705696. doi: 10.3389/ffunb.2021.705696

Giang, H. H., Viet, T. Q., Ogle, B., and Lindberg, J. E. (2012). Growth performance, digestibility, gut environment and health status in weaned piglets fed a diet supplemented with a complex of lactic acid bacteria alone or in combination with Bacillus subtilis and Saccharomyces boulardii. *Livest. Sci.* 143, 132–141. doi: 10.1016/j.livsci.2011.09.003

Haddad, S. G., and Goussous, S. N. (2005). Effect of yeast culture supplementation on nutrient intake, digestibility and growth performance of Awassi lambs. *Anim. Feed Sci. Tech.* 118, 343–348 doi: 10.1016/j.anifeedsci.2004.10.003

Hampson, D. J. (1986). Alterations in piglet small intestinal structure at weaning. *Res. Vet. Sci.* 40, 32–40. doi: 10.1016/S0034-5288(18)30482-X

Hardo, T., Brotosudarmo, P., Limantara, L., and Setiyono, E. (2020). Review article structures of astaxanthin and their consequences for therapeutic application. *Int. J. Food Sci.* 10, 1–16. doi: 10.1155/2020/2156582

He, W., Gao, Y., Guo, Z., Yang, Z., Wang, X., Liu, H., et al. (2021). Effects of fermented wheat bran and yeast culture on growth performance, immunity, and intestinal microflora in growing-finishing pigs. *J. Anim. Sci.* 9, skab308. doi: 10.1093/jas/skab308

Hu, J., Lu, W., Wang, C., Zhu, R., and Qiao, J. (2008). Characteristics of solid-state fermented feed and its effects on performance and nutrient digestibility in growing-finishing pigs. *Asian. Austral. J. Anim.* 21, 1635–1641. doi: 10.5713/ajas.2008.80032

Hu, R., He, Z., Liu, M., Tan, J., Zhang, H., and Hou, D. X. (2020). Dietary protocatechuic acid ameliorates inflammation and up-regulates intestinal tight junction proteins by modulating gut microbiota in LPS-challenged piglets. *J. Anim. Sci. Biotechn.* 11, 1–12. doi: 10.1186/s40104-020-00492-9

Hu, Y., Dun, Y., Li, S., Zhang, D., Peng, N., Zhao, S., et al. (2015). Dietary Enterococcus faecalis LAB31 improves growth performance, reduces diarrhea, and increases fecal Lactobacillus number of weaned piglets. *PLoS ONE* 10, e0116635. doi: 10.1371/journal.pone.0116635

La Ragione, R. M., Narbad, A., Gasson, M. J., and Woodward, M. J. (2004). *In vivo* characterization of *Lactobacillus johnsonii* FI9785 for use as a defined competitive exclusion agent against bacterial pathogens in poultry. *Lett. Appl. Microbiol.* 38, 197–205. doi: 10.1111/j.1472-765X.2004.01474.x

Lee, D. J., Liu, X., Sun, H. Y., Park, J. W., and Kim, I. H. (2018). Effects of yeast culture (*Saccharomyces cerevisiae*) supplementation on growth performance, fecal score, and nutrient digestibility of weaning pigs. *J. Anim. Sci.* 96, 48–49. doi: 10.1093/jas/sky073.091

Lei, Y., and Kim, I. H. (2014). Effect of *Phaffia rhodozyma* on performance, nutrient digestibility, blood characteristics, and meat quality in finishing pigs. *J. Anim. Sci.* 92, 171–176. doi: 10.2527/jas.2013-6749

Li, J., Sun, W., Ramaswamy, H. S., Yu, Y., Zhu, S., Wang, J., et al. (2017). High pressure extraction of astaxanthin from shrimp waste (Penaeus Vannamei Boone): effect on yield and antioxidant activity. *J. Food. Process. Eng.* 40, e12353. doi: 10.1111/jfpe.12353

Lin, Y., Zhao, W., Shi, Z. D., Gu, H. R., Zhang, X. T., Ji, X., et al. (2017). Accumulation of antibiotics and heavy metals in meat duck deep litter and their role in persistence of antibiotic-resistant *Escherichia coli* in different flocks on one duck farm. *Poult. Sci.* 96, 997–1006. doi: 10.3382/ps/pew368

Long, S. F., Xu, Y. T., Pan, L., Wang, Q. Q., Wang, C. L., Wu, J. Y., et al. (2018). Mixed organic acids as antibiotic substitutes improve performance, serum immunity, intestinal morphology and microbiota for weaned piglets. *Anim. Feed. Sci. Tech.* 235, 23–32. doi: 10.1016/j.anifeedsci.2017.08.018

Ma, J., Piao, X., Shang, Q., Long, S., Liu, S., and Mahfuz, S. (2021). Mixed organic acids as an alternative to antibiotics improve serum biochemical parameters and intestinal health of weaned piglets. *Anim. Nutr.* 7, 737–749. doi: 10.1016/j.aninu.2020.11.018

Matrosova, I. V., and Politaeva, A. A. (2021). Red yeast rhodotorula benthica – substitute feed base for echinoderms in factory cultivation. *E3S Web of Conf.* 265, 05010. doi: 10.1051/e3sconf/202126505010

Montagne, L., Cavaney, F. S., Hampson, D. J., Lallés, J. P., and Pluske, J. R. (2004). Effect of diet composition on postweaning colibacillosis in piglets. *J. Anim. Sci.* 82, 2364–2374. doi: 10.2527/2004.8282364x

Neuman, H., Forsythe, P., Uzan, A., Avni, O., and Koren, O. (2018). Antibiotics in early life: dysbiosis and the damage done. *FEMS Microbiol. Rev.* 42, 489–499. doi: 10.1093/femsre/fuy018

NRC (2012). *Nutrient Requirements of Swine. 11th rev. Edn.* Washington, DC: Natl. Acad. Press.

Oh, J. K., Vasquez, R., Kim, S. H., Hwang, I. C., Song, J. H., Park, J. H., et al. (2021). Multispecies probiotics alter fecal short-chain fatty acids and lactate levels in weaned pigs by modulating gut microbiota. *J. Anim. Sci. Tech.* 63, 1142–1158. doi: 10.5187/jast.2021.e94

Oliver, W. T., and Wells, J. E. (2013). Lysozyme as an alternative to antibiotics improves growth performance and small intestinal morphology in nursery pigs. *J. Anim. Sci*. 91, 3129–3136. doi: 10.2527/jas.2012-5782

Pluske, J. R., Hampson, D. J., and Williams, I. H. (1997). Factors influencing the structure and function of the small intestine in the weaned pig: a review. *Livest. Prod. Sci*. 51, 215–236. doi: 10.1016/S0301-6226(97)00057-2

Pluske, J. R., Williams, I. H., and Aherne, F. X. (1996). Villous height and crypt depth in piglets in response to increases in the intake of cows' milk after weaning. *Anim. Sci*. 62, 145–158. doi: 10.1017/S1357729800014429

Primec, M., Klemenak, M., Di Gioia, D., Aloisio, I., Cionci, N. B., Quagliariello, A., et al. (2019). Clinical intervention using Bifidobacterium strains in celiac disease children reveals novel microbial modulators of TNF-α and short-chain fatty acids. *Clin. Nutr*. 38, 1373–1381. doi: 10.1016/j.clnu.2018.06.931

Reisinger, N., Ganner, A., Masching, S., Schatzmayr, G., and Applegate, T. J. (2012). Efficacy of a yeast derivative on broiler performance, intestinalmorphology and blood profile. *Livest. Sci*.143, 195–200. doi: 10.1016/j.livsci.2011.09.013

Ren, H., Su, X., Bai, H., Yang, Y., Wang, H., and Dan, Z. (2019). Specific enrichment of microbes and increased ruminal propionate production: the potential mechanism underlying the high energy efficiency of Holstein heifers fed steam-flaked corn. *Amb. Express*. 9, 1–11. doi: 10.1186/s13568-019-0937-8

Ryazanov, V. A., Duskaev, G. K., Rakhmatullin, S. G., Miroshnikov, I. S., Miroshnikova, K. P., and Inchagova, K. S. (2021). Application of new technologies to assess the effectiveness of feed materials for ruminants. *IOP Conf. Ser. Earth Environ. Sci*. 624, 012049. doi: 10.1088/1755-1315/624/1/012049

Saied, J. M., Al-Jabary, Q. H., and Thalij, K. M. (2011). Effect of dietary supplement yeast culture on production performance and hematological parameters in broiler chicks. *Int. J. Poult. Sci*. 10, 376–380. doi: 10.3923/ijps.2011.376.380

Segata, N., Izard, J., Waldron, L., Gevers, D., Miropolsky, L., Garrett, W. S., et al. (2011). Metagenomic biomarker discovery and explanation. *Genome Biol*. 12, 1–18. doi: 10.1186/gb-2011-12-6-r60

Shuai, C., Bie, T., Xia, Y., Liao, S., Wang, M., and Jie, Y., et al. (2019). Effects of dietary gamma-aminobutyric acid supplementation on the intestinal functions in weaning piglets. *Food Funct*. 10, 366–378. doi: 10.1039/C8FO02161A

Sims, M. D., Dawson, K. A., Newman, K. E., Spring, P., and Hoogell, D. M. (2004). Effects of dietary mannan oligosaccharide, bacitracin methylene disalicylate, or both on the live performance and intestinal microbiology of turkeys. *Poult. Sci*. 83, 1148–1154. doi: 10.1093/ps/83.7.1148

Song, B., Wu, T., You, P., Wang, H., Burke, J. L., Kang, K., et al. (2021). Dietary supplementation of yeast culture into pelleted total mixed rations improves the growth performance of fattening lambs. *Front. Vet. Sci*. 8, 387. doi: 10.3389/fvets.2021.657816

Sun, J., Li, M., Tang, Z., Zhang, X., Chen, J., and Sun, Z. (2020). Effects of Rhodotorula mucilaginosa fermentation product on the laying performance, egg quality, jejunal mucosal morphology and intestinal microbiota of hens. *J. Appl. Microbiol*. 128, 54–64. doi: 10.1111/jam.14467

Tang, Z., Yin, Y., Zhang, Y., Huang, R., Sun, Z., Li, T., et al. (2008). Effects of dietary supplementation with an expressed fusion peptide bovine lactoferricin–lactoferrampin on performance, immune function and intestinal mucosal morphology in piglets weaned at age 21 d. *Brit. J. Nutr*. 101, 998–1005. doi: 10.1017/S0007114508055633

Tian, D., Xu, X., Peng, Q., Wen, Z., Zhang, Y., Wei, C., et al. (2019). In vitro fermentation of arabinoxylan from oat (*Avena sativa* L.) by Pekin duck intestinal microbiota. *3 Biotech*.9, 1–12. doi: 10.1007/s13205-019-1571-5

Upadhaya, S. D., Lee, K. Y., Serpunja, S., Song, T. H., and Kim, I. H. (2018). Growth performance, nutrient digestibility, fecal microbiota and fecal noxious gas emission in weaning pigs fed high and low density diet with and without protected organic acid blends. *Anim. Feed Sci. Tech*. 239, 1–8. doi: 10.1016/j.anifeedsci.2017.12.013

Wang, C. Y., Liu, S., Xie, X. N., and Tan, Z. R. (2017). Regulation profile of the intestinal peptide transporter 1 (PepT1). *Drug Des. Dev. Ther* 11, 3511. doi: 10.2147/DDDT.S151725

Wang, J. H., Zhao, L. Q., Liu, J. F., Wang, H., and Xiao, S. (2015). Effect of potential probiotic *Rhodotorula benthica* D30 on the growth performance, digestive enzyme activity and immunity in juvenile sea cucumber *Apostichopus japonicus*. *Fish Shellfish Immun*. 43, 330–336. doi: 10.1016/j.fsi.2014.12.028

Wang, L., Xie, J., Wu, W., Li, B., and Ou, J. (2018). Excellent microbial cultivation for astaxanthin production and its extraction by *Rhodotorula benthica*. *Med. Res*. 2, 180015. doi: 10.21127/yaoyimr20180015

Wang, T., Teng, K., Liu, Y., Shi, W., Zhang, J., Dong, E., et al. (2019). *Lactobacillus plantarum* PFM 105 promotes intestinal development through modulation of gut microbiota in weaning piglets. *Front. Microbiol*. 10, 90. doi: 10.3389/fmicb.2019.00090

Yuan, S. B., Chen, D. W., Zhang, K. Y., and Yu, B. (2007). Effects of oxidative stress on growth performance, nutrient digestibilities and activities of antioxidative enzymes of weanling pigs. *Asian. Austral. J. Anim*. 20, 1600–1605. doi: 10.5713/ajas.2007.1600

Yun, L., Wang, W., Li, Y., Xie, M., Chen, T., Hu, C., et al. (2021). Potential application values of a marine red yeast, Rhodosporidiums sphaerocarpum YLY01, in aquaculture and tail water treatment assessed by the removal of ammonia nitrogen, the inhibition to *Vibrio* spp., and nutrient composition. *PLoS ONE*. 16, e0246841. doi: 10.1371/journal.pone.0246841

Zeitz, J. O., Kaltenböck, S., Most, E., and Eder, K. (2017). Antioxidant status and expression of inflammatory genes in gut and liver of piglets fed different dietary methionine concentrations. *J. Anim. Physiolo. An. N.* 101, 1166–1174. doi: 10.1111/jpn.12633

Zeng, P. L., Yan, H. C., Wang, X. Q., Zhang, C. M., Zhu, C., Shu, G., et al. (2013). Effects of dietary lysine levels on apparent nutrient digestibility and serum amino acid absorption mode in growing pigs. *Asian. Austral. J. Anim*. 26, 1003. doi: 10.5713/ajas.2012.12555

Zhang, A. W., Lee, B. D., Lee, S. K., Lee, K. W., An, G. H., Song, K. B., et al. (2005). Effects of yeast (*Saccharomyces cerevisiae*) cell components on growth performance, meat quality, and ileal mucosa development of broiler chicks. *Poultry Sci*. 84, 1015–1021. doi: 10.1093/ps/84.7.1015

Zhang, D., Ji, H., Liu, H., Wang, S., Wang, J., and Wang, Y. (2016). Changes in the diversity and composition of gut microbiota of weaned piglets after oral administration of Lactobacillus or an antibiotic. *Appl. Microbiol. Biot*. 100, 10081–10093. doi: 10.1007/s00253-016-7845-5

Zhang, J. Y., Park, J. W., and Kim, I. H. (2019). Effect of supplementation with brewer's yeast hydrolysate on growth performance, nutrients digestibility, blood profiles and meat quality in growing to finishing pigs. *Asian. Austral. J. Anim*. 32, 1565. doi: 10.5713/ajas.18.0837

Zhang, L., Loh, K. C., Dai, Y., and Tong, Y. W. (2020). Acidogenic fermentation of food waste for production of volatile fatty acids: bacterial community analysis and semi-continuous operation. *Waste Manage*. 109, 75–84. doi: 10.1016/j.wasman.2020.04.052

Zhang, Q., Li, J., Cao, M., Li, Y., Zhuo, Y., Fang, Z., et al. (2020). Dietary supplementation of Bacillus subtilis PB6 improves sow reproductive performance and reduces piglet birth intervals. *Anim. Nutr*. 6, 278–287. doi: 10.1016/j.aninu.2020.04.002

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# Comparison of rumen bacterial communities between yaks (*Bos grunniens*) and Qaidam cattle (*Bos taurus*) fed a low protein diet with different energy levels

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The yak (*Bos grunniens*), an indigenous bovine on the Qinghai-Tibetan plateau (QTP), is reported to digest low quality forage to a greater extent and to require less protein and energy for maintenance than the introduced Qaidam cattle (*Bos taurus*). Ruminal bacteria play a major role in feed degradation, and therefore, we hypothesized that ruminal bacteria composition would differ between yaks and cattle, and confer an advantage to yaks for poor quality diets. To test our hypothesis, we determined the ruminal bacteria profiles, rumen fermentation parameters, and enzyme activities in these bovine species consuming a low-protein diet differing in energy level. Six castrated yaks (155 ± 5.8 kg) and 6 castrated Qaidam cattle (154 ± 8.0 kg) were used in two concurrent 4 × 4 Latin square designs with 2 additional animals of each species in each period. The animals were offered a low-protein diet of 70.4 g/kg dry matter (DM) and one of four metabolizable energy levels, namely 6.62, 8.02, 9.42, and 10.80 MJ/kg. Ruminal pH, concentrations of ammonia-N and total volatile fatty acids (VFAs), the molar proportion of acetate, and the ratio of acetate to propionate (A:P) were greater ($P < 0.05$), whereas the molar proportion of propionate was lesser ($P = 0.043$) in yaks than in cattle. With increasing dietary energy level, ruminal pH, the molar proportion of acetate and the ratio of A:P decreased linearly ($P < 0.05$), whereas, the concentration of total VFAs, molar proportions of propionate, butyrate, iso-butyrate, and iso-valerate and concentration of ammonia-N increased linearly ($P < 0.05$). The relative abundance (RA) of Firmicutes increased linearly ($P < 0.01$), whereas, the RA of Bacteroidetes decreased linearly

($P < 0.001$) with increasing energy level in both bovine species. The RAs of *Prevotella* and *Rikenellaceae_RC9_gut_group* decreased linearly ($P < 0.05$) with increasing energy level in both yaks and cattle. The RAs of fibrolytic (e.g., *Rikenellaceae_RC9_gut_group*), and $H_2$-incorporating (e.g., *Quinella*) bacteria were greater ($P < 0.05$) in yaks than in cattle. We concluded that the two bovines differ in ruminal bacterial profiles and rumen fermentation parameters, and confer an advantage to yaks over cattle in consuming a low protein diet with differing energy level.

KEYWORDS

**yaks, rumen bacterial community, Qaidam cattle, low protein diet, energy levels**

## Introduction

The high-altitude Qinghai-Tibetan Plateau (QTP), known as "the roof of the world," originated about 40 million years ago. The plateau is characterized by prolonged cold weather, strong ultraviolet radiation, heavy winds, and low air oxygen content. There is a short forage growing season of 120 days, with very sparse pasture of poor quality over the long winter. Yaks (*Bos grunniens*) were domesticated by the ancient Qiang people approximately 7300 years ago (Qiu et al., 2012), and are well-adapted to the extreme conditions of the QTP. There are currently more than 16 million domestic yaks worldwide, of which 15.2 million are raised on the QTP (Zhang et al., 2015). The yak has been closely linked with human civilization and daily life, providing meat, milk, leather, and dung, as well as transportation. Qaidam cattle (*Bos taurus*) were introduced to the QTP by Tibetans approximately 1700 years ago (China National Commission of Animal Genetic Resources, 2011; Chen et al., 2018), and the current population is close to 10,000. The yaks graze alpine grassland at altitudes between 3,000 and 6,000 m above sea level (a. s. l.) all year round without supplements. During the long, cold winter, yaks mobilize their body reserves as energy intake is insufficient, and lose substantial body weight. Cattle are raised in agro-pastoral transition zones at altitudes between 2,600 and 3,600 m a. s. l. and require supplements and shelter at night in winter. The two bovine species co-graze over their overlapping ranges.

Although yaks and Qaidam cattle are both capable of subsisting on high-fiber diets, it has been reported that yaks are less discriminate herbivores, digest fiber to a greater extent, and produce more volatile fatty acids (VFAs) and microbial protein than cattle when fed the same diet (Zhou et al., 2018; Liu et al., 2022). These differences between bovine species suggest they differ in their rumen microbial communities (Huang et al., 2012; Zhang et al., 2016; Huang X. D. et al., 2021), that enable yaks to cope with a low-quality diet better than cattle. It was reported that the CAZymes (degradation carbodydrates) and fibrolytic activities were greater in yaks than in cattle (Guo, 2021). Yaks also had greater relative abundances (RAs) of Thermoplamatales-affiliated Linage C (TALC), *Christensenellaceae_R7_group*, and *Lachnospiraceae_UCG_008 group* but lesser RAs of *Prevotella* and *Succiniclasticum* than cattle (Huang et al., 2012; Xin et al., 2019). We hypothesized that the ruminal bacterial profiles and fermentation parameters would differ between the two bovine species when fed different quality diets. To test this hypothesis, we determined the rumen bacterial profiles, and rumen fermentation parameters of yaks and cattle when fed a low protein diet differing in energy levels.

## Materials and methods

This experiment was carried out at Wushaoling Yak Research Facility of Lanzhou University (102°51.7'E, 37°12.4'N, altitude 3,154 m. a. s. l.), situated in the northeastern part of the QTP, Tianzhu Tibetan Autonomous County, Wuwei City, Gansu Province, China.

### Animals, design, and diets

Six yaks (155 ± 5.8 kg) and 6 Qaidam cattle (154 ± 8.0 kg), all castrated males and 2.5 years of age, were held individually in metabolic cages (2.2 m × 1.0 m). The experiment was a 2 (genotypes) × 4 (energy levels) factorial arrangements and conducted as two concurrent 4 × 4 Latin square designs balanced for carry-over effects, with 2 additional yaks and cattle in each period. Treatment sequences for the two additional animals of each species were selected randomly from columns of a separate Latin square (Bailey et al., 2012). In each period of 28 days, 4 yaks and 4 cattle received one of 4 diets differing in energy level, and the two additional animals of each species received one of the four diets (Sarraseca et al., 1998). The six animals of each species consumed all four dietary treatments.

To ensure each animal consumed all the feed offered daily, the least voluntary intake was determined before the study and offered throughout the feeding trial. Therefore, 2.75 kg/d

DM per animal of pellets (total mixed ration; Gansu Runmu Biological Engineering Co., Ltd, Jinchang, China) were offered in equal portions at 08:00 and 18:00 with water freely available. The diets were formulated to be isonitrogenous with low crude protein (CP, 74 g/kg DM), but with incremental levels of metabolizable energy (ME): 6.62, 8.02, 9.42, and 10.80 MJ/kg (**Table 1**; 14). The dietary CP content was lower than the recommendation of NRC (National Research Council, 2000) for beef cattle, but was similar to the average CP content of forage in on the QTP during the cold season (Xie et al., 1996). The diets provided 0.8, 0.9, 1.0, and 1.1 times maintenance energy requirement of growing beef cattle according to NRC (National Research Council, 2000).

TABLE 1 Ingredients and chemical composition of experimental diets.

| Items | Dietary ME level, MJ/kg DM | | | |
|---|---|---|---|---|
| | 6.62 | 8.02 | 9.42 | 10.80 |
| **Ingredient, g/kg DM** | | | | |
| Corn straw[1] | 850 | 700 | 550 | 400 |
| Corn grain, ground[2] | 46.0 | 117 | 180 | 260 |
| Corn husk | 11.0 | 55.0 | 110 | 154 |
| Cotton meal | 27.0 | 24.3 | 21.0 | 18.5 |
| Soybean meal | 27.0 | 24.0 | 21.0 | 18.0 |
| Wheat bran | 24.0 | 21.0 | 16.0 | 7.00 |
| Corn starch | – | 40.0 | 72.0 | 104 |
| Rumen bypass palm oil | – | 4.00 | 13.0 | 20.5 |
| Calcium hydrophosphate | – | – | 2.00 | 3.00 |
| Sodium chloride | 10.0 | 10.0 | 10.0 | 10.0 |
| Commercial premix[3] | 5.00 | 5.00 | 5.00 | 5.00 |
| **Chemical compositions, g/kg DM** | | | | |
| DM, g/kg as-fed | 973 | 969 | 966 | 934 |
| OM | 864 | 885 | 902 | 901 |
| CP[4] | 74.5 | 74.4 | 74.4 | 74.3 |
| aNDF | 604 | 544 | 486 | 422 |
| ADF | 322 | 276 | 232 | 186 |
| Ether extract | 55.7 | 66.8 | 80.3 | 91.6 |
| Calcium | 8.70 | 8.50 | 7.90 | 7.00 |
| Phosphorus | 2.40 | 2.30 | 2.40 | 2.40 |
| Gross energy, MJ/kg | 16.0 | 16.4 | 16.9 | 17.2 |
| ME, MJ/kg[5] | 6.62 | 8.02 | 9.42 | 10.80 |

DM, dry matter; OM, organic matter; CP, crude protein; ME, metabolizable energy; aNDF, neutral detergent fiber; ADF, acid detergent fiber.

[1]The DM, CP, EE, NDF, ADF, and Ash of corn straw were 800, 50, 12, 700, 440, and 70 g/kg DM, respectively.

[2]DM, CP, EE, NDF, ADF, and Ash of corn grain were 860, 80, 36, 99, 31, and 12 g/kg DM, respectively.

[3]The premix provided the following per kg of premix: Vitamin A 3 000 000 IU, Vitamin D 375 000 IU, Vitamin E 220 IU, biotin 12 mg, lysine 13 000 mg, Cu (as copper sulfate) 1 200 mg, Fe (as ferrous sulfate) 3 000 mg, Mn (as manganese sulfate) 2 000 mg, Zn (as zinc sulfate) 4 000 mg, I (as potassium iodide) 15 mg, Se (as sodium selenite) 20 mg.

[4]CP calculated as 6.25 × N content.

[5]The ME was calculated according to the Tables of Feed Composition and Nutritive Values in China (Xiong et al., 2018).

## Procedures and sample collection

In each period, approximately 100 g of each diet were collected daily from d 23 to 28. Two h after morning feed was offered on d 28, an oral stomach tube (Anscitech Co. Ltd., Wuhan, China) was used to collect 150 mL of rumen fluid. The first 50 mL of fluid were discarded to avoid saliva contamination. The pH of the rumen fluid was measured immediately using a pH meter (PB-10, Sartorius Co., Göttingen, Germany), and then the fluid was filtered through 4 layers of cheesecloth. Five mL of filtrate were mixed with 5 mL of deproteinizing solution (100 g metaphosphoric acid and 0.6 g crotonic acid per liter) for VFAs analysis; 5 mL of filtrate were mixed with 5 mL hydrochloric acid solution (0.5 mmol/L) for ammonia N measurement; and the rest was stored at −80°C for analyzing bacterial community and enzyme the activity.

## Feed analysis

Feed samples were dried at 65°C in a forced air oven for 72 h, and ground through a 1-mm sieve (JFSO-100, Topu Yunnong Instrument, Hangzhou, China). The DM (method 925.45), organic matter (method 942.05), and ether extract (method 920.29) were determined according to the Association of Official Analytical Chemists (AOAC, AOAC, 2006). Total nitrogen content was determined using a nitrogen analyzer (K1000, Hannon Instruments, Jinan, China), and CP was calculated as total N × 6.25. Neutral detergent fiber (aNDF) and acid detergent fiber (ADF) were determined by an automatic fiber analyzer (Ankom Technology, Fairport, NY, United States) according to Robertson and Van Soest (1981) and Van Soest et al. (1991), respectively. Ten g of sodium sulfate per aNDF solution and 0.5 g heat-stable α-amylase per sample were added to the aNDF solution. The ADF was determined on the residue of the aNDF. Gross energy of feed was determined by bomb calorimetry (6400 Calorimeter, Parr Instrument Company, Moline, Illinois, United States).

## Rumen fermentation parameters

The VFAs concentration of rumen fluids was determined by gas chromatography (GC) with a capillary column (AT-FFAP: 30 m × 0.32 mm × 0.5 μm) using a Shimadzu 2010 plus system (Shimadzu Corporation, Kyoto, Japan) following Liu et al. (2021b). Ammonia-N concentration was analyzed using a spectrometer (SpectraMax M5, Molecular Devices, San Jose, United States) at an absorbance of 630 nm, following Hristov et al. (2001).

Ruminal enzyme activities of carboxymethylcellulase (CMCase), amylase, xylanase, and pectinase were measured by commercial enzyme-linked immune sorbent assay (ELISA) kits

(product No: BYE98162, BYE98329, BYE98332, and BYE99021, respectively; Shanghai Bangyi Biological Technology Co. Ltd., Shanghai, China). The rumen fluid was centrifuged at 800 × g for 5 min at 4°C, and the supernatant was shaken ultrasonically for 3 min. The enzyme activities were determined using a spectrometer (SpectraMax M5, Molecular Devices, San Jose, United States) at an absorbance of 450 nm.

## DNA extraction, 16S rRNA gene amplification, and sequencing

After the rumen fluid was thawed on ice, total genomic DNA of rumen bacteria was extracted from 1 mL by using the E.Z.N.A® kit (Omega Bio-tek, Norcross, GA, United States), and following the manufacturer's instructions. The concentration and the purity of the extracted DNA were determined by the 260/280 nm ratio (1.8 to 2.2) using a NanoDrop 2000 UV-vis Spectrophotometer, (Thermo Scientific, Wilmington, DE, United States). The quality of the extracted DNA was tested using 1% agarose gel electrophoresis (Axygen Biosciences, Union City, CA, United States). The extracted DNA samples were stored in sterile centrifuge tubes (Thomas Scientific, Wilmington, DE, United States) at −80°C for later analysis.

The conventional polymerase chain reaction (PCR) amplification and bioinformatic analysis of extracted DNA samples were done by Shanghai Majorbio Bio-Pharm Technology Co., Ltd (Shanghai, China). The hypervariable

TABLE 2 Ruminal fermentation parameters and enzyme activities in yaks and cattle offered diets of different energy levels.

| Items | Species | Dietary ME level, MJ/kg DM[1] | | | | SEM | *P-value*[2] | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | 6.62 | 8.02 | 9.42 | 10.80 | | S | E | S × E | E-L | E-Q |
| pH | Yak | 7.20 | 7.02 | 6.84 | 6.76 | 0.090 | 0.036 | < 0.001 | 0.897 | < 0.001 | 0.412 |
| | Cattle | 7.02 | 6.86 | 6.70 | 6.64 | | | | | | |
| Ammonia-N mg/100mL | Yak | 3.93 | 4.89 | 6.46 | 7.14 | 0.200 | 0.041 | < 0.01 | 0.331 | < 0.01 | 0.210 |
| | Cattle | 3.43 | 4.47 | 6.10 | 6.33 | | | | | | |
| Total VFA, mM | Yak | 70.0 | 77.1 | 81.9 | 85.5 | 1.04 | < 0.001 | <0.001 | 0.330 | < 0.01 | <0.001 |
| | Cattle | 67.0 | 73.4 | 76.9 | 80.2 | | | | | | |
| **VFA, mol/100mol** | | | | | | | | | | | |
| Acetate (A) | Yak | 76.1 | 74.1 | 72.7 | 70.8 | 0.53 | 0.049 | < 0.001 | 0.686 | < 0.001 | 0.245 |
| | Cattle | 74.8 | 73.5 | 71.8 | 70.2 | | | | | | |
| Propionate (P) | Yak | 13.9 | 15.0 | 15.7 | 16.4 | 0.13 | 0.043 | < 0.001 | 0.673 | < 0.001 | 0.451 |
| | Cattle | 14.6 | 15.1 | 15.8 | 17.0 | | | | | | |
| Butyrate | Yak | 8.80 | 9.60 | 9.90 | 11.2 | 0.201 | 0.110 | < 0.001 | 0.264 | < 0.001 | 0.147 |
| | Cattle | 9.10 | 9.80 | 10.7 | 11.1 | | | | | | |
| Iso-butyrate | Yak | 0.37 | 0.46 | 0.51 | 0.51 | 0.052 | 0.092 | 0.013 | 0.586 | < 0.01 | 0.250 |
| | Cattle | 0.50 | 0.50 | 0.57 | 0.60 | | | | | | |
| Valerate | Yak | 0.41 | 0.44 | 0.55 | 0.56 | 0.049 | 0.904 | 0.167 | 0.261 | 0.070 | 0.302 |
| | Cattle | 0.48 | 0.50 | 0.47 | 0.51 | | | | | | |
| Iso-valerate | Yak | 0.41 | 0.45 | 0.60 | 0.61 | 0.067 | 0.148 | 0.018 | 0.791 | < 0.01 | 0.881 |
| | Cattle | 0.53 | 0.54 | 0.66 | 0.68 | | | | | | |
| A:P | Yak | 5.51 | 4.94 | 4.61 | 4.33 | 0.083 | 0.048 | < 0.001 | 0.568 | < 0.001 | 0.131 |
| | Cattle | 5.14 | 4.88 | 4.57 | 4.21 | | | | | | |
| **Digestive enzymes activities μmol $mL^{-1}$ $min^{-1}$** | | | | | | | | | | | |
| CMCase | Yak | 0.33 | 0.35 | 0.35 | 0.32 | 0.027 | 0.586 | 0.271 | 0.742 | 0.398 | 0.150 |
| | Cattle | 0.32 | 0.32 | 0.35 | 0.30 | | | | | | |
| Amylase | Yak | 9.50 | 13.4 | 13.4 | 8.70 | 0.980 | 0.938 | 0.283 | 0.512 | 0.567 | 0.167 |
| | Cattle | 10.7 | 11.5 | 12.4 | 10.4 | | | | | | |
| Xylanase | Yak | 1.75 | 1.73 | 1.64 | 1.51 | 0.160 | 0.369 | 0.912 | 0.581 | 0.235 | 0.894 |
| | Cattle | 1.62 | 1.52 | 1.51 | 1.48 | | | | | | |
| Pectinase | Yak | 1.97 | 1.98 | 1.94 | 2.12 | 0.150 | 0.752 | 0.891 | 0.492 | 0.523 | 0.647 |
| | Cattle | 1.92 | 1.9 | 1.93 | 2.05 | | | | | | |

SEM, standard error of the means; ME, metabolizable energy; VFA, volatile fatty acids.
[1]n, 6 for each group.
[2]S, animal species; E, dietary energy level; E-L, Linear effect of dietary energy levels; E-Q, Quadratic effect of dietary energy levels.

V3-V4 region of the bacterial 16S rDNA gene was amplified using primers pair 338F (5′-ACTCCTACGGGAGGCAGCAG-3′) and 806R (5′-GGACTAC HVGGGTWTCTAAT-3′). The bacterial 16S amplification and the quality-filter, cluster, and analysis of 16S rRNA sequencing data followed Liu et al. (2019). The reaction conditions and procedures of PCR amplification of the 16S rRNA gene were as follows: initial denaturation at 95°C for 3 min, followed by 27 cycles of denaturing at 95°C for 30 s, annealing at 55°C for 30 s and extension at 72°C for 45 s, and a final single extension at 72°C for 10 min. The PCR mixtures were prepared in triplicate 20 μL volumes which consisted of 4 μL of 5 × TransStart FastPfu buffer, 2 μL of 2.5 mM deoxyribonucleotides triphosphate (dNTPs), 0.8 μL of forward primer (5 μM), 0.8 μL of reverse primer (5 μM), 0.4 μL of TransStart FastPfu DNA Polymerase, 10 ng of template DNA, and dd$H_2O$ added to 20 μL. The PCR product was extracted from 2% agarose gel and purified using the AxyPrep DNA Gel Extraction Kit (Axygen Biosciences, Union City, CA, United States), according to the manufacturer's instructions and quantified using Quantus$^{TM}$ Fluorometer (Promega, Madison, WI, United States).

After amplification, purified amplicons were pooled equimolarly and paired-end sequenced (2 × 300 bp) on an Illumina MiSeq PE300 platform (Illumina, San Diego, CA, United States) by Majorbio Bio-Pharm Technology Co. Ltd. (Shanghai, China).

The raw 16S rDNA gene sequencing reads were demultiplexed and quality-filtered by Trimmomatic and merged by FLASH (version 1.2.7) according to the following criteria: (1) the 300-bp reads were truncated at any site receiving an average quality score of <20 over a 50 bp sliding window, and reads shorter than 50-bp or containing ambiguous characters were discarded; (2) only overlapping sequences longer than 10-bp were assembled according to their overlapped sequence. The maximum mismatch ratio of overlap region was 0.2. Reads that could not be assembled were discarded; and (3) individual samples were distinguished according to the barcode (exactly matching) and primers (allowing 2 nucleotide mismatches) in primer matching.

## Statistical analyses

Data were analyzed according to a 2 × 4 factorial arrangement in two concurrent 4 × 4 Latin squares using the mixed model procedure of SAS statistical package (SAS version 9.4, SAS Inst. Inc., Cary, NC). The design was not orthogonal, as there were 4 rows, 6 columns and 4 treatments, with one treatment measured three times per period (row). However, the effect of period was not significant, and, therefore, could be omitted (Sarraseca et al., 1998). Dietary energy level, animal species, and the interaction were fixed effects, and experimental animal was a random effect. When there was a significant interaction between energy level and animal species, a *t*-test was used to compare the measured variable between animal species at the same energy level. Polynomial contrasts were used to determine whether the effect of energy level on the measured variable was linear or quadratic. A level of $P < 0.05$ was accepted as significant and a trend at $0.05 \leq P < 0.10$.

The α-diversity (observed operational taxonomic units – OTU) for the rumen bacterial community of different energy levels were calculated with QIIME (Version 1.9.1) and analyzed by the Kruskal-Wallis test and Wilcoxon rank test using R package. The constrained principal coordinate analysis (CPCoA) was used to visualize classical multi-dimensional scaling of Bray-Curtis distance matrices using functions capscale and anova.cca of vegan package in R (version 3.4.1, United States), and the *P* value were calculated by permutation tests. The top 50 relatively abundant bacteria at the genus level were visualized as a heatmap using the R package "pheatmap". Spearmen's rank correlation tested the relationships between the relative abundances of the top 50 abundant ruminal bacteria (at genus level) and fermentation parameters (VFA concentration and enzyme activities) using the "corrplot" package in R (version 3.4.1, United States). Linear discriminant analysis effect size (LEfSe) determined the difference in rumen bacteria between species and among energy levels by coupling the Kruskal-Wallis Test for statistical significance with additional tests accessing biological consistency and effect relevance. Taxa with an LDA Score > 3 were considered as exhibiting a significant effect size. PICRUSt2 software predicted microbiota function and determined the differences between species and among energy levels.

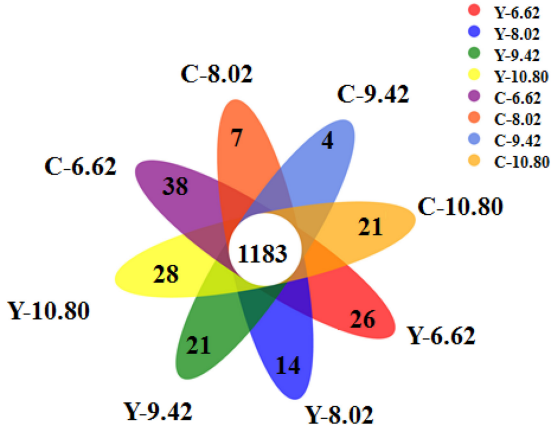


FIGURE 1
Flower plot showing different and similar OTUs in yak (Y) and Qaidam cattle (C) offered low protein diets differing energy levels (MJ ME/kg DM).

# Results

## Ruminal pH, fermentation parameters and enzyme activities

Ruminal pH (average, 6.96 vs. 6.81, $P = 0.036$), ammonia-N concentration (average, 5.61 vs. 5.08 mg/dL, $P = 0.041$), and total VFAs concentration (average, 78.6 vs. 74.4 mM, $P < 0.001$) were greater in yaks than cattle (Table 2). The molar proportion of acetate (average, 73.4 vs. 72.5 mol/100 mol, $P = 0.049$) was greater, whereas of propionate was lesser (average, 15.2 vs. 15.6 mol/100 mol, $P = 0.043$), and, as a result, the ratio of acetate to proportionate (A:P) was greater (average, 4.85 vs. 4.70, $P = 0.048$) in yaks than cattle. There was no difference ($P > 0.10$) in molar proportions of butyrate, isobutyrate, valerate, and isovalerate between yaks and cattle. With increasing energy level of the diet, ruminal pH decreased linearly ($P < 0.001$), whereas the concentrations of ruminal ammonia-N and total VFAs increased linearly ($P < 0.001$) in both bovine species. The molar proportion of acetate and the ratio of A:P decreased linearly ($P < 0.001$), while the molar proportions of propionate, butyrate, isobutyrate, and isovalerate increased linearly ($P < 0.05$) with increasing energy level. The molar proportion of valerate tended to increase linearly with increasing energy level ($P = 0.07$). There was no interaction between bovine species and dietary energy level for ruminal pH and fermentation parameters ($P > 0.10$). In addition, the activities of ruminal CMCase, amylase, xylanase, and pectinase were not affected by bovine species, energy level, or their interactions ($P > 0.10$).

## Collective sequencing data summary

A total of 1,292,848,899 raw reads were generated from the rumen fluid samples, and 3,094,821 high quality sequences remained after quality-filtering and removal of chimeric sequences. A total of 2,744 OTUs were obtained based on 97% nucleotide sequence identity analysis among reads.

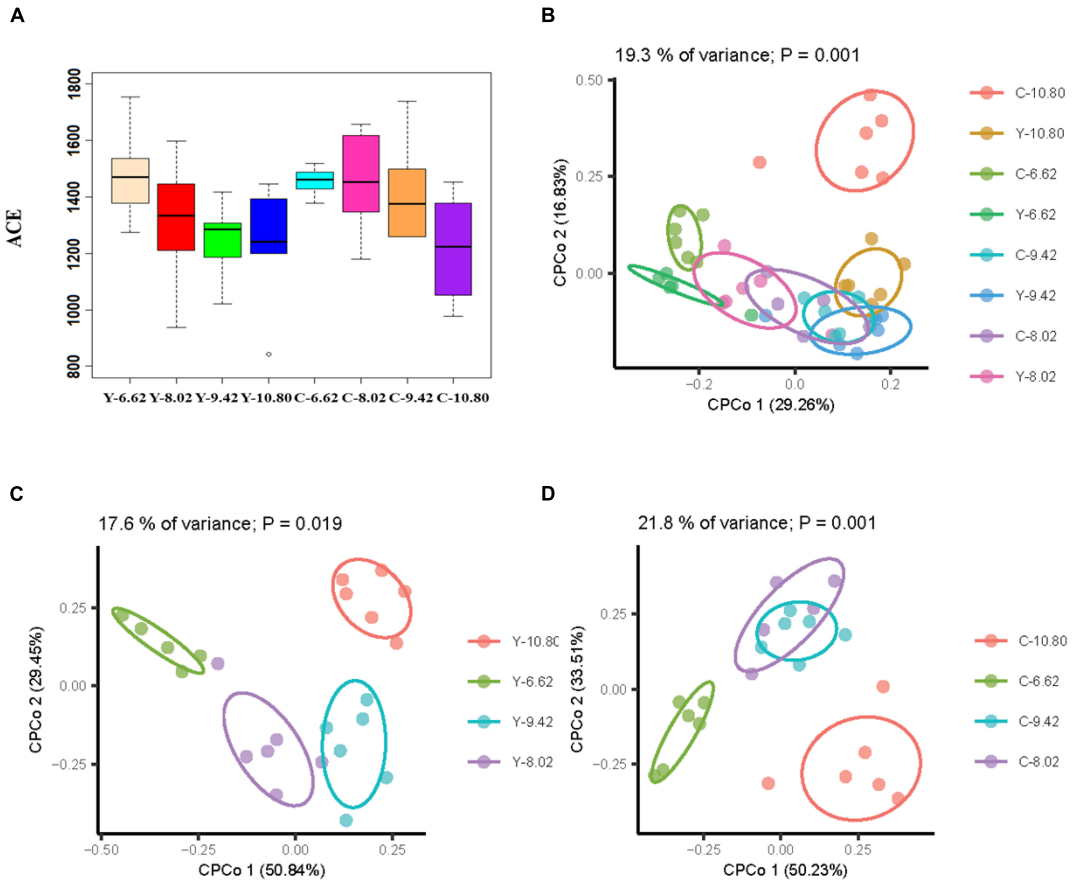


FIGURE 2
Effects of dietary energy levels on α and β diversity of the ruminal bacterial community in yak (Y) and in Qaidam cattle (C) offered low protein diets differing in energy levels (MJ ME/kg DM). **(A)** Ace index between the bovine species and among dietary energy levels; **(B)** CPCoA between the bovine species and among dietary energy levels; **(C)** CPCoA among dietary energy levels for yaks; **(D)** CPCoA among dietary energy levels for Qaidam cattle.

A total of 1,183 OTUs were shared among the 8 treatment groups, accounting for 56.6, 56.9, 60.1, and 59.7% of the total OTUs in yaks, and 54.6, 57.2, 58.5, and 61.4% of the total OTUs in cattle for the 6.62, 8.02, 9.42, and 10.80 MJ ME/kg DM diets, respectively (**Figure 1**). The number of OTUs specific to the diets containing 6.62, 8.02, 9.42, and 10.80 MJ ME/kg DM were 26, 14, 21, and 28 for yaks and 38, 7, 4, and 21 for cattle, respectively.

There was no difference in alpha diversity (ACE) between yaks and cattle, and it decreased linearly ($P < 0.05$) with increasing dietary energy level (**Figure 2A**). The CPCoA revealed differences in rumen bacterial communities between the bovine species and among dietary energy levels. Of the total variance, 19.3% was explained by species and dietary energy level ($P = 0.001$; **Figure 2B**), and 17.6% and 21.8% were explained by dietary energy level in yaks ($P = 0.019$; **Figure 2C**) and cattle ($P = 0.001$; **Figure 2D**), respectively. The rumen bacterial profiles were distinguished and affected significantly by dietary energy level, but not by bovine species (ANOSIM $R = 0.0318$, $P = 0.098$).

## Microbial community composition in the rumen fluid

A total of 22 ruminal bacterial phyla were identified across the 4 energy levels and 2 animal species. The dominant phylum was Bacteroidetes with 66.9, 57.4, 38.9, and 34.7% for yaks, and 60.9, 51.0, 45.7, and 38.0% for cattle for the diets containing 6.62, 8.02, 9.42, and 10.80 MJ ME/kg DM, respectively (**Figure 3** and **Supplementary Table 1**). Firmicutes was the second dominant phylum with 29.1, 35.9, 53.3, and 50.9% in yaks and 34.3, 43.1, 49.1, and 56.2% in cattle for the diets containing 6.62, 8.02, 9.42, and 10.80 MJ ME/kg DM, respectively. The RAs of Firmicutes and Actinobateriota increased linearly ($P < 0.05$), whereas the RA of Bacteroidetes decreased linearly ($P < 0.05$) with increasing dietary energy level. Dietary energy level was correlated negatively with the A:P ratio and positively with the Firmicutes to Bacteroidetes (F:B) ratio in both yaks and cattle (**Figure 4**).

In total, 356 bacterial genera were identified. The dominant genera were *Prevotella* with 17.3% for yaks and 18.8% for cattle, and *Rikenellaceae_RC9_gut_group* with 16.3% for yaks and 14.9% for cattle (**Figure 5** and **Supplementary Table 2**). The RAs of *Ruminococcaceae NK4A214_group*, *Butyrivibrio*, and *norank_f__Muribaculaceae* were lesser ($P < 0.05$), whereas, the RAs of *Rikenellaceae_RC9_gut_group* and *Quinella* were greater ($P < 0.05$) in yaks than in cattle. Moreover, the RAs of *Ruminococcaceae NK4A214_group*, *Succiniclasticum*, *Ruminococcus*, *Lachnospiraceae_NK3A20_group*, *Acetitomaculum*, *DNF00809*, *unclassified_f__Lachnospiraceae*, *Marvinbryantia*, *Ruminococcus gauvreauii_group*, *Anaerovibrio*, and *SP3-e08* increased linearly ($P < 0.05$), while the RAs of

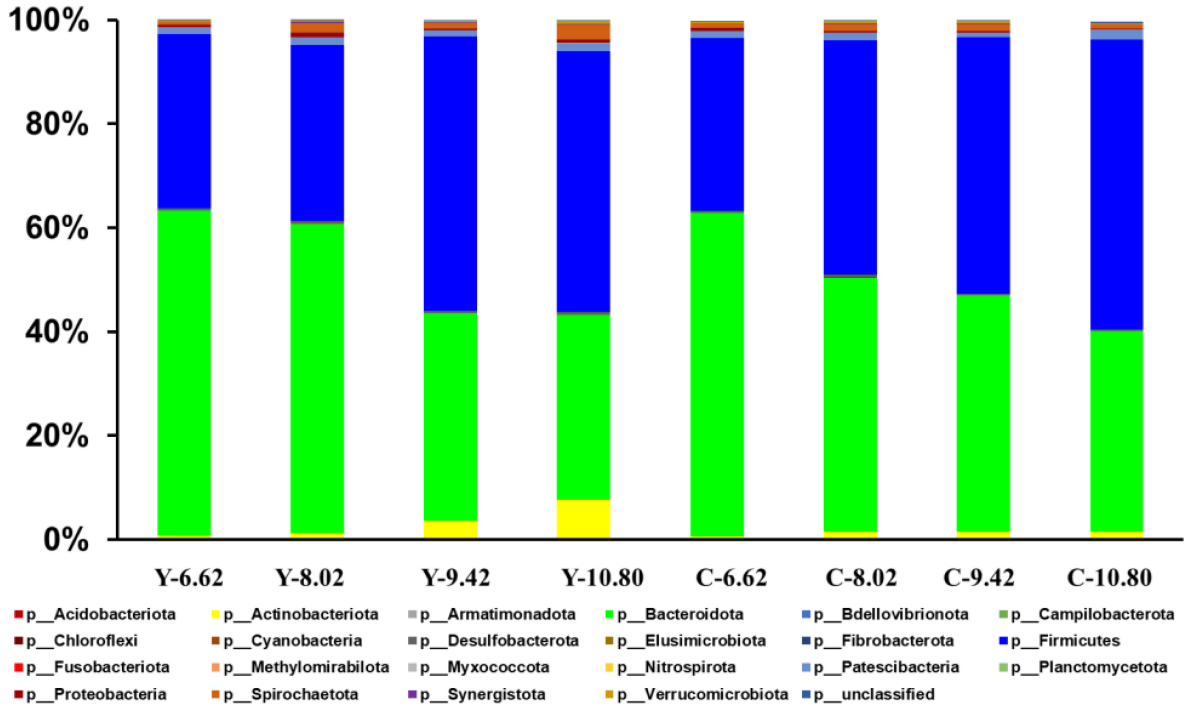


FIGURE 3
Effects of dietary energy level (MJ ME/kg DM) on the yak (Y) and Qaidam cattle (C) rumen bacterial composition at the phylum level. Each bar and color represents the average relative abundance of each phylum.

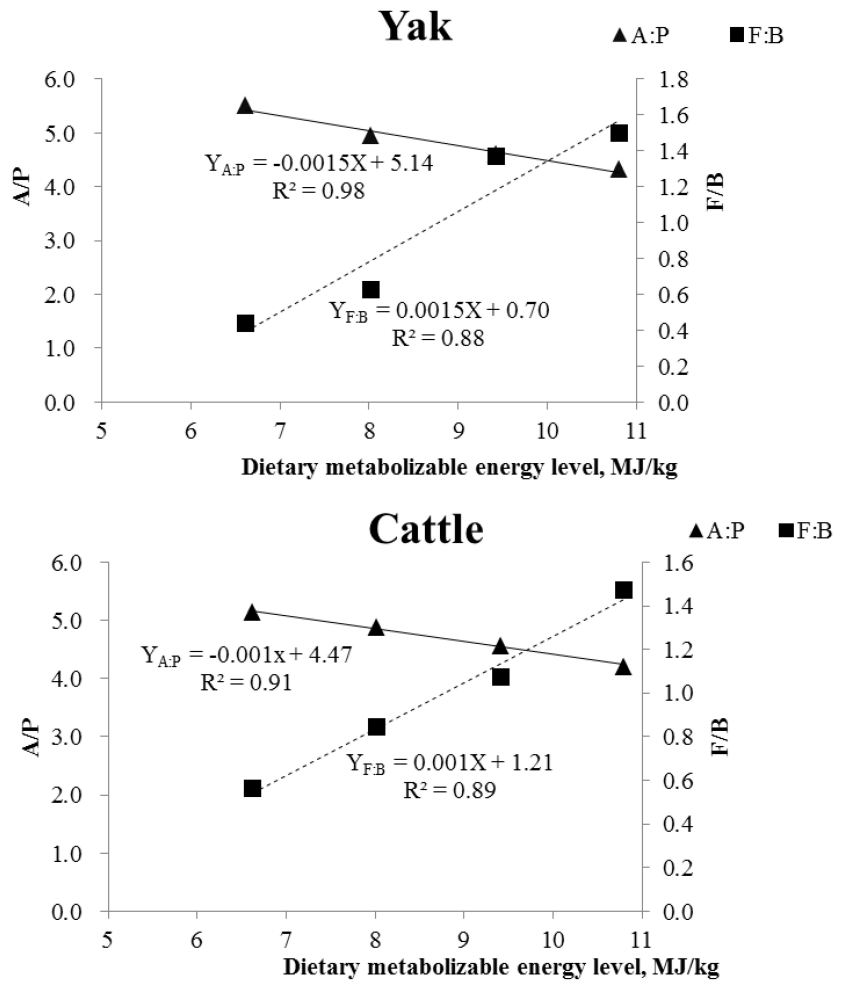


FIGURE 4
Correlation between average daily gain and Firmicutes/Bacteroidetes (F/B; ■) ratio, and acetate/propionate (A/P; ▲) ratio in yaks and in Qaidam cattle.

*Prevotella*, *Rikenellaceae_RC9_gut_group*, *Prevotellaceae_UCG-003*, *norank_f__Bacteroidales_RF16_group*, *norank_f__Bacteroidales_BS11_gut_group*, *Prevotellaceae_UCG_001*, *norank_f__Bacteroidales_UCG-001*, and *Papillibacter* decreased linearly ($P < 0.05$) with increasing dietary energy level.

Differential microbiota that varied with energy level were further identified using linear discriminant analysis effect size (LEfSe; **Figures 6A,B**). With a default LDA cut-off of ±2.0, differential taxa totaling 12, 9, 11, and 22 in yaks and 21, 4, 2, and 16 in cattle were observed for the 6.62, 8.02, 9.42, and 10.80 MJ ME/kg DM, respectively. The bacteria biomarkers in the 6.62 MJ ME/kg DM group were Bacteroidota for yaks and *Prevotellaceae_UCG-003*, *Prevotellaceae_UCG-001*, *Butyrivibrio*, and *Pseudobutyrivibrio* for cattle and in the 10.80 MJ ME/kg DM group were Actinobacteriota, *Atopobium*, *Family_XIII_AD3011_group*, *Syntrophococcus*, *Mogibacterium*, *Schwartzia*, *norank_f_norank_o_chloroplast*, *norank_f_Christensenellaceae*, *vadinHA49*, *norank_o_norank_o_Coriobacteriales*, and *Enbacterium_brachy_group* for yaks and Firmicutes, *Ruminococcus*, *Lachnospiraceae_NK3A20_group*, *Acetitomaculum*, and *Ruminococcus_gauvreauii_group* for cattle.

## Correlations between ruminal bacteria and fermentation parameters

A Spearman rank correlation tested the relationships between ruminal microbiota and rumen fermentation parameters (**Figure 7**). A total of 83 positive ($P < 0.05$) and 74 negative ($P < 0.05$) correlations emerged (**Figure 7**). *Prevotella* was correlated positively with xylanase activities ($r = 0.385$; $P < 0.001$) and negatively with the concentration of total VFAs ($r = -0.353$; $P = 0.016$). *Rinenellaceae_RC9_gut group* was correlated positively with the A:P ratio ($r = 0.311$; $P = 0.035$), but negatively with the molar proportion of propionate ($r = -0.331$;

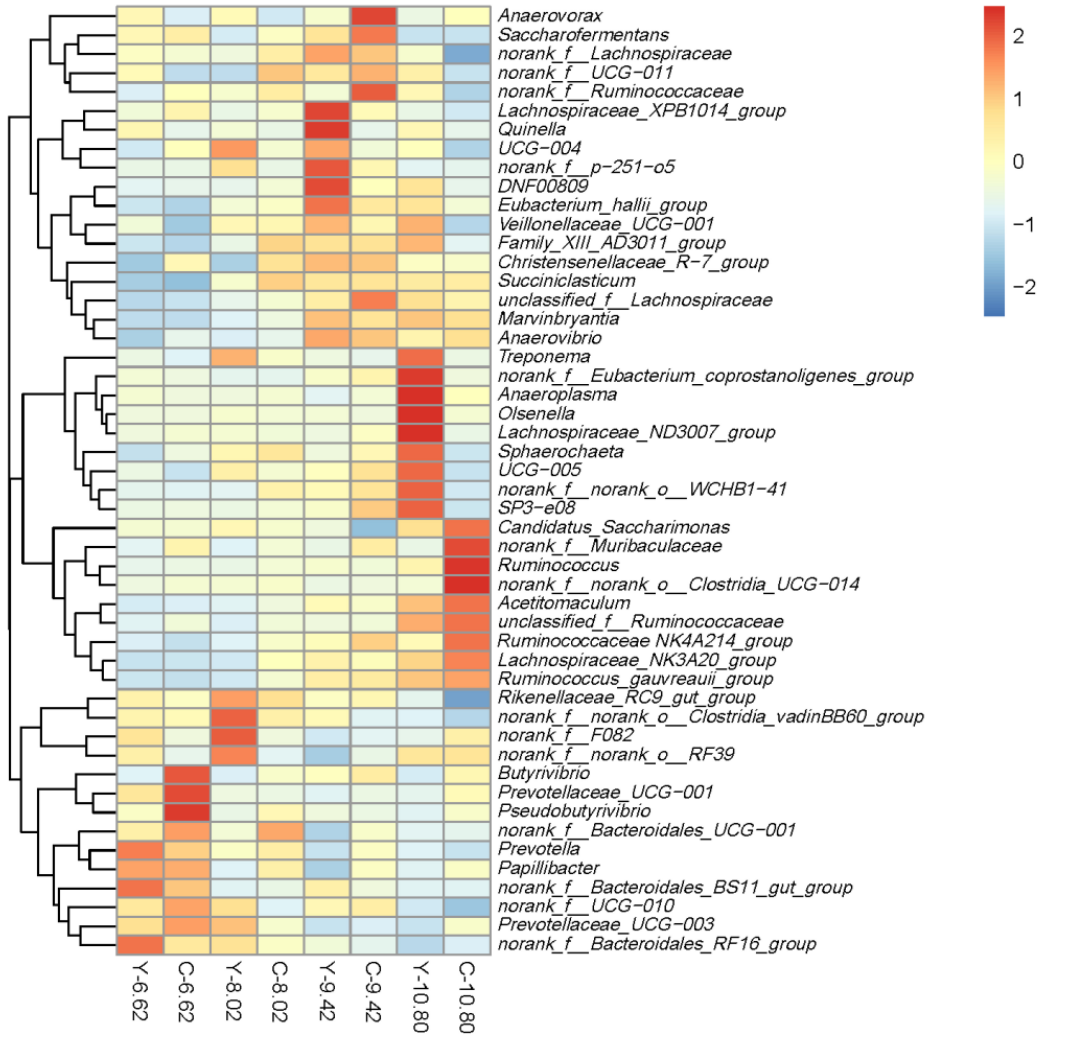


FIGURE 5
Relative abundances of the top 50 bacterial sequence variants at the genus level in the rumen of yaks (Y) and Qaidam cattle (C) fed diets differing in energy level (MJ ME/kg DM).

$P$ = 0.025) and pectinase activities ($r$ = −0.328; $P$ = 0.025). *Christensenellaceae_R-7_group* was correlated positively with the molar proportions of iso-butyrate ($r$ = 0.335; $P$ = 0.023) and iso-valerate ($r$ = 0.329; $P$ = 0.026), and negatively with xylanase activities ($r$ = −0.505; $P$ < 0.001). *Ruminococcaceae NK4A214_group* was correlated positively with the ruminal concentrations of ammonia-N ($r$ = 0.511; $P$ < 0.001) and total VFAs ($r$ = 0.431; $P$ = 0.003) and molar proportions of propionate ($r$ = 0.479; $P$ = 0.001) and isovalerate ($r$ = 0.447; $P$ = 0.002), but negatively with ruminal pH ($r$ = −0.439; $P$ = 0.002), the molar proportion of acetate ($r$ = −0.485; $P$ = 0.001), the ratio of A:P ($r$ = −0.473; $P$ = 0.001), and xylanase activities ($r$ = −0.327; $P$ = 0.026). *Prevotellaceae_UCG-003* was correlated positively with ruminal pH ($r$ = 0.425; $P$ = 0.003), the molar proportion of acetate ($r$ = 0.542; $P$ < 0.001), and the A:P ratio ($r$ = 0.460; $P$ = 0.001), and negatively with the ruminal concentrations of ammonia-N ($r$ = −0.358; $P$ = 0.018) and total VFAs ($r$ = −0.498; $P$ < 0.001), and the molar proportions of propionate ($r$ = −0.392; $P$ = 0.007), butyrate ($r$ = −0.510; $P$ < 0.001), isobutyrate ($r$ = −0.296; $P$ = 0.046), and isovalerate ($r$ = −0.336; $P$ = 0.023). *Ruminococcus*, *Lachnospiraceae_NK3A20_group*, *Acetitomaculum*, and *Ruminococcus_gauvreauii_group* were correlated positively with the ruminal concentrations of ammonia-N and total VFAs, and the molar proportions of propionate, butyrate, isobutyrate, and isovalerate, but negatively with pH, the molar proportion of acetate, the A:P ratio, and xylanase activities. *Prevotellaceae_UCG-001* was correlated positively with ruminal pH ($r$ = 0.324; $P$ = 0.020), the molar proportion of acetate ($r$ = 0.362; $P$ = 0.014) and the ratio of A:P ($r$ = 0.315; $P$ = 0.033), but negatively with the ruminal concentrations of ammonia-N ($r$ = −0. 380; $P$ = 0.011) and total VFAs ($r$ = −0.578; $P$ < 0.001), and molar proportions of butyrate

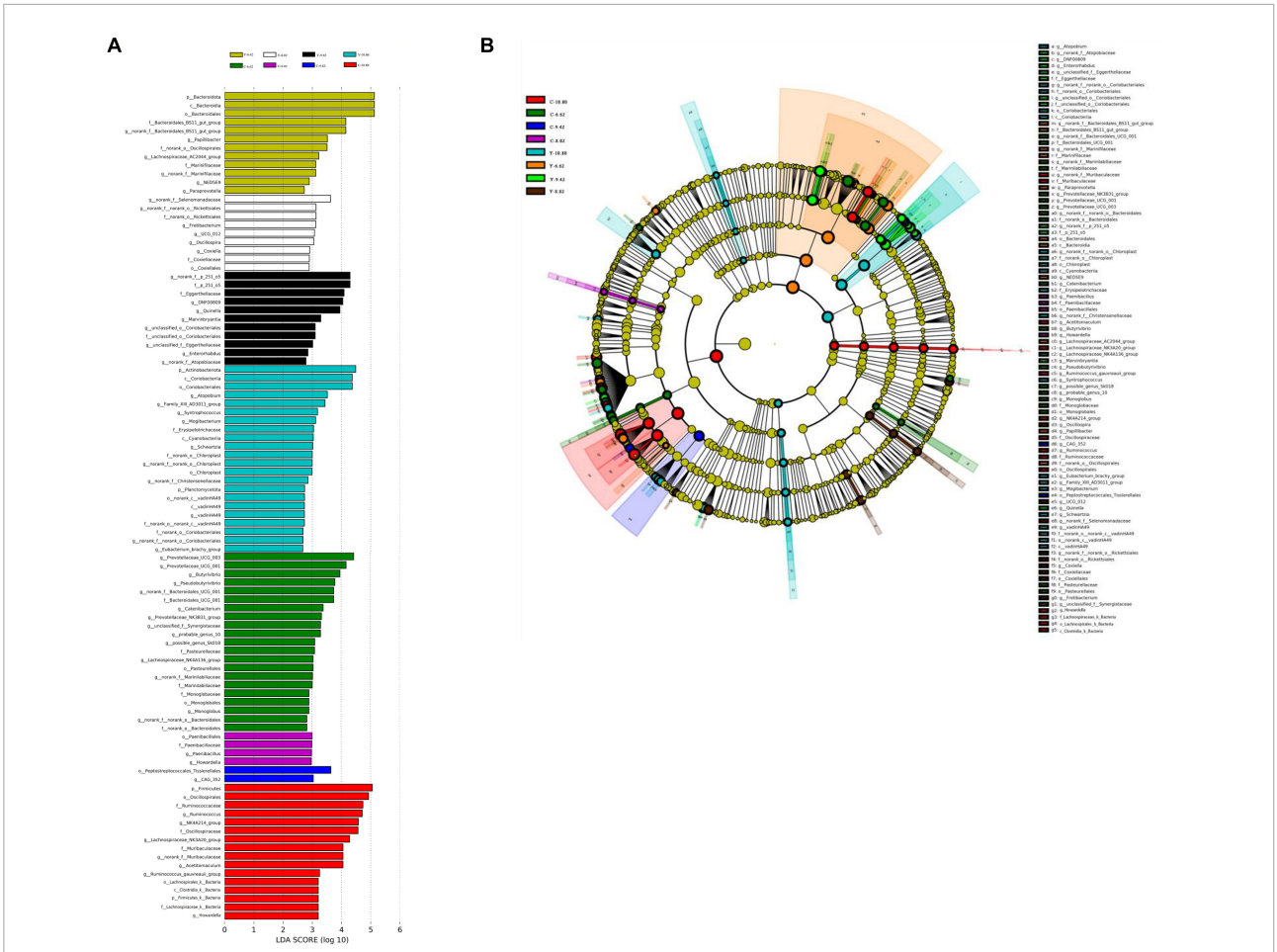


FIGURE 6
Linear discriminant analysis effect size (LEfSe) results for rumen microbiota in yaks consuming diets with different energy levels (MJ ME/kg DM). **(A)** Linear discriminant analysis. **(B)** Cladogram reported. Prefixes represent abbreviations for the taxonomic rank of each taxon, class ($c^-$) order ($o^-$), family ($f^-$) and genus ($g^-$).

($r$ = –0.374; $P$ = 0.010), isobutyrate ($r$ = –0.296; $P$ = 0.046), and isovalerate ($r$ = –0.331; $P$ = 0.025). *DNF00809* was correlated positively with the concentration of total VFAs ($r$ = 0.436; $P$ = 0.002) and the molar proportion of valerate ($r$ = 0.335; $P$ = 0.023), and negatively with ruminal pH ($r$ = –0.334; $P$ = 0.023) and the molar proportion of acetate ($r$ = –0.322; $P$ = 0.030). *Butyrivibrio* was correlated positively with the molar proportions of propionate ($r$ = 0.352; $P$ = 0.016) and isovalerate ($r$ = 0.311; $P$ = 0.035).

## PICRUSt2 function prediction

The top 40 functions of the rumen bacterial communities in yaks and cattle were predicted. There were 19 and 3 predictive metabolic pathways that were affected ($P$ < 0.05) by energy level and species, respectively, and there was no interaction between species and energy level ($P$ > 0.10; **Supplementary Table 3**). Overall, the most abundant pathway was ribosome (26.0%), followed by ABC transporters (24.8%), and then purine metabolism (22.2%). Starch and sucrose metabolisms were lesser ($P$ < 0.05), whereas valine, leucine and isoleucine biosyntheses were greater ($P$ < 0.05) in yaks than in cattle (**Figures 8A,B**). Starch and sucrose metabolisms, valine, leucine and isoleucine biosyntheses, glycolysis/gluconeogenesis, and the pentose phosphate pathway increased linearly ($P$ < 0.01) with increasing dietary energy level (**Figures 8C,D**).

# Discussion

## Effect of dietary energy on rumen fermentation parameters

Ruminal pH, which is influenced by the concentrations of VFAs and ammonia-N and by saliva secretions, affect the growth and proliferation of ruminal microbes. In the current

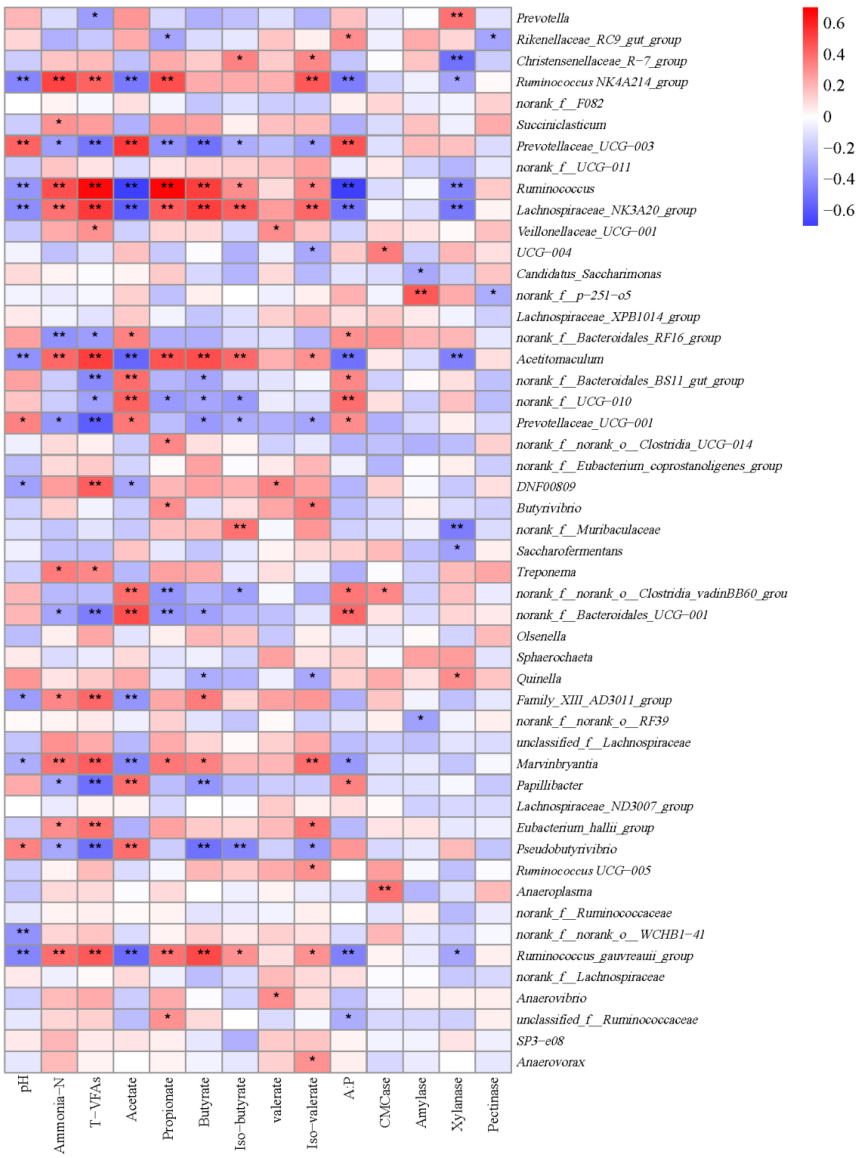


FIGURE 7
Spearman's rank correlation analysis between the top 50 bacteria at the genus level and rumen fermentation parameters. *$P < 0.05$, and **$P < 0.01$ according to Spearman's rank correlation coefficient.

study, the greater ruminal pH in yaks than in cattle across the 4 energy levels was inconsistent with the greater concentration of VFAs in yaks. It was reported that the chewing activity was greater in yaks than cattle (Ding et al., 2008; Thanner et al., 2014), and this could have led to a greater saliva secretion to the rumen in yaks than in cattle. In addition, the endogenous urea recycled to the rumen was reported to be greater in yaks than in cattle (Zhou et al., 2017). The urea is hydrolyzed to ammonia, which increases the ruminal pH. Furthermore, yaks have 36 up-regulated genes related to VFA transport and absorption compared with cattle (Zhang et al., 2016), which could increase the rate of VFA absorption and reduce the ruminal concentration of VFAs. The linear decrease in ruminal pH with an increase in energy level is consistent with the linear increase in concentration of total VFAs (Liu et al., 2021b).

Ruminal ammonia-N concentration was greater in yaks than in cattle, which implied a greater microbial protein synthesis in yaks than in cattle (Zhou et al., 2017). However, the concentrations in both bovine species consuming the 6.62 and 8.02 MJ ME/kg DM diets were below the optimal range of 5 to 25 mg/100 mL (Preston and Leng, 1987), which suggests reduced activities of fibrolytic bacteria. The ammonia-N concentration increased linearly with increasing energy level, which indicated increased protein degradability and more N

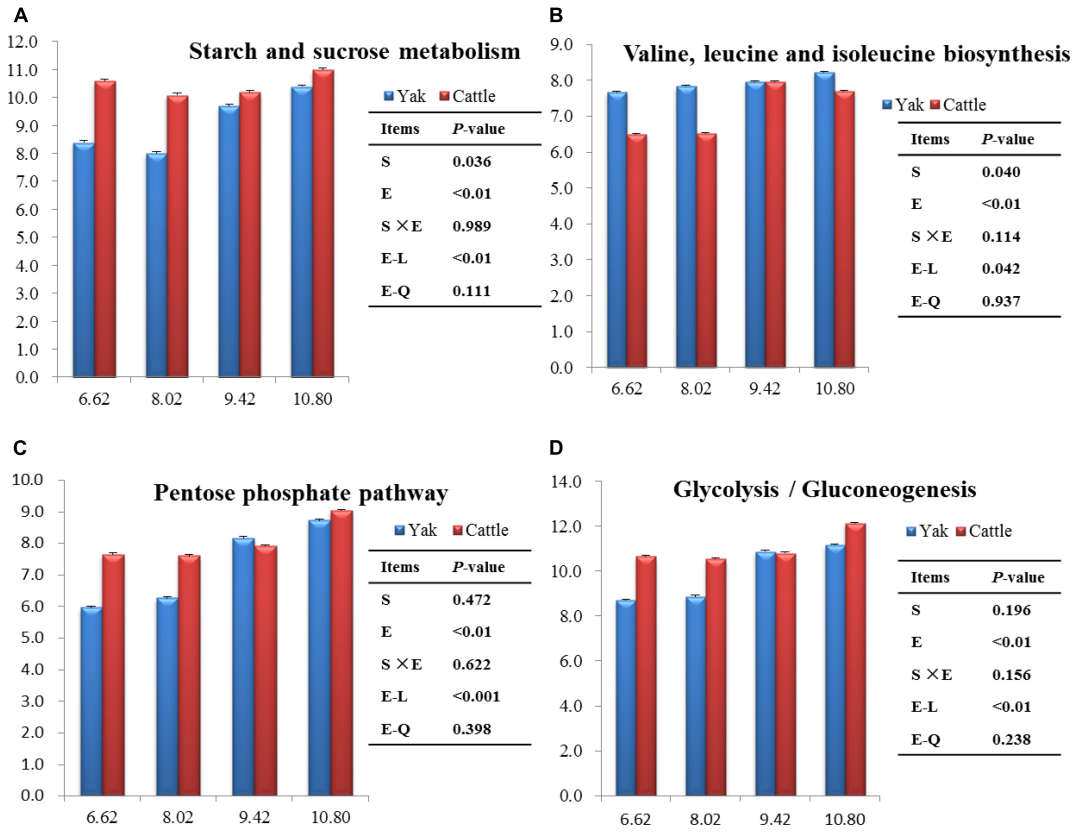


FIGURE 8
Significant differences of the functional pathways between species and among dietary energy levels (MJ ME/kg DM).

available for rumen microbial production, although the N intake was similar for all energy levels (Zhou et al., 2019).

Rumen VFAs are the end products of microbial fermentation, and are the main energy source for the host animal. The greater concentration of total VFAs in yaks than in cattle is in agreement with previous findings in an *in vivo* study when the two species were co-grazing a *Kobresia* pasture (Huang et al., 2012), and in an *in vitro* study when the same substrate was used with rumen inocula (Zhang et al., 2016). Furthermore, an ultra-deep metagenomic sequencing study demonstrated greater VFA-yielding pathways of rumen microbial genes in yaks than in cattle (Zhang et al., 2016). These findings could explain, at least in part, the greater ruminal concentration of VFAs in yaks than in cattle. The increase in concentration of ruminal total VFAs with increasing energy level was expected, as the non-fiber carbohydrate (NFC) contents of the concentrate increased (Liu et al., 2021b). A high-forage diet increased the molar proportion of acetate, whereas, a high concentrate diet increased the molar proportions of propionate and butyrate (Wang et al., 2019a). In the present study, the molar proportion of acetate decreased, whereas, of propionate and butyrate increased when the dietary energy increased. The greater molar proportion of acetate, along with the greater A:P ratio in yaks than in cattle, indicated a greater fiber digestibility in yaks (Wang, 2009), which was supported by the greater RA of fibrolytic bacteria (for example, *Rikenellaceae_RC9_gut_group*) in yaks than in cattle. Isobutyrate and isovalerate are derived mainly from hydrolyzation of branched-chain amino acids (BCAA; Apajalahti et al., 2019) and they are particularly important for fibrolytic bacteria (Van Gylswyk, 1970). The linear increase in iso-VFAs with increasing energy level indicated an increased protein degradability (Liu et al., 2022).

## Effect of dietary energy on bacterial community composition

Bacteroidetes and Firmicutes were the dominant bacteria phyla, as was reported in cattle (Xin et al., 2019), dairy cows (Wang et al., 2019b), goats (Xue et al., 2022), and sheep (Castro-Carrera et al., 2014). These findings suggest that these two phyla play important roles in feed digestion and metabolism in the rumen. The mean RAs of Firmicutes for yaks (42.3%) and cattle (45.7%), and of Bacteroidetes for yaks (49.5%) and cattle (48.9%) were either below (56% for Firmicutes) or above (31% for Bacterioidetes) values from a meta-analysis of all

curated 16S rRNA gene sequences from a NCBI database for ruminants (Kim et al., 2011). This could be due to differences in animal species, growth stage, and dietary nutritional levels and composition among studies. In the present study, the RA of Bacteroidetes decreased linearly and of Firmicutes increased linearly with increasing energy level, as was also reported in other yaks (Hu et al., 2020) and in goats (Min et al., 2019). The increased F:B ratio with increasing energy levels is in agreement with Liu et al. (2022), who also reported a linear increase in average daily gain (ADG) in yaks and cattle with increasing energy levels. A lower ratio of F:B was linked to an inhibition of fat deposition and decreased ADG of the host animal (Liu et al., 2015; Hu et al., 2020).

The maximum RA of Actinobacteria, a gram-positive bacteria, was 3% for both cattle and sheep (Sul'ák et al., 2012), and decreased with increasing energy level in Holstein heifers (Bi et al., 2018). In the present study, there was a larger variation in the RA of Actinobacteria (0.79% to 7.63%) in yaks than in cattle (0.66% to 1.57%), which suggests that Actinobacteria may be more sensitive to dietary energy change in yaks than in cattle.

*Prevotella* was the most dominant genus, which is in agreement with previous studies in steers (Xin et al., 2019), dairy cows (Wei et al., 2021), goats (Dao et al., 2021), and sheep (Huang et al., 2022). In the present study, *Prevotella* contained a number of metabolic versatile species, which secrete enzymes to degrade protein, starch, peptides, hemicellulose, and pectin into VFAs and amino acids (Stevenson and Weimer, 2007; Dao et al., 2021). This variability can be associated with the maintenance of RA balance under different fermentation conditions. The RA of *Prevotella* decreased linearly with increasing energy level, which is in agreement with a study in Angus cows (Chen et al., 2021). In addition, the RAs of *Prevotellaceae_UCG-001* and *Prevotellaceae_UCG-003*, which belong to the Prevotellaceae family, also decreased linearly with increasing energy level. The reduction in RAs of these bacteria could be explained by a decrease in dietary fiber contents with increasing energy level. The *Rikenellaceae_RC9_gut_group* was the second most dominant genus, which is consistent with a study in grazing yaks (Liu et al., 2021a) and cattle (Xin et al., 2019). *Rikenellaceae_RC9_gut_group*, a member of the Rikenellaceae family, degrades cellulose and hemicellulose, with acetate as the main end product (Sha et al., 2020), and this could explain the linear decrease in RA with an increase in energy level (i.e., decrease in fiber content). The greater RA of *Rikenellaceae_RC9_gut_group* in yaks than in cattle could explain, at least in part, the greater fiber digestibility (Liu et al., 2022) and greater molar proportion of acetate in yaks than in cattle.

*Ruminococcus* is a fibrolytic bacteria and occurs predominantly in the rumen of ruminants fed high-fiber diets (Wei et al., 2021). However, in the present study, the RA of *Ruminococcus* increased linearly with increasing energy level and decreasing fiber content. This genus also exhibits amylolytic activity and the increasing dietary energy could have stimulated its grow and proliferation (Hu et al., 2020). This could explain why *Ruminococcus* was associated positively with total VFAs concentration, and negatively with pH. *Ruminococcus_gauvreauii_group*, which increased with increasing energy level in the present study, is a gram-positive and obligate anaerobic bacteria that produces acetate as the main end-product of glucose fermentation (Domingo et al., 2008). It was correlated negatively with pH and acetate and positively with the molar proportion of propionate. The ammonia-N concentrations in both bovine species consuming the 6.62 and 8.02 MJ ME/kg DM diets were below the optimal range of 5 mg/100 mL, which could explain why the proliferation of *Ruminococcus_gauvreauii_group* was inhibited. *Ruminococcus_NK4A214 group*, another bacterium belonging to the Ruminococcus family, was associated positively with total VFAs, and the molar proportion of propionate, which is in agreement with Li et al. (2019).

*Succiniclasticum* utilizes succinate to produce propionate (Van Gylswyk, 1995). In the present study, the increased RA of *Succiniclasticum* with increasing energy level was likely associated with the increased dietary NFC. *Lachnospiraceae_NK3A20_group* was characterized by cellulose-decomposing activity and starch hydrolysis, which could produce acetate and formate (Russell and Rychlik, 2001). In addition, *Lachnospiraceae_NK3A20_group* was correlated positively with total VFAs concentration, and the molar proportions of propionate, butyrate, isobutyrate, and isovalerate, and negatively with pH, the molar proportion of acetate, the ratio of A:P, and xylanase activities. These results suggest that the fermentation pattern of *Lachnospiraceae_NK3A20_group* favor propionate production in high-energy diets when consuming a low protein diet. A low concentration of ammonia-N could inhibit the proliferation of this genus, which could explain this occurrence. *Acetitomaculum* utilizes monosaccharides to produce acetate and occurs mainly in ruminants fed a high-concentrate diet (Huang C. et al., 2021). Accordingly, the RA of *Acetitomaculum* increased with increasing energy level, which was in line with Wang et al. (2019a).

*Quinella* ferments sugars equimolarly to acetate and propionate. It was reported that a greater RA of *Quinella* was associated with a lower methane emission in ruminants (Krumholz et al., 1993). The greater RA of *Quinella* in yaks than in cattle could explain, at least in part, the lesser methane emissions from yaks than cattle (Mi et al., 2017; Bai et al., 2021). In agreement with a report by Mao et al. (2016), the RA of *Papillibacter* decreased with increasing energy levels. *Papillibacter* was correlated positively with the molar proportion of acetate and the ratio of A:P, and negatively with total VFAs concentration and the molar proportion of butyrate.

To further understand the effects of energy level and bovine species on the bacterial community, we used LEfSe

analysis. Bacteroidetes was the most differentially abundant bacteria in the yaks fed the low energy (6.62 MJ ME/kg DM) diet, inferring that Bacteroidetes was important for yaks with sparse forage availability. In addition, the RA of Firmicutes was greatest in cattle consuming the high energy (10.40 MJ ME/kg DM) diet, suggesting that a high-quality diet was linked with this phylum (Hu et al., 2020).

### Correlations between bacterial communities at genera level and fermentation parameters and PICRUSt2 prediction of functions

The *Rikenellaceae_RC9_gut_group* was correlated negatively with the molar proportion of propionate and pectinase activity, and positively with the A:P ratio, suggesting fibrolytic activity, which is in agreement with previous studies (Zhao et al., 2017; Wang et al., 2019a). In the present study, *Pseudobutyrivibrio* was correlated positively with the molar proportion of acetate and negatively with total VFAs concentration, which is in accordance with the reports that this bacterium is cellulotytic (Poonko et al., 2015). Therefore, it was correlated positively with dietary fiber content and negatively with dietary protein and starch (Hao et al., 2021). The reason for the negative correlation of *Prevotella*, *Prevotellaceae_UCG-001*, and *Prevotellaceae_UCG-003* with total VFAs concentration is not clear. *Prevotella* degrades most fibers and protein, but its function and metabolism are still unknown. There were 11 ruminal bacteria that were not associated with rumen fermentation parameters.

By using PICRUSt2 to predict the potential functions of bacteria, numerous metabolism related pathways emerged. The most prominent functional categories were ribosome and ABC transporters at the KEGG level 3 metabolic categories. These functions are vital for the survival, reproduction, and growth of livestock (Lamendella et al., 2011). In the present study, there were 17 predictive metabolic pathways that were affected by energy levels. The BCAAs, including valine, leucine, and isoleucine, are vital contributors to microbial protein synthesis (Allison et al., 1966). Ruminal microbial protein supplies up to 85% of the total amino acids absorbed in the small intestine (Westreicher-Kristen et al., 2020). In the present study, molar proportions of valine, leucine and isoleucine were greater in yaks than in cattle, and increased linearly with increasing energy level, inferring that BCAA biosynthesis in the rumen of yaks produce more microbial protein than in cattle (Zhou et al., 2018). The *Prevotella* genus has a strong relationship with BCAA synthesis (Xue et al., 2020). However, in the present study, no difference in the RA of *Prevotella* was observed between yaks and cattle.

## Conclusion

The greater ruminal concentration of total VFAs and acetate to propionate ratio in yaks than in cattle was supported by the greater RA of fibrolytic bacteria, such as *Rikenellaceae_RC9_gut_group*. In addition, Bacteroidetes and Firmicutes were the dominant phyla, and *Prevotella* and *Rikenellaceae_RC9_gut_group* were the dominant genera, regardless of energy level and bovine species. The different bacterial profiles between yaks and Qaidam cattle explain how yaks are better adapted to the poor forage on the QTP than cattle. This study provides insight in the response of ruminal bacteria in yaks and cattle to a low protein diet with differing energy levels, as is common at high altitudes.

## 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: NCBI SRA BioProject, accession no: PRJNA856780.

## Ethics statement

The protocol and procedures on the animals were approved by the Animal Care and Use Committee of Lanzhou University, Gansu, China (No. 201905201).

## Author contributions

JZ and RL: conceptualization. HL: methodology, formal analysis, data curation, and writing – original draft preparation. ZS and TR: software. JZ: validation and project administration. HL and TR: investigation. CZ: resources. JZ, ZS, TR, WY, and AD: writing – review and editing. ZS and HL: visualization. TR: supervision. JZ, TR, and RL: funding acquisition. All authors reviewed the 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

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2022.982338/full#supplementary-material

## References

Allison, M. J., Bucklin, J. A., and Robinson, I. M. (1966). Importance of the isovalerate carboxylation pathway of leucine biosynthesis in the rumen. *Appl. Microbiol.* 14, 807–814. doi: 10.1128/am.14.5.807-814

AOAC (2006). *Official methods of analysis*, 18th Edn. Arlington VA: Assoc. Off. Anal Chem.

Apajalahti, J., Vienola, K., Raatikainen, K., Holder, V., and Moran, C. A. (2019). Conversion of branched-chain amino acids to corresponding isoacids-an in vitro tool for estimating ruminal protein degradability. *Front. Vet. Med.* 6:311. doi: 10.3389/fvets.2019.00311

Bai, B. Q., Han, X. D., Degen, A. A., Hao, L. Z., Huang, Y. Y., Niu, J. Z., et al. (2021). Enteric methane emission from growing yak calves aged 8 to 16 months: Predictive equations and comparison with other ruminants. *Anim. Feed Sci. Technol.* 281:115088. doi: 10.1016/j.anifeedsci.2021.115088

Bailey, E. A., Titgemeyer, E. C., Olson, K. C., Brake, D. W., Jones, M. L., and Anderson, D. (2012). Effects of ruminal casein and glucose on forage digestion and urea kinetics in beef cattle. *J. Anim. Sci.* 90, 3505–3514. doi: 10.2527/jas.2011-4459

Bi, Y. L., Zeng, S. Q., Zhang, R., Diao, Q. Y., and Tu, Y. (2018). Effects of dietary energy levels on rumen bacterial community composition in Holstein heifers under the same forage to concentrate ratio condition. *BMC Microbiol.* 18:69. doi: 10.1186/s12866-018-1213-9

Castro-Carrera, T., Toral, P. G., Frutos, P., McEwan, N. R., Hervás, G., Abecia, L., et al. (2014). Rumen bacterial community evaluated by 454 pyrosequencing and terminal restriction fragment length polymorphism analyses in dairy sheep fed marine algae. *J. Dairy Sci.* 97, 1661–1669. doi: 10.3168/jds.2013-7243

Chen, H., Wang, C., Huasai, S., and Chen, A. (2021). Effects of dietary forage to concentrate ratio on nutrient digestibility, ruminal fermentation and rumen bacterial composition in Angus cows. *Sci. Rep.* 11:17023. doi: 10.1038/s41598-021-96580-5

Chen, N. B., Cai, Y. D., Chen, Q. M., Li, R., Wang, K., Huang, Y. Z., et al. (2018). Whole-genome resequencing reveals world-wide ancestry and adaptive introgression events of domesticated cattle in East Asia. *Nat. Commun.* 9:2337. doi: 10.1038/s41467-018-04737-0

China National Commission of Animal Genetic Resources (2011). *Animal genetic resources in China bovines* (In Chinese). Beijing: China Agriculture Press, 198–199.

Dao, T. K., Do, T. H., Le, N. G., Nguyen, H. D., Nguyen, T. Q., Le, T. T. H., et al. (2021). Understanding the role of *Prevotella* genus in the digestion of lignocellulose and other substrates in Vietnamese native goats' rumen by metagenomic deep sequencing. *Animals* 11:3257. doi: 10.3390/ani11113257

Ding, L. M., Long, R. J., Shang, Z. H., Wang, C. T., Yang, Y. G., and Xu, S. H. (2008). Feeding behaviour of yaks on spring, transitional, summer and winter pasture in the alpine region of the Qinghai-Tibetan plateau. *Appl. Anim. Behav. Sci.* 111, 373–390. doi: 10.1016/j.applanim.2007.06.008

Domingo, M. C., Huletsky, A., Boissinot, M., Bernard, K. A., Picard, F. J., and Bergeron, M. G. (2008). *Ruminococcus gauvreauii* sp. nov., a glycopeptide-resistant species isolated from a human faecal specimen. *Int. J. Syst. Evol. Microbiol.* 58, 1393–1397. doi: 10.1099/ijs.0.65259-0

Guo, W. (2021). *The development model of microbiota in the rumen of grazing yak* (In Chinese). Ph.D. thesis. Lanzhou: Lanzhou University.

Hao, Y., Gong, Y., Huang, S., Ji, S., Wang, W., Wang, Y., et al. (2021). Effects of age, diet CP, NDF, EE, and starch on the rumen bacteria community and function in dairy cattle. *Microorganisms* 9:1788. doi: 10.3390/microorganisms9081788

Hristov, A. N., Ivan, M., Rode, L. M., and McAllister, T. A. (2001). Fermentation characteristics and ruminal ciliate protozoal population in cattle fed medium- or high-concentrate barley-based diets. *J. Anim. Sci.* 79, 515–524. doi: 10.2527/2001.792515x

Hu, R., Zou, H. W., Wang, H. Z., Wang, Z. S., Wang, X. Y., Ma, J., et al. (2020). Dietary energy levels affect rumen bacterial populations that influence the intramuscular fat fatty acids of fattening yaks (*Bos grunniens*). *Animals* 10:1474. doi: 10.3390/ani10091474

Huang, C., Ge, F., Yao, X. X., Guo, X., Bao, P. J., Ma, X. M., et al. (2021). Microbiome and metabolomics reveal the effects of different feeding systems on the growth and ruminal development of yaks. *Front. Microbiol.* 12:682989. doi: 10.3389/fmicb.2021.682989

Huang, X. D., Denman, S. E., Mi, J. D., Padmanabha, J., Hao, L. Z., Long, R. J., et al. (2021). Differences in bacterial diversity across indigenous and introduced ruminants in the Qinghai Tibetan plateau. *Anim. Prod. Sci.* e33306. doi: 10.1071/AN20204

Huang, X. D., Mi, J. D., Denman, S. E., Ba, S. W. D., Ping, C. Z. D., Zhang, Q., et al. (2022). Changes in rumen microbial community composition in yak in response to seasonal variations. *J. Appl. Microbiol.* 132, 1652–1665. doi: 10.1111/jam.15322

Huang, X. D., Tan, H. Y., Long, R., Liang, J. B., and Wright, A. D. (2012). Comparison of methanogen diversity of yak (*Bos grunniens*) and cattle (*Bos taurus*) from the Qinghai-Tibetan plateau, China. *BMC Microbiol.* 12:237. doi: 10.1186/1471-2180-12-237

Kim, M., Morrison, M., and Yu, Z. (2011). Status of the phylogenetic diversity census of ruminal microbiomes. *FEMS Microbiol. Ecol.* 76, 49–63. doi: 10.1111/j.1574-6941.2010.01029.x

Krumholz, L. R., Bryant, M. P., Brulla, W. J., Vicini, J. L., Clark, J. H., and Stahl, D. A. (1993). Proposal of *Quinella ovalis* gen. nov., sp. nov., based on phylogenetic analysis. *Int. J. Syst. Bacteriol.* 43, 293–296. doi: 10.1099/00207713-43-2-293

Lamendella, R., Domingo, J. W., Ghosh, S., Martinson, J., and Oerther, D. B. (2011). Comparative fecal metagenomics unveils unique functional capacity of the swine gut. *BMC Microbiol.* 11:103. doi: 10.1186/1471-2180-11-103

Li, F. Y., Li, C. X., Chen, Y. H., Liu, J. H., Zhang, C. Y., Barry, I., et al. (2019). Host genetics influence the rumen microbiota and heritable rumen microbial features associate with feed efficiency in cattle. *Microbiome* 7:92. doi: 10.1186/s40168-019-0699-1

Liu, C., Wu, H., Liu, S. J., Chai, S. T., Meng, Q. X., and Zhou, Z. M. (2019). Dynamic alterations in yak rumen bacteria community and metabolome characteristics in response to feed type. *Front. Microbiol.* 10:1116. doi: 10.3389/fmicb.2019.01116

Liu, H., Jiang, H., Hao, L. Z., Cao, X. L., Degen, A., Zhou, J. W., et al. (2021a). Rumen bacterial community of grazing lactating yaks (*Poephagus grunniens*) supplemented with concentrate feed and/or rumen-protected lysine and methionine. *Animals* 11:2425. doi: 10.3390/ani11082425

Liu, H., Yang, G., Degen, A., Ji, K. X., Jiao, D., Liang, Y. P., et al. (2021b). Effect of feed level and supplementary rumen protected lysine and methionine on growth performance, rumen fermentation, blood metabolites and nitrogen balance in growing Tan lambs fed low protein diets. *Anim. Feed Sci. Technol.* 279:115024. doi: 10.1016/j.anifeedsci.2021.115024

Liu, H., Zhou, J. W., Degen, A., Liu, H. S., Cao, X. L., Hao, L. Z., et al. (2022). A comparison of average daily gain, apparent digestibilities, energy balance, rumen fermentation parameters, and serum metabolites between yaks (*Bos grunniens*) and Qaidam cattle (*Bos taurus*) consuming diets differing in energy level. *Anim. Nutr.* (in press).

Liu, J. H., Bian, G. R., Zhu, W. Y., and Mao, S. Y. (2015). High-grain feeding causes strong shifts in ruminal epithelial bacterial community and expression of toll-like receptor genes in goats. *Front. Microbiol.* 6:167. doi: 10.3389/fmicb.2015.00167

Mao, S. Y., Huo, W. J., and Zhu, W. Y. (2016). Microbiome-metabolome analysis reveals unhealthy alterations in the composition and metabolism of ruminal microbiota with increasing dietary grain in a goat model. *Environ. Microbiol.* 18, 525–541. doi: 10.1111/1462-2920.12724

Mi, J. D., Zhou, J. W., Huang, X. D., and Long, R. J. (2017). Lower methane emissions from yak compared with cattle in rusitec fermenters. *PLoS One* 12:e0170044. doi: 10.1371/journal.pone.0170044

Min, B. R., Gurung, N., Shange, R., and Solaiman, S. (2019). Potential role of rumen microbiota in altering average daily gain and feed efficiency in meat goats fed simple and mixed pastures using bacterial tag-encoded FLX amplicon pyrosequencing. *J. Anim. Sci.* 97, 3523–3534. doi: 10.1093/jas/skz193

National Research Council (2000). *Nutrient requirements of beef cattle*, 7th Edn. Washington, DC: National Academy of Sciences.

Poonko, S., Boonsaen, P., and Sawanon, S. (2015). Fibrolytic bacterium isolated from buffalo rumen phylogenetically closely related to *Butyrivibrios* and *Pseudobutyrivibrios*. *Kasetsart J.* 49, 547–559.

Preston, T. R., and Leng, R. A. (1987). *Matching ruminant production system with available resources in the tropical and sub-tropics*. Armidale, NSW: Penambul Books.

Qiu, Q., Zhang, G., Ma, T., Qian, W., Wang, J., Ye, Z., et al. (2012). The yak genome and adaptation to life at high altitude. *Nat. Genet.* 44, 946–949. doi: 10.1038/ng.2343

Robertson, J. B., and Van Soest, P. J. (1981). "The detergent system of analysis and its application to human foods," in *The analysis of dietary fiber in food*, eds W. P. James and O. Theander (New York, NY: Marcel Dekker), 23–158.

Russell, J. B., and Rychlik, J. L. (2001). Factors that alter rumen microbial ecology. *Science* 292, 1119–1122. doi: 10.1126/science.1058830

Sarraseca, A., Milne, E., Metcalf, M. J., and Lobley, G. E. (1998). Urea recycling in sheep: Effects of intake. *Br. J. Nutr.* 79, 79–88. doi: 10.1079/bjn19980011

Sha, Y. Z., Hu, J., Shi, B. G., Dingkao, R. Q., Wang, J. Q., Li, S. B., et al. (2020). Characteristics and functions of the rumen microbial community of cattle-yak at different ages. *Biomed Res. Int.* 2020:3482692. doi: 10.1155/2020/3482692

Stevenson, D. M., and Weimer, P. J. (2007). Dominance of *Prevotella* and low abundance of classical ruminal bacterial species in the bovine rumen revealed by relative quantification real-time PCR. *Appl. Microbiol. Biotechnol.* 75, 165–174. doi: 10.1007/s00253-006-0802-y

Sul'ák, M., Sikorová, L., Jankuvová, J., Javorský, P., and Pristaš, P. (2012). Variability of actinobacteria, a minor component of rumen microflora. *Folia Microbiol.* 57, 351–353. doi: 10.1007/s12223-012-0140-7

Thanner, S., Schori, F., Bruckmaier, R. M., and Dohme-Meier, F. (2014). Grazing behaviour, physical activity and metabolic profile of two Holstein strains in an organic grazing system. *J. Anim. Physiol. Anim. Nutr.* 98, 1143–1153. doi: 10.1111/jpn.12172

Van Gylswyk, N. O. (1970). The effect of supplementing a low-protein hay on the cellulolytic bacteria in the rumen of sheep and on the digestibility of cellulose and hemicellulose. *J. Agric. Sci.* 74, 169–180. doi: 10.1017/S0021859600021122

Van Gylswyk, N. O. (1995). *Succiniclasticum ruminis* gen. nov., sp. nov., a ruminal bacterium converting succinate to propionate as the sole energy-yielding mechanism. *Int. J. Syst. Bacteriol.* 45, 297–300. doi: 10.1099/00207713-45-2-297

Van Soest, P. J., Robertson, J. B., and Lewis, B. A. (1991). Methods for dietary fiber, neutral detergent fiber, and nonstarch polysaccharides in relation to animal nutrition. *J. Dairy Sci.* 74, 3583–3597. doi: 10.3168/jds.S0022-0302(91)78551-2

Wang, H. B., He, Y., Li, H., Wu, F., Qiu, Q. H., Niu, W. J., et al. (2019a). Rumen fermentation, intramuscular fat fatty acid profiles and related rumen bacterial populations of Holstein bulls fed diets with different energy levels. *Appl. Microbiol. Biotechnol.* 103, 4931–4942. doi: 10.1007/s00253-019-09839-3

Wang, H. B., Li, H., Wu, F., Qiu, X. J., Yu, Z. T., Niu, W. J., et al. (2019b). Effects of dietary energy on growth performance, rumen fermentation and bacterial community, and meat quality of Holstein-Friesians bulls slaughtered at different ages. *Animals* 9:1123. doi: 10.3390/ani9121123

Wang, H. C. (2009). *Urinary purine derivative excretion as method for estimation of rumen microbial protein production of yak in Qing-hai Tibetan Plateau* (In Chinese). Ph.D Dissertation. Lanzhou: Lanzhou University.

Wei, Z., Xie, X., Xue, M., Valencak, T. G., Liu, J., and Sun, H. (2021). The effects of non-fiber carbohydrate content and forage type on rumen microbiome of dairy cows. *Animals* 11:3519. doi: 10.3390/ani11123519

Westreicher-Kristen, E., Castro-Montoya, J., Hasler, M., and Susenbeth, A. (2020). Relationship of milk odd- and branched-chain fatty acids with urine parameters and ruminal microbial protein synthesis in dairy cows fed different proportions of maize silage and red clover silage. *Animals* 10:316. doi: 10.3390/ani10020316

Xie, A. Y., Chai, S. T., Wang, W. B., Xue, B., Liu, S. J., Zhao, X. P., et al. (1996). The herbage yield and the nutrient variation in mountain meadow (In Chinese). *Qinghai J. Anim. Sci. Vet. Med.* 26, 8–10.

Xin, J. W., Chai, Z. X., Zhang, C. F., Zhang, Q., Zhu, Y., Cao, H. W., et al. (2019). Comparing the microbial community in four stomach of dairy cattle, yellow cattle and three yak herds in Qinghai-Tibetan Plateau. *Front. Microbiol.* 10:1547. doi: 10.3389/fmicb.2019.01547

Xiong, B. H., Luo, Q. R., Zheng, S. S., and Zhao, Y. G. (2018). Tables of feed compostion and nutritive values in China (In Chinese). *China Feed* 22, 81–86.

Xue, B. C., Wu, M., Yue, S. M., Hu, A. H., Li, X., Hong, Q. H., et al. (2022). Changes in rumen bacterial community induced by the dietary physically effective neutral detergent fiber levels in goat diets. *Front. Microbiol.* 13:820509. doi: 10.3389/fmicb.2022.820509

Xue, M. Y., Sun, H. Z., Wu, X. H., Liu, J. X., and Guan, L. L. (2020). Multi-omics reveals that the rumen microbiome and its metabolome together with the host metabolome contribute to individualized dairy cow performance. *Microbiome* 8:64. doi: 10.1186/s40168-020-00819-8

Zhang, L., Sun, B. Z., Xie, P., Li, H. P., Su, H. W., Sha, K., et al. (2015). Using near infrared spectroscopy to predict the physical traits of *Bos grunniens* meat. *LWT Food Sci. Technol.* 64, 602–608. doi: 10.1016/j.lwt.2015.06.022

Zhang, Z. G., Xu, D. M., Wang, L., Hao, J. J., Wang, J. F., Zhou, X., et al. (2016). Convergent evolution of rumen microbiomes in high-altitude mammals. *Curr. Biol.* 26, 1873–1879. doi: 10.1016/j.cub.2016.05.012

Zhao, X. H., Zhou, S., Bao, L. B., Song, X. Z., Ouyang, K. H., Xu, L. J., et al. (2017). Response of rumen bacterial diversity and fermentation parameters in beef cattle to diets containing supplemental daidzein. *Ital. J. Anim. Sci.* 17, 643–649. doi: 10.1080/1828051x.2017.1404943

Zhou, J. W., Liu, H., Zhong, C. L., Degen, A. A., Yang, G., Zhang, Y., et al. (2018). Apparent digestibility, rumen fermentation, digestible enzyme and urinary purine derivatives in yaks and cattle offered forage-concentrate diets differing in nitrogen concentration. *Livest. Sci.* 208, 14–21. doi: 10.1016/j.livsci.2017.11.020

Zhou, J. W., Wang, W. J., Jing, X. P., Degen, A. A., Guo, Y. M., Kang, J. P., et al. (2019). Effect of dietary on digestibilities, rumen fermentation, urinary purine derivatives and serum metabolites in Tibetan and small-tailed Han sheep. *J. Anim. Physiol. Anim. Nutr.* 103, 977–987. doi: 10.1111/jpn.13098

Zhou, J. W., Zhong, C. L., Liu, H., Degen, A. A., Titgemeyer, E. C., Ding, L. M., et al. (2017). Comparison of nitrogen utilization and urea kinetics between yaks (*Bos grunniens*) and indigenous cattle (*Bos taurus*) on the Qinghai-Tibetan Plateau. *J. Anim. Sci.* 95, 4600–4612. doi: 10.2527/jas2017.1428

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# Effects of high-altitude hypoxic environment on colonic inflammation, intestinal barrier and gut microbiota in three-way crossbred commercial pigs

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In recent years, the three-way crossbred commercial pigs are extensively cultured in Tibet. However, there have been few studies about the effect of high-altitude hypoxic environment on intestinal health of them. Therefore, we selected Tibetan pigs (TP) and the three-way crossbred commercial pigs (CP-H) living in the Tibet (3,500–3,700m in altitude) as a positive control group and treatment group, respectively. The three-way crossbred commercial pigs (CP-L) living at altitudes 800–1,000m sea level were selected as a negative control group. The colonic chyme, colonic mucosa, colonic tissue and serum samples were collected for the detection of gut microbiota and intestinal inflammation. The results showed that high-altitude hypoxic environment promoted the occurrence of colonic inflammation, disrupted the colonic barrier to some extent. And Hematoxylin–Eosin (HE) staining revealed that mild inflammatory cell infiltration was observed in colon of CP-H. 16S rRNA gene sequencing revealed that the microbial community composition of CP-H was changed compared with CP-L. Gut bacterial communities formed distinctly different clusters in principal coordinates analysis (PCoA) space, and Chao 1 index of CP-H was also decreased. At the genus level, *Terrisporobacter* showed greater enrichment in the CP-H than lower-altitude pigs. *Colstridium-sensu-stricto-1* showed lower enrichment in the CP-H than lower-altitude pigs. However, the concentration of valeric acid in colonic chyme of CP-H was higher than CP-L and TP. Correlation analysis indicated that *Terrisporobacter* was positively associated with the relative mRNA expression level of *IL-1β* and the content of lipopolysaccharide (LPS), and was negatively correlated with the relative mRNA expression level of *IL-10*. The *Streptococcus* was positively associated with the concentrations of valerate. In summary, high-altitude hypoxic environment changed compositions of gut microbiota, promoted the occurrence of colonic inflammation, and disrupted intestinal barrier of the three-way crossbred commercial pigs.

KEYWORDS

**three-way crossbred commercial pigs, high-altitude hypoxic environment, intestinal barrier, gut microbiota, inflammatory cytokines, short-chain fatty acids**

# Introduction

In recent years, with the rapid development of society and the increasing demand for a better life, the demand of people living in Tibet is more diversified for meat products, especially for pork of three-way crossbred commercial pigs. To resolve this contradiction, the breeding of three-way crossbred commercial pigs was rapidly implemented under the unified leadership of the local government. According to statistics of the local government, in recent years, the scale of breeding for three-way crossbred commercial pigs has been more than 300,000 in Tibet, and the scale of breeding is steadily increasing.

At present, high-altitude hypoxic environment has been shown to increase the development of inflammation throughout the body or multiple-organ dysfunction possibly (Pham et al., 2021). For example, high-altitude hypoxic environment induces acute mountain sickness, high altitude pulmonary edema, and high altitude cerebral edema (Purushothaman et al., 2011), and can even also affect the growth and intestinal health (Jensen and Moore, 1997; Xu et al., 2014). In addition, it has also been shown that inflammatory signaling in response to high altitude hypoxic environment exist an adaptive mechanism (Pham et al., 2021). Tibetan pigs, also known as ginseng pigs, are the indigenous breed native to the Qinghai-Tibet Plateau. In order to adapt to high-altitude environments, Tibetan pigs have developed unique physiological mechanisms during the long process of evolution and have acquired stable structural and functional characteristics (Yang et al., 2021). In the last few years, the commercial pigs had also been raised in Qinghai-Tibet Plateau because of its higher growth rate, higher feed efficiency, and superior meat yield (Zhang et al., 2018). However, the three-way crossbred commercial pigs were purchased from plain areas of China soon after weaning. They may face immense living challenges from high altitude hypoxic environment due to environmental changes (Lanikova et al., 2017).

As is well known, the gut is an important physiological function organ (Brestoff and Artis, 2013). The microbiota located in the gut exerts important functions for gut homeostasis and host health. Studies have proven that intestinal microbiota is strongly linked to intestinal barrier function and systemic inflammation (Pickard et al., 2017). For example, enteric pathogen *C. rodentium* is a major hallmark of diarrheal disease for mice, its expansion can result in dramatic colonic mucosal hyperplasia and a local Th1 inflammatory response. *Clostridium difficile* infection can result in the occurrence of inflammatory bowel disease (Sokol et al., 2018). In contrast, the abundances of intestinal beneficial bacteria are positively related to the intestinal health, such as *Lactobacillus* and *Bifidobacterium* maybe improve intestinal barrier function and reduce intestinal inflammation (Wu et al., 2020). Meanwhile, gut microbiome is also influenced by many factors, such as genetic, foods and environmental factors (Goodrich et al., 2017), and it has been the widely validated. In addition to above factors, high-altitude environments affect the composition and function of the gut microbiota (Suzuki et al., 2019). At high altitude, there is a decrease in the barometric pressure and a consequent reduction in the oxygen partial pressure, which will lead to the increased abundances of the obligate anaerobes (Moreno-Indias et al., 2015). At present, a few studies have explored the microbial community composition of mammals living at different altitudes, including ruminants (Zhang et al., 2016), humans (Lan et al., 2017), and pikas (Li et al., 2016). However, the studies about that the effects of high-altitude hypoxic environment on colonic inflammation, intestinal barrier and gut microbiota in three-way crossbred commercial pigs have so far hardly been explored. Therefore, we selected Tibetan pigs and three-way crossbred commercial pigs living at 3500–3700 m as a positive control group and treatment group in the present trial, respectively. Moreover, three-way crossbred commercial pigs living at 800–1000 m were also selected as negative control group. We used 16S rRNA gene sequencing to analyze the microbiota between Tibetan pigs and three-way crossbred commercial pigs, analyzing the relationship between gut microbiota and intestinal parameters such as intestinal inflammation and intestinal barrier. This study thus sought to explore the effects of high-altitude hypoxic environment on gut microbiota and gut health of pigs.

# Materials and methods

## Ethics statement

All procedures in the current study including animal experiments and sample collection were approved by the Experimental Animal Welfare and Ethical Committee of the Institute of Animal Science, Chinese Academy of Agricultural Sciences (No. IAS2021-241).

## Animal and sample collection

Tibetan pigs (TP) and three-way crossbred commercial pigs (CP-H) were cultured under the research farm (Shannan District, Tibet, China) of Institute of Animal Science and Veterinary, Tibet Academy of Agricultural and Animal Husbandry Sciences (3,500–3,700 m above sea level). Three-way crossbred commercial pigs (CP-L; with the same genetic background as three-way crossbred commercial pigs above) were cultured under a commercial pig farm in Shanxi Province (800–1,000 m above sea level). All the pigs were reared according to the feeding standards and water *ad libitum* (Huang et al., 2021), and were fed with the same standard diet (Table 1). The pigs were euthanized for 6 randomly selected pigs per group when reached slaughter weights in this study. The blood was collected in order to obtain serum. Fresh colonic chyme and mucosa were fleetly collected, immersed in liquid nitrogen immediately, then stored at-80°C until further analysis. Finally, the colonic tissue samples were taken out and fixed in 4% formaldehyde.

## Tissue sample and intestinal morphology

The colon tissues were immersed and fixed with 4% formaldehyde. Then, they were removed from formalin and embedded in paraffin. Subsequently, the paraffin blocks were sectioned at 5 μm thick sections using a semi-automatic microtome (LONGSHOU, China). Next, they were stained with hematoxylin and eosin according to methods of Luo et al. (2021), and observed under optical microscope and calculated histologic colitis score according to a pathology score system. Methods of pathology score system are described briefly below. Inflammation score was graded from 0 to 3 depending on the severity of the inflammation and infiltration of immune cells. Inflammation extent was graded from 0 to 3 with regard to the width of colon membrane affected by colitis including mucosa, sub-mucosa and transmural layers. Crypt damage was graded from 0 to 4 considering the damage of crypt and epithelial cells. Histologic colitis score was calculated by summing inflammation severity, inflammation extent and crypt damage.

## Concentrations of SCFAs

Approximately 1 g of the colonic chyme was collected and immersed in 10 ml of dd$H_2O$ in 15-mL screw capped vials, after shaked for 30 min, refrigerated at 4°C overnight. The mixture was centrifuged at 10,000 rpm for 10 min for the concentration analysis of SCFAs. Concentrations of SCFAs were determined by gas chromatography according to the method of Wu et al. (2016).

## DNA extraction, 16S rRNA gene amplification, sequencing and analysis

Approximately 0.5–1 g the colonic chyme was collected, respectively, from each sample, and microbial community genomic DNA was extracted microbial community genomic DNA according to the manufacturer's instructions of the E.Z.N.A.® soil DNA Kit (D5625-02, Omega Bio-Tek Inc., Norcross, GA, United States). Then it was stored at-80°C until the time of analysis. In addition, the purity and DNA concentration were checked by 1% agarose gel electrophoresis and NanoDrop2000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, United States) separately. The V3-V4 regions of bacterial 16S rRNA gene were amplified with the following primer set: 338F (5′-ACTCCTACGGGAGGCAGCAG-3′) and 806R (5′-GGACTACHVGGGTWTCTAAT-3′). The reaction system, determination of amplified fragments and purification were performed according to methods of Luo et al. (2021). The raw microbial sequence data were analyzed and processed by the Majorbio Bio-Pharm Technology Co. Ltd. (Shanghai, China). The sequences were analyzed and assigned to operational taxonomic units (OTUs; 97% identity). What is more, the alpha-diversity, whose coverage was based on the Chao 1 and Shannon index within each sample was generated by QIIME (Version 174 1.7.0; Qingsen et al., 2017), and beta diversity was estimated by computing the unweighted Unifrac distance and visualized using PCoA.

TABLE 1 Composition and nutrient levels of diet.

| Ingredient composition, % | Diet |
|---|---|
| Corn | 66.40 |
| Soybean meal | 15.50 |
| Full-fat soybean | |
| Wheat bran | 15.00 |
| Soybean meal | |
| Limestone | 1.00 |
| Dried whey | |
| $CaHPO_4$ | 0.50 |
| Soybean oil | |
| NaCl | 0.30 |
| CaHPO4 | |
| L-Lys (70%) | 0.20 |
| NaCl | |
| Choline chloride | 0.10 |
| Premix[1] | 1.00 |
| Choline chloride | |
| Limestone | |
| Total | 100.00 |
| Choline chloride | |
| Calculated nutrient levels， % | |
| Lysine HCl | |
| CP | 14.83 |
| Met | |
| DE, MJ/kg | 13.90 |
| Thr | |
| NE, MJ/kg | 10.37 |
| Glucose | |
| SID Thr | 0.43 |
| SID Trp | 0.13 |
| Premixb | |
| SID Lys | 0.75 |
| SID Met | 0.23 |
| DE (MJ/kg) | |
| Ca | 0.56 |
| CP | |
| TP | 0.46 |
| Lys | |
| STTD P | 0.25 |
| Met | |

[1]Provided the following quantities per kg of diet: vitamin A, 9,140 IU; vitamin D3, 4,405 IU; vitamin E, 11 IU; menadione sodium bisulfite, 7.30 mg; riboflavin, 9.15 mg; D-pantothenic acid, 18.33 mg; niacin, 73.50 mg; choline chloride, 1,285 mg; vitamin $B_{12}$, 200 ug; biotin, 900 ug; thiamine mononitrate, 3.67 mg; folic acid, 1,650 ug; pyridoxine hydrochloride, 5.50 mg; I, 1.85 mg; Mn, 110.10 mg; Cu, 7.40 mg; Fe, 73.50 mg; Zn, 73.50 mg; Se, 500 ug.

## Genes expression

Total RNA of colonic mucosa was extracted using TransZol reagent (Ambion, UT, United States). Then the integrity and quality of total RNA were tested by electrophoresing on a 1.0% agarose gel and the Nano Drop® ND-1000 spectrophotometer (Nano-Drop Technologies, Wilmington, DE, United States),

respectively. The cDNA was synthesized by a reverse transcription kit. The cDNA was stored at-20°C for quantitative real-time PCR (qRT-PCR). All primers were designed according to the method of Luo et al. (2019), and were synthesized by Sangon Biotech Corporation (Sangon Biotech, China). The primer sequences were listed in Table 2. qRT-PCR assays were performed according to the method of Luo et al. (2021). The expression levels of all were calculated by $2^{-\Delta\Delta CT}$ methods (Luo et al., 2019).

TABLE 2 The nucleotide sequences of primer.

| Target gene | Forward sequence (5′-3′) | Reverse sequence (5′- 3′) |
|---|---|---|
| ZO-1 | CTCCAGGCCCTTACCTTTCG | GGGGTAGGGGTCCTTCCTAT |
| Occludin | CAGGTGCACCCTCCAGATTG | TATGTCGTTGCTGGGTGCAT |
| TNF-α | TAAGGGCTGCCTTGGTTCAG | AGAGGTTCAGCGATGTAGCG |
| IL-1β | ATTCAGGGACCCTACCCTCTC | CTTCTCCACTGCCACGATGA |
| P65 | CGAGAGGAGCACGGATACCA | CCCGTGTAGCCATTGATCTTG |
| IL-6 | TGAGGCAAAAGGGAAAGA | GCGCAGGATGAGAATGA |
| IL-10 | TCGGCCCAGTGAAGAGTTTC | GGAGTTCACGTGCTCCTTGA |
| GAPDH | GGGCATGAACCATGAGAAGT | GGGCATGAACCATGAGAAGT |
| β-actin | GCGTAGCATTTGCTGCATGA | GCGTGTGTGTAACTAGGGGT |

## LPS, DAO and D-lactide

LPS in serum was measured using the commercially available tachypleus amebocyte lysate kit (Chinese Horseshoe Crab Reagent Manufactory Co., Ltd., Xiamen, China) according to the method of quantitative Chromogenic Limulus Amebocyte Lysate assay. DAO and D-lactide in serum were measured by kit from Nanjing Jiancheng Bioengineering Institute (Nanjing, China).

## Statistical analysis

Morphology of colon, colonic permeability, mRNA expression, and concentrations of SCFAs date were analyzed using SPSS (23.0). The correlation matrix between the bacterial species, and intestinal barrier, inflammatory cytokines, SCFAs and blood biochemical parameters were generated using Pearson's correlation coefficient. The *p*-value below 0.05 was considered significant, and absolute value of r higher than 0.5 was considered a strong correlation factor (Cianga et al., 2021). GraphPad 8.0 was used to draw all the above data. Finally, the results were presented as means ± SEM, and with * indicating a statistically significant difference ($p < 0.05$), ** indicating a highly significant difference ($p < 0.01$).

# Results

## Histologic colitis score from colonic sections and LPS, DAO, and D-lactide in serum

The morphological observations and pathological slices of colon were shown in Figure 1A. Mild inflammatory cell infiltration was observed in colon of CP-H. There was no inflammatory cell invasion in colon of TP and CP-L. The histologic colitis score was shown in Figure 1B. The histologic colitis score of CP-H was significantly increased compared with the TP and CP-L ($p < 0.05$).

There were no significant differences in the level of D-Lac in serum (Figure 1D). The activity of DAO and the level of LPS in serum of CP-H were significantly higher compared with TP and CP-L ($p < 0.05$; Figures 1C,E).

## Concentrations of SCFAs in colonic chyme

The concentrations of acetic acid, propionic acid, isobutyric acid, butyric acid, and isovaleric acid in colonic chyme were not statistically significant (Figure 2A). In addition, concentrations of total SCFAs also revealed no significant differences (Figure 2A). However, the concentration of valeric acid in colonic chyme of CP-H was higher than CP-L and TP ($p < 0.05$; Figure 2A).

## Intestinal inflammation in colonic mucosa

Relative mRNA expression levels of *IL-1β* and *IL-6* in colonic mucosa of CP-H were higher relative to TP and CP-L ($p < 0.01$), and no significant differences were found between the two control groups (Figure 2B). Relative mRNA expression level of *p65* in CP-H was higher relative to TP ($p < 0.05$), but there were no significant differences between CP-H and CP-L (Figure 2B). Relative mRNA expression levels of *IL-10, ZO-1*, and *Occludin* in colonic mucosa of CP-H were lower relative to TP and CP-L ($p < 0.05$; Figure 2B). Furthermore, we also found that relative mRNA expression level of *IL-10* of CP-L was significantly higher than TP.

## Composition of gut microbes in colonic chyme

To further assess whether differences in gut microbiota are the causal factor for the differences in gut barrier among TP, CP-H, and CP-L. The fresh colonic chyme was obtained from TP, CP-H, and CP-L, and 16 s rRNA gene sequencing analysis was performed. Shannon index of colonic chyme in TP was higher than those in CP-H and CP-L, and a statistically significant difference was found between TP and CP-L ($p < 0.05$; Figure 3A). Chao 1 index of CP-H was significantly lower than other groups ($p < 0.05$; Figure 3B). The PCoA indicated that TP, CP-H, and CP-L were distinctly clustered separately in distribution of microbiota at the colonic chyme (Figure 3C). There were 1,405, 1,301, and 1,364 operational

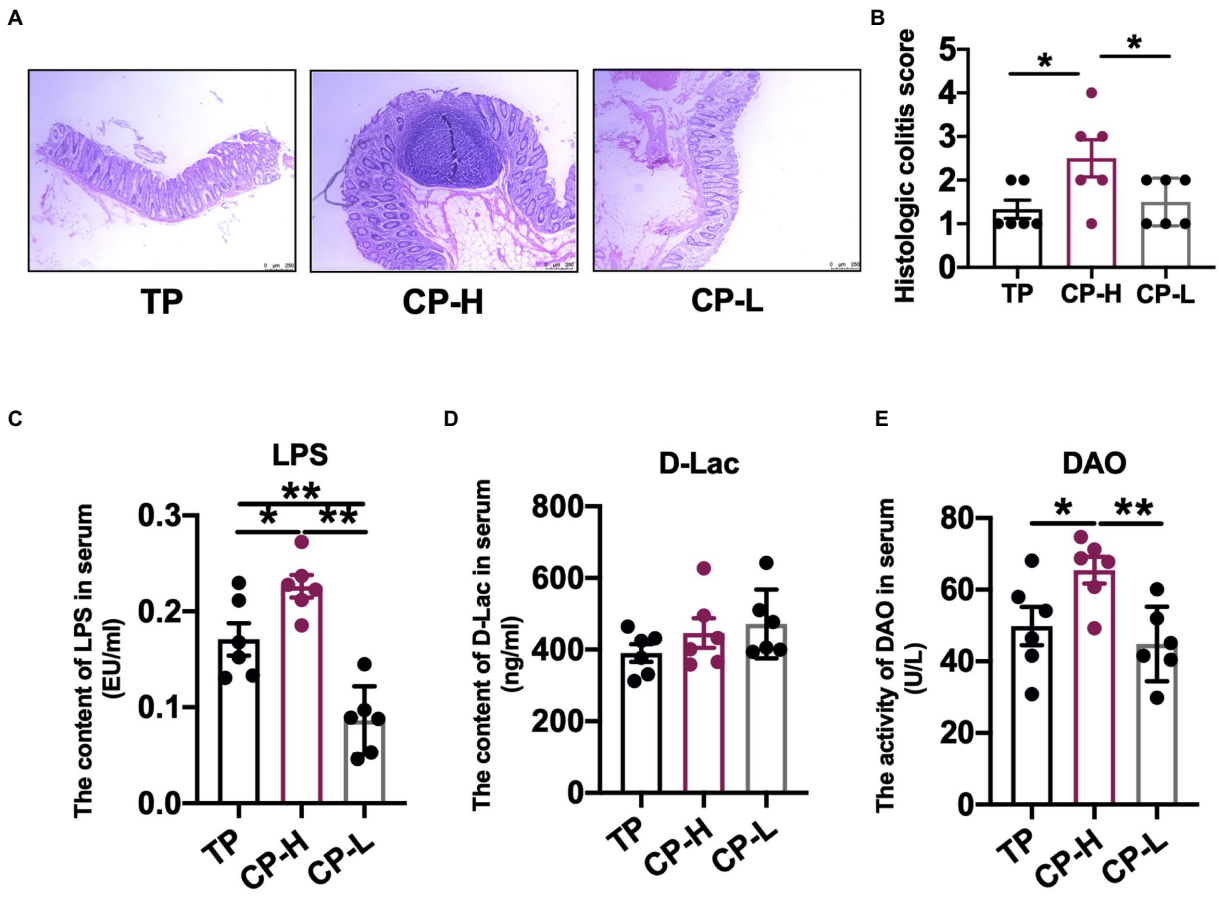


FIGURE 1
Effects of high-altitude hypoxic environment on intestinal tissue and LPS, DAO and D-lactide in serum of pig. TP: positive control group, CP-H: treatment group, CP-L: negative control group. **(A)** HE-stained pathological sections of colonic tissue from pigs of groups. **(B)** Histologic colitis score of pigs. **(C)** The content of LPS in serum of pigs. **(D)** The content of D-Lac in serum of pig. **(E)** The activity of DAO in serum of pigs. Data are expressed as mean±SEM ($n$=6). * indicating a statistically significant difference ($p$< 0.05), ** indicating a highly significant difference ($p$< 0.01).

taxonomic units (OTUs) obtained from TP, CP-H, and CP-L, respectively, of which 1,002 were common OTUs among the three experimental groups (Figure 3D).

Microbial community composition at the phylum, genus, and species level of the three groups were presented in Figures 4A–F. The results showed that colonic chyme samples comprised four major phyla including *Firmicutes*, *Bacteroidota*, *Spirochaetota*, and *Actinobacteria* (Figure 4A). *Firmicutes* and *Bacteroidetes* were the most predominant phyla in the colons of TP, CP-H, and CP-L (Figure 4A). In addition, there were also significant differences in abundance of *Cyanobacteria*, *WPS-2, Patescibacteria,* and *Fusobacteriota* among three breeds ($p < 0.05$; Figure 4B). At the genus level, the top three most abundant genus in three groups, in turn, were *Colstridium-sensu-stricto-1*, *Lactobacillus*, and *Terrisporobacter* (Figure 4C). Composition of the gut microbiota was further analyzed at the species level (Figures 4E,F). The *Lactobacillus_johnsonii* and *Lactobacillus_reuteri* were significantly enriched in TP (Figures 4F).

LEfSe analysis was performed and presented as LDA score ≥ 3.0 to further explore the differences in microbiota composition between the three groups. The results were showed in Figure 5A. A total of 43 biomarkers in TP, CP-H, and CP-L. There were 14, 13, and 16 biomarkers in TP, CP-H, and CP-L, respectively. Notably, *Terrisporobacter* was enriched in colonic chyme of CP-H, *Colstridium-sensu-stricto-1* and *Streptococcus* were enriched in colonic chyme of CP-L. The relative abundance of the three genera varied among the three groups ($p < 0.05$; Figures 5B–D).

## Correlation between microbial communities and gut barrier

Correlations between metabolites and the top 15 genus between TP, CP-H, and CP-L were obtained *via* Pearson's correlation analysis. As shown in Figure 6, the results showed that the relative abundance of *Terrisporobacter* was positively associated with the relative mRNA expression level of *IL-1β*, the content of LPS, and was negatively correlated with the relative mRNA expression level of *IL-10* (Figure 6). The relative abundance of *Streptococcus* was positively associated with the concentration of valerate (Figure 6).

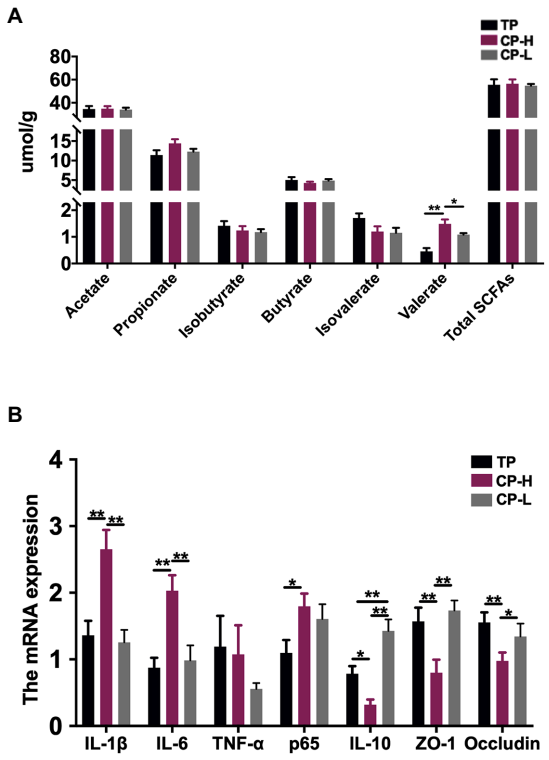


FIGURE 2
Effects of high-altitude hypoxic environment on intestinal inflammation and concentrations of SCFAs in the colonic chyme of pigs. TP: positive control group, CP-H: treatment group, CP-L: negative control group. **(A)** The concentrations of Acetate acid, Propionic acid, Isobutyric acid, Butyric acid, Isovaleric acid, Valeric acid and total SCFAs in the colonic chyme of pigs. **(B)** The mRNA expression levels of *IL-1β*, *IL-6*, *TNF-α*, *p65*, *IL-10*, *ZO-1* and *Occludin* in colonic mucosa of pigs. Data are expressed as mean±SEM ($n$=6). * indicating a statistically significant difference ($p$< 0.05), ** indicating a highly significant difference ($p$< 0.01).

## Discussion

Intestinal barrier, including ecological barrier, mechanical barrier, and immunity barrier, plays an integral role in maintaining gut homeostasis. Among them, the intestinal mechanical barrier is essentially a defensive layer composed of intestinal mucosal epithelial cells and tight junctions between cells and the bacterial membrane, and can effectively prevent intestinal injury (Odenwald and Turner, 2017). Break of mechanical barrier often show obvious mucosal ulceration, loss of crypt, goblet cells and epithelial damage, neutrophil infiltration and causes inflammation (Turner, 2009; Li et al., 2019a). In this study, the occurrence of local mild inflammatory cell infiltration was observed in the colon of CP-H group. The level of D-Lac, LPS and the activity of DAO in serum of CP-H group were higher compared with CP-L group. Moreover, we also found that relative mRNA expression levels of *IL-1β*, *IL-6* and *p65* in colonic mucosa of CP-H group were higher relative to CP-L group. These data evidenced that the colon of CP living in plateau were in the inflamed state, and the intestinal barrier was disrupted to the certain extent. This situation may be related to the altitude at which the CP were located (Yi et al., 2021). It has been reported that upon exposure to high altitude, oxygen deficit and oxidative stress can occur (Földes-Papp et al., 2005; Pichler Hefti et al., 2016). Subsequently, inflammatory factors are released in response to hypoxia and oxidative stress (Himadri et al., 2010; Querido et al., 2012). For example, Himadri et al. (2010) found that high-altitude hypoxic environment leads to activation of *NFκB* in brain of rats. The activated *NFκB* further upregulates the proinflammatory cytokines, such as *IL-1*, *IL-6*, and *TNF-α*. On the other hand, the development of the inflammatory reactions might also be involved in as well as acclimation to high altitudes, while there is a genetic basis to the adaptation of high altitude (Simonson et al., 2010; Yi et al., 2021). In one study, comparing the levels of systemic inflammatory factors in the serum of 36 healthy subjects who had been living on the Qinghai-Tibetan plateau for a short time and 24 subjects who prolonged living on the Qinghai-Tibetan plateau, the authors found that the 36 healthy subjects' levels of *IL-2*, *IL-3*, *MCP-1*, *IL-1β* in serum were higher than those in the 24 subjects (Yi et al., 2021). Simonson et al. (2010). found that high-altitude adaptation in Tibetans has resulted from local positive selection on the genes of *EGLN1* and *PPARA*. CP is not a local pig breed of Qinghai-Tibetan plateau. In this experiment, the CP were purchased from the plain area of China after weaning. Therefore, the occurrence of intestinal inflammation was observed in the colon of CP-H group, the reason may be that the change of genetic material is also very difficult to achieve in short life of CP in order to adapt to their environment.

Altitude is also an important factor that influence the gut microbiota composition. Most the studies demonstrated that significant differences of gut microbiota were observed among different altitudes (Lan et al., 2017). Researches compared the differences in intestinal microbiota between plateau pika and the low-altitude dauricus. *Scleroderma* was the most abundant genus in the plateau pika, and *Prevotella*, *Oscillospira*, *Ruminococcus*, and *yrc22* were abundant in the plateau pika. While *Proteobacteria*, *Actinobacteria*, and *Verrucomicrobia* were abundant in low-altitude dauricus. The Shannon index of the plateau pika were significantly higher than those of the low-altitude dauricus, and eight genera, including *Streptococcus* and *Pseudomonas*, increased with the altitude (Li et al., 2018, 2019b). Additionally, Zeng et al. (2020). found that *Acinetobacter*, *Pseudomonas*, and *Sphingobacterium* were the top three abundant bacterial genera in high altitude fecal samples in both humans and pigs. In this study, a large variation had also been observed in microbiota composition between CP-H group and CP-L group, including the within community diversity (alpha diversity), the between community diversity (beta diversity) and the microbial composition in the different levels. Therein, the *Terrisporobacter* and *UCG-002* were enriched in the CP-H compared with CP-L. The most likely explanation for this phenotype was that at high altitude, there was a decrease in the barometric pressure and a consequent reduction in the oxygen partial pressure, which lead to the abundances of these obligate anaerobes increased (Moreno-Indias et al., 2015).

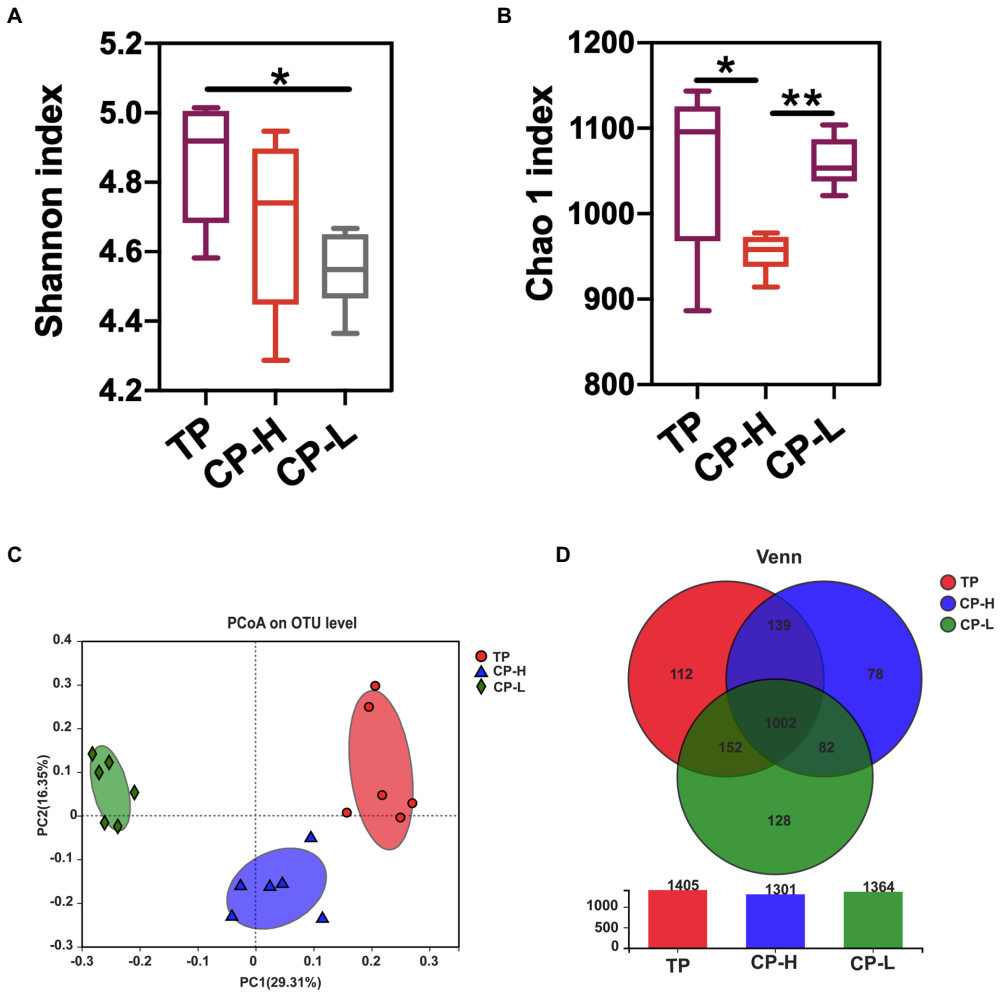


FIGURE 3
Effects of high-altitude hypoxic environment on gut microbiota diversity of pigs. TP: positive control group, CP-H: treatment group, CP-L: negative control group. **(A)** The Shannon index of microbiota in colonic chyme. **(B)** The Chao 1 index of microbiota in colonic chyme. **(C)** PCoA analysis of microbiota in colonic chyme at the OUT level. **(D)** The Wayne figures of microbiota in colonic chyme. Data are expressed as mean±SEM ($n$=6). * indicating a statistically significant difference ($p$< 0.05), ** indicating a highly significant difference ($p$< 0.01).

Under normal conditions, intact intestinal barrier can inhibit pathogenic bacterial growth (Fukuda et al., 2011). However, some conditional pathogenic bacterial have the opportunity to grow quickly when the barrier is damaged. These pathogenic bacterial destroy the intestinal microecological barrier, then accelerate intestinal damage and result in bacterial antigen translocation from the intestinal cavity to the circulatory system and finally lead to systemic inflammation (Figueredo et al., 2018; Yin et al., 2019). In this study, the abundance of *Terrisporobacter* in CP-H group was significantly higher than CP-L group, and it was positive significant correlation between *Terrisporobacter* and relative mRNA expression levels of *IL-1β*, the LPS content and the activity of DAO. Very little is currently known about *Terrisporobacter*, but there were also indicated that it has been linked to oxidative stress and inflammation in preterm infants (Cai et al., 2019; Ho et al., 2019). The *Terrisporobacter* could produce the urinary toxin, such as trimethylamine-N-oxide (Cai et al., 2019). Trimethylamine-N-oxide may be involved in the pathogenesis of IBD by impacting ATG16L1-induced autophagy and activating NLRP3 inflammasome (Yue et al., 2017; Li et al., 2022). In addition, our data indicate that *Clostridium_sensu_stricto_1* and *Streptococcus* had higher abundance in colonic chyme of CP-H compared to TP. Meanwhile, *Streptococcus* was significantly positively correlated with D-Lac. In previous studies, *Clostridium_ sensu_stricto_1* and *Streptococcus* are deemed to be opportunistic pathogens associated with colitis (Zou et al., 2020; Wen et al., 2021). Therefore, they may be another important factor to aggravate intestinal inflammation of CP-H in this study. Interestingly, we found that the *Lactobacillus_johnson* and *Lactobacillus_reuteri* were enriched in gut of TP. The *Lactobacillus_johnson* and *Lactobacillus_reuteri* is a well-known gut probiotic (Avall-Jääskeläinen and Palva, 2005; Sung et al., 2018). At present, they have been used as feed additive in swine industry, with very good results (Bampidis et al., 2021). Thus, it may be a good therapeutic candidate for intestinal diseases treatment with the

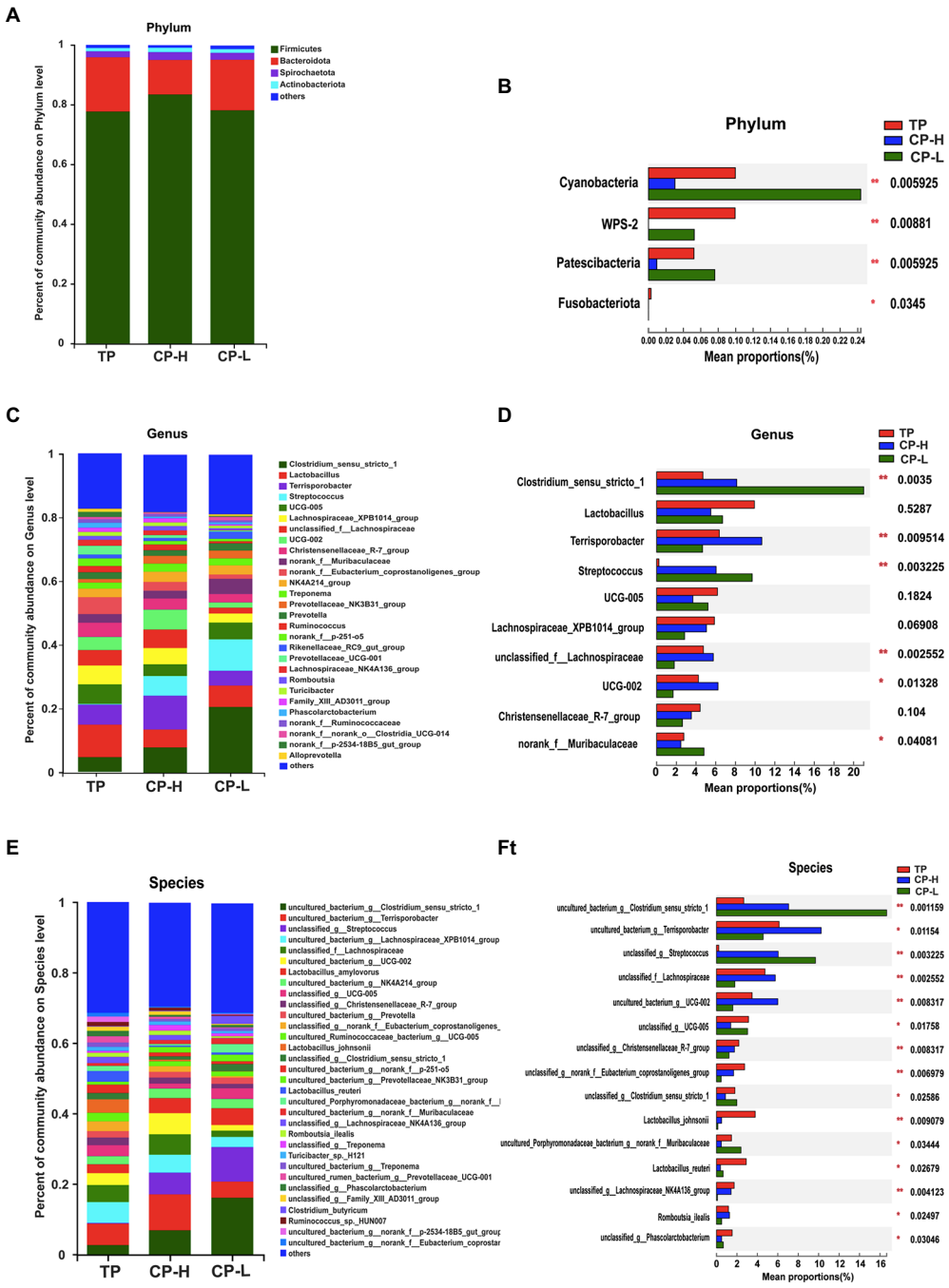


FIGURE 4
Effects of high-altitude hypoxic environment on gut microbiota community composition in the colonic chyme of pigs. TP: positive control group, CP-H: treatment group, CP-L: negative control group. **(A)** The relative abundances of microbiota in colonic chyme at the phylum level. **(B)** Analysis of variance the gut microbiota with significant differences at the phylum level. **(C)** The relative abundances of microbiota in colonic chyme at the genus level. **(D)** Analysis of variance about relative abundance of the top 10 genera in the three groups. **(E)** The relative abundances of microbiota in colonic chyme at the species level. **(F)** Analysis of variance about relative abundance of the top 15 genera with significant differences at the species level. Data are expressed as mean±SEM ($n$=6). * indicating a statistically significant difference ($p$< 0.05), ** indicating a highly significant difference ($p$< 0.01).

addition of *Lactobacillus_johnson* and *Lactobacillus_reuteri* in the CP-H feed.

Short-chain fatty acids (SCFAs) are produced by anaerobic gut bacteria through saccharolytic fermentation of complex resistant carbohydrates (Muralitharan et al., 2020). The SCFAs predominantly include acetate, propionate, and butyrate which account for approximately 80% of all SCFAs. Therein, butyrate are important substrates for maintaining the colonic epithelium,

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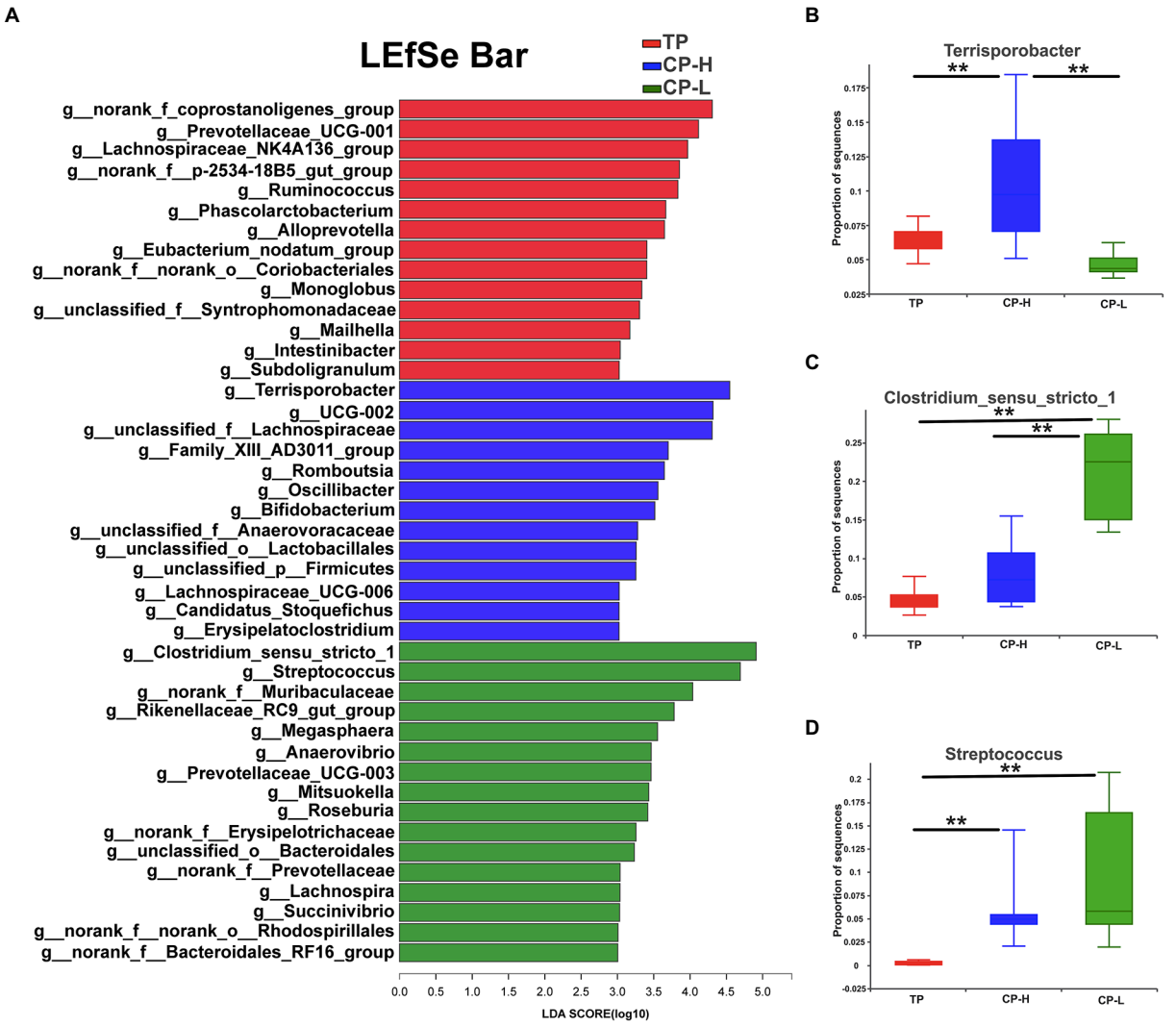


FIGURE 5
The differentially abundant taxa among the three groups by LEfSe analysis, and analysis of variance about some specific bacteria genera. TP: positive control group, CP-H: treatment group, CP-L: negative control group. **(A)** LEfSe analysis of microbiota in colonic chyme. **(B)** Analysis of variance about *Terrisporobacter*. **(C)** Analysis of variance about *Clostridium_sensu_stricto_1*. **(D)** Analysis of variance about *Streptococcusare*. Data are expressed as mean±SEM ($n$=6). ** indicating a highly significant difference ($p$< 0.01).

regulating tight junction proteins and enhancing intestinal barrier function through increasing expression of claudin-1 and ZO-1 (Wang et al., 2012). Studies have reported that the majority of butyrate are produced by a small number of gut bacteria, including *Faecalibacterium prausnitzii*, *Eubacterium rectale*, *Eubacterium hallii* and *Ruminococcus bromii* (Louis et al., 2010), of which is dominated by *Ruminococcus bromii* (Sun et al., 2017). In this study, no major strains related to butyric acid production were found in the top 15 differential bacterial genera in abundance. Thus, this may be the main reason for the no significant differences found about the concentrations of butyric acid. However, it is worth noting that the concentrations of valerate in CP-H was significantly higher than CP-L and TP. Previous studies found that valerate, which is a SCFAs mainly converted from proteins or amino acids (Gao et al., 2022), was demonstrated that could enhance intestinal barrier functions at physiological concentration (McDonald et al., 2018). However, valerate exhibited the concentration-dependent paradoxical effects on intestinal barrier function. It can exert the opposing effects on normal colonocytes at high concentrations (Burgess, 2012). Interestingly, through the correlation analysis, we found that the concentrations of valerate is significantly positively correlated with *Streptococcus* and *Clostridium_sensu_stricto_1* in this study. Based on our data, we could speculate that the most of the valerate in CP-H group was likely arose from *Streptococcus* and *Clostridium_sensu_stricto_1*. However, the mechanism underlying the production of valerate in gut is not fully understood and further investigation is needed.

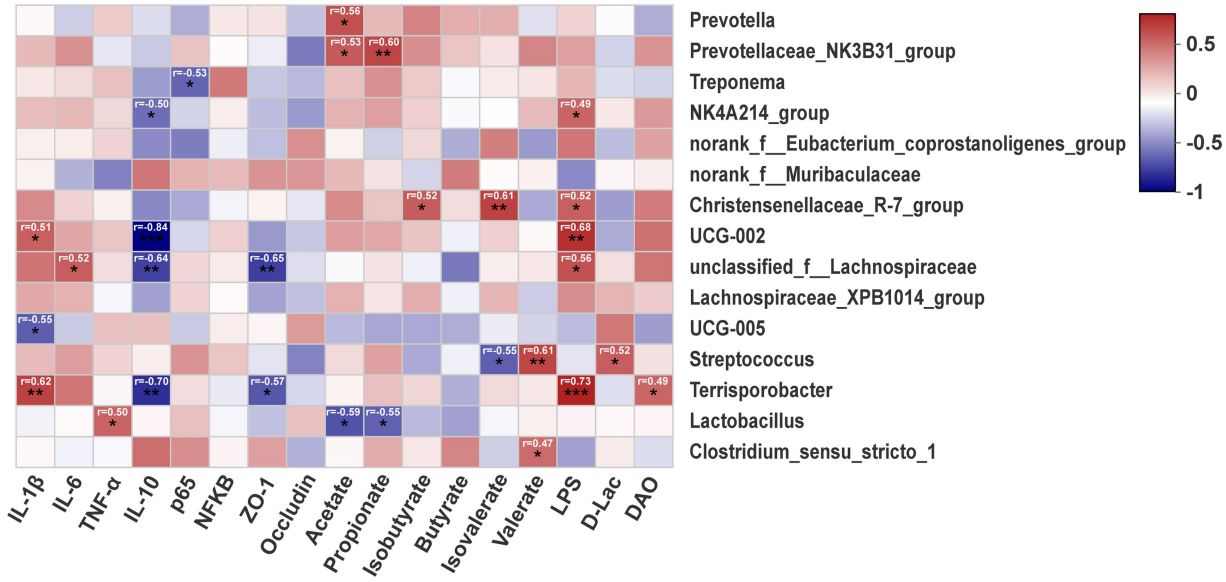


FIGURE 6
The correlation of intestinal microbiota, intestinal barrier, SCFAs, inflammatory cytokines, and blood biochemical parameters. r=Pearson correlation coefficient; *p*=significance level, **p*< 0.05, ***p*< 0.01, ****p*< 0.001. The *p*-value below 0.05 was considered significant, and absolute value of r higher than 0.5 was considered a strong correlation factor.

## Conclusion

In summary, high-altitude hypoxic environment changed compositions of gut microbiota, promoted the occurrence of colonic inflammation, and disrupted intestinal barrier of the three-way crossbred commercial pigs.

## Data availability statement

The data presented in the study are deposited in the NCBI SRA database with accession number PRJNA 872015.

## Ethics statement

The animal study was reviewed and approved by the Experimental Animal Welfare and Ethical Committee of the Institute of Animal Science, Chinese Academy of Agricultural Sciences.

## Author contributions

CL and GS designed the experiment. CL, GS, JD, and HH carried out the experiment. CL and GS wrote the manuscript. RZ, LC, BW, YZ, HZ and ZW 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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## Supplementary material

The Supplementary material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2022.968521/full#supplementary-material

## References

Avall-Jääskeläinen, S., and Palva, A. (2005). Lactobacillus surface layers and their applications. *FEMS Microbiol. Rev.* 29, 511–529. doi: 10.1016/j.femsre.2005.04.003

Bampidis, V., Azimonti, G., Bastos, M. L., Christensen, H., Dusemund, B., Fašmon Durjava, M., et al. (2021). Safety and efficacy of a feed additive consisting on Lactiplantibacillus plantarum (formerly lactobacillus plantarum) CECT 8350 and Limosilactobacillus reuteri (formerly lactobacillus reuteri) CECT 8700 (AQ02) for suckling piglets (AQUILON CYL S.L.). *EFSA J.* 19:e06631. doi: 10.2903/j.efsa.2021.6631

Brestoff, J. R., and Artis, D. (2013). Commensal bacteria at the interface of host metabolism and the immune system. *Nat. Immunol.* 14, 676–684. doi: 10.1038/ni.2640

Burgess, D. J. (2012). Metabolism: Warburg behind the butyrate paradox? *Nat. Rev. Cancer* 12, 798–799. doi: 10.1038/nrc3401

Cai, C., Zhang, Z., Morales, M., Wang, Y., Khafipour, E., and Friel, J. (2019). Feeding practice influences gut microbiome composition in very low birth weight preterm infants and the association with oxidative stress: A prospective cohort study. *Free Radic. Biol. Med.* 142, 146–154. doi: 10.1016/j.freeradbiomed.2019.02.032

Cianga, V. A., Campos Catafal, L., Cianga, P., Pavel Tanasa, M., Cherry, M., Collet, P., et al. (2021). Natural killer cell subpopulations and inhibitory receptor dynamics in Myelodysplastic syndromes and acute myeloid leukemia. *Front. Immunol.* 12:665541. doi: 10.3389/fimmu.2021.665541

Figueredo, C. M., Sete, M. R., Carlos, J. C., Lira, R., Boström, E., and Sztajnbok, F. (2018). Presence of anti-Porphyromonas gingivalis-peptidylarginine deiminase antibodies in serum from juvenile systemic lupus erythematosus patients. *Acta Reumatol. Port.* 43, 239–240. PMID: 30414375

Földes-Papp, Z., Domej, W., Demel, U., and Tilz, G. P. (2005). Oxidative stress caused by acute and chronic exposition to altitude. *Wien. Med. Wochenschr.* 155, 136–142. doi: 10.1007/s10354-005-019-3

Fukuda, S., Toh, H., Hase, K., Oshima, K., Nakanishi, Y., Yoshimura, K., et al. (2011). Bifidobacteria can protect from enteropathogenic infection through production of acetate. *Nature* 469, 543–547. doi: 10.1038/nature09646

Gao, G., Zhou, J., Wang, H., Ding, Y., Zhou, J., Chong, P. H., et al. (2022). Effects of valerate on intestinal barrier function in cultured Caco-2 epithelial cell monolayers. *Mol. Biol. Rep.* 49, 1817–1825. doi: 10.1007/s11033-021-06991-w

Goodrich, J. K., Davenport, E. R., Clark, A. G., and Ley, R. E. (2017). The relationship Between the human genome and microbiome comes into view. *Annu. Rev. Genet.* 51, 413–433. doi: 10.1146/annurev-genet-110711-155532

Himadri, P., Kumari, S. S., Chitharanjan, M., and Dhananjay, S. (2010). Role of oxidative stress and inflammation in hypoxia-induced cerebral edema: a molecular approach. *High Alt. Med. Biol.* 11, 231–244. doi: 10.1089/ham.2009.1057

Ho, J., Nicolucci, A. C., Virtanen, H., Schick, A., Meddings, J., Reimer, R. A., et al. (2019). Effect of prebiotic on microbiota, intestinal permeability, and glycemic control in children With type 1 diabetes. *J. Clin. Endocrinol. Metab.* 104, 4427–4440. doi: 10.1210/jc.2019-00481

Huang, Z., Li, Q., Li, M., and Li, C. (2021). Transcriptome analysis reveals the long intergenic noncoding RNAs contributed to skeletal muscle differences between Yorkshire and Tibetan pig. *Sci. Rep.* 11:2622. doi: 10.1038/s41598-021-82126-2

Jensen, G. M., and Moore, L. G. (1997). The effect of high altitude and other risk factors on birthweight: independent or interactive effects? *Am. J. Public Health* 87, 1003–1007. doi: 10.2105/ajph.87.6.1003

Lan, D., Ji, W., Lin, B., Chen, Y., Huang, C., Xiong, X., et al. (2017). Correlations between gut microbiota community structures of Tibetans and geography. *Sci. Rep.* 7:16982. doi: 10.1038/s41598-017-17194-4

Lanikova, L., Reading, N. S., Hu, H., Tashi, T., Burjanivova, T., Shestakova, A., et al. (2017). Evolutionary selected Tibetan variants of HIF pathway and risk of lung cancer. *Oncotarget* 8, 11739–11747. doi: 10.18632/oncotarget.14340

Li, H., Fan, C., Feng, C., Wu, Y., Lu, H., He, P., et al. (2019a). Inhibition of phosphodiesterase-4 attenuates murine ulcerative colitis through interference with mucosal immunity. *Br. J. Pharmacol.* 176, 2209–2226. doi: 10.1111/bph.14667

Li, H., Li, T., Beasley, D. E., Heděnec, P., Xiao, Z., Zhang, S., et al. (2016). Diet diversity is associated with Beta but not alpha diversity of Pika gut microbiota. *Front. Microbiol.* 7:1169. doi: 10.3389/fmicb.2016.01169

Li, H., Qu, J., Li, T., Wirth, S., Zhang, Y., Zhao, X., et al. (2018). Diet simplification selects for high gut microbial diversity and strong fermenting ability in high-altitude pikas. *Appl. Microbiol. Biotechnol.* 102, 6739–6751. doi: 10.1007/s00253-018-9097-z

Li, H., Zhou, R., Zhu, J., Huang, X., and Qu, J. (2019b). Environmental filtering increases with elevation for the assembly of gut microbiota in wild pikas. *Microb. Biotechnol.* 12, 976–992. doi: 10.1111/1751-7915.13450

Li, C., Zhu, L., Dai, Y., Zhang, Z., Huang, L., Wang, T. J., et al. (2022). Diet-induced high serum levels of Trimethylamine-N-oxide enhance the cellular inflammatory response without exacerbating acute Intracerebral hemorrhage injury in mice. *Oxidative Med. Cell. Longev.* 2022, 1599747–1599716. doi: 10.1155/2022/1599747

Louis, P., Young, P., Holtrop, G., and Flint, H. J. (2010). Diversity of human colonic butyrate-producing bacteria revealed by analysis of the butyryl-CoA: acetate CoA-transferase gene. *Environ. Microbiol.* 12, 304–314. doi: 10.1111/j.1462-2920.2009.02066.x

Luo, C., Wang, Y., Tao, S., Liao, Y., Yang, C., Cui, C., et al. (2019). Effects of replacing fish meal with mussel (Cristaria plicata) meat on growth, digestive ability, antioxidant capacity and hepatic IGF-I gene expression in juvenile Ussuri catfish (Pseudobagrus ussuriensis). *Aquac. Res.* 50, 826–835. doi: 10.1111/are.13953

Luo, C., Xia, B., Zhong, R., Shen, D., Li, J., Chen, L., et al. (2021). Early-life nutrition interventions improved growth performance and intestinal health via the gut microbiota in piglets. *Front. Nutr.* 8:783688. doi: 10.3389/fnut.2021.783688

McDonald, J. A. K., Mullish, B. H., Pechlivanis, A., Liu, Z., Brignardello, J., Kao, D., et al. (2018). Inhibiting Growth of Clostridioides difficile by Restoring Valerate, Produced by the Intestinal Microbiota. *Gastroenterology* 155, 1495.e5–1507.e15. doi: 10.1053/j.gastro.2018.07.014

Moreno-Indias, I., Torres, M., Montserrat, J. M., Sanchez-Alcoholado, L., Cardona, F., Tinahones, F. J., et al. (2015). Intermittent hypoxia alters gut microbiota diversity in a mouse model of sleep apnoea. *Eur. Respir. J.* 45, 1055–1065. doi: 10.1183/09031936.00184314

Muralitharan, R. R., Jama, H. A., Xie, L., Peh, A., Snelson, M., and Marques, F. Z. (2020). Microbial peer pressure: The role of the gut microbiota in hypertension and its complications. *Hypertension* 76, 1674–1687. doi: 10.1161/hypertensionaha.120.14473

Odenwald, M. A., and Turner, J. R. (2017). The intestinal epithelial barrier: a therapeutic target? *Nat. Rev. Gastroenterol. Hepatol.* 14, 9–21. doi: 10.1038/nrgastro.2016.169

Pham, K., Parikh, K., and Heinrich, E. C. (2021). Hypoxia and inflammation: insights From high-altitude physiology. *Front. Physiol.* 12:676782. doi: 10.3389/fphys.2021.676782

Pichler Hefti, J., Leichtle, A., Stutz, M., Hefti, U., Geiser, T., Huber, A. R., et al. (2016). Increased endothelial microparticles and oxidative stress at extreme altitude. *Eur. J. Appl. Physiol.* 116, 739–748. doi: 10.1007/s00421-015-3309-3

Pickard, J. M., Zeng, M. Y., Caruso, R., and Núñez, G. (2017). Gut microbiota: role in pathogen colonization, immune responses, and inflammatory disease. *Immunol. Rev.* 279, 70–89. doi: 10.1111/imr.12567

Purushothaman, J., Suryakumar, G., Shukla, D., Jayamurthy, H., Kasiganesan, H., Kumar, R., et al. (2011). Modulation of hypoxia-induced pulmonary vascular leakage in rats by Seabuckthorn (Hippophae rhamnoides L.). *eCAM* 2011:574524. doi: 10.1093/ecam/nep199

Qingsen, S., Guanrui, S., Meifang, Z., et al. (2017). Dietary fucoidan improves metabolic syndrome in association with increased Akkermansia population in the gut microbiota of high-fat diet-fed mice - science direct. *J. Funct. Foods* 28, 138–146. doi: 10.1016/j.jff.2016.11.002

Querido, J. S., Sheel, A. W., Cheema, R., Van Eeden, S., Mulgrew, A. T., and Ayas, N. T. (2012). Effects of 10 days of modest intermittent hypoxia on circulating measures of inflammation in healthy humans. *Sleep Breath.* 16, 657–662. doi: 10.1007/s11325-011-0555-4

Simonson, T. S., Yang, Y., Huff, C. D., Yun, H., Qin, G., Witherspoon, D. J., et al. (2010). Genetic evidence for high-altitude adaptation in Tibet. *Science* 329, 72–75. doi: 10.1126/science.1189406

Sokol, H., Jegou, S., McQuitty, C., Straub, M., Leducq, V., Landman, C., et al. (2018). Specificities of the intestinal microbiota in patients with inflammatory bowel disease and Clostridium difficile infection. *Gut Microbes* 9, 55–60. doi: 10.1080/19490976.2017.1361092

Sun, M., Wu, W., Liu, Z., and Cong, Y. (2017). Microbiota metabolite short chain fatty acids, GPCR, and inflammatory bowel diseases. *J. Gastroenterol.* 52, 1–8. doi: 10.1007/s00535-016-1242-9

Sung, V., D'Amico, F., Cabana, M. D., Chau, K., Koren, G., Savino, F., et al. (2018). Lactobacillus reuteri to treat infant colic: A meta-analysis. *Pediatrics* 141:1811. doi: 10.1542/peds.2017-1811

Suzuki, T. A., Martins, F. M., and Nachman, M. W. (2019). Altitudinal variation of the gut microbiota in wild house mice. *Mol. Ecol.* 28, 2378–2390. doi: 10.1111/mec.14905

Turner, J. R. (2009). Intestinal mucosal barrier function in health and disease. *Nat. Rev. Immunol.* 9, 799–809. doi: 10.1038/nri2653

Wang, H. B., Wang, P. Y., Wang, X., Wan, Y. L., and Liu, Y. C. (2012). Butyrate enhances intestinal epithelial barrier function via up-regulation of tight junction protein Claudin-1 transcription. *Dig. Dis. Sci.* 57, 3126–3135. doi: 10.1007/s10620-012-2259-4

Wen, X., Wang, H. G., Zhang, M. N., Zhang, M. H., Wang, H., and Yang, X. Z. (2021). Fecal microbiota transplantation ameliorates experimental colitis via gut microbiota and T-cell modulation. *World J. Gastroenterol.* 27, 2834–2849. doi: 10.3748/wjg.v27.i21.2834

Wu, H., Xie, S., Miao, J., Li, Y., Wang, Z., Wang, M., et al. (2020). Lactobacillus reuteri maintains intestinal epithelial regeneration and repairs damaged intestinal mucosa. *Gut Microbes* 11, 997–1014. doi: 10.1080/19490976.2020.1734423

Wu, W., Xie, J., and Zhang, H. (2016). Dietary fibers influence the intestinal SCFAs and plasma metabolites profiling in growing pigs. *Food Funct.* 7, 4644–4654. doi: 10.1039/c6fo01406b

Xu, C., Sun, R., Qiao, X., Xu, C., Shang, X., Niu, W., et al. (2014). Effect of vitamin e supplementation on intestinal barrier function in rats exposed to high altitude hypoxia environment. *Korean J Physiol Pharmacol* 18, 313–320. doi: 10.4196/kjpp.2014.18.4.313

Yang, Y., Gao, C., Yang, T., Sha, Y., Cai, Y., Wang, X., et al. (2021). Characteristics of Tibetan pig lung tissue in response to a hypoxic environment on the Qinghai-Tibet plateau. *Arch Anim Breed* 64, 283–292. doi: 10.5194/aab-64-283-2021

Yi, H., Yu, Q., Zeng, D., Shen, Z., Li, J., Zhu, L., et al. (2021). Serum inflammatory factor profiles in the pathogenesis of high-altitude polycythemia and mechanisms of acclimation to high altitudes. *Mediat. Inflamm.* 2021, 8844438–8844439. doi: 10.1155/2021/8844438

Yin, A., Luo, Y., Chen, W., He, M., Deng, J. H., Zhao, N., et al. (2019). FAM96A protects mice From dextran sulfate sodium (DSS)-induced colitis by preventing microbial Dysbiosis. *Front. Cell. Infect. Microbiol.* 9:381. doi: 10.3389/fcimb.2019.00381

Yue, C., Yang, X., Li, J., Chen, X., Zhao, X., Chen, Y., et al. (2017). Trimethylamine N-oxide prime NLRP3 inflammasome via inhibiting ATG16L1-induced autophagy in colonic epithelial cells. *Biochem. Biophys. Res. Commun.* 490, 541–551. doi: 10.1016/j.bbrc.2017.06.075

Zeng, B., Zhang, S., Xu, H., Kong, F., Yu, X., Wang, P., et al. (2020). Gut microbiota of Tibetans and Tibetan pigs varies between high and low altitude environments. *Microbiol. Res.* 235:126447. doi: 10.1016/j.micres.2020.126447

Zhang, Z., Xiao, Q., Zhang, Q. Q., Sun, H., Chen, J. C., Li, Z. C., et al. (2018). Genomic analysis reveals genes affecting distinct phenotypes among different Chinese and western pig breeds. *Sci. Rep.* 8:13352. doi: 10.1038/s41598-018-31802-x

Zhang, Z., Xu, D., Wang, L., Hao, J., Wang, J., Zhou, X., et al. (2016). Convergent evolution of rumen microbiomes in high-altitude mammals. *Curr. Biol.* 26, 1873–1879. doi: 10.1016/j.cub.2016.05.012

Zou, J., Shen, Y., Chen, M., Zhang, Z., Xiao, S., Liu, C., et al. (2020). Lizhong decoction ameliorates ulcerative colitis in mice via modulating gut microbiota and its metabolites. *Appl. Microbiol. Biotechnol.* 104, 5999–6012. doi: 10.1007/s00253-020-10665-1

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# Metabolome, microbiome, and gene expression alterations in the colon of newborn piglets with intrauterine growth restriction

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Newborn animals with intrauterine growth restriction (IUGR) are characterized by impaired intestinal structure and function; however, their intestinal microbiota and metabolome profiles have not been fully identified. The present study investigated the differences in colonic microbiota, metabolomics, and barrier function-related gene expression profiles between the IUGR and normal birth weight (NBW) piglets at 7, 21, and 28days of age. Forty-eight piglets (24 NBW and 24 IUGR) from 24 litters were assigned to assess the differences in colonic microbiota, metabolomics, and gene expression between IUGR and NBW piglets. Compared with the NBW piglets, IUGR piglets showed decreased Shannon index and increased Simpson index at 7days of age and Chao1 index at 21days of age ($p < 0.05$). The IUGR piglets had lower abundances of Firmicutes, *Subdoligranulum*, *Ruminococcaceae_UCG-002*, and *Ruminococcaceae_UCG-003* at 7days of age, and Bacteroidetes, *Phascolarctobacterium*, and *Ruminococcaceae_UCG-005* at 21days of age, when compared with the NBW piglets ($p < 0.05$). Metabolomics analysis showed significant changes in 147 metabolites mainly involved in organic acids and their derivatives in the colon. Six differential metabolic pathways were significantly enriched, including purine metabolism, amino sugar/nucleotide sugar metabolism, ubiquinone/other terpenoid-quinone biosynthesis, phenylalanine/tyrosine/tryptophan biosynthesis, phenylalanine metabolism, and histidine metabolism. Spearman's correlation analysis further demonstrated significant correlations between colonic microbiota and metabolites. In addition, colonic isobutyrate at 7days of age, isovalerate and total short-chain fatty acids (SCFAs) at 21days of age, and acetate, propionate, butyrate, and total SCFAs levels at 28days of age were lower and isovalerate was higher at 28days of age in the IUGR piglets than in the NBW piglets ($p < 0.05$). Furthermore, the mRNA expression of *zonula occludens* (*ZO*)-1 at 7days of age, *ZO*-1, *occludin*, and interleukin (*IL*)-4 at 21days of age were down-regulated in the IUGR piglets, whereas tumor necrosis factor (*TNF*)-α and nuclear factor-kappa B (*NF-κB*) at 28days of age were up-regulated, when compared with the NBW piglets ($p < 0.05$).

The findings suggest that the IUGR pigs present abnormal microbiota and nutrient metabolism in the colon, which may further affect the intestine barrier function by regulating gene expressions.

KEYWORDS

colonic microbiota, intrauterine growth restriction, metabolism, piglets, short-chain fatty acids

## Introduction

Intrauterine growth restriction (IUGR) has been defined as the impaired growth and development of the mammalian embryo/fetus or its organs during pregnancy (D'Inca et al., 2010). There are several factors, including genetic factors, environmental stress, insufficient uterine capacity, and maternal malnutrition, which could induce the IUGR of piglets (Wu et al., 2004). The IUGR can lead to feeding intolerance, decreased fat absorption, and digestive disorders in the early life of pigs (Gilbert and Danielsen, 2003), resulting in higher perinatal mortality and morbidity (Wu et al., 2006). Newborn piglets with IUGR have been identified with impaired intestinal structure and functions, resulting from the developmental pattern changes in their intestinal structure, transcriptomic, and proteomic profiles (Dong et al., 2016). Therefore, IUGR has attracted increasing attention in animal production (Ferenc et al., 2017).

The intestine is not only an important organ for nutrient digestion, absorption, and metabolism but also the largest immune organ protecting against pathogens in animals. The mammalian intestine is the harbor of microbes and is associated with a broad range of functions within the host. For example, the intestinal microbiota participates in the fermentation of complex carbohydrates, production of nutrients and vitamins, protection against pathogens, maintenance of immune balance, and different nutrient metabolism of the host (Marchesi et al., 2016). Early colonization and development of piglets' intestinal microbiota is a dynamic process characterized by rapid changes in microbial diversity, composition, and abundance (Matamoros et al., 2013). Several factors, such as delivery mode, diet, living environment, and diseases, can affect the early colonization and development of the intestinal microbiota of piglets (Nicholson et al., 2012). Emerging evidence confirmed that the colonization, succession process, and the balance of intestinal microbiota directly affect the intestinal barrier function (Kamada et al., 2013), metabolic reactions (Sonnenburg and Backhed, 2016), trophic effects (Sonnenburg and Backhed, 2016), and maturation of immune responses of the host (Littman and Pamer, 2011). In addition, intestinal microbiota can ferment undigested dietary components and endogenous compounds and produce short-chain fatty acids (SCFAs; Rooks and Garrett, 2016). Therefore, the gut microbiota balance and normal metabolic status are closely related to the host's health (Valdes et al., 2018).

Previous studies showed that the intestinal microbial community diversity of IUGR piglets is lower than the normal birth weight (NBW) piglets, which is easier to affect by pathogens (Jiang et al., 2019). In addition, newborn piglets with IUGR had lower microbial diversity and different taxonomic abundances in the small intestine (Zhang et al., 2019). Li et al. (2018) found that the low-birth-weight piglets also had different fecal microbial community structures and metabolome profiles. These findings suggested that the alteration of the intestinal microbiota is potentially associated with impaired growth and development of piglets. The colon is the main site of microbial fermentation in pigs (Sciascia et al., 2016), and numerous small molecular compounds are produced in this process, which affects the gene expressions related to the intestinal function of the host (Chassaing et al., 2017). Thus, we hypothesized that there might be differences in colonic microbiota and metabolites between the IUGR and NBW piglets, which may alter the intestinal barrier function-related gene expressions. Therefore, the present study was conducted to compare the differences in colonic microbiota, metabolites, and barrier function-related gene expression levels between IUGR and NBW piglets at 7, 21, and 28 days of age, using 16S rRNA gene sequencing and metabolomics technology to identify the biomarkers of intestinal microbiota and their metabolites.

## Materials and methods

### Experimental design and sample collection

Twenty-four pregnant sows with similar physical conditions with 3–5 parities were selected and herded in a pig farm located in Yongan town, Liuyang city, Hunan province, China. The sows were housed individually in gestation crates (2.2 × 0.6 m) from day 1 to day 105 of pregnancy and then housed in farrowing crates (2.2 × 1.8 m) until weaning. The sows were fed ~3 kg of food twice (at 8:00 and 17:00 h) daily during the experimental period, and the diet fluctuated with the sows' physical condition. Experimental sows had free access to drink water at all times. A total of 48 (male:female, 1:1) Large White × Landrace newborn piglets (24 NBW, 1.68 ± 0.04 kg and 24 IUGR, 0.98 ± 0.02 kg body weight; BW) with one NBW piglet and one IUGR piglet from 24 litters

were selected for this trial. Piglets with a heavier birth weight than the average birth weight per litter were identified as the NBW piglets, while those with a lower birth weight by 10% than the average birth weight of their origin were defined as the IUGR piglets (Bauer et al., 1998). Suckling piglets were kept in a warm thermal container and fed by sows freely. Piglets received commercial creep feed from 5 days of age. The piglets were weaned at 21 days of age and transferred to nursery facilities with *ad libitum* access to water and weaning diets at all times. No antibiotics were used during the experimental period. All male piglets were castrated.

At 7 (IUGR = 1.77 ± 0.08 kg and NBW = 2.79 ± 0.16 kg; BW), 21 (IUGR = 4.71 ± 0.41 kg and NBW = 6.41 ± 0.35 kg; BW), and 28 (IUGR = 5.06 ± 0.52 kg and NBW = 7.96 ± 0.34 kg; BW) days of age, 16 suckling piglets (eight pairs with one NBW piglet and one IUGR littermate) were weighed 2 h after the last suckling and then euthanized after anesthetization with sodium pentobarbital (40 mg/kg BW, Shanghai Haling Biological Technology Co., Ltd., Shanghai, China) for colonic contents and mucosa collection. The colonic contents (2 cm above the terminal colon) were collected and immediately stored at −80°C for analysis of the microbial composition, metabolome profiles, and SCFAs. The mucosa (~2 g) were sampled, quickly frozen into liquid nitrogen, and stored at −80°C for analysis of gene expressions. The overall experimental procedure is presented in Figure 1.

## DNA extraction, Illumina MiSeq sequencing, and bioinformatics analysis

Microbial DNA of the colonic contents was extracted using HiPure Stool DNA Kit (Magen, Guangzhou, China), following the manufacturer's instructions. The final concentration and purity of the extracted DNA were determined using a NanoDrop 2000 UV–vis spectrophotometer (Thermo Fisher Scientific, Waltham, MA, Unites States), and DNA quality was checked by 1% agarose gel electrophoresis. The V3-V4 hypervariable regions of the 16S rRNA gene were amplified with primers 338F (5′-ACTCCTACGGGAGGCAGCAG-3′) and 806R (5′-GGACTACHVGGGTWTCTAAT-3′) using thermocycler polymerase chain reaction (PCR) system (GeneAmp 9700, ABI, Unites States). The PCR reactions were conducted as described previously (Zhang et al., 2019). The resulting PCR products were extracted using 2% agarose gel electrophoresis, then purified using an AxyPrep DNA Gel Extraction Kit (Axygen Biosciences, Union City, CA, Unites States) and quantified using QuantiFluor™-ST (Promega, Madison, WI, Unites States) following the manufacturer's protocols. The equimolar purified amplicons were pooled, and paired-end (2 × 300) sequenced on an Illumina MiSeq platform (Illumina, San Diego, CA, Unites States), following the standard protocols by Majorbio Bio-Pharm Technology Co., Ltd. (Shanghai, China).

Raw fastq files were demultiplexed, quality-filtered by Trimmomatic, and merged by FLASH 1.2.11 (Magoc and Salzberg, 2011) with the following criteria: (i) the reads were truncated at any site receiving an average quality score < 20 and over a 50 bp sliding window; (ii) primers were exactly matched with allowing two nucleotides to mismatch, and reads containing ambiguous bases were removed; (iii) sequences of overlap >10 bp were merged according to their overlap sequence. The operational taxonomic units (OTUs) were clustered with a 97% similarity cutoff using UPARSE 7.1 and chimeric sequences were identified and removed using UCHIME (Edgar, 2013, 2016). The taxonomy of each 16S rRNA gene sequence was analyzed by RDP 11.5 Classifier algorithm against the SILVA 128 database using a confidence threshold of 70% (Wang et al., 2007). Raw sequences obtained in this study are deposited in the NCBI Sequence Read Archive database with accession number PRJNA836103.[1] Alpha diversity analysis included Shannon, Simpson, Chao1 richness estimator, and abundance-based coverage estimator (ACE) metric. Beta diversity analysis was performed to investigate the structural variation of microbial communities among samples using the principal coordinate analysis (PCoA). Partial least squares-discriminant analysis (PLS-DA) was also used as a supervised model to reveal the microbiota variation between the two groups. The taxonomic composition was investigated at the phylum and genus levels. Microbial functions were predicted by the phylogenetic investigation of communities by reconstruction of unobserved states one (PICRUSt1). All analyses were performed on the free online platform of Majorbio I-Sanger Cloud Platform.[2]

## Metabolite extraction, LC–MS analysis, and data processing

Colon contents (25 mg per sample) were weighed into 2 ml Eppendorf tubes (Eppendorf, Hamburg, Germany) and mixed with 500 µl extract solution (methanol: acetonitrile: water = 2:2:1 (v/v), containing isotopically labeled internal standard mixture). Then, the solution was vortexed for 30 s, homogenized at 35 Hz for 4 min, and sonicated for 5 min in the ice-water bath. The above treatment was repeated three times. After incubation at −40°C using a low-temperature freezer (AUCMA, Qingdao, China) for 1 h, the mixture solution was centrifuged at 10,000 × *g* and 4°C for 15 min, and then the resulting supernatant was transferred to a fresh glass vial for analysis. The quality control (QC) sample was prepared by mixing an equal aliquot of the supernatants from all samples.

The LC–MS/MS analyses were performed using an UHPLC system (Vanquish, Thermo Fisher Scientific, MA, Unites States)

1 https://www.ncbi.nlm.nih.gov/sra/PRJNA836103/
2 https://cloud.majorbio.com/

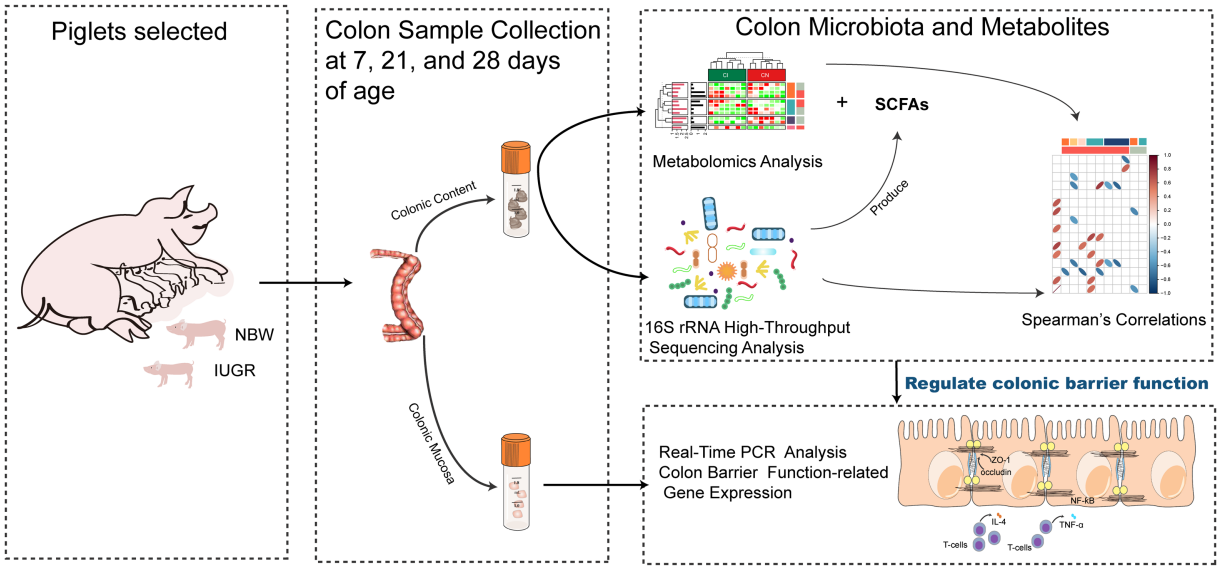


FIGURE 1
Schematic presentation of the overall experimental procedure on metabolome, microbiome, and gene expression alterations in colon of newborn piglets with intrauterine growth restriction.

with an UPLC BEH Amide column (2.1 × 100 mm, 1.7 μm) coupled with Q Exactive HFX mass spectrometer (Orbitrap MS, Thermo Fisher Scientific). The mobile phase consisted of 25 mmol/L ammonium acetate, 25 mmol/L ammonia hydroxide in water (pH = 9.75), and acetonitrile. The auto-sampler temperature was 4°C, and the injection volume was 3 μl. The Q Exactive HFX mass spectrometer was used for its ability to acquire MS/MS spectra on information-dependent acquisition (IDA) mode in the control of the acquisition software (Xcalibur, Thermo Fisher Scientific). The Electrospray ion (ESI) source conditions were set as follows: sheath gas flow rate as 30 Arb, Aux gas flow rate as 25 Arb, capillary temperature as 350°C, full MS resolution as 60,000, MS/MS resolution as 7,500, collision energy as 10/30/60 in NCE mode, and spray voltage as 3.6 kV (positive, POS) or −3.2 kV (negative, NEG), respectively.

The raw data were converted to mzXML format using ProteoWizard and processed with an in-house program, which was developed using R package and based on XCMS, for peak detection, extraction, alignment, and integration. Then an in-house secondary mass spectrometry (MS2) database (BiotreeDB V 2.1) was applied in metabolite annotation. The cutoff for annotation was set at 0.3. The SIMCA (V16.0.2, Sartorius Stedim Data Analytics AB, Umea, Sweden) was used for principal component analysis (PCA) and orthogonal partial least squares discriminant analysis (OPLS-DA). Seven-fold cross-validation was used to examine the quality of the model. Permutation tests were used to test the validity of the model. The first principal component of variable importance in the projection (VIP) and Student's *t*-test were obtained to refine the analysis. If VIP > 1 and $p < 0.05$, the variable was defined as a significantly different metabolite (SDM) between the two groups. In addition, the Kyoto Encyclopedia of Genes and Genomes (KEGG, http://www.genome.jp/kegg/) and MetaboAnalyst[3] databases were used for pathway enrichment analysis.

## Quantitation of short-chain fatty acids in colonic contents

The levels of colonic SCFAs, including acetate, propionate, isobutyrate, butyrate, isovalerate, and valerate, were detected by using Agilent 6890 gas chromatography (Agilent Technologies, Inc., Palo Alto, CA, Unites States), as described previously (Zhou et al., 2012). Briefly, colonic contents samples (~1 g) were homogenized and centrifuged in sealed tubes at 10,000 × *g* and 4°C for 10 min. A mixture of the supernatant fluid and 25% metaphosphoric acid solution (1:0.25 ml) was filtered through a 0.45-μm polysulfone microporous membrane filter and then analyzed.

## RNA extraction and barrier function-related gene expression analysis

Total RNA extraction and real-time PCR measurement were conducted as previously described (Duan et al., 2017). Briefly, the total RNA of the colonic samples was extracted using the TRIZOL reagent (Magen, Guangzhou, China). The purity and concentration of the extracted RNA were assessed using a NanoDrop ND-2000 spectrophotometer (Thermo Fisher

3 http://www.metaboanalyst.ca/

Scientific). The total RNA was reverse-transcribed into cDNA using a PrimeScript RT reagent kit with gDNA Eraser (TaKaRa Biotech. Co., Ltd., Dalian, China). An RT-PCR assay was carried out using the SYBR® Premix Ex Taq™ kit (TaKaRa Biotech. Co., Ltd.) on a 480 II system (Roche, Light Cycler® 480 II, Basel, Switzerland) with the following conditions: initial denaturation at 95°C for 30 s, followed by 40 cycles of denaturation at 95°C for 5 s, annealing at 60°C for 30 s, and a final extension at 72°C for 30 s. The primers for barrier function-related gene expression and reference gene β-actin (listed in Supplementary Table 1) were designed using the Primer-BLAST. The relative expression level of each target gene was calculated using the $2^{-\Delta\Delta Ct}$ method (Schmittgen and Livak, 2008).

## Statistical analysis

Student's *t*-test was used for the significance test of metabolites and gene expression data. Mann–Whitney *U* test was used for the microbiota data. Differences between the IUGR and NBW piglets were considered significant at $p < 0.05$. Spearman's correlation coefficient was used to assess the relationships with different microbiota, SDMs, and BW between the IUGR and NBW Piglets. Data are presented as means ± SEM (standard error of the mean). The SPSS 22.0 (SPSS Inc., Chicago, IL, Unites States), R packages ggplot2 V3.3.5 and ComplexHeatmap V2.10.0,[4] and GraphPad Prism V.6.0 (San Diego, CA, Unites States) were used for data analysis and graph preparation.

# Results

## Differences in colonic microbial community diversity between IUGR and NBW piglets

The microbial community diversity differences between the IUGR and NBW piglets are presented in Figure 2. A total of 2,832,611 high-quality reads were generated by high-throughput sequencing of 48 samples. From the high-quality reads, 1095 OTUs were detected by clustering non-repetitive sequences based on 97% similarity, including 19 phyla, 32 classes, 51 orders, 93 families, 170 genera, and 216 species. Each sample contained with an average of 50,447 sequences and 47,047 OTUs. The rarefaction curves reached the peak, indicating a near-complete sampling of the colonic microbial community (Supplementary Figure 1).

Alpha diversity was measured using the Shannon, Simpson, ACE, and Chao1 indexes (Figure 2A). The IUGR piglets had a lower ($p < 0.05$) Shannon index and a higher Simpson index at 7 days of age compared with the NBW piglets. In addition, the IUGR piglets had a lower ($p < 0.05$) Chao1 index compared with the NBW piglets at 21 days of age.

The PCoA results showed that there was no obvious separation between the IUGR and NBW piglets at 7, 21, and 28 days of age (Figure 2B). The PLS-DA showed that the intergroup score plots were clearly separated and clustered into two groups at 7, 21, and 28 days of age, indicating that there were significant differences in the colonic microbial structure between the IUGR and NBW piglets (Figure 2C).

## Differences in colonic microbial composition between IUGR and NBW piglets

The colonic microbial compositions were analyzed at the phylum and genus levels (Figure 3). At the phylum level, in the IUGR and NBW piglets, Bacteroidetes (57.50% vs. 48.72%), Firmicutes (35.23% vs. 43.87%), and Proteobacteria (6.59% vs. 4.31%) were the top three phyla at 7 days of age; Bacteroidetes (43.64% vs. 58.75%), Firmicutes (45.94% vs. 35.15%), and Spirochaetae (4.94% vs. 2.36%) were the top three phyla at 21 days of age; and Bacteroidetes (29.99% vs. 38.67%), Firmicutes (60.60% vs. 51.57%), and Proteobacteria (3.63% vs. 3.54%) were the top three phyla at 28 days of age, respectively (Figure 3A). The relative abundance of Bacteroidetes was higher ($p < 0.05$), and Firmicutes and Synergistetes were lower ($p < 0.05$) in the IUGR piglets at 7 days of age compared with the NBW piglets (Figures 3B–D). However, the relative abundance of Bacteroidetes was lower ($p < 0.05$) in the IUGR piglets compared with the NBW piglets at 21 days of age (Figure 3E). However, there was no significant ($p > 0.05$) difference in the colonic microbial composition at the phylum level between the IUGR and NBW piglets at 28 days of age.

The top 50 most abundant genera in the colon of the IUGR and NBW piglets and their differences are presented in Figure 3F. At 7 days of age, *norank_S24-7* (18.79%) was the most abundant genus, followed by *Prevotella_2* (9.90%), *Lactobacillus* (9.54%), *Bacteroides* (6.80%), and *Alloprevotella* (6.24%) in the IUGR piglets, whereas *norank_S24-7* (13.83%) was the most abundant genus, followed by *Rikenellaceae_RC9_gut_group* (9.38%), *Lactobacillus* (8.00%), *Bacteroides* (5.53%), and *Ruminococcaceae_UCG-002* (4.59%) in the NBW piglets. At 21 days of age, *Bacteroides* (9.29%) was the most abundant genus, followed by *norank_S24-7* (7.86%), *Lactobacillus* (7.86%), *norank_Erysipelotrichaceae* (5.36%), and *Prevotellaceae_NK3B31_group* (4.93%) in the IUGR piglets, whereas *norank_S24-7* (11.37%) was the most abundant genus, followed by *Bacteroides* (10.80%), *Prevotella_2* (9.46%), *Alloprevotella* (7.37%), and *Prevotellaceae_NK3B31_group* (5.19%) in the NBW piglets. At 28 days of age, *[Eubacterium]_coprostanoligenes_group* (7.78%) was the most abundant genus, followed by *Lactobacillus* (6.87%), *Bacteroides* (6.00%), *norank_S24-7* (5.41%), and *Ruminococcaceae_NK4A214_group* (4.03%) in the IUGR piglets, while *norank_Bacteroides* (9.94%) was the most abundant genus,

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

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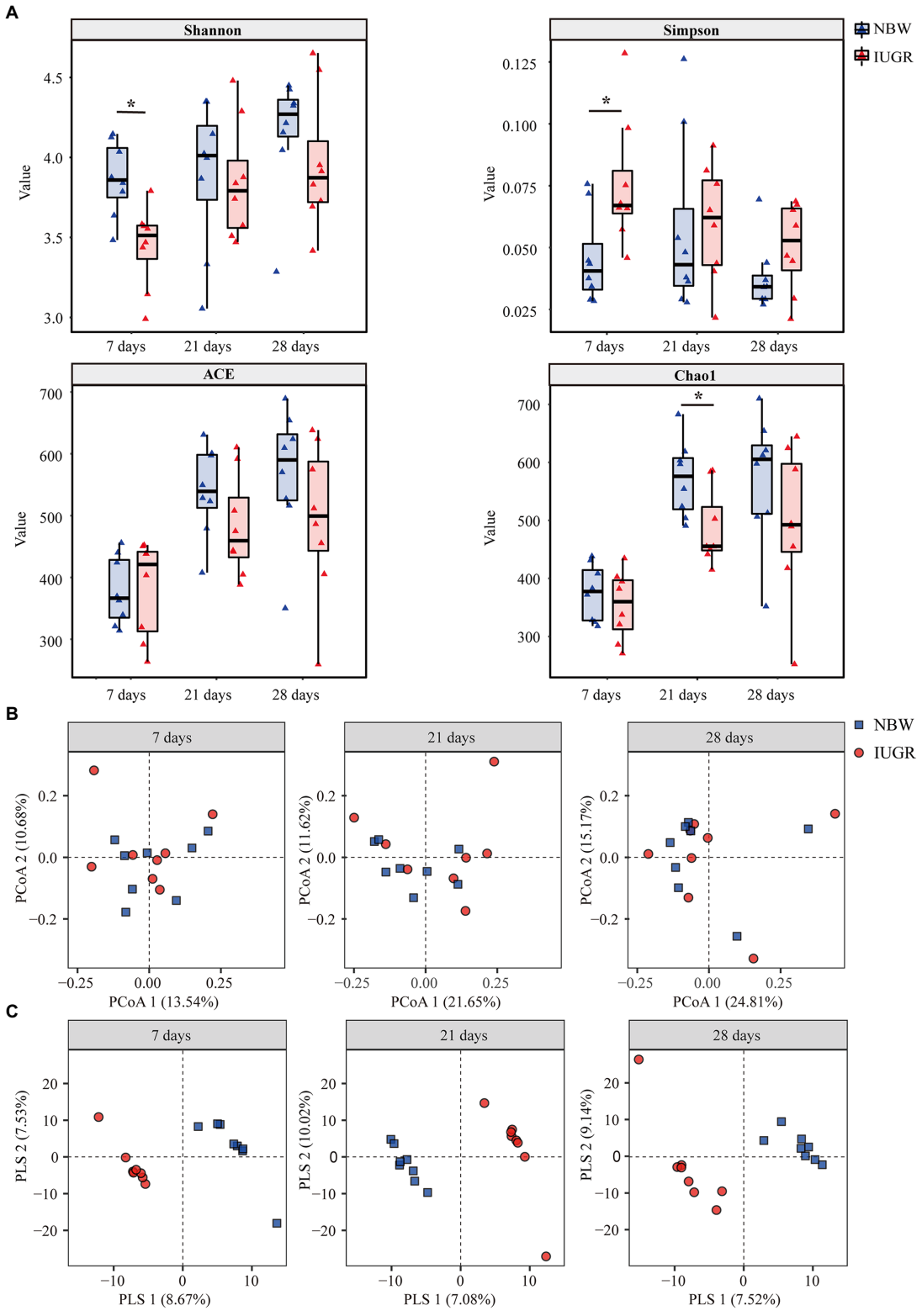


FIGURE 2
Differences in colonic microbial community diversity between the intrauterine growth restriction (IUGR) and normal birth weight (NBW) piglets at 7, 21, and 28days of age. **(A)** The microbial Alpha diversity was measured using the Shannon, Simpson, ACE, and Chao indexes ($n$=8). **(B)** Principal coordinate analysis (PCoA) on unweighted UniFrac distances ($n$=8). **(C)** Partial least square discriminant analysis (PLS-DA) score plots of colonic microbiota ($n$=8). The IUGR group and NBW group are shown along the first two PCoA/PLS axes. Each symbol represents the gut microbiota of a piglet, red represents the IUGR group, and blue represents the NBW group. *$p$ < 0.05.

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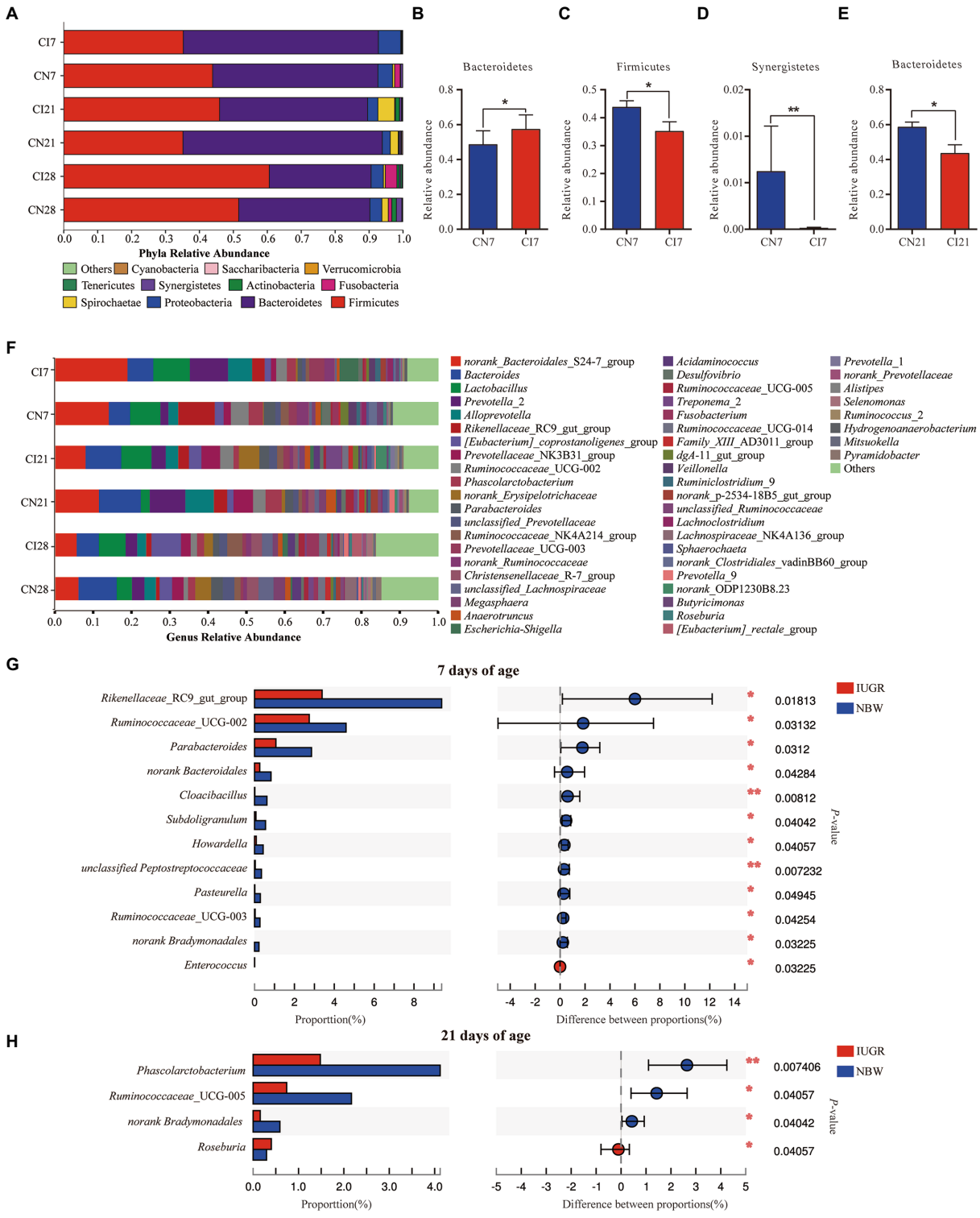


FIGURE 3
The Colonic microbial composition and taxonomic differences between the intrauterine growth restriction (IUGR) and normal birth weight (NBW) piglets at 7, 21, and 28days of age. **(A)** The relative abundances of taxa >0.01% at the phylum level are showed by bar plot. **(B–E)** The taxonomic differences in colonic microbiota at the phylum levels. **(F)** The top 50 abundant taxa are shown by heatmap. **(G,H)** The taxomonic differences in colonic microbiota at the genus levels. CI7, CI21, and CI28 represent colonic samples obtained from IUGR piglets at 7, 21, and 28days of age, respectively. CN7, CN21, and CN28 represent colonic samples obtained from NBW piglets at 7, 21, and 28days of age, respectively. Red and blue represent the IUGR and NBW piglets, respectively. The data are expressed as means±SEM ($n$=8). *$p < 0.05$; **$p < 0.01$.

followed by *norank_S24-7* (5.95%), *norank_Erysipelotrichaceae* (4.07%), *norank_Lachnospiraceae* (3.87%), and *Prevotella_2* (3.24%) in the NBW piglets (Figure 3F). The relative abundances of *Rikenellaceae_RC9_gut_group*, *Ruminococcaceae_UCG-002*, *Parabacteroides*, *norank_Bacteroidales*, *Cloacibacillus*, *Subdoligranulum*, *Howardella*, *unclassified_Peptostreptococcaceae*, *Pasteurella*, *Ruminococcaceae_UCG-003*, and *norank_Bradymonadales* were lower ($p < 0.05$), whereas *Enterococcus* was higher ($p < 0.05$) in the IUGR piglets at 7 days of age, when compared with the NBW piglets (Figure 3G). At 21 days of age, the relative abundances of *Phascolarctobacterium*, *Ruminococcaceae_UCG-005*, and *norank_Bradymonadales* were lower ($p < 0.05$), while *Roseburia* was higher ($p < 0.05$) in the IUGR piglets compared with the NBW piglets (Figure 3H). There was no significant ($p > 0.05$) difference in the colonic microbial composition between the IUGR and NBW piglets at 28 days of age.

## Differences in colonic microbial function between IUGR and NBW piglets

The PICRUSt1 was used to evaluate the metabolic function differences of gut microbiota in the colon of the IUGR and NBW piglets (Supplementary Figure 2). Compared with the NBW piglets, six pathways were up-regulated ($p < 0.05$) in the IUGR piglets at 7 days of age, including protein digestion and absorption, arachidonic acid metabolism, *Vibrio cholerae* pathogenic cycle, cellular antigens, toluene degradation, and ubiquinone/other terpenoid-quinone biosynthesis; seven pathways were down-regulated ($p < 0.05$), including arginine/proline metabolism, valine/leucine/isoleucine biosynthesis, pentose/glucuronate interconversions, chaperones and folding catalysts, butirosin and neomycin biosynthesis, phosphonate and phosphinate metabolism, and nicotinate and nicotinamide metabolism (Supplementary Figure 2A). At 21 days of age, 14 pathways were up-regulated ($p < 0.05$) in the IUGR piglets, including citrate cycle, tropane/piperidine/pyridine alkaloid biosynthesis, one carbon pool by folate, carbon fixation pathways in prokaryotes, RNA degradation, pantothenate and CoA biosynthesis, energy metabolism, glycine/serine/threonine metabolism, cysteine/methionine metabolism, translation proteins, galactose metabolism, starch and sucrose metabolism, pentose phosphate pathway, and transporters; six pathways were down-regulated ($p < 0.05$), including nitrotoluene degradation, arachidonic acid metabolism, isoquinoline alkaloid biosynthesis, biotin metabolism, riboflavin metabolism, and MAPK signaling pathway-yeast (Supplementary Figure 2B). However, there were no significant ($p > 0.05$) differences in the colonic functional metabolic pathways between the IUGR and NBW piglets at 28 days of age.

## Differences in colonic metabolome profiles between IUGR and NBW piglets

From the LC–MS/MS analysis, 8,322 and 6,132 valid peaks were obtained from the positive and negative ion modes, respectively. The unsupervised PCA was used to detect the overall changes in metabolic physiology of the IUGR and NBW piglets in the positive and negative ion modes (Supplementary Figure 3). The $R^2X$ value of the PCA model at 7, 21, and 28 days of age accounting for the variance was 0.580, 0.506, and 0.570 in the positive ion mode and 0.503, 0.528, and 0.555 in the negative ion mode, respectively (Supplementary Table 2). To maximize the discrimination between the IUGR and NBW piglets at 7, 21, and 28 days of age, the OPLS-DA was used to elucidate the different metabolic patterns (Figures 4A,B). The OPLS-DA models show goodness-of-fit ($R^2X$ and $R^2Y$) and predictability ($Q^2$), with 0.258, 0.994, and 0.344 in the positive ion mode and 0.200, 0.928, and 0.257 in the negative ion mode at 7 days of age; 0.327, 0.946, and 0.364 in the positive ion mode and 0.309, 0.947, and 0.347 in the negative ion mode at 21 days of age; and 0.253, 0.905, and 0.153 in the positive ion mode and 0.224, 0.925, and 0.239 in the negative ion mode at 28 days of age (Figure 4A; Supplementary Table 2), respectively. After 200 permutation tests, the $R^2$ and $Q^2$ intercept values at 7, 21, and 28 days of age were 0.92 and −0.12, 0.98 and −0.11, and 0.94 and −0.28 in the positive ion mode, respectively; whereas 0.89 and −0.10, 0.98 and −0.09, and 0.92 and −0.32 in the negative ion mode, respectively (Figure 4B). All samples were in the 95% confidence interval (Hotelling's *t*-squared ellipse).

A total of 378, 611, and 191 differential metabolites in the positive ion mode and 195, 373, and 154 differential metabolites in the negative ion mode were quantified at 7, 21, and 28 days of age, respectively (VIP > 1 and $p < 0.05$; Figure 4C; Supplementary Table 3). After qualitative matching by MS2, 147 metabolites were identified as SDMs in the colon of the IUGR and NBW piglets (Figures 5, 6, 7A,B). These SDMs in the colonic contents belonged to alkaloids/derivatives, benzenoids, lipids/lipid-like molecules, nucleosides/nucleotides/analogs, organic acids/derivatives, organic nitrogen compounds, organic oxygen compounds, organoheterocyclic compounds, organooxygen compounds, and phenylpropanoids/polyketides were highlighted between the IUGR and NBW piglets. Compared with the NBW piglets, at 7 days of age, 9 and 5 SDMs were up-regulated ($p < 0.05$) in the IUGR piglets, whereas 42 and 6 SDMs were down-regulated ($p < 0.05$) in the positive (Figure 5A) and negative (Figure 5B) ion modes, respectively. At 21 days of age, 19 and 5 SDMs were up-regulated ($p < 0.05$) in the IUGR piglets, whereas 30 and 8 SDMs were down-regulated ($p < 0.05$) in the positive (Figure 6A) and negative (Figure 6B) ion modes, respectively, when compared with the NBW piglets. At 28 days of age, compared with the NBW piglets, 13 and 8 SDMs were up-regulated ($p < 0.05$) in the IUGR piglets, whereas 1 and 1 SDMs were down-regulated ($p < 0.05$) in the positive (Figure 7A) and negative (Figure 7B) ion modes, respectively.

The KEGG database was used to explore the metabolism pathways and metabolite markers in the IUGR piglets compared with the NBW piglets. A total of 22 metabolism pathways were enriched at 7, 21, and 28 days of age in the IUGR piglets

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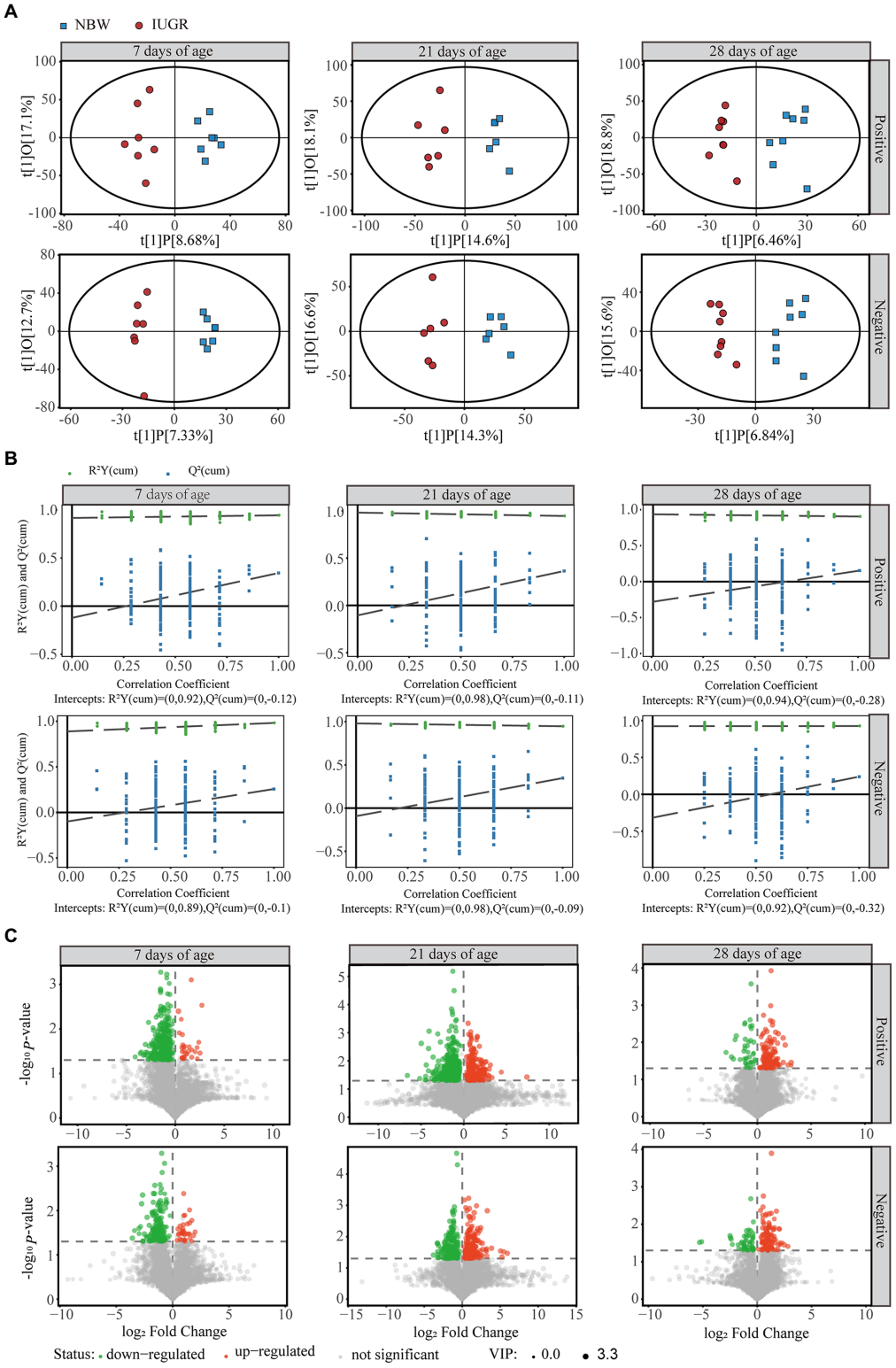


FIGURE 4
Differences in colonic metabolome profiles between the intrauterine growth restriction (IUGR) and normal birth weight (NBW) piglets at 7 (*n*=7), 21 (*n*=6), and 28 (*n*=8) days of age. **(A)** The score plots of Orthogonal projections to latent structures-discriminant analysis (OPLS-DA) model based on the non-target metabolomics for the data from positive and negative ion modes of the colonic contents. Red and blue represent the IUGR and NBW piglets, respectively. **(B)** The permutation test results of OPLS-DA. **(C)** Volcano plots of positive and negative ion modes based on the non-target metabolomics of the colonic contents. Each symbol represents the identified metabolite, orange represents significantly up-regulation of different metabolites, green represents significantly down-regulation of different metabolites, and gray represents the metabolites those did not differ.

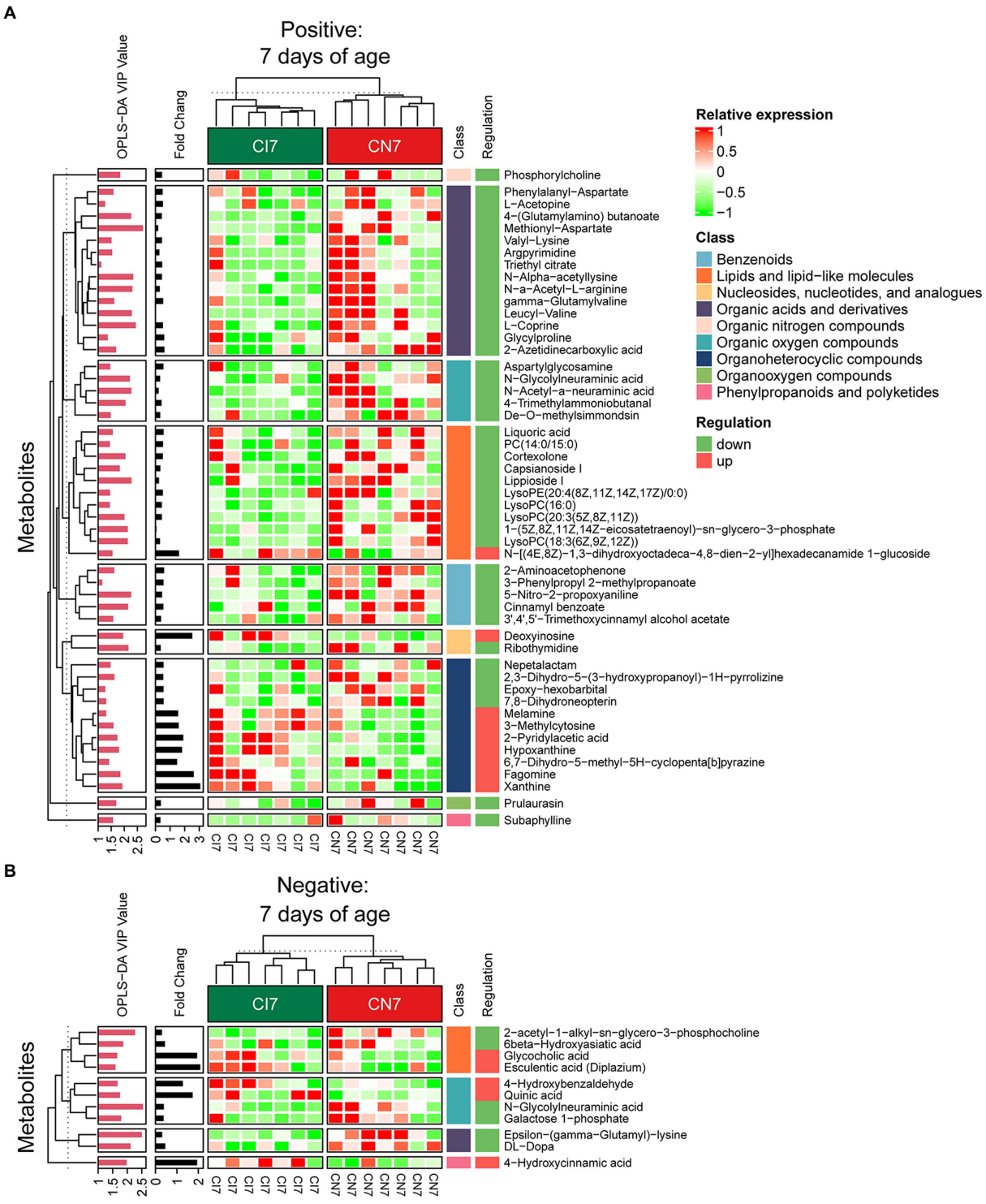


FIGURE 5
Hierarchical clustering analysis for different metabolites with MS2 based on the non-target metabolomics of the colonic contents between the intrauterine growth restriction (IUGR) and normal birth weight (NBW) piglets at 7days of age ($n$=7). **(A,B)** Represent positive and negative ion models, respectively. The relative metabolite level is depicted according to the color scale. Red indicates significant up-regulation ($p < 0.05$), and green indicates significant down-regulation ($p < 0.05$).

compared with the NBW piglets (Figures 7C–H; Table 1). Six of these metabolic pathways were enriched ($p < 0.05$; Table 1). Compared with the NBW piglets, purine metabolism was up-regulated ($p < 0.05$) in the positive ion mode, whereas amino sugar and nucleotide sugar metabolism were down-regulated ($p < 0.05$) in the negative ion mode in the IUGR piglets at 7 days

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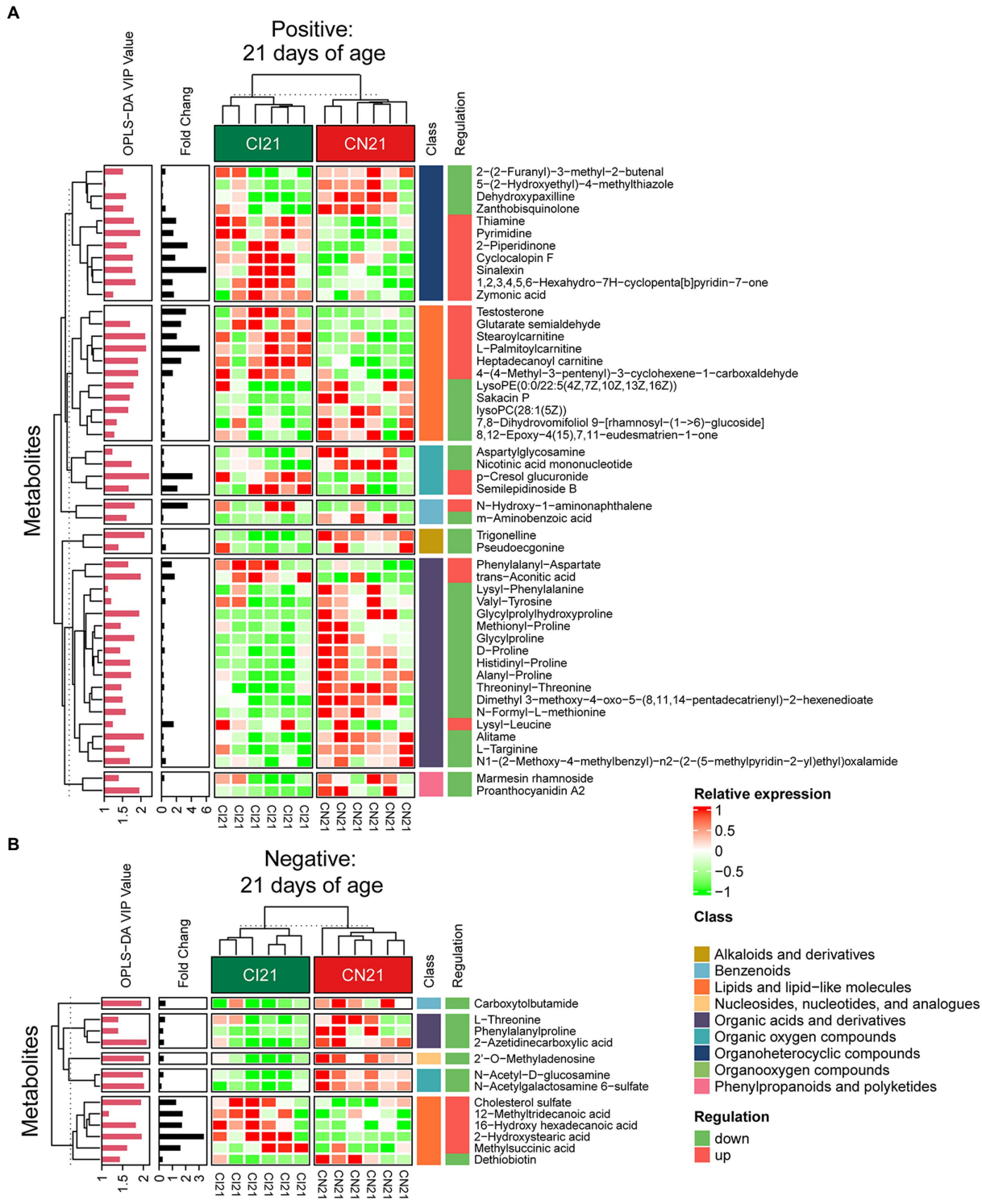


FIGURE 6
Hierarchical clustering analysis for different metabolites with MS2 based on the non-target metabolomics of the colonic contents between the intrauterine growth restriction (IUGR) and normal birth weight (NBW) piglets at 21days of age ($n$=6). **(A,B)** represent positive and negative ion models, respectively. The relative metabolite level is depicted according to the color scale. Red indicates significant up-regulation ($p < 0.05$), and green indicates significant down-regulation ($p < 0.05$).

of age. At 21 days of age, there were no significant different metabolism pathways ($p > 0.05$). At 28 days of age, ubiquinone/other terpenoid-quinone biosynthesis, phenylalanine/tyrosine/tryptophan biosynthesis, phenylalanine metabolism, and histidine metabolism were up-regulated ($p < 0.05$) in the IUGR piglets in the positive ion mode compared with the NBW piglets

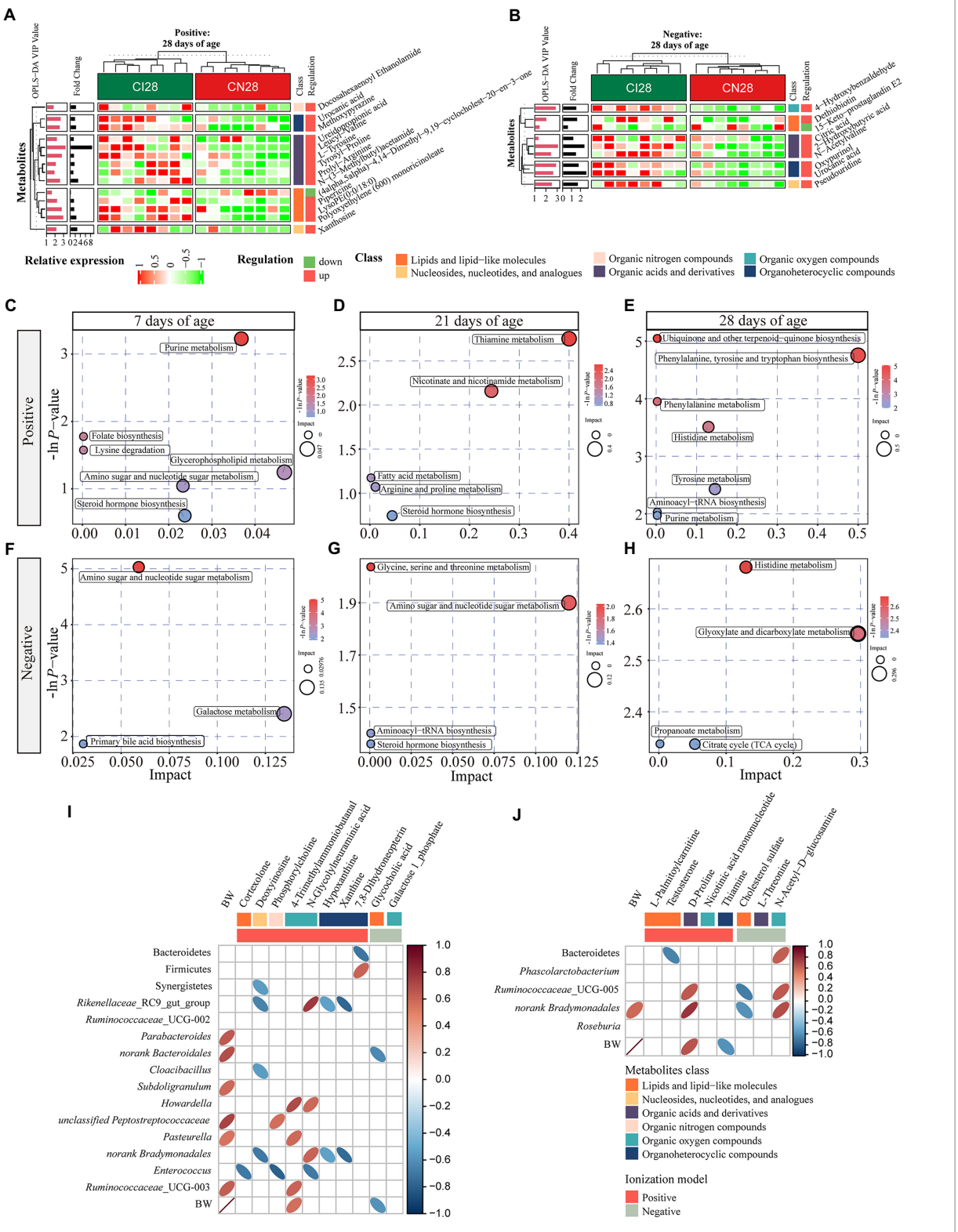


FIGURE 7
**(A,B)** Hierarchical clustering analysis for different metabolites with MS2 based on the non-target metabolomics of the colonic contents between the intrauterine growth restriction (IUGR) and normal birth weight (NBW) piglets at 28days of age ($n$=8; Positive: **A**, Negative: **B**). The relative metabolite level is depicted according to the color scale. Red indicates significant up-regulation ($p < 0.05$), and green indicates significant down-regulation ($p < 0.05$). **(C–H)** Metabolome view map of significant metabolic pathways characterized in the colonic contents between the IUGR and NBW piglets at 7, 21, and 28days of age (Positive: **(C–E)**, Negative: **(F–H)**). Significantly changed pathways based on enrichment and topology analysis are shown. The x-axis represents pathway impact, and the y-axis represents pathway enrichment. Large size and red colors represent major pathway enrichment and high pathway impact values, respectively. **(I,J)** Spearman's rank correlations between colonic microbiota and metabolites at 7 **(I)** and 21 **(J)** days of age. Ellipses in the graph represent significant correlations, red represents significant positive ($p < 0.05$) correlations, and blue represents significant negative ($p < 0.05$) correlations.

(Figures 7C–H). These metabolism pathways included seven SDMs: xanthine, deoxyinosine, hypoxanthine, L-tyrosine, urocanic acid, galactose 1-phosphate, and N-glycolylneuraminic acid (Table 1).

## Correlation analysis among different microbiota, SDMs, and BW

Spearman's correlations among the colonic microbiota abundance, SDMs, and BW (previously published from our research group; Zhang et al., 2019) are shown in Figures 7I,J. At 7 days of age (Figure 7I), the BW was positively correlated with *Parabacteroides*, *norank_Bacteroidales*, *Subdoligranulum*, *unclassified_Peptostreptococcaceae*, *Pasteurella*, and *Ruminococcaceae_UCG*-003 abundances and 4-trimethylammoniobutanal, while negatively correlated with glycocholic acid ($p < 0.05$). The cortexolone was negatively correlated with *Enterococcus* abundance ($p < 0.05$). Deoxyinosine was negatively correlated with Synergistetes, *Rikenellaceae_RC9_gut_group*, *Cloacibacillus,* and *norank_Bradymonadales* abundances ($p < 0.05$). The phosphorylcholine was positively correlated with *unclassified_Peptostreptococcaceae* but negatively correlated with *Enterococcus* abundances ($p < 0.05$), whereas 4-trimethylammoniobutanal was positively correlated with *Howardella*, *Pasteurella,* and *Ruminococcaceae_UCG_*003 abundances ($p < 0.05$). The N-glycolylneuraminic acid was positively correlated with *Rikenellaceae_RC9_gut_group*, *Howardella,* and *norank_Bradymonadales* abundances but negatively correlated with *Enterococcus* abundance ($p < 0.05$). Hypoxanthine and xanthine were negatively correlated with *Rikenellaceae_RC9_gut_group* and *norank_Bacteroidales* abundances ($p < 0.05$). Moreover, 7,8-dihydroneopterin was positively correlated with Bacteroidetes but negatively correlated with Firmicutes abundances ($p < 0.05$), whereas galactose 1-phosphate was negatively correlated with *norank_Bradymonadales* abundance ($p < 0.05$). At 21 days of age (Figure 7J), the BW was positively correlated with *norank_Bradymonadales* abundance and D-proline while negatively correlated with thiamine ($p < 0.05$). The testosterone was negatively correlated with Bacteroidetes abundance ($p < 0.05$), whereas D-proline was positively correlated with *Ruminococcaceae_UCG*-005 and *norank_Bradymonadales* abundances ($p < 0.05$). Moreover, cholesterol sulfate was negatively correlated with *Ruminococcaceae_UCG*-005 and *norank_Bradymonadales* abundances ($p < 0.05$), whereas N-acetyl-D-glutamate was positively correlated with *Bacteroidetes*, *Ruminococcaceae_UCG*-005, and *norank_Bradymonadales* abundances ($p < 0.05$).

## Differences in colonic short-chain fatty acids levels between IUGR and NBW piglets

Colonic SCFAs levels of the IUGR and NBW piglets at 7, 21, and 28 days of age are presented in Table 2. The levels of colonic isobutyrate at 7 days of age and isovalerate and total SCFAs at 21 days of age were lower ($p < 0.05$) in the IUGR piglets compared with the NBW piglets. In addition, the levels of acetate, propionate, butyrate, and total SCFAs were higher, whereas the level of isovalerate was lower in the IUGR piglets compared with the NBW piglets at 28 days of age ($p < 0.05$).

## Differences in the mRNA expression of colonic health-related genes between IUGR and NBW piglets

The mRNA expression of intestinal health-related genes of the IUGR and NBW piglets at 7, 21, and 28 days of age are shown in Table 3. Compared with the NBW piglets, colonic zonula occludens (*ZO*)*-1* expression in the IUGR piglets was down-regulated ($p < 0.05$) at 7 days of age. At 21 days of age, *ZO-1*, *occludin*, and interleukin (*IL*)*-4* expressions were down-regulated ($p < 0.05$) in the IUGR piglets compared with the NBW piglets. Moreover, tumor necrosis factor *(TNF)-α* and nuclear factor kappa B (*NF-κB*) expressions were up-regulated ($p < 0.05$) in the IUGR piglets compared with the NBW piglets at 28 days of age.

# Discussion

The mammalian gut harbors a complex and diverse microbial community, which influences host's normal physiology and disease susceptibility through their metabolic activity and the interactions with host (Rooks and Garrett, 2016). The present study compared the differences in colonic microbiota composition, metabolic profiles, and barrier function-related gene expression levels between the IUGR and NBW piglets. The findings indicate that the IUGR pigs present abnormal microbiota and nutrient metabolism in the colon, which may further affect the intestinal barrier function by regulating gene expression.

Gut microbial diversity is highly associated with the host's health (Clarke et al., 2014). It has been reported that the higher diversity contributed to the gut microbiota maturation and host health (Le Chatelier et al., 2013). However, the decreased diversity of intestinal microbiota is considered a marker in gut dysbiosis (Clarke et al., 2014) and easily contributes to the increased risk of several intestine diseases (Weiss and Hennet, 2017). In the present study, the colonic microbial alpha diversity was decreased in the IUGR piglets at 7 and 21 days of age, which was consistent with our previous study on the small intestinal microbiota of IUGR piglets (Zhang et al., 2019), suggesting that the intestinal dysbiosis occurred in the IUGR piglets. In addition, the beta diversity analysis showed that colonic microbiota structure between the IUGR and NBW piglets were divided into two groups at 7, 21, and 28 days of age in the present study, indicating that the IUGR significantly altered the structure of colonic microbiota of piglets.

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TABLE 1 Metabolite pathways and significantly different metabolites (SDMs) markers between the IUGR and NBW piglets in the positive and negative ion models at 7, 21, and 28days of age.

| Ion model | Pathway | *p*-value | Impact | SDMs |
|---|---|---|---|---|
| 7 days of age | | | | |
| Positive | Purine metabolism | 0.040 | 0.037 | Xanthine; Deoxyinosine; Hypoxanthine |
| Positive | Folate biosynthesis | 0.170 | 0.000 | 7,8-Dihydroneopterin |
| Positive | Lysine degradation | 0.208 | 0.000 | 4-Trimethylammoniobutanal cpd |
| Positive | Glycerophospholipid metabolism | 0.287 | 0.047 | Phosphorylcholine |
| Positive | Amino sugar and nucleotide sugar metabolism | 0.352 | 0.023 | N-Glycolylneuraminic acid |
| Positive | Steroid hormone biosynthesis | 0.548 | 0.024 | Cortexolone |
| Negative | Amino sugar and nucleotide sugar metabolism | 0.007 | 0.059 | Galactose 1-phosphate; N-Glycolylneuraminic acid |
| Negative | Galactose metabolism | 0.090 | 0.135 | Galactose 1-phosphate |
| Negative | Primary bile acid biosynthesis | 0.155 | 0.030 | Glycocholic acid |
| 21 days of age | | | | |
| Positive | Thiamine metabolism | 0.064 | 0.400 | Thiamine |
| Positive | Nicotinate and nicotinamide metabolism | 0.115 | 0.244 | Nicotinic acid mononucleotide |
| Positive | Fatty acid metabolism | 0.310 | 0.000 | L-Palmitoylcarnitine |
| Positive | Arginine and proline metabolism | 0.343 | 0.011 | D-Proline |
| Positive | Steroid hormone biosynthesis | 0.475 | 0.045 | Testosterone |
| Negative | Glycine, serine, and threonine metabolism | 0.131 | 0.000 | L-Threonine |
| Negative | Amino sugar and nucleotide sugar metabolism | 0.150 | 0.120 | N-Acetyl-D-glucosamine |
| Negative | Aminoacyl-tRNA biosynthesis | 0.247 | 0.000 | L-Threonine |
| Negative | Steroid hormone biosynthesis | 0.257 | 0.000 | Cholesterol sulfate |
| 28 days of age | | | | |
| Positive | Ubiquinone and other terpenoid-quinone biosynthesis | 0.006 | 0.000 | L-Tyrosine |
| Positive | Phenylalanine, tyrosine and tryptophan biosynthesis | 0.009 | 0.500 | L-Tyrosine |
| Positive | Phenylalanine metabolism | 0.019 | 0.000 | L-Tyrosine |
| Positive | Histidine metabolism | 0.030 | 0.130 | Urocanic acid |
| Positive | Tyrosine metabolism | 0.088 | 0.145 | L-Tyrosine |
| Positive | Aminoacyl-tRNA biosynthesis | 0.132 | 0.000 | L-Tyrosine |
| Positive | Purine metabolism | 0.140 | 0.000 | Xanthosine |
| Negative | Histidine metabolism | 0.069 | 0.130 | Urocanic acid |
| Negative | Glyoxylate and dicarboxylate metabolism | 0.078 | 0.296 | Citric acid |
| Negative | Propanoate metabolism | 0.097 | 0.000 | 2-Hydroxybutyric acid |
| Negative | Citrate cycle | 0.097 | 0.054 | Citric acid |

IUGR, intrauterine growth restriction; NBW, normal birth weight.

Bacteroidetes and Firmicutes were the two most predominant phyla in piglets (Li et al., 2018). In addition, Firmicutes/Bacteroidetes ratio plays an important role in digesting polysaccharide-rich diets and protecting against gut inflammation and colonic diseases (Ni et al., 2021). The sequencing of Bacteroidetes genomes confirms the presence of numerous carbohydrate-active enzymes for degrading high molecular weight organic matter, such as proteins and carbohydrates (Azad et al., 2018). Furthermore, Bacteroidetes play an important role in maintaining the host's gut health by producing butyrate (Kim and Milner, 2007), which interact with the immune system to activate T-cell mediated responses (Wen et al., 2008) and prevent the potentially pathogenic bacteria from colonizing in the gut (Mazmanian et al., 2008). In the present study, a lower Bacteroidetes abundance was detected in the IUGR piglets at 21 days of age, suggesting the IUGR piglets had a lower efficiency of proteins and carbohydrates utilization from the diet compared with the NBW piglets. However, a higher Bacteroidetes proportion was found in the IUGR piglets at 7 days of age, which may be related to other bacteria composition changes in the IUGR piglets and still need further research. Firmicutes is associated with energy intake from diets (Turnbaugh et al., 2006), and a higher Firmicutes proportion was found in both obese children and adults (Ley et al., 2006). In the present study, a higher Firmicutes abundance was found in the NBW piglets at 7 days of age, suggesting that the IUGR piglets had lower efficiency of energy intake from the diets compared with the NBW piglets.

*Ruminococcaceae* contains numerous carbohydrate-active enzymes that can ferment the undigested complex carbohydrates and produce SCFAs, such as butyrate, which plays a key role in

TABLE 2 Differences in colonic short-chain fatty acids (SCFAs) levels between the IUGR and NBW piglets (mg/g).

| Items | 7 days of age | | 21 days of age | | 28 days of age | |
|---|---|---|---|---|---|---|
| | NBW | IUGR | NBW | IUGR | NBW | IUGR |
| Acetate | 1.10 ± 0.10 | 0.98 ± 0.07 | 1.33 ± 0.14 | 1.01 ± 0.10 | 2.19 ± 0.18 | 1.13 ± 0.12** |
| Propionate | 0.42 ± 0.05 | 0.31 ± 0.04 | 0.48 ± 0.05 | 0.39 ± 0.05 | 0.76 ± 0.08 | 0.52 ± 0.05* |
| Isobutyrate | 0.07 ± 0.01 | 0.04 ± 0.01** | 0.07 ± 0.01 | 0.08 ± 0.01 | 0.06 ± 0.01 | 0.07 ± 0.01 |
| Butyrate | 0.16 ± 0.02 | 0.12 ± 0.02 | 0.18 ± 0.02 | 0.19 ± 0.02 | 0.29 ± 0.03 | 0.19 ± 0.03* |
| Isovalerate | 0.09 ± 0.01 | 0.07 ± 0.01 | 0.16 ± 0.01 | 0.13 ± 0.01* | 0.09 ± 0.01 | 0.13 ± 0.01* |
| Valerate | 0.09 ± 0.01 | 0.07 ± 0.01 | 0.10 ± 0.01 | 0.10 ± 0.01 | 0.09 ± 0.01 | 0.09 ± 0.01 |
| Total SCFAs | 1.92 ± 0.16 | 1.58 ± 0.10 | 2.32 ± 0.16 | 1.90 ± 0.11* | 3.48 ± 0.24 | 2.14 ± 0.12** |

Data are presented as means ± SEM ($n = 8$). IUGR, intrauterine growth restriction; NBW, normal birth weight.
*$p < 0.05$; **$p < 0.01$.

TABLE 3 Differences in mRNA expression of colonic mucosal genes related to barrier function between the IUGR and NBW piglets at 7, 21, and 28days of age.

| Items | 7 days of age | | 21 days of age | | 28 days of age | |
|---|---|---|---|---|---|---|
| | NBW | IUGR | NBW | IUGR | NBW | IUGR |
| *ZO-1* | 1.00 ± 0.06 | 0.81 ± 0.05* | 1.00 ± 0.16 | 0.40 ± 0.10** | 1.00 ± 0.14 | 0.67 ± 0.17 |
| *Occludin* | 1.00 ± 0.12 | 0.80 ± 0.07 | 1.00 ± 0.11 | 0.40 ± 0.07*** | 1.00 ± 0.19 | 0.78 ± 0.16 |
| *IL-4* | 1.00 ± 0.10 | 0.78 ± 0.10 | 1.00 ± 0.16 | 0.52 ± 0.08* | 1.00 ± 0.24 | 0.47 ± 0.29 |
| *TNF-α* | 1.00 ± 0.11 | 0.78 ± 0.09 | 1.00 ± 0.14 | 1.07 ± 0.13 | 1.00 ± 0.07 | 1.41 ± 0.16* |
| *NF-κB* | 1.00 ± 0.24 | 1.53 ± 0.22 | 1.00 ± 0.14 | 1.10 ± 0.20 | 1.00 ± 0.30 | 2.38 ± 0.12** |

Data are presented as means ± SEM ($n = 8$). IUGR, intrauterine growth restriction; NBW, normal birth weight; ZO, zonula occludens; IL, interleukin; TNF-α, tumor necrosis factor alpha; NF-κB, nucelar factor kappa B.
*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.

maintaining gut health (Biddle et al., 2013). In addition, a higher *Ruminococcaceae* abundance was detected in the obese mice (Kim et al., 2012). At the genus level, the present study found several low-abundant bacterial taxa, including *Ruminococcaceae_UCG-002*, *Ruminococcaceae_UCG-003*, and *Ruminococcaceae_UCG-005* in the colon of IUGR piglets at 7 and 21 days of age, implying that the IUGR did not allow the host to access indigestible energy sources to obtain extra energy for the growth and development. Some species of *Parabacteroides* genus, such as *P. goldsteinii* and *P. distasonis*, play a predominant role in anti-obesity effects (Wang et al., 2019; Wu et al., 2019). The present study showed that the *Parabacteroides* abundance in the IUGR piglets was higher compared to the NBW piglets, indicating that several members of *Parabacteroides* are negatively correlated with BW. In addition, *Subdoligranulum* and *Phascolarctobacterium* can produce SCFAs by fermenting carbohydrates to provide nutrients and energy for the host and play an important role in maintaining gut health (Deldot et al., 1993; Holmstrom et al., 2004). In the present study, colonic *Subdoligranulum* and *Phascolarctobacterium* had lower abundances in the IUGR piglets compared with the NBW piglets at 7 and 21 days of age, suggesting that the function of colonic epithelial cells of IUGR piglets may be adversely affected.

The PICRUSt1 is based on 16S rRNA gene sequencing to predicate the microbiota metabolic function (Langille et al., 2013). Amino acids are essential precursors for protein biosynthesis (Wu, 2009). Vitamins and cofactors are critical for converting nutrients to energy (Hu et al., 2016). In the present study, arginine/proline metabolism, valine/leucine/isoleucine biosynthesis, and nicotinate/nicotinamide metabolism were down-regulated in the IUGR piglets compared with the NBW piglets at 7 days of age, indicating that the growth and development might be suppressed in the IUGR piglets. Interestingly, amino acid, vitamin, glucose, and energy metabolisms were up-regulated in the IUGR piglets compared with the NBW piglets at 21 days of age; however, further in-depth studies are needed to explore the underlying mechanism of these metabolisms in IUGR piglets.

The colonic metabolome differences between the IUGR and NBW piglets were profiled using LC–MS/MS metabolomics analysis in the present study. According to HMDB database classification, the highest changing metabolites in the IUGR piglets compared with the NBW piglets were annotated to organic acids and their derivatives, which have been considered to be related to feed utilization in previous studies (Thacker et al., 1992). In animal production, organic acids are usually used as feed additives to promote growth, inhibit pathogens, and supply energy (Grilli et al., 2015). In addition, as important components of organic acids and their derivatives, amino acids can promote the intestinal development and health of piglets

(Mou et al., 2019). Interestingly, in the present study, most of the SDMs of organic acids and derivatives in the colon of IUGR piglets were significantly decreased at 7 and 21 days of age while increased at 28 days of age compared with NBW piglets. Davila et al. (2013) reported that pigs with a high feed utilization rate had higher colonic concentrations of organic acids. These findings suggested that the feed utilization rate of IUGR piglets was lower, and this situation was alleviated with the increase of age.

In order to reveal the specific effect of SDMs in the colon of IUGR piglets, the KEGG database was used to characterize the most influential metabolism pathways. Recent studies showed that intestinal microbiota can improve experimental colitis in mice by regulating purine metabolism (Wu et al., 2020). In the present study, the purine metabolism was up-regulated in the IUGR piglets compared with the NBW piglets at 7 days of age, as well as the xanthine, hypoxanthine, and deoxyinosine metabolisms suggesting that inflammation might be present in the colon. The amino sugar/nucleotide sugar metabolism was negatively correlated with intestinal permeability (Liu et al., 2019). In the present study, the amino sugar/nucleotide sugar metabolism was down-regulated in the IUGR piglets compared with the NBW piglets at 7 days of age, as well as the galactose 1-phosphate and N-Glycolylneuraminic acid metabolisms, indicating that intestinal microbiota in the IUGR piglets were more likely to enter the circulation, resulting in dysregulated micro-ecological balance. However, there were no significantly different metabolic pathways in the IUGR piglets compared with the NBW piglets at 21 days of age. These findings were distinct from the predicted microbial functions analysis, suggesting the limitation and deviation of the analysis of the IUGR piglet's colon microbial functions based solely on microbial data.

Ubiquinone is vital for cellular energy production (Søballe and Poole, 2000). Tyrosine is an essential amino acid for animals and can be replenished by the metabolic conversion of phenylalanine (Wang et al., 2022). In the present study, ubiquinone/other terpenoid-quinone biosynthesis, phenylalanine/tyrosine/tryptophan biosynthesis, phenylalanine metabolism, and histidine metabolism were up-regulated in the IUGR piglets compared with the NBW piglets at 28 days of age, as well as the L-tyrosine and urocanic acid indicating that the ability of the microbiota to utilize assimilable substrates is enhanced in IUGR piglets.

The SCFAs are produced by the microbial fermentation of undigested carbohydrates and nitrogenous substances (Rooks and Garrett, 2016). These metabolites are important for the host, such as inhibiting pathogenic bacteria growth, improving intestinal function, maintaining body fluid and electrolyte balances, and providing energy (Duncan et al., 2004; Tremaroli and Backhed, 2012). In the present study, the IUGR piglets had lower colonic isobutyrate level at 7 days of age, isovalerate and total SCFAs levels at 21 days of age, and acetate, propionate, butyrate, and total SCFAs levels at 28 days of age, suggesting that the colonic microbiota of IUGR piglets cannot effectively utilize carbohydrates and nitrogenous substances from diets. These findings were consistent with lower abundances of *Ruminococcaceae_UCG-002*, *Ruminococcaceae_UCG-003*, and *Ruminococcaceae_UCG-005* in the colon of IUGR piglets at 7 and 21 days of age in the present study. Moreover, the decreased SCFAs may also be related to the lower abundances of colonic Firmicutes and Bacteroidetes in the IUGR piglets, thus causing the IUGR piglets to be unable to obtain additional energy from these metabolites to meet their growth and development. However, the IUGR piglets had a higher isovalerate level at 28 days of age compared with the NBW piglets in the present study. The possible reason might be related to the age and gut maturation on energy absorption capacity from diets. However, it warranted further study to determine the exact mechanism.

The intestinal barrier can regulate the homeostasis of the body and resist the invasion of pathogens and foodborne antigens, which is important for the maintenance of the intestinal health of animals (Luissint et al., 2016). Tight junction proteins, such as *ZO*-1 and *occludin*, are key molecules that determine intestinal mucosal permeability (Zihni et al., 2016). In the present study, the IUGR piglets had lower mRNA expression levels of colonic *ZO-1* at 7 and 21 days of age and *occludin* at 21 days of age, suggesting that IUGR piglets had impaired intestinal integrity. This decrease may be related to the decrease in microbial diversity and the increase in pathogen abundance in the IUGR piglets (Zhang et al., 2019).

Cytokines are involved in the immune response and intestinal barrier function (Andrews et al., 2018). Inflammatory cytokines are closely related to the changes in tight junction proteins (Al-Sadi et al., 2009). In the present study, the IUGR piglets had a lower mRNA expression level of *IL-4* at 21 days of age while a higher *TNF-α* expression level at 28 days of age, suggesting that there was an intestinal inflammatory reaction in the IUGR piglets.

## Conclusion

In summary, the IUGR could affect intestinal micro-ecological dysbiosis of piglets during their early growth stage by decreasing the microbiota diversity and abundances, leading to impaired intestinal mucosal integrity. The IUGR was associated with the alterations of colonic microbial abundances of Bacteroidetes, Firmicutes, and other bacteria (taxonomically belong to the *Ruminococcaceae* family) that may be involved in the digestion, absorption, and metabolism of nutrients of the IUGR piglets. In addition, the IUGR piglets also showed dysregulated metabolomics profiles related to protein synthesis and the growth and development of piglets. Furthermore, there is a tight cross-talk between gut microbiota and metabolomic biomarkers. These findings will provide the crucial guiding significance for further research into dietary nutrients for IUGR piglets at the early stage.

## 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: NCBI Sequence Read Archive (SRA) with under the accession number PRJNA836103.

## Ethics statement

All aspects of this research were conducted in accordance with the Chinese Guidelines for Animal Welfare and was approved by the Animal Care and Use Committee of the Institute of Subtropical Agriculture, Chinese Academy of Sciences (No. ISA-2017-016).

## Author contributions

WT, WZ, and XK conceived and designed the experiment. WT, WZ, MA, CM, and QZ performed the experiment. WT and WZ processed the data. WT, WZ, and MA prepared and drafted the manuscript. MA and XK 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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## Supplementary material

The Supplementary material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2022.989060/full#supplementary-material

## References

Al-Sadi, R., Boivin, M., and Ma, T. (2009). Mechanism of cytokine modulation of epithelial tight junction barrier. *Front. Biosci.* 14, 2765–2778. doi: 10.2741/3413

Andrews, C., McLean, M. H., and Durum, S. K. (2018). Cytokine tuning of intestinal epithelial function. *Front. Immunol.* 9:1270. doi: 10.3389/fimmu.2018.01270

Azad, M. A. K., Bin, P., Liu, G., Fang, J., Li, T., and Yin, Y. (2018). Effects of different methionine levels on offspring piglets during late gestation and lactation. *Food Funct.* 9, 5843–5854. doi: 10.1039/c8fo01343h

Bauer, R., Walter, B., Hoppe, A., Gaser, E., Lampe, V., Kauf, E., et al. (1998). Body weight distribution and organ size in newborn swine (*sus scrofa domestica*) – a study describing an animal model for asymmetrical intrauterine growth retardation. *Exp. Toxicol. Pathol.* 50, 59–65. doi: 10.1016/s0940-2993(98)80071-7

Biddle, A., Stewart, L., Blanchard, J., and Leschine, S. (2013). Untangling the genetic basis of fibrolytic specialization by *Lachnospiraceae* and *Ruminococcaceae* in diverse gut communities. *Diversity* 5, 627–640. doi: 10.3390/d5030627

Chassaing, B., Van de Wiele, T., De Bodt, J., Marzorati, M., and Gewirtz, A. T. (2017). Dietary emulsifiers directly alter human microbiota composition and gene expression *ex vivo* potentiating intestinal inflammation. *Gut* 66, 1414–1427. doi: 10.1136/gutjnl-2016-313099

Clarke, S. F., Murphy, E. F., O'Sullivan, O., Lucey, A. J., Humphreys, M., Hogan, A., et al. (2014). Exercise and associated dietary extremes impact on gut microbial diversity. *Gut* 63, 1913–1920. doi: 10.1136/gutjnl-2013-306541

Davila, A. M., Blachier, F., Gotteland, M., Andriamihaja, M., Benetti, P. H., Sanz, Y., et al. (2013). Intestinal luminal nitrogen metabolism: role of the gut microbiota and consequences for the host. *Pharmacol. Res.* 68, 95–107. doi: 10.1016/j.phrs.2012.11.005

Deldot, T., Osawa, R., and Stackebrandt, E. (1993). *Phascolarctobacterium faecium* gen-nov, spec nov, a novel taxon of the *Sporomusa* group of bacteria. *Sys. Appl. Microbiol.* 16, 380–384. doi: 10.1016/S0723-2020(11)80269-9

D'Inca, R., Kloareg, M., Gras-Le Guen, C., and Le Huerou-Luron, I. (2010). Intrauterine growth restriction modifies the developmental pattern of intestinal structure, transcriptomic profile, and bacterial colonization in neonatal pigs. *J. Nutr.* 140, 925–931. doi: 10.3945/jn.109.116822

Dong, L., Zhong, X., He, J. T., Zhang, L. L., Bai, K. W., Xu, W., et al. (2016). Supplementation of tributyrin improves the growth and intestinal digestive and barrier functions in intrauterine growth-restricted piglets. *Clin. Nutr.* 35, 399–407. doi: 10.1016/j.clnu.2015.03.002

Duan, Y. H., Zeng, L. M., Li, F. N., Wang, W. L., Li, Y. H., Guo, Q. P., et al. (2017). Effect of branched-chain amino acid ratio on the proliferation, differentiation, and expression levels of key regulators involved in protein metabolism of myocytes. *Nutrition* 36, 8–16. doi: 10.1016/j.nut.2016.10.016

Duncan, S. H., Holtrop, G., Lobley, G. E., Calder, A. G., Stewart, C. S., and Flint, H. J. (2004). Contribution of acetate to butyrate formation by human faecal bacteria. *Br. J. Nutr.* 91, 915–923. doi: 10.1079/bjn20041150

Edgar, R. C. (2013). UPARSE: highly accurate OTU sequences from microbial amplicon reads. *Nat. Methods* 10, 996–998. doi: 10.1038/nmeth.2604

Edgar, R. C. (2016). UNOISE2: improved error-correction for Illumina 16S and ITS amplicon sequencing. *BioRXiv*. 081257. doi: 10.1101/081257

Ferenc, K., Pilzys, T., Skrzypek, T., Garbicz, D., Marcinkowski, M., Dylewska, M., et al. (2017). Structure and function of enterocyte in intrauterine growth retarded pig neonates. *Dis. Markers* 2017:5238134. doi: 10.1155/2017/5238134

Gilbert, W. M., and Danielsen, B. (2003). Pregnancy outcomes associated with intrauterine growth restriction. *Am. J. Obstet. Gynecol.* 188, 1596–1601. doi: 10.1067/mob.2003.384

Grilli, E., Tugnoli, B., Passey, J. L., Stahl, C. H., Piva, A., and Moeser, A. J. (2015). Impact of dietary organic acids and botanicals on intestinal integrity and inflammation in weaned pigs. *BMC Vet. Res.* 11:96. doi: 10.1186/s12917-015-0410-0

Holmstrom, K., Collins, M. D., Moller, T., Falsen, E., and Lawson, P. A. (2004). *Subdoligranulum variable* gen. nov., sp nov from human feces. *Anaerobe* 10, 197–203. doi: 10.1016/j.anaerobe.2004.01.004

Hu, J., Nie, Y., Chen, J., Zhang, Y., Wang, Z., Fan, Q., et al. (2016). Gradual changes of gut microbiota in weaned miniature piglets. *Front. Microbiol.* 7:1727. doi: 10.3389/fmicb.2016.01727

Jiang, L., Feng, C., Tao, S., Li, N., Zuo, B., Han, D., et al. (2019). Maternal imprinting of the neonatal microbiota colonization in intrauterine growth restricted piglets: a review. *J. Anim. Sci. Biotechnol.* 10:88. doi: 10.1186/s40104-019-0397-7

Kamada, N., Chen, G. Y., Inohara, N., and Nunez, G. (2013). Control of pathogens and pathobionts by the gut microbiota. *Nat. Immunol.* 14, 685–690. doi: 10.1038/ni.2608

Kim, K. A., Gu, W., Lee, I. A., Joh, E. H., and Kim, D. H. (2012). High fat diet-induced gut microbiota exacerbates inflammation and obesity in mice via the TLR4 signaling pathway. *PLoS One* 7:e47713. doi: 10.1371/journal.pone.0047713

Kim, Y. S., and Milner, J. A. (2007). Dietary modulation of colon cancer risk. *J. Nutr.* 137, 2576S–2579S. doi: 10.1093/jn/137.11.2576S

Langille, M. G. I., Zaneveld, J., Caporaso, J. G., McDonald, D., Knights, D., Reyes, J. A., et al. (2013). Predictive functional profiling of microbial communities using 16S rRNA marker gene sequences. *Nat. Biotechnol.* 31, 814–821. doi: 10.1038/nbt.2676

Le Chatelier, E., Nielsen, T., Qin, J. J., Prifti, E., Hildebrand, F., Falony, G., et al. (2013). Richness of human gut microbiome correlates with metabolic markers. *Nature* 500, 541–546. doi: 10.1038/nature12506

Ley, R. E., Turnbaugh, P. J., Klein, S., and Gordon, J. I. (2006). Microbial ecology – human gut microbes associated with obesity. *Nature* 444, 1022–1023. doi: 10.1038/4441022a

Li, N., Huang, S. M., Jiang, L. L., Wang, W., Li, T. T., Zuo, B., et al. (2018). Differences in the gut microbiota establishment and metabolome characteristics between low- and normal-birth-weight piglets during early-life. *Front. Microbiol.* 9:1798. doi: 10.3389/fmicb.2018.01798

Littman, D. R., and Pamer, E. G. (2011). Role of the commensal microbiota in normal and pathogenic host immune responses. *Cell Host Microbe* 10, 311–323. doi: 10.1016/j.chom.2011.10.004

Liu, X., Zheng, H., Lu, R., Huang, H., Zhu, H., Yin, C., et al. (2019). Intervening effects of total alkaloids of bunting on rats with antibiotic-induced gut microbiota dysbiosis based on 16S rRNA gene sequencing and untargeted metabolomics analyses. *Front. Microbiol.* 10:1151. doi: 10.3389/fmicb.2019.01151

Luissint, A. C., Parkos, C. A., and Nusrat, A. (2016). Inflammation and the intestinal barrier: leukocyte-ypithelial nell interactions, cell junction remodeling, and mucosal repair. *Gastroenterology* 151, 616–632. doi: 10.1053/j.gastro.2016.07.008

Magoc, T., and Salzberg, S. L. (2011). FLASH: fast length adjustment of short reads to improve genome assemblies. *Bioinformatics* 27, 2957–2963. doi: 10.1093/bioinformatics/btr507

Marchesi, J. R., Adams, D. H., Fava, F., Hermes, G. D. A., Hirschfield, G. M., Hold, G., et al. (2016). The gut microbiota and host health: a new clinical frontier. *Gut* 65, 330–339. doi: 10.1136/gutjnl-2015-309990

Matamoros, S., Gras-Leguen, C., Le Vacon, F., Potel, G., and de La Cochetiere, M. F. (2013). Development of intestinal microbiota in infants and its impact on health. *Trends Microbiol.* 21, 167–173. doi: 10.1016/j.tim.2012.12.001

Mazmanian, S. K., Round, J. L., and Kasper, D. L. (2008). A microbial symbiosis factor prevents intestinal inflammatory disease. *Nature* 453, 620–625. doi: 10.1038/nature07008

Mou, Q., Yang, H. S., Yin, Y. L., and Huang, P. F. (2019). Amino acids influencing intestinal development and health of the piglets. *Animals* 9:302. doi: 10.3390/ani9060302

Ni, H., Long, L., Bin, P., Azad, M. A. K., Xu, K., Zhou, X., et al. (2021). Maternal cysteine intake influenced oxidative status and lipid-related gut microbiota and plasma metabolomics in male suckling piglets. *Anim. Feed Sci. Technol.* 276:114947. doi: 10.1016/j.anifeedsci.2021.114947

Nicholson, J. K., Holmes, E., Kinross, J., Burcelin, R., Gibson, G., Jia, W., et al. (2012). Host-gut microbiota metabolic interactions. *Science* 336, 1262–1267. doi: 10.1126/science.1223813

Rooks, M. G., and Garrett, W. S. (2016). Gut microbiota, metabolites and host immunity. *Nat. Rev. Immunol.* 16, 341–352. doi: 10.1038/nri.2016.42

Schmittgen, T. D., and Livak, K. J. (2008). Analyzing real-time PCR data by the comparative $C_T$ method. *Nat. Protoc.* 3, 1101–1108. doi: 10.1038/nprot.2008.73

Sciascia, Q., Das, G., and Metges, C. C. (2016). Review: the pig as a model for humans: effects of nutritional factors on intestinal function and health. *J. Anim. Sci.* 94, 441–452. doi: 10.2527/jas.2015-9788

Søballe, B., and Poole, R. K. (2000). Ubiquinone limits oxidative stress in *Escherichia coli*. *Microbiology* 146, 787–796. doi: 10.1099/00221287-146-4-787

Sonnenburg, J. L., and Backhed, F. (2016). Diet-microbiota interactions as moderators of human metabolism. *Nature* 535, 56–64. doi: 10.1038/nature18846

Thacker, P. A., Campbell, G. L., and Grootwassink, J. (1992). The effect of organic-acids and enzyme supplementation on the performance of pigs fed barley-based diets. *Can. J. Anim. Sci.* 72, 395–402. doi: 10.4141/cjas92-047

Tremaroli, V., and Backhed, F. (2012). Functional interactions between the gut microbiota and host metabolism. *Nature* 489, 242–249. doi: 10.1038/nature11552

Turnbaugh, P. J., Ley, R. E., Mahowald, M. A., Magrini, V., Mardis, E. R., and Gordon, J. I. (2006). An obesity-associated gut microbiome with increased capacity for energy harvest. *Nature* 444, 1027–1031. doi: 10.1038/nature05414

Valdes, A. M., Walter, J., Segal, E., and Spector, T. D. (2018). Role of the gut microbiota in nutrition and health. *BMJ* 361:k2179. doi: 10.1136/bmj.k2179

Wang, Q., Garrity, G. M., Tiedje, J. M., and Cole, J. R. (2007). Naive Bayesian classifier for rapid assignment of rRNA sequences into the new bacterial taxonomy. *Appl. Environ. Microbiol.* 73, 5261–5267. doi: 10.1128/aem.00062-07

Wang, K., Liao, M. F., Zhou, N., Bao, L., Ma, K., Zheng, Z. Y., et al. (2019). Parabacteroides distasonis alleviates obesity and metabolic dysfunctions via production of succinate and secondary bile acids. *Cell Rep.* 26, 222.e5–235.e5. doi: 10.1016/j.celrep.2018.12.028

Wang, C., Wei, S., Jin, M., Liu, B., Yue, M., and Wang, Y. (2022). Integrated microbiomic and metabolomic dynamics of fermented corn and soybean by-product mixed substrate. *Front. Nutr.* 9:831243. doi: 10.3389/fnut.2022.831243

Weiss, G. A., and Hennet, T. (2017). Mechanisms and consequences of intestinal dysbiosis. *Cell. Mol. Life Sci.* 74, 2959–2977. doi: 10.1007/s00018-017-2509-x

Wen, L., Ley, R. E., Volchkov, P. Y., Stranges, P. B., Avanesyan, L., Stonebraker, A. C., et al. (2008). Innate immunity and intestinal microbiota in the development of type 1 diabetes. *Nature* 455, 1109–1113. doi: 10.1038/nature07336

Wu, G. Y. (2009). Amino acids: metabolism, functions, and nutrition. *Amino Acids* 37, 1–17. doi: 10.1007/s00726-009-0269-0

Wu, G. Y., Bazer, F. W., Cudd, T. A., Meininger, C. J., and Spencer, T. E. (2004). Maternal nutrition and fetal development. *J. Nutr.* 134, 2169–2172. doi: 10.1093/jn/134.9.2169

Wu, G., Bazer, F. W., Wallace, J. M., and Spencer, T. E. (2006). BOARD-INVITED REVIEW: intrauterine growth retardation: implications for the animal sciences. *J. Anim. Sci.* 84, 2316–2337. doi: 10.2527/jas.2006-156

Wu, T. R., Lin, C. S., Chang, C. J., Lin, T. L., Martel, J., Ko, Y. F., et al. (2019). Gut commensal *Parabacteroides goldsteinii* plays a predominant role in the anti-obesity effects of polysaccharides isolated from *Hirsutella sinensis*. *Gut* 68, 248–262. doi: 10.1136/gutjnl-2017-315458

Wu, J. W., Wei, Z. H., Cheng, P., Qian, C., Xu, F. M., Yang, Y., et al. (2020). Rhein modulates host purine metabolism in intestine through gut microbiota and ameliorates experimental colitis. *Theranostics.* 10, 10665–10679. doi: 10.7150/thno.43528

Zhang, W., Ma, C., Xie, P., Zhu, Q., Wang, X., Yin, Y., et al. (2019). Gut microbiota of newborn piglets with intrauterine growth restriction have lower diversity and different taxonomic abundances. *J. Appl. Microbiol.* 127, 354–369. doi: 10.1111/jam.14304

Zhou, X. L., Kong, X. F., Yang, X. J., and Yin, Y. L. (2012). Soybean oligosaccharides alter colon short-chain fatty acid production and microbial population *in vitro*. *J. Anim. Sci.* 90, 37–39. doi: 10.2527/jas.50269

Zihni, C., Mills, C., Matter, K., and Balda, M. S. (2016). Tight junctions: from simple barriers to multifunctional molecular gates. *Nat. Rev. Mol. Cell Biol.* 17, 564–580. doi: 10.1038/nrm.2016.80

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# Effects of *Allium mongolicum* Regel ethanol extract on three flavor-related rumen branched-chain fatty acids, rumen fermentation and rumen bacteria in lambs

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The objective of this study was to evaluate the effect of *Allium mongolicum* Regel ethanol extract (AME) on the concentration of three branched-chain fatty acids (BCFAs) related to flavor, fermentation parameters and the bacteria and their correlations in the rumen of lambs. A total of thirty 3-month-old male, Small-tailed Han sheep (33.60 ± 1.23 kg) were randomly distributed into 2 groups as follows: control group (CON) was fed a basal diet and AME group was fed a basal diet supplemented with 2.8 g·lamb$^{-1}$·d$^{-1}$ *A. mongolicum* Regel ethanol extract. AME supplementation decreased ($P$ = 0.022) 4-methyloctanoic acid (MOA) content and tended to lower ($P$ = 0.055) 4-methylnonanoic acid (MNA) content in the rumen. Compared to CON group, the ruminal concentrations of valerate and isovalerate were higher ($P$ = 0.046 and $P$ = 0.024, respectively), and propionate was lower ($P$ = 0.020) in the AME group. At the phylum level, the AME group had a lower abundance of *Bacteroidetes* ($P$ = 0.014) and a higher abundance of *Firmicutes* ($P$ = 0.020) than the CON group. At the genus level, the relative abundances of *Prevotella* ($P$ = 0.001), *Christensenellaceae_R-7_group* ($P$ = 0.003), *Succiniclasticum* ($P$ = 0.004), and *Selenomonas* ($P$ = 0.001) were significantly lower in the AME group than in the CON group, while the relative abundances of *Ruminococcus* ($P$ < 0.001), *Quinella* ($P$ = 0.013), and *Lachnospiraceae_XPB1014_group* ($P$ = 0.001) were significantly higher. The relative abundances of *Prevotella* ($P$ = 0.029, $R$ = 0.685; $P$ = 0.009, $R$ = 0.770), *Christensenellaceae_R-7_group* ($P$ = 0.019, $R$ = 0.721; $P$ = 0.029, $R$ = 0.685), and *Succiniclasticum* ($P$ = 0.002, $R$ = 0.842; $P$ = 0.001, $R$ = 0.879) was positively correlated with MOA and MNA levels, and the relative abundance of *Lachnospiraceae_XPB1014_group* ($P$ = 0.033, $R$ = −0.673) was negatively correlated with MOA. The relative abundance of *Christensenellaceae_R-7_group* ($P$ = 0.014, $R$ = −0.744) and *Prevotellaceae_UCG-003* ($P$ = 0.023, $R$ = −0.706) correlated negatively

with the EOA content. In conclusion, these findings suggest that the AME affected the concentration of BCFAs, fermentation parameters and the rumen bacteria in the rumen of lambs.

KEYWORDS

*Allium mongolicum* Regel ethanol extract, branched-chain fatty acids, mutton flavor, rumen bacteria, lamb

## Introduction

Sheep meat and milk have a characteristic "mutton flavor," which is mainly associated with branched-chain fatty acids (BCFAs), especially 4-methyloctanoic acid (MOA), 4-ethyloctanoic acid (EOA) and 4-methylnonanoic acid (MNA) (Salles et al., 2002). MOA and MNA are *de novo* synthesized by the use of methylmalonyl-CoA in chain lengthening with acetyl-CoA using propionate as a precursor in the liver (Massart et al., 1983; Vlaeminck et al., 2006). Propionate from rumen fermentation through the rumen epithelium into the portal vein arrives at the liver, and then the liver synthesize BCFAs when propionate concentrations exceed the capacity of gluconeogenesis (Wong et al., 1975; Aschenbach et al., 2010). The biosynthesis pathway of EOA is less well known and may follow the same biosynthesis pathway of MOA and MNA, which uses butyrate and ethylmalonyl-CoA instead of propionate and methylmalonyl-CoA (Kaffarnik et al., 2015). Methylmalonyl-CoA and ethylmalonyl-CoA originate from the carboxylation of propionyl-CoA and butyryl-CoA, respectively, by acetyl-CoA carboxylase (Dewulf et al., 2019). In addition, ruminal microbiota also participate in the formation of BCFAs, which by using the carbon skeleton of iso-butyrate, iso-valerate and branched-chain amino acids (Shivani et al., 2016).

The composition of a diet is one of the most important factors affecting the concentration of BCFAs. High-concentrate diets lead to a higher proportion of VFAs, especially propionate, in the rumen than low-concentrate diets (Plaizier et al., 2008), which will provide sufficient precursors for the synthesis of BCFAs. Young et al. (2003) reported that ram lambs fed with an alfalfa pellet or a corn-based diet had greater concentrations of MOA and MNA in the subcutaneous fat than ram lambs grazing ryegrass/clover pasture. Teng et al. (2020) found that milk from lambs offered perennial ryegrasses/white clover has lower levels of MOA and MNA than milk from lambs offered Lucerne silage and soy meal. In recent years, researchers have made substantial efforts to reduce mutton flavor by adding natural plant extracts, such as tannins, flavonoids, and essential oils, to lamb diets. Vasta et al. (2013) stated that essential oils from *Rosmarinus officinalis* or *Artemisia herba alba* can change the volatile compound composition in lamb meat. Del Bianco et al. (2021) reported that supplementation with 4% tannins from *Acacia mearnsii*, *Castanea sativa*, or *Caesalpinia spinosa* in the diet decreased the concentrations of MOA and 8-methylnonanoic acid, which also conferred a "mutton" flavor (Brennand et al., 1989) to the perirenal fat of Sarda × Comisana lambs.

*Allium Mongolicum* Regel is a traditional Mongolian medicinal herb belonging to the genus *Allium* of the *Liliaceae* family and grows extensively in northwest China (Wang et al., 2019). *A. Mongolicum* Regel contains polysaccharides, flavonoids, polyphenols, and other bioactive compounds (Li et al., 2019). Our previous studies indicated that *A. Mongolicum* Regel ethanol extract (AME) significantly decreased the concentration of BCFAs in the *longissimus dorsi* muscle and *dorsal subcutaneous, omental* and *perirenal* adipose tissues of Small-tailed Han sheep (Liu et al., 2019; Liu and Ao, 2021). However, it is not clear whether AME affects the synthesis of three BCFAs related to flavor in the rumen of lambs. Thus, the objective of this study was to investigate the effects of AME on the concentrations of three rumen BCFAs related to flavor, rumen fermentation parameters, the diversity of rumen bacteria and their correlations in lambs.

## Materials and methods

All experimental procedures involving animals were evaluated and approved according to the guidelines of the Animal Care and Use Committee of Inner Mongolia Agriculture University (Hohhot, China). This study was conducted at a commercial farm in Bayannaoer, Inner Mongolia Autonomous Region, China (latitude 40°13′–42°28′; longitude 105°12′–109°53′) between April and June 2021.

### Animals and experimental design

A total of thirty 3-month-old male, Small-tailed Han sheep (33.60 ± 1.23 kg) were randomly distributed into 2 groups as follows: control group (CON) was fed a basal diet and AME group was fed a basal diet supplemented with 2.8 g·lamb$^{-1}$·d$^{-1}$ *A. Mongolicum* Regel ethanol extract. One group was composed of three pens, with five lambs in each pen (3.95 m × 5.12

m). *A. Mongolicum* Regel powder was purchased from Hao Hai Biological Company (Alxa League, Inner Mongolia, China), and the ethanol extraction process was conducted as previously described by Ding et al. (2021). The obtained AME primarily contained 26.43% flavonoids, 18.57% organic acids and their derivatives, 14.43% nucleotides and their derivatives, 11.14% amino acids and others. The dose of the ethanol extract (2.8 g·lamb$^{-1}$·d$^{-1}$) in the diet has been proven to be most beneficial for the lamb based on our previous studies (Zhao et al., 2021). We mixed 2.8 g AME with 50 g concentrate for each lamb. Thus, the total dose of five lambs (a pen) is 14 g AME mixed with 250 g concentrate, divided into two equal amounts, and provided for each pen twice daily to ensure that the lambs completely consumed the AME. Each pen of CON group was fed 250 g of concentrate without AME in the same manner. Then, total mixed ration was provided, and the lambs were allowed to feed *ad libitum*. The basal diet met the requirements for sheep, as described by the National Research Council (NRC, 2007), and its composition and nutritional level are shown in **Table 1**.

The experimental period lasted for 75 days, with 15 days of adaptation to the experimental environment and 60 days for the experimental feeding period. The lambs were given free access to water and TMR was provided twice daily at 7:00 and 18:00. During the experimental period, feed was provided to the lambs and refusals were recorded to calculate the dry matter intake (DMI). The body weight of each lamb was also recorded before morning feeding at 0, 15, 30, 45, and 60 days, to calculate average daily gain (ADG), meanwhile, initial body weight (IBW), and final body weight (FBW) were recorded.

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

| Item | Content (%) |
|---|---|
| Chinese wildrye | 25.00 |
| Caragana | 17.80 |
| Whole corn silage | 23.60 |
| Wheat bran | 5.15 |
| Sunflower seed meal | 21.35 |
| Pea stem and leaf | 2.64 |
| Red jujube | 2.04 |
| $CaHPO_4$ | 0.74 |
| NaCl | 0.68 |
| Premix[a] | 1.0 |
| Total | 100.00 |
| Nutrient level | |
| DE[b] (MJ/kg) | 13.46 |
| CP | 16.87 |
| NDF | 38.72 |
| ADF | 27.51 |
| Ca | 1.33 |
| P | 0.53 |

[a]Nutritional composition of premix per kilogram: Mn 30.00 mg, Fe 25.00 mg, Zn 29.00 mg, Cu 8.00 mg, Co 0.10 mg, I 0.04 mg, VA 3200 IU, VD 1200 IU, VE 20 IU.
[b]DE was a calculated value, and the others were measured values; DE, digestible energy.

## Sampling

At 60 days of the experimental period, rumen fluid samples were collected before the morning feeding using an oral stomach tube connected to a 50 mL syringe from six randomly selected lambs from each group. The initial part of the rumen fluid was discarded to avoid contamination with saliva, and then rumen fluid (approximately 50 mL) was collected and filtered through four layers of gauze. The pH was measured using a portable pH meter (PHB-4; INESA Scientific Instrument Co., Ltd, Shanghai, China). 10 mL of rumen fluid was mixed with 2 mL of 25% $HPO_3$ and stored at −20°C until volatile fatty acid (VFA) analysis. 10 mL of rumen fluid was stored at −20°C until ammonium nitrogen ($NH_3$-N) analysis. The remaining rumen fluid was immediately frozen in liquid nitrogen and stored at −80°C until BCFAs analysis and microbial community analysis.

## Chemical analyses of feeds

Samples of feed were dried at 55°C for 48 h and then ground to pass through a 2 mm sieve before chemical analysis. Crude protein (N × 6.25, method 981.10) and minerals content were measured using an analytical method provided by the Association of Official Agricultural Chemists (AOAC, 2000). The neutral detergent fiber, acid detergent fiber were determined as described by Van Soest et al. (1991).

## Branched-chain fatty acid analyses

Three BCFAs, namely, MOA, MNA, and EOA, in the rumen were analyzed by gas chromatography–mass spectrometry (GC–MS). Prior to GC–MS analysis, fatty acids in the rumen were converted to their corresponding methyl esters, which was carried out according to the method of Kaffarnik et al. (2014). Undecanoic acid (Sigma–Aldrich, USA) was used as an internal standard.

A GC–MS system (Thermo TRANCE GC 1300) equipped with an autosampler was used in EI mode (70 eV). Solutions (1 μL) were injected in split mode (split ratio 10:1) onto a TG-WAX column (30 m, 0.25 mm i.d., 0.25 μm film thickness). The GC oven was held at 60°C for 2 min, and then, the temperature was raised at 20°C min$^{-1}$ to 250°C and held for 5 min. The flow rate of the helium carrier gas was 1.2 mL/min. The mass spectrometer transfer line was operated at 250°C. Mass spectra were acquired using an ion source temperature of 220°C. GC–MS analyses were performed in full-scan mode (m/z 50–350).

## Ruminal fermentation analyses

Ruminal $NH_3$-N concentrations were analyzed using a microplate reader (Epoch, BioTek Instruments, Inc., USA) according to the method described by Chaney and Marbach (1962). The concentration of VFA was determined using a gas chromatograph (GC-2014; Shimadzu, Japan) with a fused silica column (60 m × 0.25 mm × 0.50 μm; DB-FFAP, Agilent Technologies, USA) according to Erwin et al. (1961). 2-Ethyl butyric acid was used as an internal standard. The column temperature was increased from 80 to 180°C at 20°C/min and held for 3 min. The injector and detector temperatures were set at 220 and 250°C, respectively.

## 16S rDNA extraction and sequencing

Microbial community genomic DNA was extracted from rumen fluid samples using the E.Z.N.A.® soil DNA Kit (Omega Bio-Tek, Norcross, GA, U.S.) according to the manufacturer's instructions. The DNA quality and quantity were determined by a NanoDrop 2000 UV–vis spectrophotometer (Thermo Fisher Scientific, Wilmington, USA). The V3–V4 region of the bacterial 16S rRNA gene was amplified with the primer pair 338F (5′-ACTCCTACGGGAGGCAGCAG-3′) and 806R (5′-GGACTACHVGGGTWTCTAAT-3′) by an ABI GeneAmp® 9700 PCR thermocycler (ABI, CA, USA). The PCR amplification of the 16S rRNA gene was performed as follows: initial denaturation at 95°C for 3 min, followed by 27 cycles of denaturing at 95°C for 30 s, annealing at 55°C for 30 s and extension at 72°C for 45 s, and a single extension at 72°C for 10 min, ending at 4°C. The PCR mixtures contained 4 μL of 5 × *TransStart* FastPfu buffer, 2 μL of 2.5 mM deoxynucleoside triphosphates, 0.8 μL of each primer (5 μM), 0.4 μL of *TransStart* FastPfu DNA polymerase, 10 ng of template DNA, and $ddH_2O$ to reach a total volume of 20 μL. The PCR products were purified using the AxyPrep DNA Gel Extraction Kit (Axygen Biosciences, Union City, CA, USA) and quantified using a Quantus™ Fluorometer (Promega, USA). Purified amplicons were sequenced on an Illumina MiSeq PE300 platform (Illumina, San Diego, USA).

## Sequencing data analyses

Fastp v0.19.6 software[1] and FLASH v1.2.11 software[2] were used for quality control and splicing, respectively. UPARSE v7.0.1090 software was used to cluster operational taxonomic units (OTUs) with 97% similarity (Edgar, 2013). The taxonomy of each OTU representative sequence was analyzed by ribosomal database project (RDP) classifier v2.11[3] compared with Silva v138[4] using a comparison threshold of 70% (Han et al., 2015). Alpha diversity analysis was performed by Mothur v1.30.2[5].

## Statistical analysis

Sequencing data followed normality (Shapiro–Wilk's tests, $P > 0.2$) after log transformations, and their differences were homogenous (Levene's test, $P > 0.05$). Non-transformed values are shown in this study. Data for growth performance, the concentration of BCFAs, rumen fermentation parameters and bacterial communities between CON and AME group lambs were analyzed using one-way ANOVA by SPSS 26.0 (IBM, New York, USA). The statistical model was $Y_i = \mu + A_i + e$, where $Y_i$ is the dependent variable; $\mu$ is the overall mean; $A_i$ is the fixed effect of treatment ($i$ = 1.2, CON or AME); and e is the random effect. The degrees of freedom of ANOVA are as follows: total: $df_T = n - 1$ ($n$ = total sample size); group: $df_G = k - 1$ ($k$ = total group size); error: $df_E = df_T - df_G$. Statistical significance was defined at $P < 0.05$, and trending toward significance was defined at $0.05 \leq P \leq 0.1$. Spearman's rank correlation coefficient analysis between BCFA levels, VFA levels and rumen bacterial taxa abundances was carried out using tools on the Majorbio platform (Majorbio Bio-Pharm Technology Co., Ltd.; Shanghai, China)[6]. Significant correlations were defined at $P < 0.05$.

# Results

## Growth performance

The results of the effect of AME on the growth performance of lambs are presented in **Table 2**. There were no differences in

1 https://github.com/OpenGene/fastp

2 https://ccb.jhu.edu/software/FLASH/index.shtml

3 https://sourceforge.net/projects/rdp-classifier/

4 https://www.arb-silva.de/

5 https://www.mothur.org/

6 www.majorbio.com

TABLE 2 Effect of AME on the growth performance of lambs.

| Items | CON | AME | SEM | *P*-value |
|---|---|---|---|---|
| IBW[a], kg | 33.40 | 34.14 | 0.37 | 0.342 |
| FBW[b], kg | 50.22 | 51.28 | 1.14 | 0.526 |
| ADG[c], g | 240.6 | 287.2 | 0.01 | 0.098 |
| DMI[d], g/d | 1095.23 | 1117.38 | 21.42 | 0.659 |

CON, basal diet; AME, basal diet + 2.8 g·lamb$^{-1}$·d$^{-1}$ AME.
[a]IBW, initial body weight.
[b]FBW, final body weight.
[c]ADG, average daily gain.
[d]DMI, dry matter intake.

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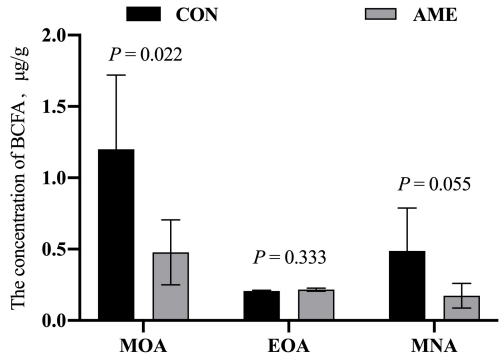


FIGURE 1
Effect of AME on the concentration of BCFAs in the rumen of lambs. CON, basal diet; AME, basal diet + 2.8 g·lamb$^{-1}$·d$^{-1}$ *A. mongolicum* Regel ethanol extract (AME).

FBM and DMI between the CON and AME groups. Compared with the CON group, AME supplementation tended to increase ($P$ = 0.098) the ADG of lambs.

## Three branched-chain fatty acids concentrations and fermentation parameters in the rumen

There was no difference in the concentration of EOA in rumens between the CON and AME groups (Figure 1). AME supplementation decreased ($P$ = 0.022) the concentration of MOA and tended to lower ($P$ = 0.055) the concentration of MNA in the rumen.

The results of the rumen fermentation parameters are presented in Table 3. There were no differences in ruminal pH, TVFA level, acetate level, butyrate level, iso-butyrate level, or acetate/propionate ration between the CON and AME groups. The concentration of rumen $NH_3$-N ($P$ = 0.078) tended to be lower in the AME group than in the CON group. Lambs consuming the AME had higher ruminal concentrations of valerate and iso-valerate ($P$ = 0.046 and $P$ = 0.024, respectively) than lambs consuming the CON diet and had a lower ruminal concentration of propionate ($P$ = 0.020).

TABLE 3 Effect of AME on rumen fermentation in lambs.

| Items | CON | AME | SEM | *P*-value |
|---|---|---|---|---|
| pH | 7.00 | 7.17 | 0.07 | 0.276 |
| $NH_3$-N[a], mg/dL | 7.12 | 6.51 | 0.17 | 0.078 |
| TVFA[b], mmol/L | 61.60 | 60.42 | 0.81 | 0.424 |
| Acetate, mmol/L | 37.94 | 37.03 | 0.54 | 0.494 |
| Propionate, mmol/L | 12.07 | 10.84 | 0.29 | 0.020 |
| Butyrate, mmol/L | 8.13 | 8.58 | 0.36 | 0.565 |
| Iso-butyrate, mmol/L | 1.27 | 1.35 | 0.05 | 0.376 |
| Valerate, mmol/L | 0.67 | 0.77 | 0.03 | 0.046 |
| Iso-valerate, mmol/L | 1.52 | 1.84 | 0.08 | 0.024 |
| Acetate/Propionate | 3.16 | 3.42 | 0.09 | 0.150 |

CON, basal diet; AME, basal diet + 2.8 g·lamb$^{-1}$·d$^{-1}$ AME.
[a]$NH_3$-N, ammonia nitrogen.
[b]TVFA, total volatile fatty acid.

## Abundance and diversity of rumen bacteria

In total, 664,062 raw sequence reads were obtained by sequencing from the lamb ruminal fluid in the CON and AME groups. After quality control and filtering, a total of 556,763 clean tags were generated, and an average of 46,397 clean tags were analyzed for each sample. According to 97% similarity, 1,243 OTUs were identified in the two groups (Table 4). No significant differences were found in the OTU number, ACE, Chao 1 index, or Shannon and Simpson diversity indices between the two treatment groups. The coverage ($P$ = 0.07) tended to higher in AME group than CON group.

At the phylum level, the relative abundances of the top 10 rumen bacterial phyla are presented in Table 5. *Bacteroidetes* and *Firmicutes* were the predominant phyla for the two groups and accounted for approximately 96% of the total bacterial

TABLE 4 Effect of AME on OTUs and alpha indices in lambs.

| Items | CON | AME | SEM | *P*-value |
|---|---|---|---|---|
| OTU[a] | 1,108 | 1,150 | 4.62 | 0.358 |
| ACE[b] | 766.02 | 711.42 | 5.90 | 0.480 |
| Coverage | 99.70% | 99.79 | 0.0002 | 0.070 |
| Chao1 | 770.60 | 714.82 | 7.29 | 0.487 |
| Shannon | 4.30 | 4.38 | 0.11 | 0.766 |
| Simpson | 0.04 | 0.04 | 0.01 | 0.905 |

CON, basal diet; AME, basal diet + 2.8 g·lamb$^{-1}$·d$^{-1}$ AME.
[a]OTUs, operational taxonomic units.
[b]ACE, abundance-based coverage estimator.

TABLE 5 Effect of AME on bacterial abundance at the phylum level in the rumen of lambs (%).

| Items | CON | AME | SEM | *P*-value |
|---|---|---|---|---|
| Bacteroidetes | 55.18 | 36.84 | 4.13 | 0.014 |
| Firmicutes | 42.35 | 59.12 | 3.92 | 0.020 |
| Actinobacteriota | 0.66 | 0.56 | 0.18 | 0.801 |
| Desulfobacterota | 0.56 | 0.92 | 0.10 | 0.052 |
| Synergistota | 0.36 | 0.51 | 0.14 | 0.623 |
| Spirochaetota | 0.31 | 0.25 | 0.05 | 0.578 |
| Proteobacteria | 0.18 | 0.72 | 0.12 | 0.001 |
| Patescibacteria | 0.17 | 0.34 | 0.07 | 0.266 |
| unclassified_k__norank_d__Bacteria | 0.07 | 0.07 | 0.01 | 0.860 |
| Cyanobacteria | 0.06 | 0.25 | 0.04 | 0.001 |

CON, basal diet; AME, basal diet + 2.8 g·lamb$^{-1}$·d$^{-1}$ AME.

abundance. The relative composition of the bacterial phyla was changed when the diet was supplemented with AME. Compared to that in the CON group, the richness of *Bacteroidetes* ($P$ = 0.014) was significantly decreased in the AME group, while the richness of *Firmicutes* ($P$ = 0.020) was significantly increased. The relative abundances of *Proteobacteria* ($P$ = 0.001) and *Cyanobacteria* ($P$ = 0.001) were higher in the AME group than in the CON group, and that of *Desulfobacterota* ($P$ = 0.052) tended to be higher.

At the genus level, 22 rumen bacterial genera with a relative abundance > 1% in at least one group are presented in Table 6. The dominant bacterial genera were *Prevotella*, *norank_f_bacteroidales_RF16_group*, *Rikenellaceae_RC9_gut_group* and *Ruminococcus* in both groups. The relative abundances of *Prevotella* ($P$ = 0.001), *norank_f_bacteroidales_RF16_group* ($P$ = 0.001), *Christensenellaceae_R-7_group* ($P$ = 0.003), *norank_f_p-251-o5* ($P$ = 0.006), *Succiniclasticum* ($P$ = 0.004), and *Selenomonas* ($P$ = 0.001) were significantly lower in the AME group than in the CON group. In addition, the relative abundances of *Ruminococcus* ($P$ < 0.001), *Quinella* ($P$ = 0.013), *norank_f_Muribaculaceae* ($P$ < 0.001), *unclassified_f_Ruminococcus* ($P$ < 0.001), and *Lachnospiraceae_XPB1014_group* ($P$ = 0.001) were significantly higher in the AME group than in the CON group.

TABLE 6 Effect of AME on bacterial abundance at the genus level in the rumen for lambs (>1% at least in one group).

| Items | CON | AME | SEM | *P*-value |
|---|---|---|---|---|
| Prevotella | 22.47 | 11.99 | 2.02 | 0.001 |
| norank_f__Bacteroidales_RF16_group | 8.63 | 3.90 | 0.90 | 0.001 |
| Rikenellaceae_RC9_gut_group | 7.00 | 5.65 | 0.46 | 0.155 |
| Ruminococcus | 5.56 | 16.39 | 1.60 | < 0.001 |
| Christensenellaceae_R-7_group | 4.50 | 1.71 | 0.56 | 0.003 |
| norank_f__p-251-o5 | 3.89 | 1.35 | 0.53 | 0.006 |
| norank_f__F082 | 3.53 | 2.58 | 0.32 | 0.150 |
| Prevotellaceae_UCG-003 | 3.46 | 2.57 | 0.31 | 0.167 |
| Succiniclasticum | 2.94 | 1.93 | 0.21 | 0.004 |
| Quinella | 2.77 | 9.55 | 1.51 | 0.013 |
| Selenomonas | 2.76 | 0.76 | 0.20 | 0.001 |
| Veillonellaceae_UCG-001 | 2.65 | 1.82 | 0.25 | 0.102 |
| Veillonella | 2.65 | 1.82 | 0.31 | 0.199 |
| unclassified_f__Selenomonadaceae | 2.24 | 1.63 | 0.35 | 0.422 |
| Eubacterium_Coprostanoligenes_group | 2.21 | 2.02 | 0.22 | 0.701 |
| NK4A214_group | 2.21 | 2.11 | 0.36 | 0.901 |
| Prevotellaceae_UCG-001 | 2.18 | 2.59 | 0.43 | 0.657 |
| Lachnospiraceae_NK3A20_group | 2.08 | 1.79 | 0.28 | 0.635 |
| Anaerovibrio | 1.47 | 2.00 | 0.40 | 0.541 |
| norank_f__Muribaculaceae | 1.39 | 3.52 | 0.38 | < 0.001 |
| unclassified_f__Ruminococcaceae | 1.38 | 4.24 | 0.53 | < 0.001 |
| Lachnospiraceae_XPB1014_group | 0.37 | 2.12 | 0.33 | 0.001 |

CON, basal diet; AME, basal diet + 2.8 g·lamb$^{-1}$·d$^{-1}$ AME.

## Relationships between volatile fatty acid levels, rumen bacteria and branched-chain fatty acid levels

The relationships between BCFA levels, rumen fermentation parameters and the bacterial community are presented in Figure 2. Iso-butyrate ($P$ = 0.015, $R$ = −0.736; $P$ = 0.003, $R$ = −0.827) and iso-valerate ($P$ = 0.011, $R$ = −0.758; $P$ = 0.001, $R$ = −0.879) levels correlated negatively with the MOA and MNA levels. At the phylum level, the relative abundances of *Desulfobacterota* ($P$ = 0.016, $R$ = −0.733) and *Proteobacteria* ($P$ = 0.025, $R$ = −0.697) correlated negatively with the MOA content. The relative abundances of *Desulfobacterota* ($P$ = 0.009, $R$ = −0.770) and *Cyanobacteria* ($P$ = 0.038, $R$ = −0.661) negatively correlated with the MNA content.

At the genus level, there were positive correlations among the following: MOA content with the *Prevotella* ($P$ = 0.029, $R$ = 0.685), *norank_f_Bacteroidales_RF16_group* ($P$ = 0.009, $R$ = 0.770), *Christensenellaceae_R-7_group* ($P$ = 0.019, $R$ = 0.721), *Succiniclasticum* ($P$ = 0.002, $R$ = 0.842), and *unclassified_f_Selenomonadaceae* ($P$ = 0.022, $R$ = 0.709) abundances and MNA content with the *Prevotella* ($P$ = 0.009, $R$ = 0.770), *norank_f_Bacteroidales_RF16_group* ($P$ = 0.022, $R$ = 0.709), *Christensenellaceae_R-7_group* ($P$ = 0.029, $R$ = 0.685), and *Succiniclasticum* ($P$ = 0.001, $R$ = 0.879) abundances. Moreover, there were negative correlations between the MOA content with the *norank_f__Muribaculaceae* ($P$ = 0.009, $R$ = −0.770), *unclassified_f__Ruminococcaceae* ($P$ = 0.019, $R$ = −0.721), and *Lachnospiraceae_XPB1014_group* ($P$ = 0.033, $R$ = −0.673) abundances; EOA content with the *Christensenellaceae_R-7_group* ($P$ = 0.014, $R$ = −0.744) and *Prevotellaceae_UCG-003* ($P$ = 0.023, $R$ = −0.706) abundances; and MNA content with the *norank_f__Muribaculaceae* ($P$ = 0.006, $R$ = −0.794) and *unclassified_f__Ruminococcaceae* ($P$ = 0.022, $R$ = −0.709) abundances.

# Discussion

## Effect of *Allium mongolicum* Regel ethanol extract on the growth performance of Lambs

In this study, there were no differences in the FBW and DMI between the CON and AME groups. In accordance with this study, Ding et al. (2021) reported that AME did not affect the FBW and DMI, which suggested that AME had no effect on the palatability of the diet. AME supplementation tended to increase the ADG of lambs, which may be attributed to the high content of flavonoids in the AME. Muqier et al. (2017) reported that flavonoids from *Allium Mongolicum* Regel promote the secretion of growth hormone, insulin-like growth factor and

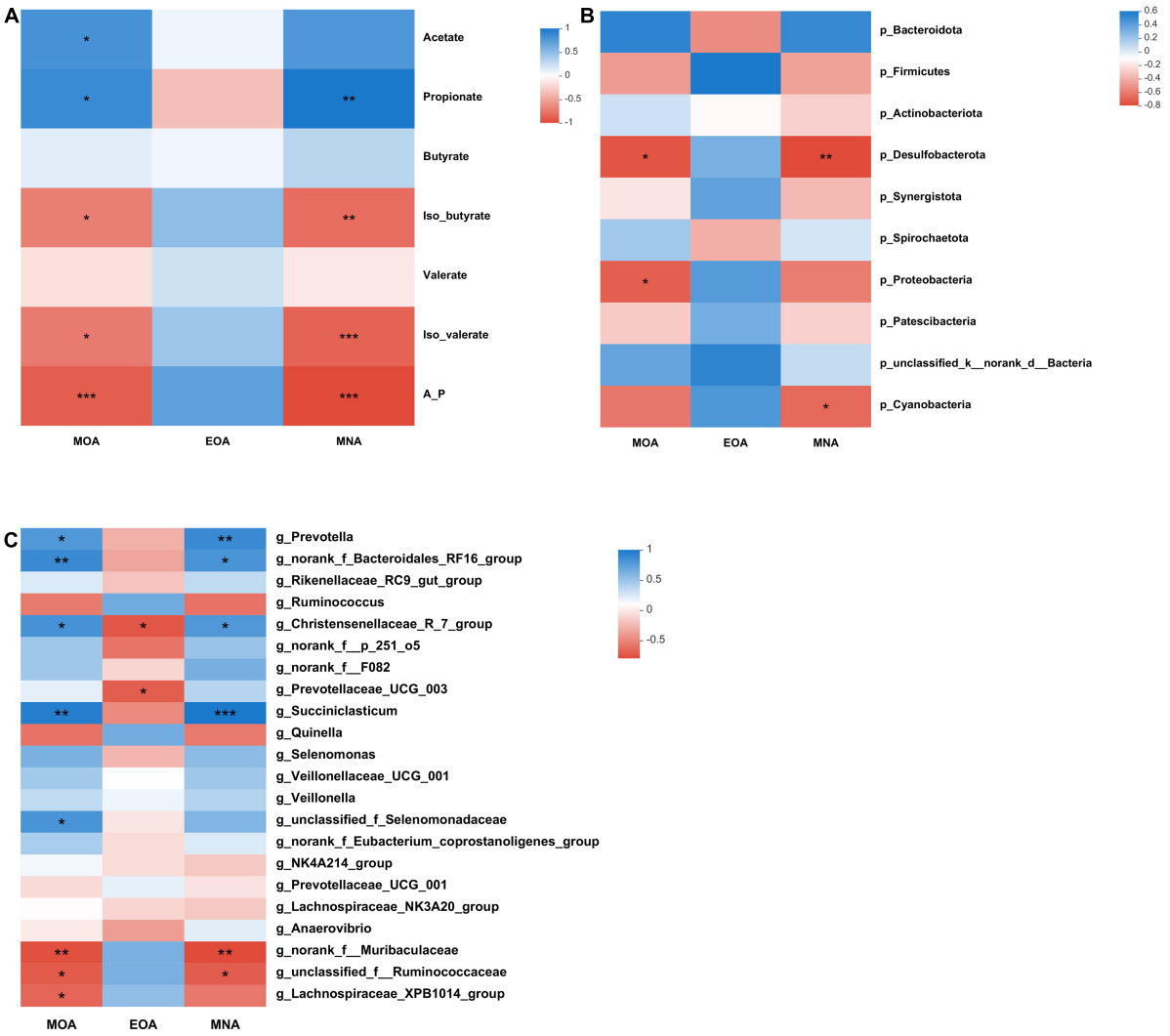


FIGURE 2
Correlations between VFA contents, microbial taxa and BCFA contents **(A–C)**. **(A)** Correlation between the concentration of BCFAs and VFAs. **(B)** Correlation between the concentration of BCFAs and the top 10 microbial taxa at the phylum level. **(C)** Correlation between the concentration of BCFAs and genera with relative abundances > 1% (at least in one group). Significant correlations are shown by $*P < 0.05$, $**P < 0.01$, and $***P < 0.001$. Blue represents positive correlation coefficients. Red represents negative correlation coefficients.

adrenocorticotropic hormone, leading to a significant increase in the ADG. This result proved that AME could improve the growth performance of lambs.

## Effect of *Allium mongolicum* Regel ethanol extract on three branched-chain fatty acids concentrations and fermentation parameters in the rumen of lambs

The rumen is a complex ecosystem that has a strong relationship with the formation of BCFAs. Acetate, propionate and butyrate are produced by rumen fermentation, which through the rumen epithelium into the portal vein arrives at the liver to increase the synthesis of BCFAs in the liver of lambs (Chilliard et al., 2003). Berthelot et al. (2001) further verified this view and suggested that a diet supplemented with propionate significantly increased the content of BCFAs in *subcutaneous* and visceral fat of lambs. This could also explain why concentrate-based diets promote animal growth, while meat has a strong mutton flavor. In the present study, we observed that AME supplementation decreased the concentrations of MOA and MNA in the rumen of lambs. The lower concentrations of MOA and MNA in the AME group might be due to the active ingredients, such as flavonoids and organic acids, of the AME inhibiting

the isomerization of unsaturated fatty acids to BCFAs after triglyceride degradation.

In the present study, there was no significant difference in pH between the CON and AME groups. However, the pH was in the optimal range between 6.2 and 7.2, which reflected the stability of the rumen microbial ecosystem (Calsamiglia et al., 2002). $NH_3$-N is an essential indicator of rumen function and is the primary substrate for microbial protein synthesis (Wallace et al., 1994). The AME group had a lower concentration of $NH_3$-N, which may be attributed to the high content of flavonoids in the AME. A similar result was found by Jelali and Ben Salem (2014) in a study with buffalo calves offered *Moringa oleifera* leaf that is rich in flavonoids. Liu et al. (2020) also reported that flavonoids decrease ruminal $NH_3$-N concentrations in *in vitro* experiments. This would indicate that the AME may reduce ruminal ammonia concentrations by increasing the utilization of amino acids or promoting the synthesis of microbial proteins.

VFAs produced by ruminal microorganisms degrade nutrients, which meet 60–80% of the energy requirements of ruminants (Lane and Jesse, 1997). In the current study, there were no differences in the TVFA, acetate, butyrate, or iso-butyrate levels or acetate/propionate ratio among the treatments. In accordance with this study, Seradj et al. (2018) suggested that citrus flavonoids did not affect the concentrations of TVFAs, butyrate and isobutyrate *in vitro*. The AME did not affect the concentration of TVFAs, probably due to a significant lack of effect on ruminal pH (Gabel et al., 2002). Furthermore, we also found that supplementation with AME in the diet decreased the concentration of propionate and increased the concentration of valerate and iso-valerate. Consistent with our results, Oskoueian et al. (2013) reported that the addition of 4.5% flavonoid substrate caused a lower propionate concentration *in vitro*. Moreover, there was no difference in acetate/propionate ratio between the CON and AME groups, although the concentration of propionate was lower in the AME group than in the CON group. Ruminal BCFAs (iso-butyrate and iso-valerate) originate from the microbial deamination of branched amino acids (Apajalahti et al., 2019). A relatively high valerate content is conducive to improving the growth performance of ruminants (Bergman, 1990).

## Effects of *Allium mongolicum* Regel ethanol extract on the abundance and diversity of rumen bacteria in lambs

Previous studies have suggested that extracts from natural plants could affect the composition of rumen microorganisms in ruminants (Jiao et al., 2021; Wang et al., 2021). *Bacteroidetes* and *Firmicutes* are the predominant phyla in the ruminal microbial community, which is consistent with our results (Scharen et al., 2017). *Proteobacteria* are positively correlated with fiber intake (Faniyi et al., 2019). In the current study, we observed that the abundances of *Bacteroidetes* were lower in the AME group than in the CON group, and the abundances of *Firmicutes, Proteobacteria,* and *Cyanobacteria* were higher. Zhan et al. (2017) reported that supplementation with flavonoid extracts from alfalfa increased the ruminal abundance of *Firmicutes* in dairy cows. Jiang et al. (2021) observed that the relative abundances of *Firmicutes* and *Proteobacteria* were relatively high in yak calves offered root extracts from *Codonopsis pilosula* that contained high concentrations of flavonoids. Consequently, the flavonoids of the AME could explain the decreased abundance of *Bacteroidetes* and increased abundances of *Firmicutes* and *Proteobacteria* in the rumen of lambs.

At the genus level, *Prevotella*, *Succiniclasticum,* and *Selenomonas* are associated with propionate production (Henderson et al., 2015; Yuste et al., 2020). In the present study, adding AME to the diet significantly decreased the relative abundances of *Prevotella*, *Succiniclasticum,* and *Selenomonas*. The reduction in the abundances of *Prevotella*, *Succiniclasticum,* and *Selenomonas* resulted in a lower concentration of propionate in the rumen. The decrease in the relative abundance of *Prevotella* (within *Bacteroidetes*) could be due to the AME decreasing the relative abundance of *Bacteroidetes* at the phylum level. In addition, the abundance of *Prevotella* was negatively associated with the ruminal pH (Chiquette et al., 2012). In this study, AME increased the ruminal pH but not significantly, which may also have causes a decrease in the relative abundance of *Prevotella*. *Charistensenellaceae_R-7_group* is involved in biofilm formation (Mao et al., 2015). Studies have suggested that flavonoids can increase the relative abundance of the *Charistensenellaceae_R-7_group* (Harlow et al., 2018; Jiang et al., 2021). However, in the current study, the relative abundance of *Charistensenellaceae_R-7_group* was lower in the AME group than in the CON group, which could be due to its growth being inhibited by other active compounds of the AME.

## The relationships of volatile fatty acid levels, rumen bacteria and branched-chain fatty acid levels

The phenotypic characteristics of ruminants are determined to some extent by rumen microbiota (Scharen et al., 2018). The feed was fermented by microorganisms in the rumen to produce precursors (e.g., propionate and butyrate) of BCFA biosynthesis. Some of the precursors are formed into BCFAs in the rumen, while the others are absorbed into the blood through the rumen wall and then transported to the liver to synthesize BCFAs (Victoria and Robert, 1993). Therefore, rumen VFAs and microorganisms are key factors that directly or indirectly affect BCFA biosynthesis. In the present study, Spearman's rank correlation coefficient analysis was conducted to analyze the relationip between VFA levels, rumen bacteria

and BCFA levels related to mutton flavor. We found that MOA and MNA levels negatively correlated with iso-butyrate and iso-valerate levels. Moreover, the relative abundances of *Prevotella*, *Christensenellaceae_R-7_group,* and *Succiniclasticum* correlated positively with MOA and MNA contents, and the *Lachnospiraceae_XPB1014_group* abundance correlated negatively with MOA and MNA levels. The relative abundance of *Lachnospiraceae_XPB1014_group* correlated negatively with the EOA content. These findings reveal the relationship between the rumen bacteria and three flavor-related BCFAs in the rumen of lambs.

## Conclusion

In the present study, a diet supplemented with AME significantly decreased the concentrations of MOA and MNA in the rumen of lambs. AME supplementation also changes the rumen fermentation pattern and the composition of rumen bacteria. Moreover, this study also found that MOA and MNA were positively correlated with *Prevotella*, *Christensenellaceae_R-7_group,* and *Succiniclasticum* but negatively correlated with iso-butyrate, iso-valerate, and *Lachnospiraceae_XPB1014_group*.

## 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

This animal study was reviewed and approved by the Animal Care and Use Committee of Inner Mongolia Agriculture University.

## Author contributions

YZ involved in investigation, formal analysis, and writing—original draft. YmZ involved in data curation. EK involved in writing—review and editing. CB involved in supervision. CA involved in project administration and funding acquisition. 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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## References

AOAC (2000). *AOAC Official Methods of Analysis*, 17th Edn. Washington, D.C: Association of Official Agricultural Chemists.

Apajalahti, J., Vienola, K., Raatikainen, K., Holder, V., and Moran, C. A. (2019). Conversion of branched-chain amino acids to corresponding isoacids - an in vitro tool for estimating ruminal protein degradability. *Front. Vet. Sci.* 6:311. doi: 10.3389/fvets.2019.00311

Aschenbach, J. R., Kristensen, N. B., Donkin, S. S., Hammon, H. M., and Penner, G. B. (2010). Gluconeogenesis in dairy cows: the secret of making sweet milk from sour dough. *Iubmb Life.* 62, 869–877. doi: 10.1002/iub.400

Berthelot, V., Bas, P., Schmidely, P., and Duvaux-Ponter, C. (2001). Effect of dietary propionate on intake patterns and fatty acid composition of adipose tissues in lambs. *Small Rumin Res.* 40, 29–39. doi: 10.1016/s0921-4488(00)00217-0

Bergman, E. N. (1990). Energy contributions of volatile fatty acids from the gastrointestinal tract in various species. *Physiol. Rev.* 70, 567–590. doi: 10.1007/BF00370249

Brennand, C. P., Ha, J. K., and Lindsay, R. C. (1989). Aroma properties and thresholds of some branched-chain and other minor volatile fatty acids occurring in milkfat and meat lipids. *J. Sens Stud.* 4, 105–120.

Calsamiglia, S., Ferret, A., and Devant, M. (2002). Effects of pH and pH fluctuations on microbial fermentation and nutrient flow from a dual-flow continuous culture system. *J. Dairy Sci.* 85, 574–579. doi: 10.3168/jds.S0022-0302(02)74111-8

Chaney, A. L., and Marbach, E. P. (1962). Modified reagents for determination of urea and ammonia. *Clin. Chem.* 8, 130–132. doi: 10.1093/clinchem/8.2.130

Chilliard, Y., Ferlay, A., Rouel, J., and Lamberet, G. (2003). A review of nutritional and physiological factors affecting goat milk lipid synthesis and lipolysis. *J. Dairy Sci.* 86, 1751–1770. doi: 10.3168/jds.S0022-0302(03)73761-8

Chiquette, J., Allison, M. J., and Rasmussen, M. (2012). Use of Prevotella bryantii 25A and a commercial probiotic during subacute acidosis challenge in midlactation dairy cows. *J. Dairy Sci.* 95, 5985–5995. doi: 10.3168/jds.2012-5511

Del Bianco, S. A., Natalello, G., Luciano, B., Valenti, L., Campidonico, V., Gkarane, F., et al. (2021). Influence of dietary inclusion of tannin extracts from mimosa, chestnut and tara on volatile compounds and flavour in lamb meat. *Meat Sci.* 172:108336. 2020.108336 doi: 10.1016/j.meatsci

Dewulf, J. P., Gerin, I., Rider, M. H., Veiga-da-Cunha, M., Van Schaftingen, E., and Bommer, G. T. (2019). The synthesis of branched-chain fatty acids is limited by enzymatic decarboxylation of ethyl- and methylmalonyl-CoA. *Biochem. J.* 476, 2427–2447. doi: 10.1042/BCJ20190500

Ding, H., Liu, W., Erdene, K., Du, H., and Ao, C. (2021). Effects of dietary supplementation with *Allium mongolicum* Regel extracts on growth performance, serum metabolites, immune responses, antioxidant status, and meat quality of lambs. *Anim. Nutr.* 7, 530–538. doi: 10.1016/j.aninu.2021.04.001

Edgar, R. C. (2013). UPARSE: highly accurate OTU sequences from microbial amplicon reads. *Nat. Methods.* 10, 996–998. doi: 10.1038/nmeth.2604

Erwin, E. S., Marco, G. J., and Emery, E. M. (1961). Volatile fatty acid analyses of blood and rumen fluid by gas chromatography. *J. Dairy Sci.* 44, 1768–1771. doi: 10.3168/jds.S0022-0302(61)89956-6

Faniyi, T. O., Adegbeye, M. J., Elghandour, M., Pilego, A. B., Salem, A. Z. M., Olaniyi, T. A., et al. (2019). Role of diverse fermentative factors towards microbial community shift in ruminants. *J. Appl. Microbiol.* 127, 2–11. doi: 10.1111/jam.14212

Gabel, G., Aschenbach, J. R., and Muller, F. (2002). Transfer of energy substrates across the ruminal epithelium: implications and limitations. *Anim. Health Res. Rev.* 3, 15–30. doi: 10.1079/ahrr200237

Han, X., Yang, Y., Yan, H., Wang, X., Qu, L., and Chen, Y. (2015). Rumen bacterial diversity of 80 to 110-day-old goats using 16S rRNA sequencing. *PLoS One.* 10:e0117811. doi: 10.1371/journal.pone.0117811

Harlow, B. E., Flythe, M. D., and Aiken, G. E. (2018). Biochanin a improves fibre fermentation by cellulolytic bacteria. *J. Appl. Microbiol.* 124, 58–66. doi: 10.1111/jam.13632

Henderson, G., Cox, F., Ganesh, S., Jonker, A., Young, W., Collaborators, G. R. C., et al. (2015). Rumen microbial community composition varies with diet and host, but a core microbiome is found across a wide geographical range. *Sci. Rep.* 5:14567. doi: 10.1038/srep14567

Jelali, R., and Ben Salem, H. (2014). Daily and alternate day supplementation of *Moringa oleifera* leaf meal or soyabean meal to lambs receiving oat hay. *Livest. Sci.* 168, 84–88. doi: 10.1016/j.livsci.2014.07.005

Jiang, C., Ding, L., Dong, Q., Wang, X., Wei, H., Hu, C., et al. (2021). Effects of root extracts of three traditional Chinese herbs as dietary supplements on dry matter intake, average daily gain, rumen fermentation and ruminal microbiota in early weaned yak calves. *Anim. Feed Sci. Technol.* 278:115002. doi: 10.1016/j.anifeedsci.2021.115002

Jiao, P., Hu, G., Liang, G., Chen, M., An, N., Wang, Z., et al. (2021). Dietary supplementation with *Macleaya cordata* extract inclusion affects growth performance, rumen fermentation, bacterial communities, and immune responses of weaned lambs. *Anim. Feed Sci. Technol.* 282:115127. doi: 10.1016/j.anifeedsci.2021.115127

Kaffarnik, S., Heid, C., Kayademir, Y., Eibler, D., and Vetter, W. (2015). High enantiomeric excess of the flavor relevant 4-alkyl-branched Fatty acids in milk fat and subcutaneous adipose tissue of sheep and goat. *J. Agric. Food Chem.* 63, 469–475. doi: 10.1021/jf505452u

Kaffarnik, S., Kayademir, Y., Heid, C., and Vetter, W. (2014). Concentrations of volatile 4-alkyl-branched fatty acids in sheep and goat milk and dairy products. *J. Food Sci.* 79, C2209–C2214. doi: 10.1111/1750-3841.12673

Lane, M. A., and Jesse, B. W. (1997). Effect of volatile fatty acid infusion on development of the rumen epithelium in neonatal sheep. *J. Dairy Sci.* 80, 740–746. doi: 10.3168/jds.S0022-0302(97)75993-9

Li, M. Y., Guo, W. Q., Guo, G. L., Zhu, X. M., Niu, X. T., Shan, X. F., et al. (2019). Effect of sub-chronic exposure to selenium and *Allium mongolicum* Regel flavonoids on *Channa argus*: Bioaccumulation, oxidative stress, immune responses and immune-related signaling molecules. *Fish Shellfish Immunol.* 91, 122–129. doi: 10.1016/j.fsi.2019.05.002

Liu, S., Zhang, Z., Hailemariam, S., Zheng, N., Wang, M., Zhao, S., et al. (2020). Biochanin A inhibits ruminal nitrogen-metabolizing bacteria and alleviates the decomposition of amino acids and urea in vitro. *Animals* 10:368. doi: 10.3390/ani10030368

Liu, W., Ding, H., Erdene, K., Chen, R., Mu, Q., Ao, C., et al. (2019). Effects of flavonoids from *Allium mongolicum* Regel as a dietary additive on meat quality and composition of fatty acids related to flavor in lambs. *Can. J. Anim Sci.* 99, 15–23. doi: 10.1139/cjas-2018-0008

Liu, W. J., and Ao, C. J. (2021). Effect of dietary supplementation with *Allium mongolicum* Regel extracts on growth performance, carcass characteristics, and the fat color and flavor-related branched-chain fatty acids concentration in ram lambs. *Anim Biosci.* 34, 1134–1145. doi: 10.5713/ajas.20.0246

Mao, S., Zhang, M., Liu, J., and Zhu, W. (2015). Characterising the bacterial microbiota across the gastrointestinal tracts of dairy cattle: membership and potential function. *Sci. Rep.* 5:16116. doi: 10.1038/srep16116

Massart, A. M., Roets, E., Peeters, G., and Verbeke, R. (1983). Propionate for fatty acid synthesisby the mammary glandof the lactating goat. *J. Dairy Sci.* 66, 1145–1154. doi: 10.3168/jds.S0022-0302(83)81958-4

Muqier, S., Qi, T., Wang, R. W., Chen, C. F., Wang, C., and Ao, C. (2017). Effects of flavonoids from *Allium Mongolicum* Regel on growth performance and growth-related hormones in meat sheep. *Anim Nutr.* 3, 33–38. doi: 10.1016/j.aninu.2017.01.003

NRC (2007). *Nutrient requirements of small ruminants: sheep, goats, cervids, and new world camelids*, 6th Edn. Washington, DC: National Academies Press.

Oskoueian, E., Abdullah, N., and Oskoueian, A. (2013). Effects of flavonoids on rumen fermentation activity, methane production, and microbial population. *Biomed Res. Int.* 2013:349129. doi: 10.1155/2013/349129

Plaizier, J. C., Krause, D. O., Gozho, G. N., and McBride, B. W. (2008). Subacute ruminal acidosis in dairy cows: the physiological causes, incidence and consequences. *Vet. J.* 176, 21–31. doi: 10.1016/j.tvjl.2007.12.016

Shivani, S., Srivastava, A., Shandilya, U. K., Kale, V., and Tyagi, A. K. (2016). Dietary supplementation of *Butyrivibrio fibrisolvens* alters fatty acids of milk and rumen fluid in lactating goats. *J. Sci. Food Agr.* 96, 1716–1722. doi: 10.1002/jsfa.7277

Salles, C., Sommerer, N., Septier, C., Issanchou, S., Chabanet, C., Garem, A., et al. (2002). Goat cheese flavor sensor evaluation of branched-chain fatty acids and small peptides. *J. Food Sci.* 67, 835–841. doi: 10.1111/j.1365-2621.2002.tb10686.x

Scharen, M., Drong, C., Kiri, K., Riede, S., Gardener, M., Meyer, U., et al. (2017). Differential effects of monensin and a blend of essential oils on rumen microbiota composition of transition dairy cows. *J. Dairy Sci.* 100, 2765–2783. doi: 10.3168/jds.2016-11994

Scharen, M., Frahm, J., Kersten, S., Meyer, U., Hummel, J., Breves, G., et al. (2018). Interrelations between the rumen microbiota and production, behavioral, rumen fermentation, metabolic, and immunological attributes of dairy cows. *J. Dairy Sci.* 101, 4615–4637. doi: 10.3168/jds.2017-13736

Seradj, A. R., Gimeno, A., Fondevila, M., Crespo, J., Armengol, R., and Balcells, J. (2018). Effects of the citrus flavonoid extract Bioflavex or its pure components on rumen fermentation of intensively reared beef steers. *Anim Prod. Sci.* 58, 553–560. doi: 10.1071/an15146

Teng, F., Reis, M. G., Broadhurst, M., Lagutin, K., Samuelsson, L., Ma, Y., et al. (2020). Factors affecting levels of volatile 4-alkyl branched-chain fatty acids in sheep milk from 2 contrasting farming systems in New Zealand. *J. Dairy Sci.* 103, 2419–2433. doi: 10.3168/jds.2019-17192

Van Soest, P. J., Robertson, J. B., and Lewis, B. A. (1991). Methods for dietary fiber, neutral detergent fiber, and nonstarch polysaccharides in relation to animal nutrition. *J. Dairy Sci.* 74, 3583–3597. doi: 10.3168/jds.S0022-0302(91)78551-2

Vasta, V., Aouadi, D., Brogna, D. M., Scerra, M., Luciano, G., Priolo, A., et al. (2013). Effect of the dietary supplementation of essential oils from rosemary and artemisia on muscle fatty acids and volatile compound profiles in Barbarine lambs. *Meat Sci.* 95, 235–241. doi: 10.1016/j.meatsci.2012.12.021

Victoria, L., and Robert, C. L. (1993). Metabolic conjugates as precursors for characterizing flavor compounds in ruminant milks. *J. Agric. Food Chem.* 41, 446–454. doi: 10.1021/jf00027a01

Vlaeminck, B., Fievez, V., Cabrita, A. R. J., Fonseca, A. J. M., and Dewhurst, R. J. (2006). Factors affecting odd- and branched-chain fatty acids in milk: A review. *Anim. Feed Sci. Technol.* 131, 389–417. doi: 10.1016/j.anifeedsci.2006.06.017

Wallace, R. J., Arthaud, L., and Newbold, C. J. (1994). Influence of *Yucca shidigera* extract on ruminal ammonia concentrations and ruminal microorganisms. *Appl. Environ. Microbiol.* 60, 1762–1767. doi: 10.1128/aem.60.6.1762-1767.1994

Wang, W., Li, J., Zhang, H., Wang, X., Fan, J., and Zhang, X. (2019). Phenolic compounds and bioactivity evaluation of aqueous and methanol extracts of *Allium mongolicum* Regel. *Food Sci. Nutr.* 7, 779–787. doi: 10.1002/fsn3.926

Wang, Y., Nan, X., Zhao, Y., Jiang, L., Wang, H., Hua, D., et al. (2021). Dietary supplementation with inulin improves lactation performance and serum lipids by regulating the rumen microbiome and metabolome in dairy cows. *Anim. Nutr.* 7, 1189–1204. doi: 10.1016/j.aninu.2021.09.007

Wong, E., Nixon, L. N., and Johnson, C. B. (1975). Volatile medium chain fatty acids and mutton flavor. *J. Agric. Food Chem.* 23, 495–498. doi: 10.1021/jf60199a044

Zhao, Y. X., Erdene, K., Changjin, A., Zhibi, B., Hongxi, D., Zejun, F., et al. (2021). Effects of *Allium mongolicum* Regel and its extracts supplementation on the growth performance, carcass parameters and meat quality of sheep. *Ital J. Anim Sci.* 20, 1899–1908. doi: 10.1080/1828051x.2021.1971572

Young, O. A., Lane, G. A., Priolo, A., and Fraser, K. (2003). Pastoral and species flavour in lambs raised on pasture, lucerne or maize. *J. Sci. Food Agr.* 83, 93–104. doi: 10.1002/jsfa.1282

Yuste, S., Amanzougarene, Z., de la Fuente, G., Fondevila, M., and de Vega, A. (2020). Effects of partial substitution of barley with maize and sugar beet pulp on growth performance, rumen fermentation and microbial diversity shift of beef calves during transition from a milk and pasture regimen to a high-concentrate diet. *Livest. Sci.* 238:104071. doi: 10.1016/j.livsci.2020.104071

Zhan, J., Liu, M., Wu, C., Su, X., Zhan, K., and Zhao, G. Q. (2017). Effects of alfalfa flavonoids extract on the microbial flora of dairy cow rumen. *Anim. Biosci.* 30, 1261–1269. doi: 10.5713/ajas.16.0839

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# Supplementation with paraformic acid in the diet improved intestinal development through modulating intestinal inflammation and microbiota in broiler chickens

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The aim of this study was to explore the effects of supplementing paraformic acid (PFA) to the diet of broiler chickens on intestinal development, inflammation, and microbiota. A total of 378 healthy 1-day-old Arbor Acres broilers with similar birth weight were used in this study, and randomly assigned into two treatment groups. The broiler chickens were received a basal diet or a basal diet supplemented with 1,000 mg/kg PFA. Results showed that PFA supplementation increased ($P < 0.05$) small intestinal villus height and villus height/crypt depth ratio, elevated intestinal mucosal factors (mucin 2, trefoil factor family, and zonula occludens-1) concentrations, and upregulated mNRA expression of y + L amino acid transporter 1. Moreover, PFA supplementation decreased ($P < 0.05$) the concentrations of inflammatory cytokines (tumor necrosis factor-alpha, interleukin-1beta, interleukin-6, and interleukin-10), activities of caspase-3 and caspase-8, and mNRA expressions of Toll-like Receptor 4, nuclear factor-kappa B, *Bax*, and *Bax*/*Bcl-2* ratio in small intestinal mucosa. Dietary PFA supplementation also increased ($P < 0.05$) alpha diversity of cecal microbiota and relative abundance of *Alistipes*. The present study demonstrated that supplementation of 1,000 mg/kg PFA showed beneficial effects in improving intestinal development, which might be attributed to the suppression of intestinal inflammation and change of gut microbiota composition in broiler chickens. These findings will aid in our knowledge of the mechanisms through which dietary PFA modulates gut development, as well as support the use of PFA in poultry industry.

KEYWORDS

**chickens, inflammation, microbiota, organic acid, small intestine**

# Introduction

A healthy and well-developed gut is essential for nutrient absorption and serves as a necessary barrier against pathogen invasion (Kogut et al., 2017). However, with the rapid expansion of the scale of intensive farming worldwide, poultry production faces increasing challenges, such as pathogenic bacteria, environmental variables, and feed hygiene, which raise the risks of intestinal diseases in broiler chickens (Qian et al., 2018; Caekebeke et al., 2020). Previous studies have demonstrated that intestinal diseases can limit intestinal development, cause dysfunction of intestinal digestion and absorption, and induce an intestinal flora imbalance, resulting in growth restriction, disease, and even mortality in broiler chickens (Roberts et al., 2015; Shi et al., 2018). For many years, the subtherapeutic use of antibiotic growth promoters (AGP) has been an economically viable means of enhancing animal performance (Bedford, 2000). With the prohibition of AGP due to its significant negative effects on environmental conditions and human health, it is critical to find new feed additives that promote intestinal health and development in poultry production (Castanon, 2007; Muaz et al., 2018).

Acidifiers such as pure organic acids have been used as feed preservatives for decades to prevent microbial and fungal destruction of feedstuffs (Hu et al., 2019; Zhang et al., 2019; Abd El-Hack et al., 2022). Growing studies suggested that organic acids could be used as powerful tool in maintaining gut health by suppressing the proliferation of acid intolerance bacteria, such as *E. coli* and *Clostridium perfringens*, leading to improvement of growth performance in poultry (Gharib et al., 2012; Ateya et al., 2019; Hu et al., 2019). It was reported that dietary supplementation with formic acid (FA) benefited to growth performance, immune function, intestinal development, and microbiological characteristics of broilers (Hernández et al., 2006; Garcia et al., 2007; Ragaa and Korany, 2016). However, FA has a strong, pungent odor and corrosiveness to gastrointestinal tract, which limit its usage in animal husbandry (Luise et al., 2020). Recently, FA salts and its derivatives, such as calcium formate, benzoic acid, and potassium diformate, have deserved more and more attention in poultry production due to their little or no corrosive effect and also efficient against harmful microorganisms (Izat et al., 1990; Józefiak et al., 2010; Ragaa and Korany, 2016). Paraformic acid (PFA) is a hyperpolymer formed by dehydration polymerization between two FA molecules. It has not been reported that whether PFA can promote intestinal development of broiler chickens.

Therefore, the aim of this study was to explore the effects of supplementing PFA to the diet of broiler chickens on intestinal development, and provide a reference for the use of PFA in poultry industry.

# Materials and methods

## Animals and diets

A total of 378 healthy 1-day-old Arbor Acres broilers with similar birth weight (48.47 ± 0.43 g) were used in this study. All broilers were randomly assigned into two treatment groups with seven replicates of 27 broiler chickens each and housed in three-level wired cages placed in a light- and temperature-controlled room with constant illumination in a 42-day study. The treatment groups were as follows: broiler chickens received a basal diet (CON group), or broiler chickens received a basal diet supplemented with 1,000 mg/kg PFA (PFA group). The basal diets (**Supplementary Table 1**) were formulated based on nutrient requirements of the National Research Council [NAC] (1994), and PFA was provided by Omega Nutrition Group (Spain) & Numega Nutrition Pte. Ltd. The broilers were fed according to a two-phase feeding program (0–21 day and 21–42 day), and had free access to feed and water throughout the experiment. A Newcastle disease vaccine and an inactivated infectious bursal disease vaccine were inoculated on days 7 and 14 of the trial, respectively. The temperature of the room was maintained at 35°C at the first week, and then gradually reduced to 21°C at the rate of 0.5°C daily.

## Sample collection

On day 42 of experiment, one broiler from each replicate (7 birds per group) with similar body weight (BW) to the cage average were selected to collect intestinal tissue samples after being narcotized by $CO_2$ asphyxiation. Intestinal segments with a length of 2 cm were cut from the medium of small intestine, and fixed in 4% paraformaldehyde for 24 h after being flushed gently with a 0.9% saline solution. Then mucosal tissue was carefully scraped with a sterile glass slide from the medium of small intestine that had been washed using ice-cold saline solution, and subsequently stored at −80°C after being chilled in liquid nitrogen. Besides, the cecal contents were collected and placed in sterile bags immediately, and stored at -80°C for microbiological analysis.

## Measurements of intestinal morphology

The fixed intestinal segments were dehydrated in ethanol and xylene solutions, and embedded according to conventional paraffin-embedding protocol, followed by being cut into 5-μm thin slices using Leica semi-automatic microtome (Leica Co., Wetzlar, Germany). Then the slices were processed by hematoxylin and eosin staining. The intestinal morphology was examined, and villus height (VH), crypt depth (CD) and VH/CD

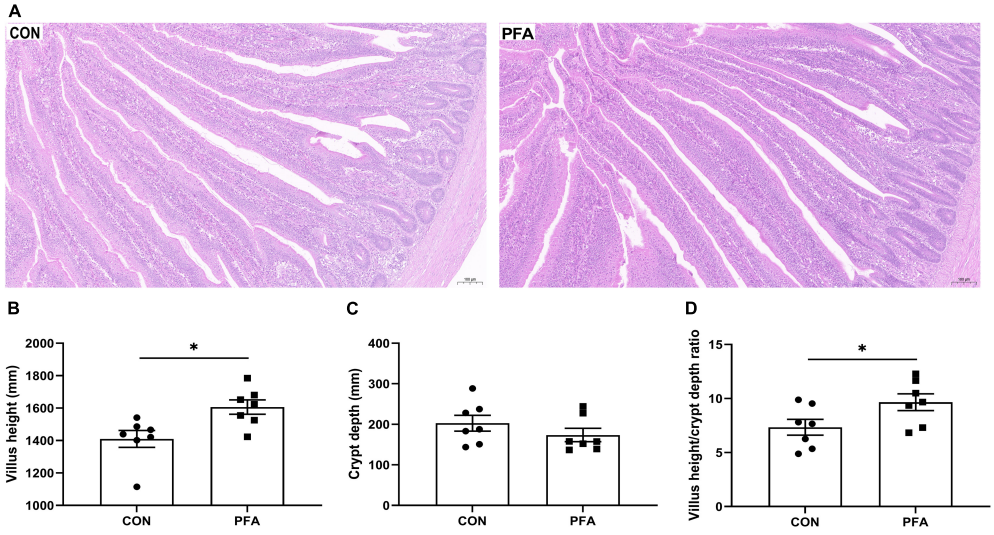


FIGURE 1
Effect of dietary paraformic acid supplementation on intestinal morphology in broiler chickens. **(A)** Hematoxylin and eosin photomicrographs obtained at 100 × magnification; **(B)** Villus height; **(C)** Crypt depth; **(D)** Villus height/crypt depth ratio. CON, broiler chickens fed basal diet; PFA, broiler chickens fed basal diet supplemented with 1,000 mg/kg paraformic acid. Values are mean ± standard error ($n$ = 7). Differences between treatments were displayed by $^{*}P < 0.05$.

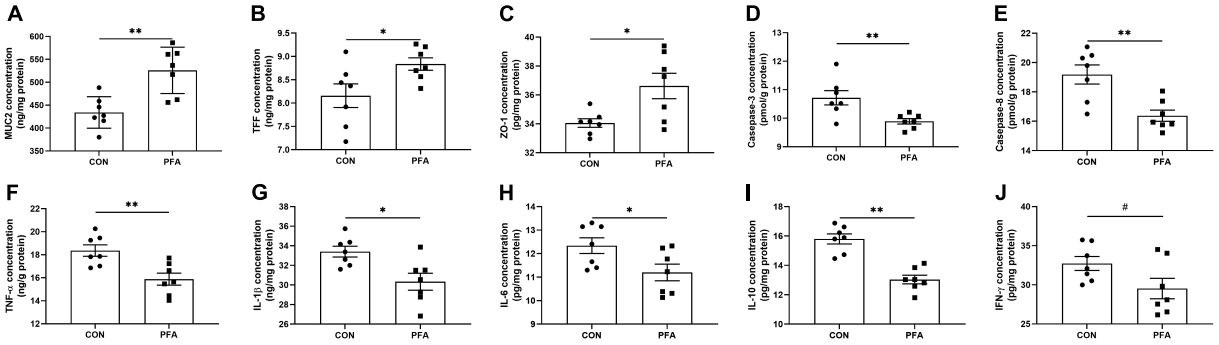


FIGURE 2
Effects of dietary paraformic acid supplementation on intestinal mucosal barrier functions, apoptosis regulators, and inflammatory factors concentrations in broiler chickens. **(A)** Mucin (MUC2); **(B)** Trefoil factor family (TFF); **(C)** Zonula occludens-1 (ZO-1); **(D)** Caspase-3; **(E)** Caspase-9; **(F)** Tumor necrosis factor-alpha (TNF-α); **(G)** Interleukin-1beta (IL-1β); **(H)** Interleukin-6 (IL-6); **(I)** Interleukin-10 (IL-10); **(J)** Interferon-γ (IFN-γ). CON, broiler chickens fed basal diet; PFA, broiler chickens fed basal diet supplemented with 1,000 mg/kg paraformic acid. Values are mean ± standard error ($n$ = 7). Differences between treatments were displayed by $^{\#}0.05 \leq P < 0.10$, $^{*}P < 0.05$, and $^{**}P < 0.01$.

ratio were analyzed according to the method described in Chen et al. (2021).

## Determination of intestinal mucosal barrier factors and total protein concentrations

Intestinal mucosal barrier factors including mucin 2 (MUC2), trefoil factor family (TFF), transforming growth factor-α (TGF-α), and zonula occludens-1 (ZO-1) were determined with the specific ELISA kits (Jiangsu Meimian Industrial Co., Ltd., Jiangsu, China), following the manufacturer's instructions strictly. Briefly, the supernatants of intestinal mucosa samples were harvested after being homogenized in ice-cold saline solution (1:9, wt/vol) and centrifugated at 12,000 × $g$ for 15 min. Then 50 μl of diluted standard solutions and supernatants were added to the prepared microplates, respectively, and incubated 30 min at 37°C after being sealed with microplate sealers. The HRP-Conjugate reagent (50 μl) was added to each well after the microplate was washed five times with Wash Buffer, and the microplate was incubated 30 min at 37°C again. After being washed five times again, the wells were added with Chromogenic solution

A and B (50 μl each) in order, and the microplate was allowed to stand at 37°C for 10 min under dark condition after gently shaking. Finally, the optical density of each well was determined in 15 min using a microplate reader set at 450 nm, following addition of Stop Solution (50 μl). The concentrations of MUC2, TFF, TGF-α, and ZO-1 were normalized to each sample's total protein concentration which was quantified with a BCA protein assay (Jiangsu Meimian Industrial Co., Ltd.) (Lang et al., 2022).

## Determination of intestinal mucosal caspases activities

Intestinal mucosal activities of caspase-3, caspase-8, and caspase-9 were determined with the specific ELIAS kits purchased from Beyotime Biotech (Shanghai, China) as descried previously (Chen et al., 2021), and normalized to each sample's total protein concentration.

## Determination of intestinal mucosal inflammatory cytokines and secretory immunoglobulin A concentrations

The intestinal mucosal concentrations of tumor necrosis factor-alpha (TNF-α), interleukin-1beta (IL-1β), interleukin-6 (IL-6), interleukin-10 (IL-10), interferon-γ (IFN-γ), and secretory immunoglobulin A (SIgA) were examined with ELISA kits (R&D Systems Inc., Minneapolis, MN, United States) according to the detection steps of ELISA operation described in Chen et al. (2021). Inflammatory cytokines and SIgA concentrations in intestinal mucosa were normalized to each sample's total protein concentration.

## Determination of relative mRNA expression in intestinal mucosa

The mRNA expression levels of occludin (*OCLN*), claudin 2 (*CLDN2*), claudin 3 (*CLDN3*), *ZO-1*, glucose transporter 2 (*GLUT2*), Na + /glucose cotransporter (*SGLT1*), y + L amino acid transporter 1 (*y + LAT1*), fatty acid binding protein (*FABP1*), cationic amino acid transporter 1 (*CAT1*), Bax, Bcl-2, Toll-like Receptor 4 (*TLR4*), and nuclear factor-kappa B (*NF-κB*) in intestinal mucosa samples were assessed using a CFX-96 real-time PCR detection system (Bio-Rad, Hercules, CA, United States). Primer sequences used for real-time PCR in this study are shown in **Supplementary Table 2**. The detailed procedure of the relative mRNA expression determination was described in Zhang P. et al. (2021). The β-actin gene was amplified in parallel as the internal control for gene normalization and quantification. The $2^{-\Delta\ \Delta\ Ct}$ method was used to calculate the relative mRNA abundances of target genes in intestinal mucosa samples. All samples were measured in triplicate, and product sizes and quantities were determined by agarose gel electrophoresis.

## Determination of pH values of cecal digesta

The determination of pH values of cecal digesta was conducted as previously described in Li et al. (2020) using the pH meter (PHS-3C PH, Shanghai, China).

## Microbial analysis

Total genomic DNA was isolated from cecal digesta by the Omega Bio-tek E.Z.N.A. $^{TM}$ stool DNA kit (Norcross, GA, United States), followed by DNA concentration and purity examination by agarose gel electrophoresis (Chen et al., 2021). Briefly, the V4 hypervariable region of 16S rDNA was amplified by using 515F and 806R primer (Li et al., 2019). The produced library quality was examined using a Qubit 2.0 Fluorometer (Thermo Fisher Scientific, United Kingdom). Sequencing of the library was carried out on the Illumina HiSeq PE2500 platform at the Novogene Bioinformatics Technology Co., Ltd. (Beijing, China), after which 250 bp paired-end sequences were generated. Paired-end sequences were merged using FLASH (v1.2.7) (Magoè and Salzberg, 2011), and the chimera sequences were removed through comparing with the Silva database using UCHIME algorithm to obtain the effective sequences after quality filtering on the raw tags (Edgar et al., 2011). Sequences were clustered into operational taxonomic units (OTUs) at least 97% sequence similarity using Uparse software (Edgar, 2013), and taxonomic information was annotated using the Silva Database based on Mothur algorithm (Quast et al., 2013). Alpha diversity (including observed species, Shannon index, Simpson index, ACE index, and Chao 1 index) and beta diversity were used to analyze complexity of species diversity for a sample and differences of samples in species complexity, respectively. Principal Coordinate Analysis (PCoA) was performed based on bray-curtis distances to visualize the dissimilarity matrices of OTUs, and the analysis of similarity (ANOSIM) was applied to examine the significant differences among the microbial communities.

## Statistical analyses

The individual chicken data were used to access the effects on all variables. Statistical analyses for all data were performed with *t*-test of SAS (9.4 Inst. Inc., Cary, NC, United States). The Shapiro-Wilk W statistic was applied to check normality of the data, and the data that were not normally distributed were

transformed to achieve approximated normality. Spearman's correlation and linear regression analysis were used to assess the associations between bacterial abundance and intestinal mucosal concentrations of immunological markers, as well as between microorganisms. Values are expressed as mean ± standard error. Statistical significance was set at $P < 0.05$, and a trend toward significance was considered at $0.05 \leq P < 0.10$.

# Results

## Intestinal morphology

The effects of PFA supplementation on intestinal morphology are presented in Figure 1. Compared with CON group, the villus in PFA group showed thicker and denser (Figure 1A). Broilers fed the diet supplemented with PFA had significantly higher ($P < 0.05$) intestinal VH (Figure 1B) and VH/CD ratio (Figure 1D) than broilers fed the CON diet. There was no significant difference ($P > 0.05$) in intestinal CD (Figure 1C) between broilers fed the CON diet and the PFA diet.

## Intestinal mucosal barrier functions, apoptosis regulators, and inflammatory factors concentrations

Dietary supplementation with 1,000 mg/kg PFA significantly increased ($P < 0.05$) MUC2 (Figure 2A), TFF (Figure 2B), and ZO-1 (Figure 2C) concentrations, and significantly decreased ($P < 0.05$) activities of caspase-3 (Figure 2D) and caspase-8 (Figure 2E) and concentrations of TNF-α (Figure 2F), IL-1β (Figure 2G), IL-6 (Figure 2H), and IL-10 (Figure 2I) in intestinal mucosa of broilers. Besides, PFA supplementation tended to decrease ($P < 0.10$) intestinal mucosal IFN-γ concentration compared with CON group (Figure 2J). There were no significant differences ($P > 0.05$) in TGF-α and SIgA concentrations as well as caspase-9 activity in intestinal mucosa of broilers (Supplementary Figure 1).

## Genes expressions in intestinal mucosa

As shown in Figure 4, PFA supplementation in broiler diet significantly increased ($P < 0.05$) *ZO-1* (Figure 3A) and *y + LAT1* (Figure 3B) mRNA expressions, and tended to increase ($P < 0.05$) *CAT1* (Figure 3C) and *Bcl-2* (Figure 3E) mRNA expression in intestinal mucosa. Besides, PFA group showed significantly lower ($P < 0.05$) mucosal *Bax* (Figure 3D), *TLR4* (Figure 3G) and *NF-κB* (Figure 3H) mRNA expressions as well as *Bax*/*Bcl-2* ratio (Figure 3F). There were no significant differences ($P > 0.05$) in the mRNA expressions of *OCLN*, *CLDN2*, *CLDN3*, *GLUT2*, *SGLT1*, and *FABP1* in intestinal mucosa (Supplementary Figure 2).

## Cecal pH of digesta

Effect of dietary paraformic acid supplementation on pH values of cecal digesta in broiler chickens is shown in Table 1. Broilers in PFA group had significantly lower ($P < 0.05$) cecal pH values than those in CON group.

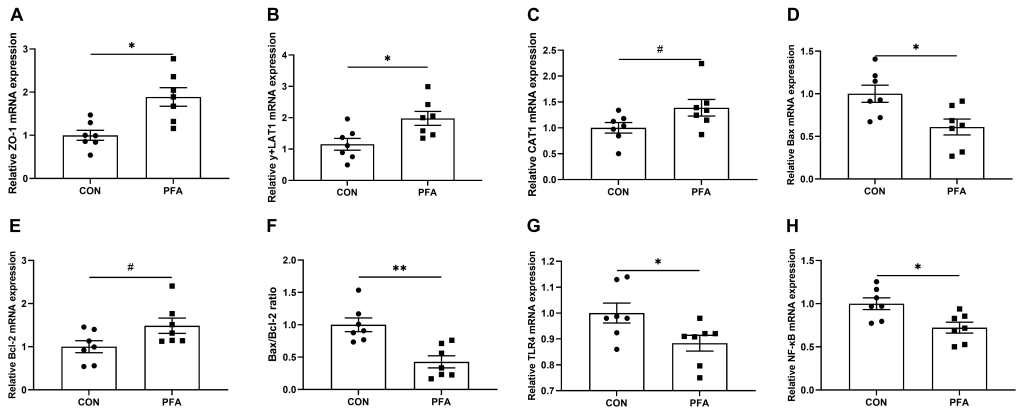


FIGURE 3
Effect of dietary paraformic acid supplementation on relative mRNA expression in intestinal mucosa of broilers. **(A)** Zonula occludens-1 (ZO-1); **(B)** y + L amino acid transporter 1 (y + LAT1); **(C)** Cationic amino acid transporter 1 (CAT1); **(D)** Bax; **(E)** Bcl-2; **(F)** Bax/Bcl-2 ratio; **(G)** Toll-like Receptor 4 (TLR4); **(H)** Nuclear factor-kappa B (NF-κB). CON, broiler chickens fed basal diet; PFA, broiler chickens fed basal diet supplemented with 1,000 mg/kg paraformic acid. Values are mean ± standard error ($n = 7$). Differences between treatments were displayed by $^{\#}0.05 \leq P < 0.10$, $^{*}P < 0.05$, and $^{**}P < 0.01$.

TABLE 1 Effect of dietary paraformic acid supplementation on pH values of cecal digesta in broiler chickens.

| Items, % | Treatment[1] | | *P*-value |
|---|---|---|---|
| | CON | PFA | |
| pH value | 6.70 ± 0.07 | 6.43 ± 0.08 | 0.034 |

Values are mean ± standard error ($n$ = 7). Differences were considered statistically significant when $P < 0.05$.
[1]CON, broilers fed a basal diet; PFA, broilers fed a basal diet supplemented with 1,000 mg/kg paraformic acid.

## Microbial diversity in cecal digesta

As shown in Supplementary Table 3, a total of 825,033 total tags, 778,291 taxon tags, 13 unclassified tags, 56,729 unique tags, and 12,147 OTUs were obtained from 14 cecal digesta samples of two treatment groups. Moreover, the species accumulation curves (Supplementary Figure 3) tend to flatten with analyzed sequences number increasing up to 14, demonstrating that our samples were sufficient for OTU testing and prediction of species richness of samples. The bacteria community diversity and richness are shown in Figure 4. The overall OTUs of cecal bacteria differ between groups, and broilers in PFA group showed an increased number of OTUs (Figure 4A). The two groups shared 1,129 common OTUs. Compared with CON group, PFA group showed significantly higher ($P < 0.05$) observed species (Figure 4B) and ACE index (Figure 4E). No significant differences were observed ($P > 0.05$) in Shannon index (Figure 4C), Simpson index (Figure 4D), and Chao 1 index (Figure 4F). The PCoA plot (Figure 4G) drawn based on the bray_curtis distances revealed that the PFA samples dispersed far apart with the CON samples, and ANOSIM

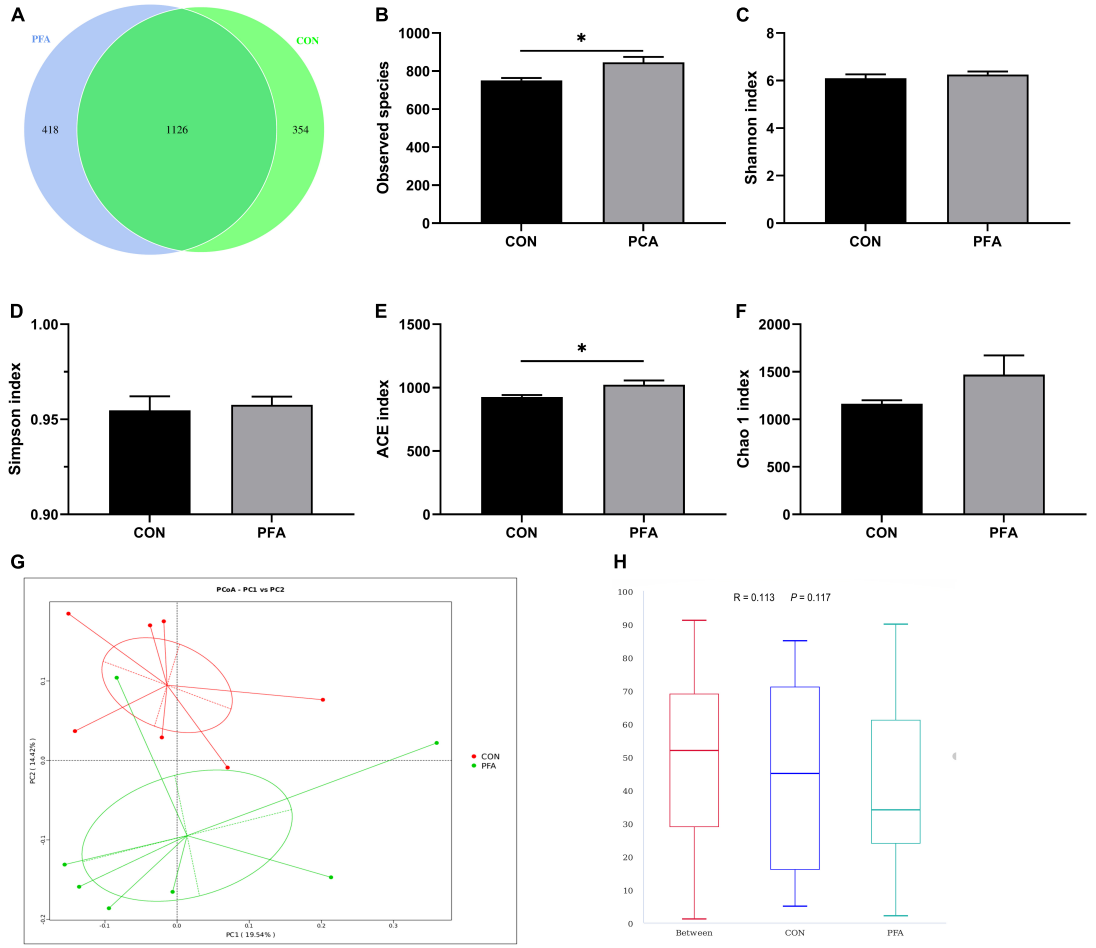


FIGURE 4
Effect of dietary paraformic acid supplementation on diversity and richness of cecal microbiota in broilers. **(A)** A Venn diagram generated to display the common and unique operational taxonomic units between two groups; **(B)** Observed species; **(C)** Shannon index; **(D)** Simpson index; **(E)** ACE index; **(F)** Chao 1 index; **(G)** The principal coordinate analysis (PCoA) profile of bray_curtis distance; **(H)** Analysis of similarity. CON, broiler chickens fed basal diet; PFA, broiler chickens fed basal diet supplemented with 1,000 mg/kg paraformic acid. Values are mean ± standard error ($n$ = 7). Differences between treatments were displayed by $^*P < 0.05$.

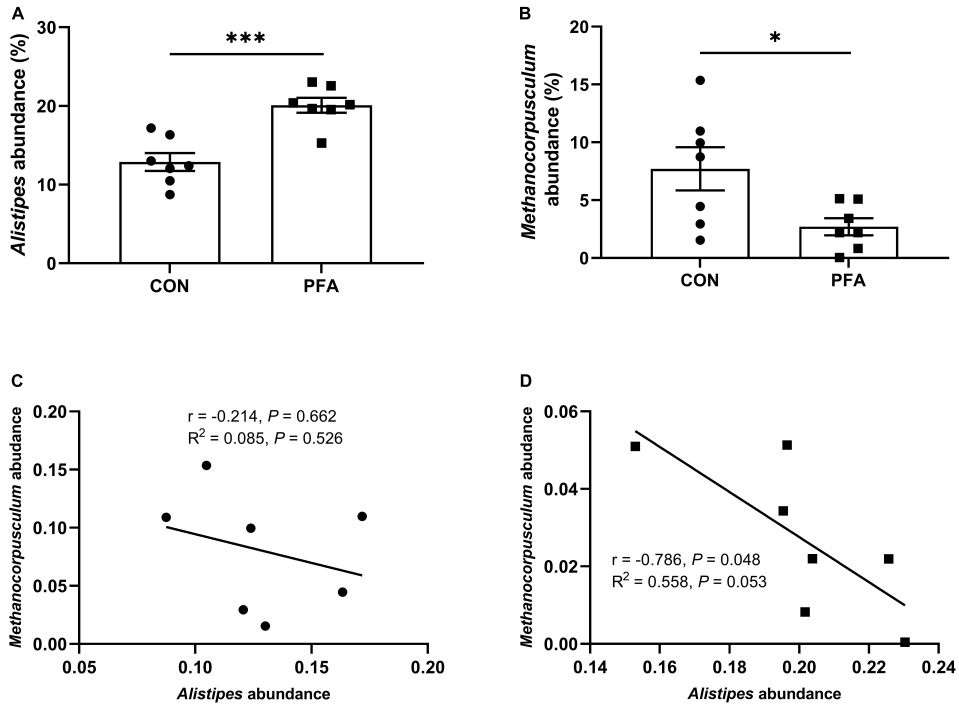


FIGURE 5
Relative abundance of cecal microbiota (at the genus level) that are significantly different between two treatments in broilers. **(A,B)** Relative abundances of *Alistipes* and *Methanocorpusculum*. Differences between treatments were displayed by $^{*}P < 0.05$ and $^{***}P < 0.001$. Values are mean ± standard error. **(C,D)** The correlation between the *Alistipes* and *Methanocorpusculum* abundances for CON group and PFA group based on Spearman's correlation test and simple linear regression analysis. Statistical significance was set at $P < 0.05$. CON, broiler chickens fed basal diet; PFA, broiler chickens fed basal diet supplemented with 1,000 mg/kg paraformic acid, $n = 7$.

(Figure 4H) showed the two groups had significantly different bacterial community structures ($P < 0.05$).

## Relative abundance of cecal microbiota

The top 10 phyla in relative abundance of cecal microbiota are shown in shown in Supplementary Table 4. The most predominant phyla in cecal samples of broilers are Bacteroidetes and Firmicutes. Dietary PFA supplementation significantly decreased ($P < 0.05$) Halobacterota abundance, and tended to decrease ($P < 0.10$) Campylobacterota abundance.

The relative abundance at genus level in broiler cecal microbiota (top 35 genera) in shown in Figure 5 and Supplementary Table 5. The identified most plentiful genera in cecal samples were *Alistipes*, *Bacteroides*, and *Desulfovibrio*. PFA group had significantly higher ($P < 0.05$) *Alistipes* abundance and significantly lower ($P < 0.05$) *Methanocorpusculum* abundance than CON group. Besides, PFA group tended to had lower ($P < 0.10$) abundances of *Helicobacter*, *Christensenellaceae_R-7_group*, and *Ruminococcus* in cecal samples than CON group. In PFA group, the *Alistipes* abundance was significantly negatively ($P = 0.048$) correlated with *Methanocorpusculum* abundance, and the *Methanocorpusculum* abundance tended to ($P = 0.053$) be linearly decreased with the *Alistipes* abundance increasing. No significant correlation ($P > 0.05$) between *Alistipes* abundance and *Methanocorpusculum* abundance was observed in cecal digesta of CON group.

## Spearman's correlation analysis between *Alistipes* and *Methanocorpusculum* abundances and mucosal immunological markers concentrations

As shown in Figure 6 and Supplementary Figure 4, intestinal mucosal concentrations of IL-1β and IL-6 tended to decrease ($P < 0.10$) with increasing relative abundance of *Alistipes* in broilers of PFA group. The relative abundance of *Methanocorpusculum* was significantly positively ($P < 0.05$) correlated with mucosal TNF-α concentration of PFA group and IL-6 concentration of CON group, and intestinal mucosal TNF-α concentration increased linearly ($P = 0.037$) as *Methanocorpusculum* abundance increased in PFA group.

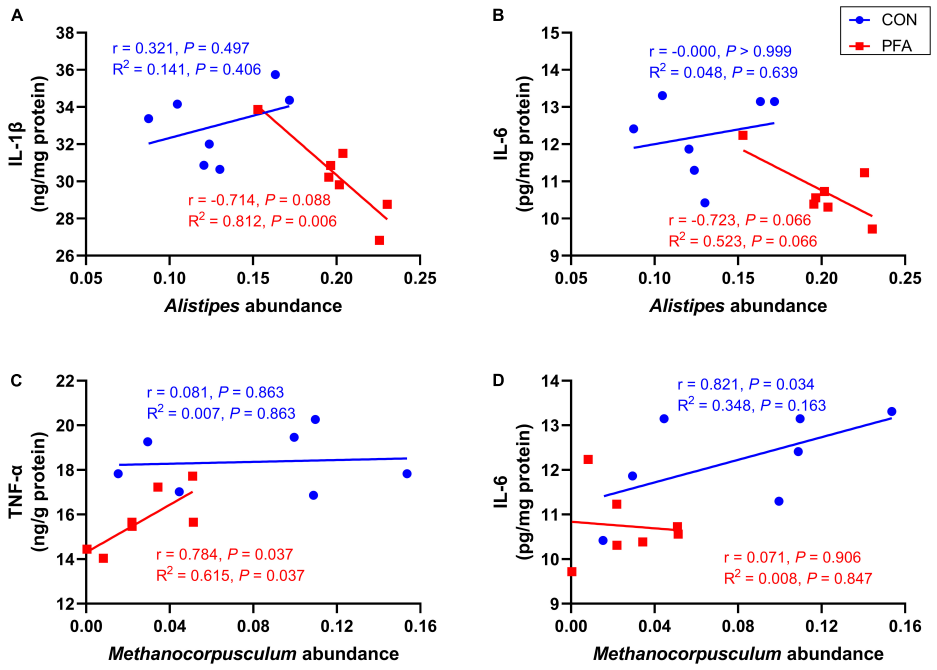


FIGURE 6
Correlation analysis between *Alistipes* and *Methanocorpusculum* abundances and mucosal immunological markers concentrations. **(A,B)** Correlation analysis between *Alistipes* and interleukin-1beta (IL-1β) and interleukin-6 (IL-6) for CON group and PFA group; **(C,D)** Correlation analysis between *Methanocorpusculum* and tumor necrosis factor-alpha (TNF-α) and IL-6 for CON group and PFA group. Correlation analysis was based on Spearman's test and simple linear regression. Statistical significance was set at $P < 0.05$, $n = 7$.

## Discussion

Intestinal morphology and barrier integrity are two crucial indicators of intestinal development (Li et al., 2020; Beumer and Clevers, 2021). In the present study, PFA supplementation in the diet increased VH and VH/CD ratio of small intestine. Previous study also demonstrated that broilers fed the diets supplemented with FA or potassium diformate had higher intestinal VH than those fed a basal diet (Abd El-Hack et al., 2022). The small intestine is the principal organ in charge of nutrient absorption, and the crypt-villus structure is responsible for efficient nutrition intake (Beumer and Clevers, 2021). The VH and VH/CD are related to the digestion and absorption capacity of the intestine (Chen et al., 2021). Consistently, compared with CON group, PFA group had higher intestinal expressions of *y + LAT1* and *CAT1* which were responsible for amino acids transport (Closs et al., 2006; del Amo et al., 2008). Garcia et al. (2007) reported that dietary FA supplementation significantly increased the digestibility of crude protein. It might suggest that PFA supplementation was helped to enhance the digestion and absorption capacity of the intestine in this study. Besides, we also found that PFA supplementation increased ZO-1, MUC2, and TFF levels in small intestinal mucosa in the current study. As a regulator of paracellular permeability in epithelia and endothelia, the major component of tight junction protein (TJP) ZO-1 plays an important role in maintaining intestinal barrier function and defensing systemic inflammatory diseases through interacting with the gap, actin cytoskeleton, and adherens junction proteins (Dai et al., 2020; Lai et al., 2021). Trefoil factor family and mucins are typical exocrine products of mucous epithelia. Mucin2, predominantly produced by the goblet cells, is the principal mucin in the small intestine and located throughout the surface of the intestinal epithelium (Liu et al., 2020). Recent researches demonstrated that MUC2 is involved in intestinal barrier protection, microbiome homeostasis regulation, and diseases prevention (Kim and Khan, 2013; Liu et al., 2020). Trefoil factor family peptides are typically co-secreted together with mucins, and play vital roles in mucosal innate immune defense, mucosal repair, and prevention of the infiltration of microorganisms (Hoffmann, 2020). It was also reported that organic acids (mainly containing FA, formate ammonia, propionate, acetate, and lactate) supplementation enhanced duodenal TJPs (including *ZO-1* and *CLDN2*) in broilers (Ma et al., 2021). A compound acidifier blend of calcium formate, calcium citrate and calcium lactate with 7:2:1 ratio could increase the expressions of *MUC2*, *OCLN*, and *CLDN3* in jejunal mucosa of broilers (He et al., 2020). Above all, dietary PFA supplementation could promote small intestinal development through improving intestinal morphology and barrier integrity.

Excessive apoptosis of epithelial cells is an important cause of intestinal mucosal barrier damage (Chen et al., 2021). Caspases are an evolutionary conserved family of cysteine proteases which are centrally involved in inflammation responses and cell death (Abd El-Hack et al., 2022). In the present study, we found that supplementation of 1,000 mg/kg PFA decreased activities of caspase-3 and caspase-8 in small intestinal mucosa. Caspase-8, serving as an initiator caspase, is important player in activation of extrinsic apoptotic pathway (Weng et al., 2014). Caspase-3, an executioner caspase, can be activated by caspase-8 and initiate the process of apoptosis (Van Opdenbosch and Lamkanfi, 2019). Pro-apoptotic Bax and anti-apoptotic Bcl-2 are two important proteins in the Bax family, and upregulation of Bax/Bcl-2 ratio has been proven to increase the activation of caspase-3 and caspase-8 cascade (Du et al., 2014). Consistently, dietary PFA supplementation decreased the expressions of *Bax*, *Bcl-2*, and *Bax*/*Bcl-2* ratio in current study. Therefore, our results of this study suggested that PFA supplementation in the diet could improve intestinal morphology and barrier integrity through suppressing the activation of extrinsic apoptotic pathway in small intestinal mucosa.

Inflammatory response is usually associated with excessive cell apoptosis, leading to the intestinal mucosal barrier damage and gastrointestinal disorders (Van Opdenbosch and Lamkanfi, 2019). It was reported that inflammatory cytokines such as TNF-α, IL-1β, IFN-γ, and IL-6 could induce cell apoptosis (Idriss and Naismith, 2000; Kuo et al., 2021; Ren et al., 2021). In the present study, dietary PFA supplementation decreased concentrations of TNF-α, IL-1β, IL-6, IL-10, and IFN-γ in small intestinal mucosa of broilers. Tumor necrosis factor-alpha, IL-1β, and IFN-γ are three major pro-inflammatory cytokines implicated in the pathogenesis of many inflammatory-associated diseases (Idriss and Naismith, 2000; Jorgovanovic et al., 2020; Kuo et al., 2021). Moreover, TNF-α, IL-1β, and IFN-γ can also activate the generation of pro-inflammatory IL-6 which contributes to the pathogenesis of various diseases, such as inflammation, autoimmunity, and cancers (Suzuki et al., 2000; Raeburn et al., 2002; Ishimaru et al., 2013; Yao et al., 2014). Interleukin-10 is a cytokine with anti-inflammatory properties. Under inflammatory condition, increased IL-6 concentration often accompanied an increased IL-10 concentration (Chun et al., 2007). Therefore, the results of our study indicated that dietary PFA supplementation decreased inflammatory response in small intestinal mucosa of broilers. To further explore the mechanisms underlying the inhibition of intestinal inflammatory response by PFA in broiler chickens, TLR4/NF-κB signaling pathway-related genes expressions were examined in this study. Toll-like receptor 4 is a crucial regulator of inflammatory reactions, whose activation triggers its downstream effector NF-κB, which translocates to the nucleus and upregulates the expressions of pro-inflammatory cytokines such as TNF-α, IL-1β, IL-6, and IFN-γ (Wei et al., 2015; Ye et al., 2021). In the present study, we found that PFA supplementation inhibited the expressions of *TLR4* and *NF-κB* in small intestinal mucosa of broilers. Previous study has demonstrated that encapsulated essential oils and organic acids mixture (containing 4% carvacrol, 4% thyme, 0.5% hexanoic, 3.5% benzoic, and 0.5% butyric acid) supplementation can inhibit necrotic enteritis-induced increase in genes expressions of *TLR4*, *IL-1*β, and *IFN-γ* in the jejunum (Pham et al., 2022). Overall, dietary PFA supplementation could suppress the inflammation response in small intestinal mucosa partially via inhibiting TLR4/NF-κB signaling pathway.

Gut microbiota plays an important role in host gut health by improving gastrointestinal development, enhancing the immune function, and competitively suppressing pathogens (Ley et al., 2008; Camara-Lemarroy et al., 2018; Zhang S. et al., 2021). It has been proven that FA is efficient against pathogenic bacteria through reducing intestinal pH, leading to improvement of gut health in poultry (Hernández et al., 2006; Gharib et al., 2012; Ateya et al., 2019). In the present study, we also found that supplementation of 1,000 mg/kg PFA decreased pH value in cecal digesta of broilers. Besides, PFA supplementation increased the observed species and ACE index of cecal microbiota in broilers. Observed species and ACE index are two important indicators of alpha diversity, and used to calculate unique OTUs and estimate community richness, respectively (Li et al., 2020; Chen et al., 2021). Gut dysbiosis and gastrointestinal inflammatory disease are usually characterized by reduced bacterial richness (Clemente et al., 2018). The results in Song et al. (2022) showed that necrotic enteritis challenge increased intestinal inflammatory cytokine gene expression levels, inhibited intestinal development, and caused intestinal damage, as well as reduced alpha diversity of microbiota in ileum of broilers. Bacteroidetes and Firmicutes were the most predominant phyla in cecal samples of broilers in this study, which was in accord with the results of previous study (Cui et al., 2021). Importantly, dietary PFA supplementation increased the abundance of *Alistipes* that was the dominate genus, and decreased *Methanocorpusculum* abundance in cecal digesta of broilers. Zhang et al. (2022) also found that *Alistipes* was the most plentiful genera in cecum of broilers. *Alistipes* is classified as Gram-negative anaerobic rods, and found primarily in the gut of healthy humans (Shkoporov et al., 2015). Previous study showed that *Alistipes finegoldii* supplementation could decrease the severity of the colitis in mice (Dziarski et al., 2016). Besides, Zhang et al. (2022) reported that dietary rhamnolipids addition could enhance the immunity, improve intestinal barrier function, and increase the cecal abundance of *Alistipes* in broilers. The results suggested that *Alistipes* genus might have a protective role in inflammatory bowel disease. The correlation analysis in this study also showed that the relative abundance of *Alistipes* was negatively correlated with intestinal mucosal concentrations of pro-inflammatory IL-1β and IL-6 which were significantly decreased in PFA group. Previous studies

suggesting that *Alistipes* was a short-chain fatty acids (SCFAs) producer (Oliphant and Allen-Vercoe, 2019), which might be contributed to the decreased pH in cecal digesta in this study. Short-chain fatty acids are identified as a principal energy source for intestinal epithelial cells and are known to strengthen the gut barrier function (Parada Venegas et al., 2019). It was reported that SCFAs could lower inflammation and oxidative stress through reducing intestinal permeability and circulating endotoxins (Kim et al., 2018). Besides, SCFAs may signal through cell surface G-protein coupled receptors to activate signaling cascades that regulate immune functions and production of cytokines (Vinolo et al., 2011). *Methanocorpusculum* was the predominant methanogen (Duan et al., 2014), and *Methanocorpusculum* abundance was positively correlated with intestinal mucosal pro-inflammatory TNF-α and IL-6 concentration. The decrease of *Methanocorpusculum* in PFA group might suggested that PFA supplementation benefited to the mitigation of methane emissions in poultry production and the decrease of inflammatory response in intestine of broilers. Therefore, the increased microbial diversity and beneficial bacteria might be another reason for the improved intestinal development in PFA broilers.

## Conclusion

In conclusion, the present study demonstrated that supplementation of 1,000 mg/kg PFA showed beneficial effects in improving intestinal development and function including intestinal morphology, barrier integrity, and nutrients transformation. It might be attributed to the suppression of apoptosis though inhibiting intestinal inflammation partially via inactivating TLR4/NF-κB signaling pathway and change of gut microbiota composition in broiler chickens. These findings will aid in our knowledge of the mechanisms through which dietary PFA modulates gut development, as well as support the use of PFA in poultry industry.

## Data availability statement

The data presented in this study are deposited in the NCBI Sequence Read Archive repository, accession number: PRJNA847797.

## Ethics statement

This animal study was reviewed and approved by the Care and Use Committee of Shandong Agricultural University (protocol code SDAUA-2021-019).

## Author contributions

NJ, WY, and YLi: conceptualization. JL, YLiu, and JN: data curation and project administration. YLiu and CJ: formal analysis. WY and YLi: funding acquisition, supervision, and writing – review and editing. CJ, NJ, LY, and YLi: investigation. YLiu, JN, NJ, LH, SJ, and YLi: methodology. JN, LH, and LY: resources. JL and YLiu: software and writing – original draft. SJ and YLi: validation. CJ and WY: visualization. All authors contributed to the article and approved the submitted version.

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## Conflict of interest

JL was employed by Qingdao Huaxin Feed Co., Ltd. CJ was employed by Shandong Chinwhiz Co., Ltd. LY was employed by Shandong New Hope Liuhe Group 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/fmicb.2022.975056/full#supplementary-material

# References

Abd El-Hack, M. E., El-Saadony, M. T., Salem, H. M., El-Tahan, A. M., Soliman, M. M., Youssef, G. B. A., et al. (2022). Alternatives to antibiotics for organic poultry production: types, modes of action and impacts on bird's health and production. *Poult. Sci.* 101:101696. doi: 10.1016/j.psj.2022.101696

Ateya, A. I., Arafat, N., Saleh, R. M., Ghanem, H. M., Naguib, D., Radwan, H. A., et al. (2019). Intestinal gene expressions in broiler chickens infected with *Escherichia coli* and dietary supplemented with probiotic, acidifier and synbiotic. *Vet. Res. Commun.* 43, 131–142. doi: 10.1007/s11259-019-09753-z

Bedford, M. (2000). Removal of antibiotic growth promoters from poultry diets: implications and strategies to minimise subsequent problems. *World's Poult. Sci. J.* 56, 347–365. doi: 10.1079/WPS20000024

Beumer, J., and Clevers, H. (2021). Cell fate specification and differentiation in the adult mammalian intestine. *Nat. Rev. Mol. Cell Biol.* 22, 39–53. doi: 10.1038/s41580-020-0278-0

Caekebeke, N., Ringenier, M., De Meyer, F., Ducatelle, R., Ongena, N., Van Immerseel, F., et al. (2020). A study on risk factors for macroscopic gut abnormalities in intensively reared broiler chickens. *Avian. Pathol.* 49, 193–201. doi: 10.1080/03079457.2019.1711019

Camara-Lemarroy, C. R., Metz, L. M., and Yong, V. W. (2018). Focus on the gut-brain axis: Multiple sclerosis, the intestinal barrier and the microbiome. *World J. Gastroenterol.* 24, 4217–4223. doi: 10.3748/wjg.v24.i37.4217

Castanon, J. I. (2007). History of the use of antibiotic as growth promoters in European poultry feeds. *Poult. Sci.* 86, 2466–2471. doi: 10.3382/ps.2007-00249

Chen, J., Li, F., Yang, W., Jiang, S., and Li, Y. (2021). Supplementation with exogenous catalase from *Penicillium notatum* in the diet ameliorates lipopolysaccharide-induced intestinal oxidative damage through affecting intestinal antioxidant capacity and microbiota in weaned pigs. *Microbiol. Spectr.* 9:e0065421. doi: 10.1128/Spectrum.00654-21

Chun, H. Y., Chung, J. W., Kim, H. A., Yun, J. M., Jeon, J. Y., Ye, Y. M., et al. (2007). Cytokine IL-6 and IL-10 as biomarkers in systemic lupus erythematosus. *J. Clin. Immunol.* 27, 461–466. doi: 10.1007/s10875-007-9104-0

Clemente, J. C., Manasson, J., and Scher, J. U. (2018). The role of the gut microbiome in systemic inflammatory disease. *Bmj* 360:j5145. doi: 10.1136/bmj.j5145

Closs, E. I., Boissel, J. P., Habermeier, A., and Rotmann, A. (2006). Structure and function of cationic amino acid transporters (CATs). *J. Membr. Biol.* 213, 67–77. doi: 10.1007/s00232-006-0875-7

Cui, L., Zhang, X., Cheng, R., Ansari, A. R., Elokil, A. A., Hu, Y., et al. (2021). Sex differences in growth performance are related to cecal microbiota in chicken. *Microb. Pathog.* 150:104710. doi: 10.1016/j.micpath.2020.104710

Dai, W., Nadadur, R. D., Brennan, J. A., Smith, H. L., Shen, K. M., Gadek, M., et al. (2020). ZO-1 regulates intercalated disc composition and Atrioventricular node conduction. *Circ. Res.* 127, e28–e43. doi: 10.1161/circresaha.119.316415

del Amo, E. M., Urtti, A., and Yliperttula, M. (2008). Pharmacokinetic role of L-type amino acid transporters LAT1 and LAT2. *Eur. J. Pharm. Sci.* 35, 161–174. doi: 10.1016/j.ejps.2008.06.015

Du, L., Mei, H. F., Yin, X., and Xing, Y. Q. (2014). Delayed growth of glioma by a polysaccharide from Aster tataricus involve upregulation of Bax/Bcl-2 ratio, activation of caspase-3/8/9, and downregulation of the Akt. *Tumour. Biol.* 35, 1819–1825. doi: 10.1007/s13277-013-1243-8

Duan, Y. F., Al-Soud, W. A., Brejnrod, A., Sørensen, S. J., Elsgaard, L., Petersen, S. O., et al. (2014). Methanotrophs, methanogens and microbial community structure in livestock slurry surface crusts. *J. Appl. Microbiol.* 117, 1066–1078. doi: 10.1111/jam.12584

Dziarski, R., Park, S. Y., Kashyap, D. R., Dowd, S. E., and Gupta, D. (2016). Pglyrp-regulated gut microflora *Prevotella falsenii*, *Parabacteroides distasonis* and *Bacteroides eggerthii* enhance and *Alistipes finegoldii* attenuates colitis in mice. *PLoS One* 11:e0146162. doi: 10.1371/journal.pone.0146162

Edgar, R. C. (2013). UPARSE: highly accurate OTU sequences from microbial amplicon reads. *Nat. Methods* 10, 996–998. doi: 10.1038/nmeth.2604

Edgar, R. C., Haas, B. J., Clemente, J. C., Quince, C., and Knight, R. (2011). UCHIME improves sensitivity and speed of chimera detection. *Bioinformatics* 27, 2194–2200. doi: 10.1093/bioinformatics/btr381

Garcia, V., Catala-Gregori, P., Hernandez, F., Megias, M., and Madrid, J. (2007). Effect of formic acid and plant extracts on growth, nutrient digestibility, intestine mucosa morphology, and meat yield of broilers. *J. Appl. Poult. Res.* 16, 555–562. doi: 10.3382/japr.2006-00116

Gharib, N. K., Rahimi, S., and Khaki, P. (2012). Comparison of the effects of probiotic, organic acid and medicinal plant on *Campylobacter jejuni* challenged broiler chickens. *J. Agric. Sci. Technol.* 14, 1485–1496.

He, J., Ma, L., Qiu, J., Lu, X., Hou, C., Liu, B., et al. (2020). Effects of compound organic acid calcium on growth performance, hepatic antioxidation and intestinal barrier of male broilers under heat stress. *Asian-Australas J. Anim. Sci.* 33, 1156–1166. doi: 10.5713/ajas.19.0274

Hernández, F., García, V., Madrid, J., Orengo, J., Catalá, P., and Megías, M. D. (2006). Effect of formic acid on performance, digestibility, intestinal histomorphology and plasma metabolite levels of broiler chickens. *Br. Poult. Sci.* 47, 50–56. doi: 10.1080/00071660500475574

Hoffmann, W. (2020). Trefoil Factor Family (TFF) peptides and their diverse molecular functions in mucus barrier protection and more: changing the paradigm. *Int. J. Mol. Sci.* 21:4535. doi: 10.3390/ijms21124535

Hu, Y., Wang, L., Shao, D., Wang, Q., Wu, Y., Han, Y., et al. (2019). Selectived and reshaped early dominant microbial community in the cecum with similar proportions and better homogenization and species diversity due to organic acids as agp alternatives mediate their effects on broilers growth. *Front. Microbiol.* 10:2948. doi: 10.3389/fmicb.2019.02948

Idriss, H. T., and Naismith, J. H. (2000). TNF alpha and the TNF receptor superfamily: structure-function relationship(s). *Microsc. Res. Tech.* 50, 184–195.

Ishimaru, M., Tsukimoto, M., Harada, H., and Kojima, S. (2013). Involvement of P2Y11 receptor in IFN-γ-induced IL-6 production in human keratinocytes. *Eur. J. Pharmacol.* 703, 67–73. doi: 10.1016/j.ejphar.2013.02.020

Izat, A. L., Adams, M. H., Cabel, M. C., Colberg, M., Reiber, M. A., Skinner, J. T., et al. (1990). Effects of formic acid or calcium formate in feed on performance and microbiological characteristics of broilers. *Poult. Sci.* 69, 1876–1882. doi: 10.3382/ps.0691876

Jorgovanovic, D., Song, M., Wang, L., and Zhang, Y. (2020). Roles of IFN-γ in tumor progression and regression: a review. *Biomark Res.* 8:49. doi: 10.1186/s40364-020-00228-x

Józefiak, D., Kaczmarek, S., and Rutkowski, A. (2010). The effects of benzoic acid supplementation on the performance of broiler chickens. *J. Anim. Physiol. Anim. Nutr.* 94, 29–34. doi: 10.1111/j.1439-0396.2008.00875.x

Kim, J. J., and Khan, W. I. (2013). Goblet cells and mucins: role in innate defense in enteric infections. *Pathogens* 2, 55–70. doi: 10.3390/pathogens2010055

Kim, Y. A., Keogh, J. B., and Clifton, P. M. (2018). Probiotics, prebiotics, synbiotics and insulin sensitivity. *Nutr. Res. Rev.* 31, 35–51. doi: 10.1017/S095442241700018X

Kogut, M. H., Yin, X., Yuan, J., and Broom, L. (2017). Gut health in poultry. *CAB Rev.* 12, 1–7.

Kuo, W. C., Lee, C. C., Chang, Y. W., Pang, W., Chen, H. S., Hou, S. C., et al. (2021). Structure-based Development of Human Interleukin-1β-Specific Antibody That Simultaneously Inhibits Binding to Both IL-1RI and IL-1RAcP. *J. Mol. Biol.* 433:166766. doi: 10.1016/j.jmb.2020.166766

Lai, W. T., Lee, H. C., Huang, Y. H., Lo, M. H., and Kuo, H. C. (2021). Tight junction protein ZO-1 in Kawasaki disease. *BMC Pediatr.* 21:157. doi: 10.1186/s12887-021-02622-2

Lang, W., Cheng, M., Zheng, X., Zhao, Y., Qu, Y., Jia, Z., et al. (2022). Forsythiaside A alleviates methotrexate-induced intestinal mucositis in rats by modulating the NLRP3 signaling pathways. *Int. Immunopharmacol.* 103:108466. doi: 10.1016/j.intimp.2021.108466

Ley, R. E., Lozupone, C. A., Hamady, M., Knight, R., and Gordon, J. I. (2008). Worlds within worlds: evolution of the vertebrate gut microbiota. *Nat. Rev. Microbiol.* 6, 776–788. doi: 10.1038/nrmicro1978

Li, Y., Liu, H., Zhang, L., Yang, Y., Lin, Y., Zhuo, Y., et al. (2019). Maternal dietary fiber composition during gestation induces changes in offspring antioxidative capacity, inflammatory response, and gut microbiota in a sow model. *Int. J. Mol. Sci.* 21:31. doi: 10.3390/ijms21010031

Li, Y., Zhao, X., Zhang, L., Zhan, X., Liu, Z., Zhuo, Y., et al. (2020). Effects of a diet supplemented with exogenous catalase from *Penicillium notatum* on intestinal development and microbiota in weaned piglets. *Microorganisms* 8:391. doi: 10.3390/microorganisms8030391

Liu, Y., Yu, X., Zhao, J., Zhang, H., Zhai, Q., and Chen, W. (2020). The role of MUC2 mucin in intestinal homeostasis and the impact of dietary components on MUC2 expression. *Int. J. Biol. Macromol.* 164, 884–891. doi: 10.1016/j.ijbiomac.2020.07.191

Luise, D., Correa, F., Bosi, P., and Trevisi, P. (2020). A review of the effect of formic acid and its salts on the gastrointestinal microbiota and performance of pigs. *Animals* 10:887. doi: 10.3390/ani10050887

Ma, J., Mahfuz, S., Wang, J., and Piao, X. (2021). Effect of dietary supplementation with mixed organic acids on immune function, antioxidative characteristics, digestive enzymes activity, and intestinal health in broiler chickens. *Front. Nutr.* 8:673316. doi: 10.3389/fnut.2021.673316

Magoè, T., and Salzberg, S. L. (2011). FLASH: fast length adjustment of short reads to improve genome assemblies. *Bioinformatics* 27, 2957–2963. doi: 10.1093/bioinformatics/btr507

Muaz, K., Riaz, M., Akhtar, S., Park, S., and Ismail, A. (2018). Antibiotic residues in chicken meat: global prevalence. threats, and decontamination strategies: a review. *J. Food Prot.* 81, 619–627. doi: 10.4315/0362-028x.Jfp-17-086

National Research Council [NAC] (1994). *Nutrient requirements of poultry*, 9th edn. Washington, DC: National Academy Press.

Oliphant, K., and Allen-Vercoe, E. (2019). Macronutrient metabolism by the human gut microbiome: major fermentation by-products and their impact on host health. *Microbiome* 7:91. doi: 10.1186/s40168-019-0704-8

Parada Venegas, D., De la Fuente, M. K., Landskron, G., González, M. J., Quera, R., Dijkstra, G., et al. (2019). Short chain fatty acids (SCFAs)-mediated gut epithelial and immune regulation and its relevance for inflammatory bowel diseases. *Front. Immunol.* 10:277. doi: 10.3389/fimmu.2019.00277

Pham, V. H., Abbas, W., Huang, J., He, Q., Zhen, W., Guo, Y., et al. (2022). Effect of blending encapsulated essential oils and organic acids as an antibiotic growth promoter alternative on growth performance and intestinal health in broilers with necrotic enteritis. *Poult. Sci.* 101:101563. doi: 10.1016/j.psj.2021.101563

Qian, Y., Song, K., Hu, T., and Ying, T. (2018). Environmental status of livestock and poultry sectors in China under current transformation stage. *Sci. Total Environ.* 1, 702–709. doi: 10.1016/j.scitotenv.2017.12.045

Quast, C., Pruesse, E., Yilmaz, P., Gerken, J., Schweer, T., Yarza, P., et al. (2013). The SILVA ribosomal RNA gene database project: improved data processing and web-based tools. *Nucleic Acids Res.* 41, D590–D596. doi: 10.1093/nar/gks1219

Raeburn, C. D., Sheppard, F., Barsness, K. A., Arya, J., and Harken, A. H. (2002). Cytokines for surgeons. *Am. J. Surg.* 183, 268–273. doi: 10.1016/s0002-9610(02)00781-x

Ragaa, N. M., and Korany, R. M. S. (2016). Studying the effect of formic acid and potassium diformate on performance, immunity and gut health of broiler chickens. *Anim. Nutr.* 2, 296–302. doi: 10.1016/j.aninu.2016.08.003

Ren, Y., Yan, Y., Zhen, L., Cao, C., Wang, Q., Zhang, Y., et al. (2021). Zhike Pingchuan Granule suppresses interleukin (IL)-6 or the medium of M2 macrophages induced apoptosis in human bronchial epithelial cells. *Bioengineered* 12, 7694–7703. doi: 10.1080/21655979.2021.1982309

Roberts, T., Wilson, J., Guthrie, A., Cookson, K., Vancraeynest, D., Schaeffer, J., et al. (2015). New issues and science in broiler chicken intestinal health: Emerging technology and alternative interventions. *J. Appl. Poult. Res.* 24, 257–266. doi: 10.3382/japr/pfv023

Shi, S., Wu, S., Shen, Y., Zhang, S., Xiao, Y., He, X., et al. (2018). Iron oxide nanozyme suppresses intracellular Salmonella Enteritidis growth and alleviates infection in vivo. *Theranostics* 8, 6149–6162. doi: 10.7150/thno.29303

Shkoporov, A. N., Chaplin, A. V., Khokhlova, E. V., Shcherbakova, V. A., Motuzova, O. V., Bozhenko, V. K., et al. (2015). *Alistipes inops* sp. nov. and *Coprobacter secundus* sp. nov., isolated from human faeces. *Int. J. Syst. Evol. Microbiol.* 65, 4580–4588. doi: 10.1099/ijsem.0.000617

Song, B., Li, P., Yan, S., Liu, Y., Gao, M., Lv, H., et al. (2022). Effects of dietary astragalus polysaccharide supplementation on the Th17/Treg balance and the gut microbiota of broiler chickens challenged with necrotic enteritis. *Front. Immunol.* 13:781934. doi: 10.3389/fimmu.2022.781934

Suzuki, M., Tetsuka, T., Yoshida, S., Watanabe, N., Kobayashi, M., Matsui, N., et al. (2000). The role of p38 mitogen-activated protein kinase in IL-6 and IL-8 production from the TNF-alpha- or IL-1beta-stimulated rheumatoid synovial fibroblasts. *FEBS Lett.* 465, 23–27. doi: 10.1016/s0014-5793(99)01717-2

Van Opdenbosch, N., and Lamkanfi, M. (2019). Caspases in cell death, inflammation, and disease. *Immunity* 50, 1352–1364. doi: 10.1016/j.immuni.2019.05.020

Vinolo, M. A., Rodrigues, H. G., Nachbar, R. T., and Curi, R. (2011). Regulation of inflammation by short chain fatty acids. *Nutrients* 3, 858–876. doi: 10.3390/nu3100858

Wei, W., Dejie, L., Xiaojing, S., Tiancheng, W., Yongguo, C., Zhengtao, Y., et al. (2015). Magnolol inhibits the inflammatory response in mouse mammary epithelial cells and a mouse mastitis model. *Inflammation* 38, 16–26. doi: 10.1007/s10753-014-0003-2

Weng, D., Marty-Roix, R., Ganesan, S., Proulx, M. K., Vladimer, G. I., Kaiser, W. J., et al. (2014). Caspase-8 and RIP kinases regulate bacteria-induced innate immune responses and cell death. *Proc. Natl. Acad. Sci. U.S.A.* 111, 7391–7396. doi: 10.1073/pnas.1403477111

Yao, X., Huang, J., Zhong, H., Shen, N., Faggioni, R., Fung, M., et al. (2014). Targeting interleukin-6 in inflammatory autoimmune diseases and cancers. *Pharmacol. Ther.* 141, 125–139. doi: 10.1016/j.pharmthera.2013.09.004

Ye, Y., Wang, P., and Zhou, F. (2021). miR-489-3p inhibits TLR4/NF-κB signaling to prevent inflammation in psoriasis. *Exp. Ther. Med.* 22:744. doi: 10.3892/etm.2021.10176

Zhang, P., Jing, C., Liang, M., Jiang, S., Huang, L., Jiao, N., et al. (2021). Zearalenone exposure triggered cecal physical barrier injury through the TGF-β1/Smads signaling pathway in weaned piglets. *Toxins* 13:902. doi: 10.3390/toxins13120902

Zhang, R., Zhang, H., Liu, J., Zeng, X., Wu, Y., and Yang, C. (2022). Rhamnolipids enhance growth performance by improving the immunity, intestinal barrier function, and metabolome composition in broilers. *J. Sci. Food Agric.* 102, 908–919. doi: 10.1002/jsfa.11423

Zhang, S., Shen, Y. R., Wu, S., Xiao, Y. Q., He, Q., and Shi, S. R. (2019). The dietary combination of essential oils and organic acids reduces Salmonella enteritidis in challenged chicks. *Poult. Sci.* 98, 6349–6355. doi: 10.3382/ps/pez457

Zhang, S., Zhong, G., Shao, D., Wang, Q., Hu, Y., Wu, T., et al. (2021). Dietary supplementation with *Bacillus subtilis* promotes growth performance of broilers by altering the dominant microbial community. *Poult. Sci.* 100:100935. doi: 10.1016/j.psj.2020.12.032

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# Dietary supplementation with *Cyberlindnera jadinii* improved growth performance, serum biochemical Indices, antioxidant status, and intestinal health in growing raccoon dogs (*Nyctereutes procyonoides*)

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This study was conducted to investigate whether different dietary *Cyberlindnera jadinii* levels affect growth performance, serum immunity, antioxidant capacity, and intestinal microbiota in growing raccoon dogs. Forty-five healthy male raccoon dogs were randomly assigned to three treatment groups, with 15 raccoon dogs per group. Each raccoon dog was housed in an individual cage. The raccoon dogs in the three groups were fed diets supplemented with *Cyberlindnera jadinii* at dosages of 0 (N group), $1 \times 10^9$ (L group) and $5 \times 10^9$ CFU/g (H group). A 7-day pretest period preceded a formal test period of 30 days. The results showed that *Cyberlindnera jadinii* in the L and H groups improved average daily gain (ADG) ($P < 0.05$) and decreased the ratio of feed to weight (F/G) ($P < 0.05$). Serum immunoglobulins A and G levels were increased in the L and H groups compared to the N group ($P < 0.05$). *Cyberlindnera jadinii* in the L and H groups increased serum superoxide dismutase activity ($P < 0.05$), and serum glutathione peroxidase activity was increased in the L group compared to the N group ($P < 0.05$). The relative abundance of Firmicutes and Actinobacteriota were increased, and the relative abundance of Bacteroidota was decreased in the L and H groups compared to the N group ($P < 0.05$). The relative abundance of Proteobacteria and Cyanobacteria was increased in the H group compared to the other two groups ($P < 0.05$). The ratio of Firmicutes to Bacteroidetes in the *Cyberlindnera jadinii* supplementation groups increased compared with the N group ($P < 0.05$). The relative abundance of *Megasphaera* and *Bifidobacterium* were increased, and the relative abundance of *Prevotella* was decreased in the L and H groups

compared to the N group ($P < 0.05$). The relative abundance of *Dialister* was increased, while the relative abundance of *Blautia* was decreased in the H group compared to the other two groups ($P < 0.05$). The relative abundance of *Agathobacter* was decreased in the H group compared to the N group ($P < 0.05$). In conclusion, dietary supplementation with *Cyberlindnera jadinii* increased growth performance, serum immunity, antioxidant capacity, and improved intestinal microbiota in growing raccoon dogs. *Cyberlindnera jadinii* can therefore be used as a growth promoter in raccoon dogs.

KEYWORDS

***Cyberlindnera jadinii* (*Candida utilis*), growth performance, immunity, antioxidant, microbiota, raccoon dogs**

## Introduction

Adding antibiotics to animal feed can improve growth and feed conversion efficiency, and prevent infection (Gadde et al., 2017). However, the use of antibiotics leads to antibiotic resistance and the destruction of beneficial bacteria in the gut, which pose a potential threat to animal and public health safety. Therefore, the development of effective antibiotic alternatives is a pressing need. Probiotics are an effective and eco-friendly alternative to antibiotics (Zorriehzahra et al., 2016; Alagawany et al., 2018). Among them, yeast is a promising feed additive that may replace frequently used antimicrobial growth promoters (Alizadeh et al., 2016). Research has shown that feeding yeast and its products can modulate growth performance, and gut microbiota, enhance gut development and integrity, support the immune system, and improve nutrient utilization (Gao et al., 2008; Jiang et al., 2015; Bilal et al., 2021).

The yeast *Cyberlindnera jadinii* is a close relative of *Candida utilis*, which is currently used in the food and feed industries (Rupp et al., 2015). *Cyberlindnera jadinii* can produce valuable bioproducts that are an attractive source of biomass enriched in protein and vitamins (Sousa-Silva et al., 2021). Partially replacing conventional protein sources with inactivated *Cyberlindnera jadinii* yeast in for the diet of young pigs is possible without compromising energy and protein metabolism (Cruz et al., 2020). More importantly, feeding *Cyberlindnera jadinii* yeast can improve post-weanling gut homeostasis and result in more robust piglets (Håkenåsen et al., 2020). *Cyberlindnera jadinii* can also improve growth performance, reduce diarrhea rates, improve intestinal health, and increase the diversity and abundance of cecal microbiota in weaned piglets. Therefore, it may be used as antibiotic alternative feed additive in the production of weaned piglets (Yang et al., 2021). However, there is no research data available regarding the use of *Cyberlindnera jadinii* in the diets of canines.

The Ussuri raccoon dog is the most easily bred fur animal and has high economic value. Unlike in herbivores, carnivorous gut microbiota are specialized to degrade protein as an energy source (Muegge et al., 2011). The gut microbiota is a central regulator of host metabolism, and the composition and function of the gut microbiota is dynamic and affected by dietary properties (Schoeler and Caesar, 2019). The effect of *Cyberlindnera jadinii* on the Ussuri raccoon dog has not been studied. Thus, the present study examined the effects of *Cyberlindnera jadinii* supplementation on growth performance, serum immunity, antioxidant status, and gut microbiota of Ussuri raccoon dogs during the growing period. We hypothesized that *Cyberlindnera jadinii* may affect the growth and gut microbiota of raccoon dogs.

## Materials and methods

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

### Fungal strain

*Cyberlindnera jadinii* were preserved by the microbiology laboratory of the Chifeng University. The liquid potato dextrose culture medium (PDB) (Potato extract powder 20 g/L, glucose 20 g/L, distilled water 1 L) was used to resuscitate and passage cultures for 24–48 h at 37°C.

### Experimental design

Forty-five 60 (±5) day-old healthy male raccoon dogs of a similar body weight (1.98 ± 0.15 kg) were randomly

assigned to three treatment groups, with 15 replicates per group. *Cyberlindnera jadinii* was either not added to the diet (N group) or was supplemented at $1 \times 10^9$ CFU/g (L group) or $5 \times 10^9$ CFU/g (H group). The basal diet was formulated based on the management guide of the National Research Council (National Research Council, 1982); the composition and nutrient levels of the basal diet are shown in **Table 1**. All animals were individually housed in conventional cages (1.0 m × 0.8 m × 0.8 m). The raccoon dogs were fed twice each day at 7:00 and 15:00 and had free access to water. After 7 days of adaptation, the experimental diets were fed for 30 days.

## Sample collection and preparation

Body weight was measured at the beginning and end of the experiment, and the feed intake of each animal was recorded daily. The average daily gain, average daily feed intake and the ratio of feed to gain (F/G) were calculated according to the following formula: Average daily gain (ADG) (g/d) = (final body weight-initial body weight) (g)/days (d); Average daily feed intake (ADFI) (g/d) = total feed intake (g)/days (d); Ratio of feed to gain (F/G) = average daily feed intake (g)/average daily weight gain (g). After the 30-day supplementation, the raccoon dogs were restrained for blood sampling and approximately 5 mL blood was collected in a vacuum tube from the posterior limb vein. Blood was centrifuged at 3,000 × *g* for 15 min at 4°C to isolate serum which was stored at −20°C until analysis. In addition, fresh feces from each raccoon dog was collected on the same day using 5 mL sterile centrifuge tubes, and stored at −80°C for gut microbiota analyses.

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

| Items | Content (%) | Items | Content (%) |
|---|---|---|---|
| **Ingredients** | | **Nutrient levels**[2] | |
| Extruded corn | 40.84 | Metabolizable energy (MJ/kg)[2] | 14.66 |
| Corn | 5.11 | Crude protein | 21.66 |
| Corn germ meal | 9.20 | Ether extract | 9.43 |
| Rice bran meal | 3.07 | Ash | 6.59 |
| Soybean oil | 5.62 | Calcium | 0.96 |
| Soybean meal | 14.82 | phosphorus | 0.72 |
| Distillers dried grains with solubles | 9.20 | Lysine | 1.26 |
| Dried porcine soluble | 1.02 | Methionine | 0.93 |
| Fish meal | 1.63 | Methionine + Cysteine | 1.22 |
| Meat and bone meal | 6.54 | | |
| Pork plasma protein powder | 1.02 | | |
| NaCl | 0.20 | | |
| Lysine | 0.46 | | |
| Methionine | 0.61 | | |
| Choline | 0.05 | | |
| Premix[1] | 0.61 | | |
| Total | 100.00 | | |

[1]The premix provided the following per kg of diets: VA 18 997 IU, $VB_1$ 53 mg, $VB_2$ 23 mg, $VB_6$ 23 mg, $VB_{12}$ 0.08 mg, $VD_3$ 2 658 IU, VE 152 mg, $VK_3$ 2.3 mg, biotin 0.66 mg, folic acid 1.2 mg, D-pantothenic acid 21 mg, nicotinamide 46 mg, antioxidant 0.6 mg, Cu (as copper sulfate) 31 mg, Fe (as ferrous sulfate) 114 mg, Mn (as manganese sulfate) 60 mg, Zn (as zinc sulfate) 97 mg, I (as calcium iodate) 0.3 mg, Se (as sodium selenite) 0.23 mg.
[2]Metabolizable energy was a calculated value; the other values were measured.

## Serum sample analysis

The serum immunoglobulins (Ig)A, IgM, and IgG were measured using ELISA kits (Shanghai Shuangying Biotechnology Co., Ltd., Shanghai, China). Glutathione peroxidase (GSH-Px), superoxide dismutase (SOD), total antioxidant capacity (T-AOC) and maleic dialdehyde (MDA) were determined using diagnostic kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, China).

## Microbiota analysis based on 16S RNA high-throughput sequencing

Six fecal samples from the raccoon dogs in each group were chosen for microbiota analysis. Total bacterial DNA was extracted from approximately 0.25 g of feces using a Qiagen magnetic bead extraction Kit (Qiagen, Valencia, California, USA) according to the manufacturer's instructions. The 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.

The paired-end reads were merged into raw tags using FLASH version 1.2.7 (Magoè and Salzberg, 2011). Quality filtering of the raw tags was strictly filtered (< 30 Phred score) to obtain high-quality clean tags using QIIME (version 1.9.1) (Caporaso et al., 2010; Bokulich et al., 2013). The tags were compared with the SILVA database (version 138), and the chimera of effective tags were identified and removed using the UCHIME algorithm (Edgar et al., 2011; Haas et al., 2011). The Uparse algorithm (Uparse version 7.0.1001) was used to cluster the effective tags from all samples, and the sequences were clustered *via* default parameters with 97% identity into operational taxonomic units (OTUs) (Edgar, 2013). The sequence with the highest frequency of occurrence in OTUs was selected as the representative sequence of OTUs for further annotation. OTUs abundance information was normalized using a standard of sequence number corresponding to the sample with the fewest sequences. Subsequent analysis of alpha diversity and beta diversity was performed based on this output normalized data. Chao1, ACE, Shannon and Simpson indices were calculated with the software package

QIIME (version 1.9.1). Beta diversity analysis was performed using QIIME (version 1.9.1). The PCoA analyses were used to reveal the differences in the bacterial communities among the three groups (Minchin, 1987). The 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) (Stat et al., 2013). Correlations were analyzed by using Spearman's correlation in R software (version 2.15.3) with the R psych package and pheatmap for the heat map.

## Statistical analysis

All graphs were generated using GraphPad Prism version 8 and Adobe Illustrator 2022. All statistical analyses were performed using SPSS 26.0 software. The differences among groups were compared using One-way analysis of variance (ANOVA) and Bonferroni multiple comparison test. Data are represented as mean ± standard error. $P < 0.05$ indicates a significant difference. STAMP software (*t*-test) was used to analyze the differences of microbiota abundance between groups, 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 ($P > 0.05$) among the three groups. ADG was increased in the L and H groups compared to the N group ($P < 0.05$). F/G was decreased in the L and H groups compared to the N group ($P < 0.05$).

## Serum immune levels

As shown in Table 3, the serum IgA and IgG levels were increased in the L and H groups compared to the N group ($P < 0.05$). No significant differences in serum IgM levels were observed among the three groups ($P > 0.05$).

## Serum antioxidant capacity

As shown in Table 4, the activity of SOD in the L and H groups was increased compared to in the N group ($P < 0.05$), but no significant differences were observed between the L and H groups ($P > 0.05$). GSH-Px activity was increased in the L group compared with the N group ($P < 0.05$). No significant differences in serum T-AOC and MDA were observed among the three groups ($P > 0.05$).

## Summary of high-throughput sequencing and alpha diversity

The present study obtained a total of 1,436,812 16S rRNA gene sequences from three groups. After clustering at the 97% similarity level, sequences were assigned to 1,346 OTUs. Good's coverage, ranging from 0.998 to 0.999, demonstrated an adequate sequencing depth for all samples. The number of OTUs was not significantly different among the three groups ($P > 0.05$, $N = 456.17 \pm 129.11$, $L = 447.33 \pm 119.64$ and $H = 420.44 \pm 97.52$, respectively). As shown in Figure 1, the Shannon and Simpson index values in the N group were increased compared to the L and H groups ($P < 0.05$). There was no difference in the Chao1 and ACE indices among the three groups ($P > 0.05$).

## Composition and comparison of the gut microbiota in raccoon dogs

PCoA was applied to examine differences in taxonomic community composition and structure in the gut of the raccoon dog. The PCoA based on the Bray–Curtis distance (Figure 2A) and weighted UniFrac distance (Figure 2B) showed that the N group was separated from the L and H groups [Table 5, Adonis: $P < 0.05$ (N vs. L, N vs. H)]. Whereas the PCoA based on the binary Jaccard distance (Figure 2C) and unweighted UniFrac distance (Figure 2D) showed that the H group was separated from the N and L groups [Table 5, Adonis: $P < 0.05$ (N vs. H, L vs. H)].

At the phylum level, Bacteroidota ($N = 48.17 \pm 3.22\%$, $L = 24.98 \pm 4.16\%$), Firmicutes ($N = 38.67 \pm 2.76\%$, $L = 51.89 \pm 3.77\%$), Actinobacteriota ($N = 6.33 \pm 1.18\%$, $L = 18.07 \pm 4.82\%$), unidentified_Bacteria ($N = 3.45 \pm 3.40\%$, $L = 1.64 \pm 0.19\%$), and Proteobacteria ($N = 1.73 \pm 0.56\%$, $L = 1.56 \pm 0.47\%$) were the most abundant phyla in both the N and L groups, while Firmicutes (49.48 ± 3.96%), Bacteroidota (22.75 ± 6.67%), Actinobacteriota (16.42 ± 3.61%), Proteobacteria (6.20 ± 1.89%), and Fusobacteria (2.83 ± 1.81%) were the five most abundant phyla in the H group (Figure 2E). The ratio of Firmicutes to Bacteroidetes in the *Cyberlindnera jadinii* supplementation groups ($L = 3.09 \pm 0.60$, $H = 3.41 \pm 0.90$) were significantly increased compared with the N group (0.73 ± 0.07). At the genus level, *Prevotella* was the dominant genus in all three groups ($N = 41.48 \pm 2.73\%$, $L = 20.80 \pm 3.58\%$, $H = 19.16 \pm 5.72\%$). *Megasphaera* (8.74 ± 1.83%), *Alloprevotella* (5.26 ± 1.27%), *Bifidobacterium* (5.03 ± 1.07%), and *Blautia* (4.32 ± 0.75%) were the most abundant genera in the N group. *Bifidobacterium* (16.75 ± 4.73%), *Megasphaera* (17.31 ± 2.13%), *Lactobacillus* (4.08 ± 1.32%), and *Alloprevotella* (3.13 ± 0.54%) were the most abundant genera in the L group, and *Megasphaera* (14.88 ± 2.92%), *Bifidobacterium* (15.38 ± 3.68%), *Streptococcus*

(12.28 ± 5.57%), and *Lactobacillus* (4.70 ± 0.40%) were the most abundant genera in the H group (Figure 2F).

Furthermore, we also compared the bacterial taxa among the three groups. The relative abundance of Firmicutes and Actinobacteriota was increased in the L and H groups compared to the N group, while the relative abundance of Bacteroidota and unidentified Bacteria were decreased in the L and H groups compared to the N group ($P < 0.05$) (Figures 3A,B). In addition, the relative abundance of Proteobacteria and Cyanobacteria in the H group was increased compared to the N and L groups ($P < 0.05$) (Figures 3B,C). At the genus level, the relative abundance of *Bifidobacterium* and *Megasphaera* was increased while the relative abundance of *Prevotella* was decreased in the L and H groups compared with the N group ($P < 0.05$) (Figures 3D,E). The relative abundance of *Agathobacter* was decreased in the H group compared with the N group ($P < 0.05$) (Figure 3E). The relative abundance of *Dialister* was increased where the relative abundance of *Blautia* decreased in the H group compared with the N and L groups ($P < 0.05$) (Figures 3E,F).

## Correlation between growth performance or serum markers and gut microbiota

Spearman's rank correlation analysis was performed to evaluate the potential relationship between alterations in gut microbiota composition and growth performance and serum markers of raccoon dogs. Sixteen genera, including *Prevotella*, *Bifidobacterium* and *Dialister*, showed significant correlations with the serum markers (Figure 4). The genus *Prevotella* showed

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

| Items | Groups | | | *P*-value |
|---|---|---|---|---|
| | N | L | H | |
| IBW, kg | 1.98 ± 0.05 | 1.96 ± 0.04 | 1.99 ± 0.11 | 0.968 |
| FBW, kg | 4.88 ± 0.35 | 5.47 ± 0.06 | 5.47 ± 0.25 | 0.152 |
| ADG, g/day | 43.10 ± 4.89$^{b}$ | 60.46 ± 1.13$^{a}$ | 60.00 ± 2.85$^{a}$ | 0.002 |
| ADFI, g/day | 343.89 ± 6.64 | 334.69 ± 7.13 | 341.59 ± 6.75 | 0.620 |
| F/G | 8.09 ± 0.83$^{a}$ | 5.53 ± 0.21$^{b}$ | 5.82 ± 0.23$^{b}$ | 0.008 |

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, $1 \times 10^9$ CFU/g *Cyberlindnera jadinii*; H group, $5 \times 10^9$ 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 ($P < 0.05$).

TABLE 3 Effects of *Cyberlindnera jadinii* on serum immune indices in growing raccoon dogs.

| Items | Groups | | | *P*-value |
|---|---|---|---|---|
| | N | L | H | |
| IgA, μg/ml | 36.28 ± 0.98$^{b}$ | 41.72 ± 0.73$^{a}$ | 41.00 ± 0.99$^{a}$ | 0.001 |
| IgG, μg/ml | 324.844 ± 4.96$^{b}$ | 368.31 ± 9.12$^{a}$ | 367.20 ± 5.35$^{a}$ | 0.001 |
| IgM, μg/ml | 19.34 ± 0.39 | 18.05 ± 0.45 | 19.43 ± 0.39 | 0.065 |

IgA, Immunoglobulin A; IgG, Immunoglobulin G; IgM, Immunoglobulin M; N group, 0 CFU/g *Cyberlindnera jadinii*; L group, $1 \times 10^9$ CFU/g *Cyberlindnera jadinii*; H group, $5 \times 10^9$ 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 ($P < 0.05$).

TABLE 4 Effects of *Cyberlindnera jadinii* on serum antioxidant indices in growing raccoon dogs.

| Items | Groups | | | *P*-value |
|---|---|---|---|---|
| | N | L | H | |
| GSH-Px, U/mL | 364.63 ± 20.21$^{b}$ | 469.51 ± 32.08$^{a}$ | 393.90 ± 25.86$^{ab}$ | 0.036 |
| SOD, U/mL | 28.56 ± 1.61$^{b}$ | 36.85 ± 0.75$^{a}$ | 36.79 ± 1.07$^{a}$ | < 0.001 |
| T-AOC, U/mL | 2.79 ± 0.29 | 2.98 ± 0.24 | 2.86 ± 0.25 | 0.871 |
| MDA, nmol/mL | 10.19 ± 1.20 | 11.90 ± 1.07 | 10.86 ± 0.85 | 0.524 |

GSH-Px, Glutathione peroxidase; SOD, Superoxide dismutase; T-AOC, Total antioxidant capacity; MDA, Malondialdehyde; N group, 0 CFU/g *Cyberlindnera jadinii*; L group, $1 \times 10^9$ CFU/g *Cyberlindnera jadinii*; H group, $5 \times 10^9$ 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 ($P < 0.05$).

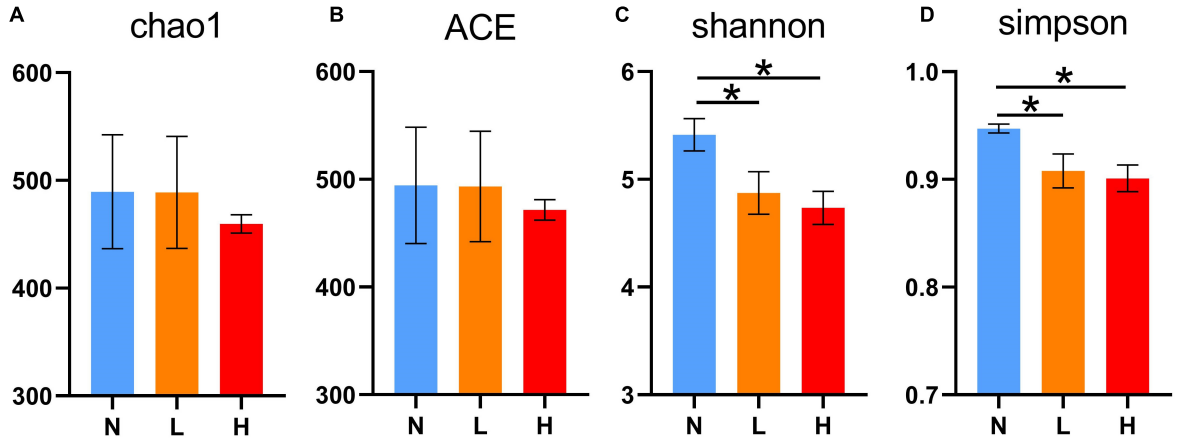


FIGURE 1
Comparisons of the alpha diversity indices of the raccoon dog gut microbiota among the three groups. Chao1 index **(A)**, ACE index **(B)**, Shannon index **(C)**, and Simpson index **(D)**. N group, 0 CFU/g *Cyberlindnera jadinii*; L group, $1 \times 10^9$ CFU/g *Cyberlindnera jadinii*; H group, $5 \times 10^9$ CFU/g *Cyberlindnera jadinii*. *$P < 0.05$.

a significant negative correlation with ADG ($P < 0.05$), the genus *Bifidobacterium* showed a significant positive correlation with ADG and serum T-AOC ($P < 0.05$), and *Dialister* showed a significant positive correlation with serum IgA and T-AOC ($P < 0.05$) (**Figure 4**).

## Discussion

Previous studies have suggested that *Cyberlindnera jadinii* can improve the growth performance of weaned piglets (Yang et al., 2021). Our results showed that *Cyberlindnera jadinii* increased ADG and decreased the F/G in raccoon dogs. Additionally, the dietary supplementation of *Cyberlindnera jadinii* at $1 \times 10^9$ CFU/g resulted in the highest ADG and the lowest F/G. This may be because yeast can bind metal ions in the environment and permanently integrate into their cellular structure. Due to active transport mechanisms, absorbed elements can be transferred to the inside of the cell and accumulate in the form of metal. This can lead to the formation of stable complexes with proteins. Due to these connections, nutrient elements are more readily collected, transported, and absorbed in the digestive tracts of animals (Kieliszek et al., 2017).

Serum immunoglobulin can be used as a parameter reflecting the immune state of animals. Research has shown that diets supplemented of *Saccharomyces cerevisiae* increased the level of serum IgA in piglets (Trckova et al., 2014; Jiang et al., 2015). In addition, diets supplemented with *Saccharomyces cerevisiae* and yeast culture increased the levels of IgA, IgG and IgM in lambs during the growing period (Mahmoud et al., 2020). Our results showed that *Cyberlindnera jadinii* improved the levels of IgA and IgG in raccoon dogs. The changes in serum immunoglobulins observed in our studies may be related to β-glucans found in yeast cell walls, which have immunomodulatory properties (Raghebian et al., 2016). β-glucans increase the host immune defense by activating the complement system and enhancing macrophage and natural killer cell function (Akramiene et al., 2007). In addition, oxidative stress is a common condition which can produce variety of oxygen free radicals. An excess of oxygen free radicals can damage proteins, nucleic acids, and other biological macromolecules, thus causing tissue damage and tissue mitochondrial damage (Bai et al., 2018). In our study, *Cyberlindnera jadinii* dietary supplementation increased serum SOD and GSH-Px activities. An increase in SOD activity is an element of the natural antioxidant defense system (Yousefi et al., 2020), and the increased GSH-Px activity may be due to the presence of glutathione-bound selenium compounds in yeasts. Yeasts are characterized by a relatively high capacity to accumulate selenium (Kieliszek et al., 2016, 2017). Selenium is an essential constituent of the GSH-Px, and GSH-Px catalyzes the reduction of hydrogen peroxide by glutathione (Patching and Gardiner, 1999). The GSH and GSH-Px enzymes relieve oxidative damage by eliminating excessive free radicals (Bai et al., 2018). The results of the current study showed that *Cyberlindnera jadinii* supplementation improved the immune state and antioxidant activity in raccoon dogs.

The intestinal microbiota is closely related to the growth and development of animals. The gut microbiota of the raccoon dogs was dominated by sequences representative of Firmicutes, Bacteroidetes, Actinobacteria, and Proteobacteria. The phyla Firmicutes, Proteobacteria, Bacteroidetes, and Actinobacteria are also widely present in the gastrointestinal tracts of other carnivore species such as mink, Eurasian otters, leopard cats, blue fox and silver fox (An et al., 2017; Peng et al., 2019; Liu et al., 2020; Nan et al., 2021). Our results showed that at the

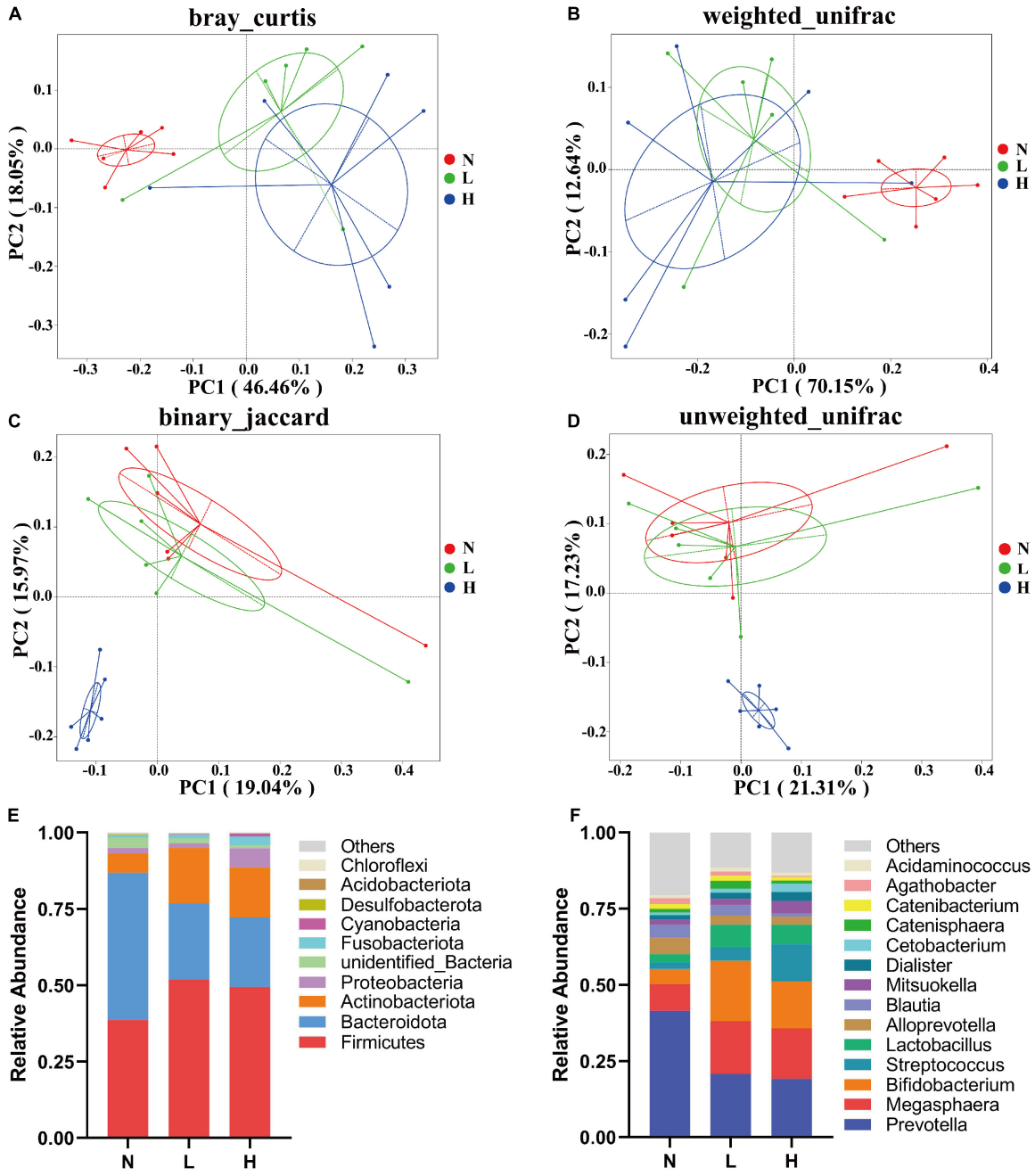


FIGURE 2
Composition and comparisons of the raccoon dog gut microbiota among the three groups. PCoA reveals the separation of the gut microbiota in the three groups based on the Bray–Curtis distance **(A)**, weighted UniFrac distance **(B)**, binary Jaccard distance **(C)** unweighted UniFrac distance **(D)**. Microbial composition in the gut of raccoon dogs from the N, L, and H groups at the phylum **(E)** and genus **(F)** levels. N group, 0 CFU/g *Cyberlindnera jadinii*; L group, $1 \times 10^9$ CFU/g *Cyberlindnera jadinii*; H group, $5 \times 10^9$ CFU/g *Cyberlindnera jadinii*.

phylum level, *Cyberlindnera jadinii* supplementation increased the relative abundance of Firmicutes and Actinobacteria but decreased the relative abundance of Bacteroidetes. In addition, $5 \times 10^9$ CFU/g *Cyberlindnera jadinii* also improved the relative abundance of Proteobacteria and Cyanobacteria. Moreover, the Firmicutes/Bacteroidetes ratio (F/B ratio) was increased in the *Cyberlindnera jadinii* supplementation groups. The Firmicutes phyla contain genes that are related to energy metabolism and the decomposition of substances (Kaakoush, 2015; Zhang et al., 2019), and Bacteroidetes are associated with the degradation of proteins and carbohydrates (Thomas et al., 2011; Waite and Taylor, 2014). Previous studies found

TABLE 5 Adonis analysis of the bacterial communities in the gut of growing raccoon dogs.

| Group | Bray–Curtis | | Weighted UniFrac | | Binary Jaccard | | Unweighted UniFrac | |
|---|---|---|---|---|---|---|---|---|
| | $R^2$ | *P*-value | $R^2$ | *P*-value | $R^2$ | *P*-value | $R^2$ | *P*-value |
| N vs. L | 0.321 | 0.012 | 0.483 | 0.009 | 0.075 | 0.625 | 0.064 | 0.705 |
| N vs. H | 0.394 | 0.007 | 0.498 | 0.004 | 0.220 | 0.003 | 0.223 | 0.001 |
| L vs. H | 0.125 | 0.212 | 0.089 | 0.413 | 0.179 | 0.005 | 0.179 | 0.003 |

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

that the F/B ratio is proportional to body weight (Singh et al., 2013). A high F/B ratio is beneficial for gut microbiota-mediated energy harvesting in animals (Li et al., 2016), which assists with the maintenance of metabolic balance and better growth performance (Ley et al., 2006; Murphy et al., 2010). Therefore, this study suggests that the gut microbiota following *Cyberlindnera jadinii* supplementation had a strong effect on energy metabolism and the decomposition of substances. This strong ability to obtain energy may also be one reason for their strong growth performance. In addition, the composition of the gut microbiota is influenced by endogenous and environmental factors (Laparra and Sanz, 2010). In general, the diet is considered a major driver of changes in gut microbial diversity, which may affect its functional relationship with the host (Ley et al., 2008; Laparra and Sanz, 2010). However, multiple dietary components can interact non-additively to influence gut microbial diversity (Bolnick et al., 2014). In the current study, the *Cyberlindnera jadinii* supplementation decreased the alpha diversity of the gut microbiota as shown by the Shannon and Simpson indices. It may be that *Cyberlindnera jadinii* supplementation has indirect effects on raccoon dog physiology and immunity, which may regulate the diversity of the gut microbiota. Alternatively, *Cyberlindnera jadinii* supplementation might increase the activity of certain bacteria that affect the presence or growth of certain gut microbiota. For example, Actinobacteria can produce secondary metabolites, many of which have antibacterial and antifungal properties (Ul-Hassan and Wellington, 2009). *Cyanobacteria* is a diverse source of compounds with antimicrobial activity (Swain et al., 2017). Consequently, the alpha diversity of the gut microbiota may decrease with *Cyberlindnera jadinii* supplementation. Given that an imbalanced gut microbiota often arises from a sustained increase in abundance of the phylum Proteobacteria, the natural gut microbiota normally contains only a minor proportion of this phylum (Shin et al., 2015). The anaerobic Proteobacteria are usually associated with an impaired microbiota, or dysbiosis (Litvak et al., 2017). Thus, dietary supplementation of $1 \times 10^9$ CFU/g *Cyberlindnera jadinii* may be the most beneficial dose for the balance of intestinal microbiota in growing raccoon dogs.

Our results showed that at the genus level, *Cyberlindnera jadinii* increased the relative abundance of *Megasphaera* and *Bifidobacterium* and decreased the relative abundance of *Prevotella*. Some strains of *Megasphaera* can produce several short-chain fatty acids (SCFAs) such as acetate, propionate, butyrate, and valerate (Yoshikawa et al., 2018). These SCFAs may provide energy sources for animal growth. The increase of *Bifidobacterium* may be due to the presence of bifidus factors in yeast that promote the growth of *Bifidobacterium* (Ghoddusi and Tamime, 2014). Spearman correlation analysis also showed that ADG exhibited a significant, positive correlation with the relative abundance of *Bifidobacterium*. From a metabolic point of view, Bifidobacteria use the fructose 6-phosphate pathway for the metabolism of glucose and lactose, which can also provide energy sources for animal growth (González-Rodríguez et al., 2013; Hidalgo-Cantabrana et al., 2017). Moreover, the abundance of *Prevotella* is negatively correlated with weight changes (Christensen et al., 2019), which provids further support for the increase of ADG in raccoon dogs. Some strains of *Prevotella,* identified as active microbes, are associated with plant-rich diets and can express various genes encoding carbohydrate-degrading enzymes (Dai et al., 2015). Our results showed that the gut microbiota following *Cyberlindnera jadinii* supplementation had a lower abundance of cellulose-degrading bacteria, which may be more suitable to the dietary habits of carnivores. In addition, adding $5 \times 10^9$ CFU/g *Cyberlindnera jadinii* in the feed also increased the relative abundance of *Dialister* and decreased the relative abundance of *Blautia* and *Agathobacter*. *Dialister*, a potential microbial marker of disease activity, has been shown to exhibit a positive correlation with disease activity (Tito et al., 2017). Moreover, we observed a significant positive correlation between the abundance of the *Dialister* and serum IgA concentration, as shown by spearman correlation analysis. This may be an immune defense mechanism of the host. *Blautia* is a genus of anaerobic bacteria with probiotic characteristics that occur widely in the feces and intestines of mammals. *Blautia* has been shown to play a role in metabolic diseases, inflammatory diseases, and biotransformation (Liu et al., 2021). The main fermentation product of *Agathobacter* is butyrate (Hua et al., 2020). Butyrate is a short-chain fatty acid that plays an important role in gut physiology. Butyrate can enhance the barrier function of the gut and represses inflammatory responses through inhibition of NF-kB activation

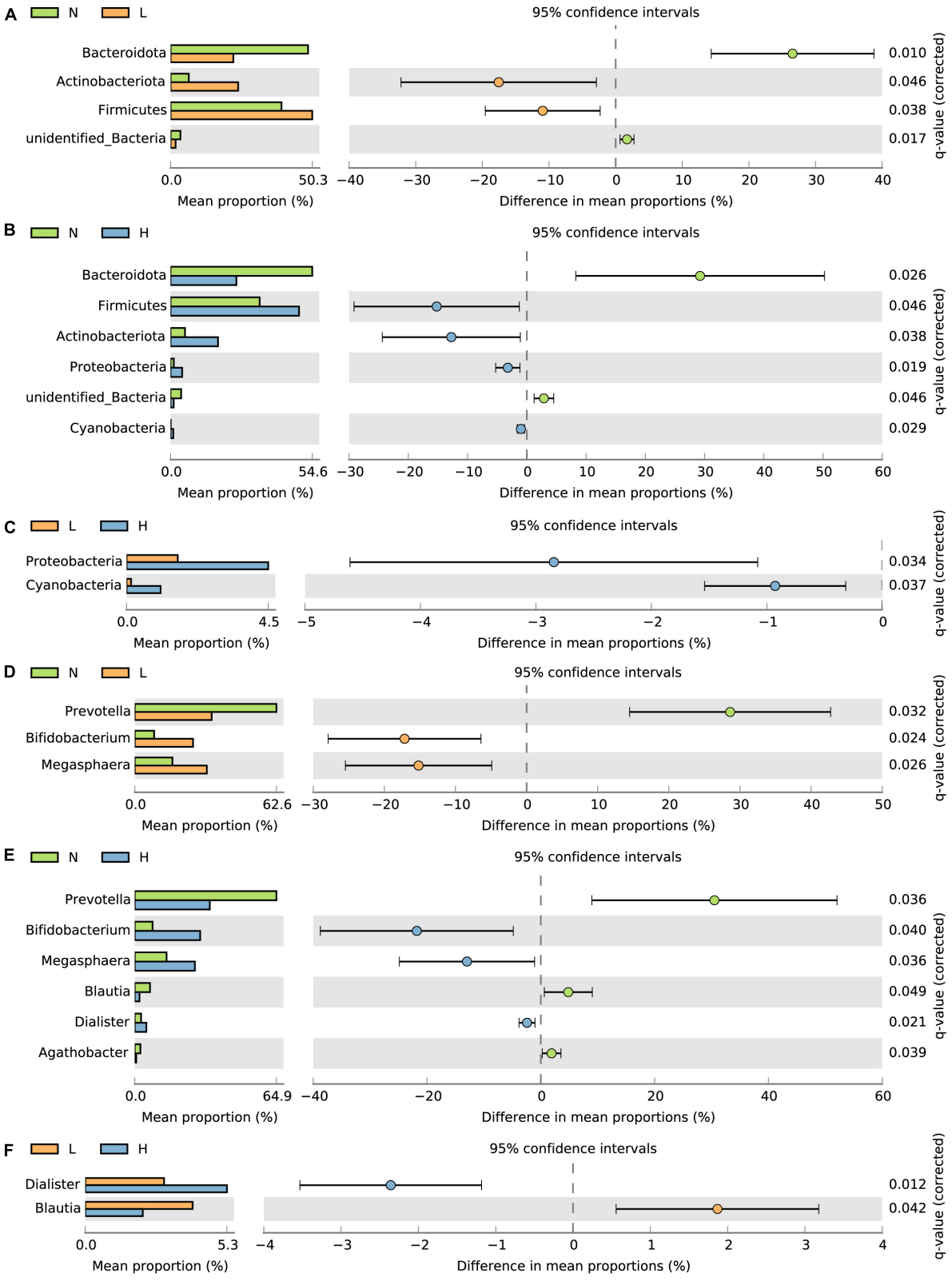


FIGURE 3
*T*-test bar plots showing differences in the relative abundance of bacteria at the phylum **(A–C)** and genus **(D–F)** levels. N group, 0 CFU/g *Cyberlindnera jadinii*; L group, $1 \times 10^9$ CFU/g *Cyberlindnera jadinii*; H group, $5 \times 10^9$ CFU/g *Cyberlindnera jadinii*.

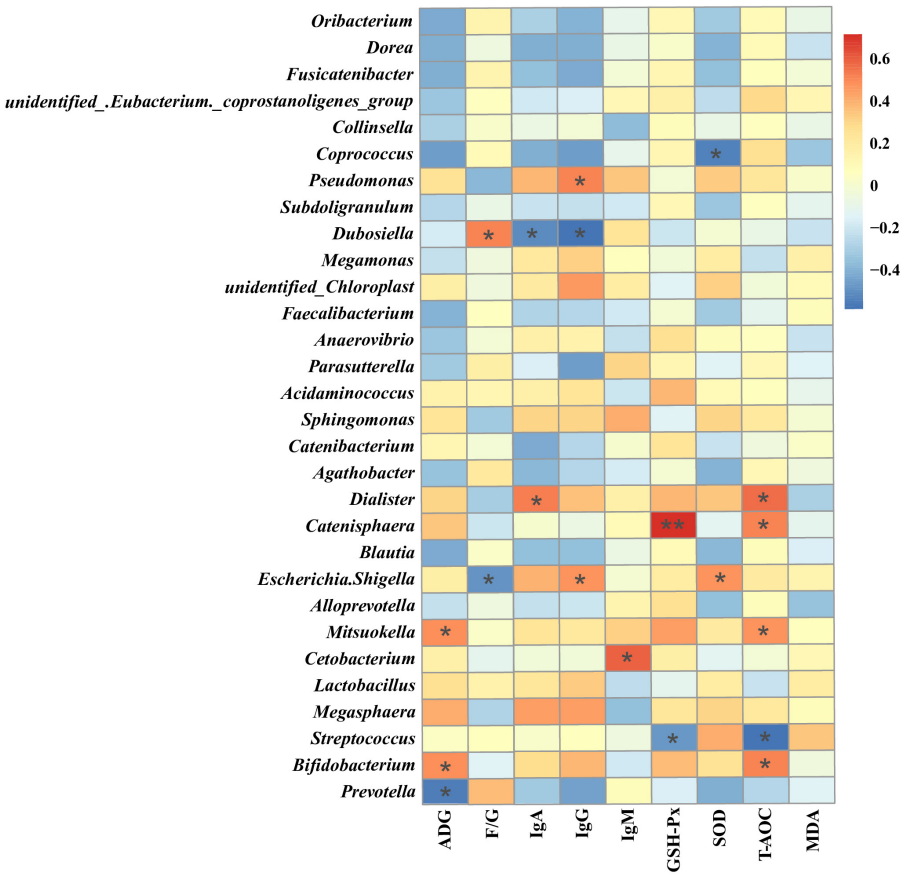


FIGURE 4
Heatmap of the Spearman rank correlation between the growth performance or serum markers and gut microbiota. ADG, Average daily gain; F/G, ratio of feed to gain; IgA, Immunoglobulin A; IgG, Immunoglobulin G; IgM, Immunoglobulin M; GSH-Px, Glutathione peroxidase; SOD, Superoxide dismutase; T-AOC, Total antioxidant capacity; MDA, Malondialdehyde. *$P < 0.05$,**$P < 0.01$ (following the Spearman correlation analysis).

(Macfarlane and Macfarlane, 2011). Results of the current study suggest that adding $5 \times 10^9$ CFU/g *Cyberlindnera jadinii* may decrease the abundance of beneficial bacteria and increase the abundance of microbiota related to intestinal inflammation. Therefore, *Cyberlindnera jadinii* may play an active role in growth performance by regulating intestinal microbiota, and this effect was greater when the addition level was $1 \times 10^9$ CFU/g.

## Conclusion

The present research demonstrated that dietary *Cyberlindnera jadinii* supplementation improved growth performance, serum antioxidant capacity and immunity, and intestinal microbiota in growing raccoon dogs. Among the concentrations tested, $1 \times 10^9$ CFU/g was the most effective level of supplementation. Thus, *Cyberlindnera jadinii* has potential as an efficient antibiotic alternative in raccoon dog feed.

## 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: NCBI BioProject – PRJNA853184.

## 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: funding acquisition, methodology, project administration, and writing—review and editing. HZ: methodology, validation, and resources. KL and XZ: data curation, investigation, and supervision. QL, YW, TZ, and QZ: 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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## References

Akramiene, D., Kondrotas, A., Didziapetriene, J., and Kevelaitis, E. (2007). Effects of beta-glucans on the immune system. *Medicina (Kaunas)* 43, 597–606.

Alagawany, M., Abd El-Hack, M. E., Farag, M. R., Sachan, S., Karthik, K., and Dhama, K. (2018). The use of probiotics as eco-friendly alternatives for antibiotics in poultry nutrition. *Environ. Sci. Pollut. Res. Int.* 25, 10611–10618. doi: 10.1007/s11356-018-1687-x

Alizadeh, M., Rodriguez-Lecompte, J. C., Yitbarek, A., Sharif, S., Crow, G., and Slominski, B. A. (2016). Effect of yeast-derived products on systemic innate immune response of broiler chickens following a lipopolysaccharide challenge. *Poult. Sci.* 95, 2266–2273. doi: 10.3382/ps/pew154

An, C., Okamoto, Y., Xu, S., Eo, K. Y., Kimura, J., and Yamamoto, N. (2017). Comparison of fecal microbiota of three captive carnivore species inhabiting Korea. *J. Vet. Med. Sci.* 79, 542–546. doi: 10.1292/jvms.16-0472

Bai, K., Feng, C., Jiang, L., Zhang, L., Zhang, J., Zhang, L., et al. (2018). Dietary effects of Bacillus subtilis fmbj on growth performance, small intestinal morphology, and its antioxidant capacity of broilers. *Poult. Sci.* 97, 2312–2321. doi: 10.3382/ps/pey116

Bilal, R. M., Hassan, F. U., Saeed, M., Rafeeq, M., Zahra, N., Fraz, A., et al. (2021). Role of yeast and yeast-derived products as feed additives in broiler nutrition. *Anim. Biotechnol.* 1–10. doi: 10.1080/10495398.2021.1942028 [Epub ahead of print].

Bokulich, N. A., Subramanian, S., Faith, J. J., Gevers, D., Gordon, J. I., Knight, R., et al. (2013). Quality-filtering vastly improves diversity estimates from Illumina amplicon sequencing. *Nat. Methods* 10, 57–59. doi: 10.1038/nmeth.2276

Bolnick, D. I., Snowberg, L. K., Hirsch, P. E., Lauber, C. L., Knight, R., Caporaso, J. G., et al. (2014). Individuals' diet diversity influences gut microbial diversity in two freshwater fish (threespine stickleback and Eurasian perch). *Ecol. Lett.* 17, 979–987. doi: 10.1111/ele.12301

Caporaso, J. G., Kuczynski, J., Stombaugh, J., Bittinger, K., Bushman, F. D., Costello, E. K., et al. (2010). QIIME allows analysis of high-throughput community sequencing data. *Nat. Methods* 7, 335–336. doi: 10.1038/nmeth.f.303

Christensen, L., Vuholm, S., Roager, H. M., Nielsen, D. S., Krych, L., Kristensen, M., et al. (2019). Prevotella abundance predicts weight loss success in healthy, overweight adults consuming a whole-grain diet ad libitum: A post hoc analysis of a 6-Wk randomized controlled trial. *J. Nutr.* 149, 2174–2181. doi: 10.1093/jn/nxz198

Cruz, A., Tauson, A.-H., Matthiesen, C. F., Mydland, L. T., and Øverland, M. (2020). Cyberlindnera jadinii yeast as a protein source for growing pigs: Effects on protein and energy metabolism. *Livest. Sci.* 231:103855. doi: 10.1016/j.livsci.2019.103855

Dai, X., Tian, Y., Li, J., Luo, Y., Liu, D., Zheng, H., et al. (2015). Metatranscriptomic analyses of plant cell wall polysaccharide degradation by microorganisms in the cow rumen. *Appl. Environ. Microbiol.* 81, 1375–1386. doi: 10.1128/AEM.03682-14

Edgar, R. C. (2013). UPARSE: Highly accurate OTU sequences from microbial amplicon reads. *Nat. Methods* 10, 996–998. doi: 10.1038/nmeth.2604

Edgar, R. C., Haas, B. J., Clemente, J. C., Quince, C., and Knight, R. (2011). UCHIME improves sensitivity and speed of chimera detection. *Bioinformatics* 27, 2194–2200. doi: 10.1093/bioinformatics/btr381

Gadde, U., Kim, W. H., Oh, S. T., and Lillehoj, H. S. (2017). Alternatives to antibiotics for maximizing growth performance and feed efficiency in poultry: A review. *Anim. Health Res. Rev.* 18, 26–45. doi: 10.1017/S1466252316000207

Gao, J., Zhang, H. J., Yu, S. H., Wu, S. G., Yoon, I., Quigley, J., et al. (2008). Effects of yeast culture in broiler diets on performance and immunomodulatory functions. *Poult. Sci.* 87, 1377–1384. doi: 10.3382/ps.2007-00418

Ghoddusi, H. B., and Tamime, A. Y. (2014). "MICROFLORA OF THE INTESTINE | Biology of Bifidobacteria," in *Encyclopedia of Food Microbiology (Second Edition)*, eds C. A. Batt and M. L. Tortorello (Oxford: Academic Press), 639–645. doi: 10.1016/B978-0-12-384730-0.00208-1

González-Rodríguez, I., Gaspar, P., Sánchez, B., Gueimonde, M., Margolles, A., and Neves, A. R. (2013). Catabolism of glucose and lactose in bifidobacterium animalis subsp. lactis, studied by 13 C nuclear magnetic resonance. *Appl. Environ. Microbiol.* 79, 7628–7638. doi: 10.1128/AEM.02529-13

Haas, B. J., Gevers, D., Earl, A. M., Feldgarden, M., Ward, D. V., Giannoukos, G., et al. (2011). Chimeric 16S rRNA sequence formation and detection in Sanger and 454-pyrosequenced PCR amplicons. *Genome Res.* 21, 494–504. doi: 10.1101/gr.112730.110

Håkenåsen, I. M., Øverland, M., Ånestad, R., Åkesson, C. P., Sundaram, A. Y. M., Press, C. M., et al. (2020). Gene expression and gastrointestinal function is altered in piglet small intestine by weaning and inclusion of Cyberlindnera jadinii yeast as a protein source. *J. Funct. Foods* 73:104118. doi: 10.1016/j.jff.2020.104118

Hidalgo-Cantabrana, C., Delgado, S., Ruiz, L., Ruas-Madiedo, P., Sánchez, B., and Margolles, A. (2017). Bifidobacteria and their health-promoting effects. *Microbiol. Spectr.* 5:5.3.21. doi: 10.1128/microbiolspec.BAD-0010-2016

Hua, X., Zhu, J., Yang, T., Guo, M., Li, Q., Chen, J., et al. (2020). The gut microbiota and associated metabolites are altered in sleep disorder of children with autism spectrum disorders. *Front. Psychiatry* 11:855. doi: 10.3389/fpsyt.2020.00855

Jiang, Z., Wei, S., Wang, Z., Zhu, C., Hu, S., Zheng, C., et al. (2015). Effects of different forms of yeast Saccharomyces cerevisiae on growth performance, intestinal development, and systemic immunity in early-weaned piglets. *J. Anim. Sci. Biotechnol.* 6:47. doi: 10.1186/s40104-015-0046-8

Kaakoush, N. O. (2015). Insights into the role of Erysipelotrichaceae in the human host. *Front. Cell. Infect. Microbiol.* 5:84. doi: 10.3389/fcimb.2015.00084

Kieliszek, M., Błażejak, S., and Płaczek, M. (2016). Spectrophotometric evaluation of selenium binding by Saccharomyces cerevisiae ATCC MYA-2200 and Candida utilis ATCC 9950 yeast. *J. Trace Elem. Med. Bio.* 35, 90–96. doi: 10.1016/j.jtemb.2016.01.014

Kieliszek, M., Kot, A. M., Bzducha-Wróbel, A., Błażejak, S., Gientka, I., and Kurcz, A. (2017). Biotechnological use of Candida yeasts in the food industry: A review. *Fungal Biol. Rev.* 31, 185–198. doi: 10.1016/j.fbr.2017.06.001

Laparra, J. M., and Sanz, Y. (2010). Interactions of gut microbiota with functional food components and nutraceuticals. *Pharmacol. Res.* 61, 219–225. doi: 10.1016/j.phrs.2009.11.001

Ley, R. E., Lozupone, C. A., Hamady, M., Knight, R., and Gordon, J. I. (2008). Worlds within worlds: Evolution of the vertebrate gut microbiota. *Nat. Rev. Microbiol.* 6, 776–788. doi: 10.1038/nrmicro1978

Ley, R. E., Turnbaugh, P. J., Klein, S., and Gordon, J. I. (2006). Microbial ecology: Human gut microbes associated with obesity. *Nature* 444, 1022–1023. doi: 10.1038/4441022a

Li, H., Li, T., Beasley, D. E., Hedìnec, P., Xiao, Z., Zhang, S., et al. (2016). Diet diversity is associated with beta but not alpha diversity of pika gut microbiota. *Front. Microbiol.* 7:1169. doi: 10.3389/fmicb.2016.01169

Litvak, Y., Byndloss, M. X., Tsolis, R. M., and Bäumler, A. J. (2017). Dysbiotic *Proteobacteria* expansion: A microbial signature of epithelial dysfunction. *Curr. Opin. Microbiol.* 39, 1–6. doi: 10.1016/j.mib.2017.07.003

Liu, H., Li, Z., Si, H., Zhong, W., Fan, Z., and Li, G. (2020). Comparative analysis of the gut microbiota of the blue fox (*Alopex lagopus*) and raccoon dog (*Nyctereutes procyonoides*). *Arch. Microbiol.* 202, 135–142. doi: 10.1007/s00203-019-01721-0

Liu, X., Mao, B., Gu, J., Wu, J., Cui, S., Wang, G., et al. (2021). Blautia —a new functional genus with potential probiotic properties? *Gut Microbes* 13:1875796. doi: 10.1080/19490976.2021.1875796

Macfarlane, G. T., and Macfarlane, S. (2011). Fermentation in the human large intestine: Its physiologic consequences and the potential contribution of prebiotics. *J. Clin. Gastroenterol.* 45, S120–S127. doi: 10.1097/MCG.0b013e31822fecfe

Magoè, T., and Salzberg, S. L. (2011). FLASH: Fast length adjustment of short reads to improve genome assemblies. *Bioinformatics* 27, 2957–2963. doi: 10.1093/bioinformatics/btr507

Mahmoud, M. M., Youssef, I. M. I., Abd El-Tawab, M. M., Bakr, H. A., Eissa, N. A., Hassan, M. S., et al. (2020). Influence of probiotic and yeast culture supplementation on selected biochemical and immunological parameters of growing lambs. *Pol. J. Vet. Sci.* 23, 5–12. doi: 10.24425/pjvs.2019.131413

Minchin, P. R. (1987). An evaluation of the relative robustness of techniques for ecological ordination. *Plant Ecol.* 69, 89–107. doi: 10.1007/BF00038690

Muegge, B. D., Kuczynski, J., Knights, D., Clemente, J. C., González, A., Fontana, L., et al. (2011). Diet drives convergence in gut microbiome functions across mammalian phylogeny and within humans. *Science* 332, 970–974. doi: 10.1126/science.1198719

Murphy, E. F., Cotter, P. D., Healy, S., Marques, T. M., O'Sullivan, O., Fouhy, F., et al. (2010). Composition and energy harvesting capacity of the gut microbiota: Relationship to diet, obesity and time in mouse models. *Gut* 59, 1635–1642. doi: 10.1136/gut.2010.215665

Nan, W., Si, H., Yang, Q., Shi, H., Zhang, T., Shi, Q., et al. (2021). Effect of Vitamin a supplementation on growth performance, serum biochemical parameters, intestinal immunity response and gut microbiota in American mink (*Neovison vison*). *Animals (Basel)* 11:1577. doi: 10.3390/ani11061577

National Research Council (1982). *Nutrient requirements of mink and foxes, Second Revised Edition*. Washington, DC: National Academies Press.

Patching, S. G., and Gardiner, R. H. E. (1999). Recent developments in selenium metabolism and chemical speciation: A review. *J. Trace Elem. Med. Biol.* 13, 193–214. doi: 10.1016/S0946-672X(99)80037-6

Peng, Y., Shi, Q., Wang, Y., Zhang, F., Ji, Z., and Zhang, J. (2019). Dietary probiotics have different effects on the composition of fecal microbiota in farmed raccoon dog (*Nyctereutes procyonoides*) and silver fox (*Vulpes vulpes* fulva). *BMC Microbiol.* 19:109. doi: 10.1186/s12866-019-1491-x

Raghebian, M., Babaei Yazdi, A., Dabiri, N., Hajimohammadi, A., Hatami, P., Raghebian, A., et al. (2016). Effect of different levels of live yeast in a high concentrate diet on performance, blood constituents and immune system status of Zandi lambs. *Iran. J. Appl. Anim. Sci.* 6, 833–840.

Rupp, O., Brinkrolf, K., Buerth, C., Kunigo, M., Schneider, J., Jaenicke, S., et al. (2015). The structure of the *Cyberlindnera jadinii* genome and its relation to Candida utilis analyzed by the occurrence of single nucleotide polymorphisms. *J. Biotechnol.* 211, 20–30. doi: 10.1016/j.jbiotec.2015.06.423

Schoeler, M., and Caesar, R. (2019). Dietary lipids, gut microbiota and lipid metabolism. *Rev. Endocr. Metab. Disord* 20, 461–472. doi: 10.1007/s11154-019-09512-0

Shin, N.-R., Whon, T. W., and Bae, J.-W. (2015). *Proteobacteria*: Microbial signature of dysbiosis in gut microbiota. *Trends Biotechnol.* 33, 496–503. doi: 10.1016/j.tibtech.2015.06.011

Singh, P., Karimi, A., Devendra, K., Waldroup, P. W., Cho, K. K., and Kwon, Y. M. (2013). Influence of penicillin on microbial diversity of the cecal microbiota in broiler chickens. *Poult. Sci.* 92, 272–276. doi: 10.3382/ps.2012-02603

Sousa-Silva, M., Vieira, D., Soares, P., Casal, M., and Soares-Silva, I. (2021). Expanding the knowledge on the skillful yeast Cyberlindnera jadinii. *J. Fungi (Basel)* 7:36. doi: 10.3390/jof7010036

Stat, M., Pochon, X., Franklin, E. C., Bruno, J. F., Casey, K. S., Selig, E. R., et al. (2013). The distribution of the thermally tolerant symbiont lineage (*Symbiodinium* clade D) in corals from Hawaii: Correlations with host and the history of ocean thermal stress. *Ecol. Evol.* 3, 1317–1329. doi: 10.1002/ece3.556

Swain, S. S., Paidesetty, S. K., and Padhy, R. N. (2017). Antibacterial, antifungal and antimycobacterial compounds from cyanobacteria. *Biomed. Pharmacother.* 90, 760–776. doi: 10.1016/j.biopha.2017.04.030

Thomas, F., Hehemann, J.-H., Rebuffet, E., Czjzek, M., and Michel, G. (2011). Environmental and gut bacteroidetes: The food connection. *Front. Microbiol.* 2:93. doi: 10.3389/fmicb.2011.00093

Tito, R. Y., Cypers, H., Joossens, M., Varkas, G., Van Praet, L., Glorieus, E., et al. (2017). Brief report: *Dialister* as a microbial marker of disease activity in spondyloarthritis. *Arthritis Rheumatol.* 69, 114–121. doi: 10.1002/art.39802

Trckova, M., Faldyna, M., Alexa, P., Sramkova Zajacova, Z., Gopfert, E., Kumprechtova, D., et al. (2014). The effects of live yeast *Saccharomyces cerevisiae* on postweaning diarrhea, immune response, and growth performance in weaned piglets. *J. Anim. Sci.* 92, 767–774. doi: 10.2527/jas.2013-6793

Ul-Hassan, A., and Wellington, E. M. (2009). "Actinobacteria," in *Encyclopedia of microbiology*, (3rd Edition) Edn, (Amsterdam: Elsevier), 25–44.

Waite, D. W., and Taylor, M. W. (2014). Characterizing the avian gut microbiota: Membership, driving influences, and potential function. *Front. Microbiol.* 5:223. doi: 10.3389/fmicb.2014.00223

Yang, Z., Wang, Y., He, T., Ziema Bumbie, G., Wu, L., Sun, Z., et al. (2021). Effects of dietary Yucca schidigera extract and oral Candida utilis on growth performance and intestinal health of weaned piglets. *Front. Nutr.* 8:685540. doi: 10.3389/fnut.2021.685540

Yoshikawa, S., Araoka, R., Kajihara, Y., Ito, T., Miyamoto, H., and Kodama, H. (2018). Valerate production by *Megasphaera* elsdenii isolated from pig feces. *J. Biosci. Bioeng.* 125, 519–524. doi: 10.1016/j.jbiosc.2017.12.016

Yousefi, S., Monsef Shokri, M., Allaf Noveirian, H., and Hoseinifar, S. H. (2020). Effects of dietary yeast cell wall on biochemical indices, serum and skin mucus immune responses, oxidative status and resistance against *Aeromonas hydrophila* in juvenile Persian sturgeon (*Acipenser persicus*). *Fish Shellfish Immunol.* 106, 464–472. doi: 10.1016/j.fsi.2020.08.007

Zhang, W., Ma, C., Xie, P., Zhu, Q., Wang, X., Yin, Y., et al. (2019). Gut microbiota of newborn piglets with intrauterine growth restriction have lower diversity and different taxonomic abundances. *J. Appl. Microbiol.* 127, 354–369. doi: 10.1111/jam.14304

Zorriehzahra, M. J., Delshad, S. T., Adel, M., Tiwari, R., Karthik, K., Dhama, K., et al. (2016). Probiotics as beneficial microbes in aquaculture: An update on their multiple modes of action: A review. *Vet. Q.* 36, 228–241. doi: 10.1080/01652176.2016.1172132

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# Association between rumen microbiota and marbling grade in Hu sheep

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The marbling fat regulates the flavor of mutton and measures the fat density in the loin eye and is the most important parameter of carcass grading. The objective of this study was to explore the relationship of rumen microbiota and mutton marbling grade. One hundred and eighty-seven feedlot-finished Hu male lambs (Age: 180 day; Final BW: 46.32 ± 6.03 kg) were slaughtered, and ruminal contents and marbling grade were collected. Ruminal microbial DNA extraction and 16S rRNA gene sequencing was performed to investigate microbial composition and to predict microbial metabolic pathways. The animal cohort was then grouped based on marbling grades [low marbling (LM), marbling grade $\leq$ 1; Medium marbling (MM), 1 $<$ marbling grade $\leq$ 3; High Marbling (HM), 3 $<$ marbling grade $\leq$ 5] and intramuscular fat-associated microorganisms were pinpointed using LEfSe and random forest classification model. Intramuscular fat content had significantly differences among the three groups ($P < 0.05$), and was significantly correlated with VFAs profiling. HM sheep showed a higher abundance of one bacterial taxon (*Kandleria*), and two taxa were overrepresented in the MM sheep (*Pseudobutyrivibrio* and *Monoglobus*), respectively. In addition, the main intramuscular fat deposition pathway was found to involve peroxisome proliferator-activated receptor (PPAR) fatty acid synthesis. By studying the effect of the ruminal microbiome on the marbling of sheep, the present study provides insights into the production of high-quality mutton.

KEYWORDS

**16S rRNA, Hu sheep, marbling score, rumen microbiota, muscle chemical composition**

## Introduction

China has rich sheep breed resources, where its mutton production ranks first in the world. Mutton possesses an unique flavor that is linked to the intramuscular fat or marbling fat (Li et al., 2020). Marbling is defined as the appearance of visible white flecks or streaks in intramuscular fat (IMF), which can be detected visually between the bundles of muscle (Nguyen et al., 2021). In the beef industry, the marbling grade is an important index to evaluate the quality of beef. Marbling can be assessed by ultrasound imaging or be visually evaluated by an expert (Nguyen et al., 2017; Harris et al., 2018). Marbling affects the color, flavor, tenderness and juiciness of meat (Stewart et al., 2021). Therefore, a higher marbling grade can obtain greater economic benefits. Particularly in Korea and Japan, producers have attempted to increase IMF to enhance the economic value of beef (Sasaki et al., 2009; Kim et al., 2020). Experts use genetic methods to screen related genes and single nucleotide polymorphisms (SNP)(Sasaki et al., 2009; Kwon et al., 2016). In addition, various nutritional methods are also used, such as oil supplements, vitamin A supplementation and high-grain diet feeding (Schmid et al., 2006; Gorocica-Buenfil et al., 2007; Pickworth et al., 2012; Kim et al., 2020). Previous studies have shown that the formation of marbling is affected by many factors, such as genetic, sexual, nutritional and management factors (Nguyen et al., 2021). Fat infiltration within skeletal muscle is known as IMF, which increases with aging (Yoshiko et al., 2017). The marbling is accumulated through IMF cell hyperplasia and hypertrophy (Harris et al., 2018). Fibro/adipogenic progenitor cells (FAPs) are the main reservoir of IMF cells, and IMF cells primarily develop during the fetal and neonatal stages (Harris et al., 2018; Costa et al., 2021). Research has shown that IMF favors the use of glucose for fat synthesis at early ages (Smith and Crouse, 1984). During later phases of growth, IMF cells may continue to grow and use acetate as a carbon source (Nguyen et al., 2021). In addition, recent research has shown that propionic acid is also involved in fat formation (Zhang Y. et al., 2021). As rumen volatile fatty acids (VFAs) can provide a carbon source for the formation of adipose tissue (IMF), the change of rumen microbiota may be related to the formation of marbling. In addition, several studies have shown that rumen biohydrogenating bacteria, including *Prevotellaceae*, *Lachnospiraceae* and *Rikenellaceae,* provide VFAs (acetic, propionic and butyric acids), which can be positively or negatively affect fat deposition (Zhang Y. et al., 2021). Biohydrogenating bacteria *Butyrivibrio* spp., *Megasphaera elsdenii* and *Propionibacterium* spp. have related fatty acid metabolism, which can affect adipocyte differentiation (Kim et al., 2020).

Many studies have investigated the contribution of rumen microorganisms to growth factors and immunity (Berlutti et al., 2011; Ban and Guan, 2021) and the potential effect of bovine rumen microbiota on marbling (Kim et al., 2020). However, research on this subject, the potential effect of rumen microbiota on marbling, has mainly focused on cattle, with few studies being carried out on sheep. Thus, it is important to reveal the characteristics and functions of the Hu sheep rumen microbiota on marbling. This study aims to research the potential effect of the rumen microbiome on IMF deposition or marbling grade in sheep, which is of great significance to promote the production, research and maximally processed of high-quality mutton in China.

## Materials and methods

### Ethics statement

All experiments in this study were ratified by the Animal Welfare and Ethics Committee of Gansu Agricultural University and implemented in compliance with the Food and Drug Supervision and Administration Regulations of the People's Republic of China (permit number for conducting animal experiments: NO. 2012-2-159).

### Animals

In present study, 187 Hu sheep from Gansu Wuwei Minqin Defu Agriculture Co., Ltd. (Minqin, China). All sheep were fed in a single pen (0.8 × 1.5 × 1.0 m) and provided with adequate pellet feed and water. Commodity feed was purchased from Gansu Sanyangjinyuan Husbandry Co., Ltd., and the dietary information is listed **Supplementary Table 1**. The acclimation period was 14 days, the pretest period was 10 days and the trial period was 100 days. The Hu sheep included were slaughtered before morning feeding at 6 months of age, with an average weight of 46.32 kg. All sheep were slaughtered under the supervision of a qualified veterinarian (**Supplementary Table 7**).

### Marbling grade and muscle chemical composition determination

Visual marbling grades were estimated according to the marbling scoring standard formulated by the agricultural industry standard of the People's Republic of China (NY/T 630—2002 *Lamb and mutton evaluation and grading*).

After slaughter, the longissimus dorsi of the Hu sheep were obtained, placed at 4°C for 12 h, and then stored at –20°C to test the muscle chemical composition. Meat samples were scanned by reflectance spectroscopy (NIRS) using a FoodScan2 Near-Infrared Meat Fast Analyzer (Foss Science Technology and Trading Co., Ltd., Beijing, China). Samples were scanned twice in duplicate re-packing (resulting in six spectra per sample).

Experimental animals were grouped which based on marbling grade. three marbling grade groups are as follows: low marbling (LM) grade $\leq 1$; MM, $1 <$ medium marbling (MM) grade $\leq 3$; HM, $3 <$ high marbling (HM) grade $\leq 5$.

## Microbiological and volatile fatty acids analyses

### 16S rRNA gene sequencing

After slaughter, the rumen contents were collected from all sheep. Rumen fluid was obtained by four layers of cheesecloth filtration and stored at −80° for subsequent microbiological and VFAs analysis. Microbiological DNA extraction was performed using an EasyPure Stool Genomic DNA Kit (EE301-01; TransGen Biotech, Beijing, China), according to the manufacturer's guidelines. The 16S rRNA V3-V4 regions were amplified using the primers: 341F: 5′-CCTAYGGGRBGCASCAG-3′ and 806R: 5′-GGACTACNNGGGTATCTAAT-3′. The PCR amplification system has been described previously (Zhang Y. K. et al., 2021). A TruSeq® DNA PCR-Free Sample Preparation Kit (Illumina, USA) was used to obtain the sequence libraries (Schloss et al., 2009). The qualified amplicon libraries were sequenced by using paired-end sequencing on the Illumina NovaSeq PE250 platform. FLASH[1] was used to splice and assemble reads. Fastqc[2] was used for quality filtering. The detection and removal of chimeras were performed by UCHIME.[3]

### Taxonomic and diversity analyses

The available plugins within QIIME2 were used to be taxonomic, such as the DADA2 plugin was used to inspect the quality of the sequence and denoise the reads. Then, the abundance table of amplicon sequencing variant (ASV) was determined. The classify-sklearn plugin was used to train the feature classifier. The naïve Bayesian taxonomic classifier was used to annotate the ASV taxonomy. Alpha Diversity Profiling, Abundance Profiling, Interactive Pie Chart analysis, linear discriminant analysis (LDA) effect size (LEfSe) and random forest analysis were formed using the MicrobiomeAnalyst platform.[4] LEfSe analysis was used to identify microbial biomarkers, using false discovery rate (FDR) values of 0.05 and a LDA threshold score of 2. Tax4Fun software was used to analyze the functional enrichment of bacterial communities. Kyoto Encyclopedia of Genes and Genomes (KEGG) was used to predict microbial function.

1 http://ccb.jhu.edu/software/FLASH/

2 https://www.bioinformatics.babraham.ac.uk/projects/fastqc/

3 http://www.drive5.com/usearch/manual/uchime_algo.html

4 https://www.microbiomeanalyst.ca/MicrobiomeAnalyst/home.xhtml

### Volatile fatty acid analysis

We followed previous research methods when treating rumen liquid (Zhang Y. et al., 2021). VFAs were detected by gas chromatography (ThermoFisher Scientific, Shanghai, China), where 1 μL Samples were injected into a DB-FFAP capillary column (15 m × 0.32 mm × 0.25 μm). The samples were run at a split ratio of 50: 1, with a column temperature of 50–220°C (heating rate = 10°C/min). The injector and detector temperatures were both at 240°C. Peak integration was performed using Chromeleon® Software.

## RNA extraction and real-time fluorescence quantification

The mRNA levels of genes in sheep rumen tissue were quantified for high-marbling grade and low-marbling grade groups by using real-time fluorescence quantification (RT-qPCR). Primers were designed by Oligo 7.0 software (**Table 1**), total RNA extraction was performed using TransZol (TransGen Biotech, Beijing, China). RT-PCR was performed using a cDNA Synthesis Kit (Yeasen Biotechnology, Shanghai, China), according to the manufacturer's instructions. RT-qPCR was performed using a Roche LightCycler 480 (Roche Applied Science) and SYBR Green assay (Yeasen Biotechnology, Shanghai, China). The reaction volume was 20 μL, containing 6.4 μL of sterile water, 2 μL of cDNA (100 ng/μL), 0.8 μL of each primer and 10 μL of 2 μL qPCR SYBR Green Master Mix (Yeasen Biotechnology, Shanghai, China). The qPCR conditions were as described in our previous study (Wang et al., 2021). Each gene underwent four technical replicates. The gene expression levels were normalized to that of *ACTB* to determine relative gene expression by using the $2^{-\Delta\Delta Ct}$ value method (Zhang et al., 2019).

## Statistical analysis

For data related to phenotypes, descriptive statistics (means, SD). The comparison of groups was conducted using SPSS 26.0 software (SPSS, Chicago, IL).[5] One Way ANOVA and Least—Significant Difference was used to find significant differences among the group. Spearman's correlation was used to perform correlation analysis by R software. Linear regression fit was performed to determine relations between marbling and fat data sets (*lm4* in R). And the Linear regression fit model is as follows:

$$y_{ijk} = \mu + m_i + b_j + n_k + e_{ijk}$$

$y_{ijk}$ is the IMF content, $\mu$ is the average value, $m_i$ is the marbling grade ($i$ = 0,1,2,3,4,5), $b_j$ is the birthplace ($j$ = PD, RL,

5 www.ibm.com/software/analytics/SPSS

TABLE 1 Primer pairs designed for target genes.

| Primer name | Primer sequence (5′-3′) | GenBank accession number | Annealing temperature (°C) | Size (bp) |
|---|---|---|---|---|
| *ACADL*-F | ATTGGCAAGATCCATAAGTGA | XM_027965150 | 51 | 183 |
| *ACADL*-R | TACTAAAATGATGCTGGCAGT | | | |
| *ALDH2*-F | AGGAGATCAGCCACAAGACCA | XM_004017409 | 58 | 180 |
| *ALDH2*-R | CACTCTGAAAGCCAGTAGCAGT | | | |
| *FGFRL1*-F | CTGAGAGTAAGATGCCTTCCAC | XM_027971341 | 58 | 131 |
| *FGFRL1*-R | TCCAAAGGCATTACATGGTGA | | | |
| *NHE1*-F | GACCCCTTGCTACCTATGTCC | XM_004005085 | 60 | 138 |
| *NHE1*-R | CACCACAAGCAACGACGGAA | | | |
| *PCCA*-F | CCGTGAAGCATGTTCCTCA | XM_015098256 | 57 | 128 |
| *PCCA*-R | CGCAATTTCTCCTCTATTAGCAA | | | |
| *PLCB1*-F | ACTTTGAATTTACTGTTCCGCTT | XM_042230393 | 58 | 219 |
| *PLCB1*-R | TGGAAGAAACATCATGCCACA | | | |
| *PPARD*-F | TGGCAAAATTCTTCCCTCTGGT | XM_042237247 | 60 | 112 |
| *PPARD*-R | AGAAGAGCTGCATTCCTCAGT | | | |
| *SIX1*-F | ACGCGCATAACCCTTACCCCTC | NM_001174113 | 63 | 144 |
| *SIX1*-R | CGGTGTTCTCCCTTTCCTTGGC | | | |
| *ACTB*-F | TCCGTGACATCAAGGAGAAGC | NM_001009784 | 58 | 267 |
| *ACTB*-R | CCGTGTTGGCGTAGAGGT | | | |

YS, ZS), $n_k$ is the time ($k$ = 2019, 2020), and $e_{ijk}$ was the residual with a distribution assumption N(0, $\sigma^2$).

# Results

## Marbling grade

Based on the marbling scoring standard formulated by the agricultural industry standard of the People's Republic of China, members of our team optimized and specified the marbling scoring standard for mutton (shown in Figure 1). 187 Hu sheep were divided into 3 groups based on marbling grade (LM, marbling grade $\leq$ 1; MM, 1 $<$ marbling grade $\leq$ 3; HM, 3 $<$ marbling grade $\leq$ 5), the HM, MM, LM sample sizes were 27, 101, 60, respectively; the average initial body weight were 20.91, 18.89, 17.20 kg, respectively; the average final body weight were 49.17, 46.1, 43.7 kg, respectively (shown in Supplementary Table 7).

## The different analysis with the muscle chemical composition among the marbling grade groups

The difference of the muscle chemical composition among the marbling grade groups were showed that the fat of HM was significantly higher than LM and MM ($P < 0.05$; Table 2), indicating that it is possible for marbling grades to predict IMF content in sheep. While, there are no significant differences in moisture, salt, protein and collagen among groups.

## Comparing the major classified taxa and analyzing the differential rumen microbiota

This study obtained 19,449,515 raw reads by 16S rDNA sequencing. After checking for chimeras and picking ASVs, obtained 10,808,820 clean reads with successful retention with an average of 57,801 clean reads per sample. In the rumen, 37 bacterial phyla, 102 bacterial classes, 238 bacterial orders, 412 bacterial families, 893 bacterial genera and 1,324 bacterial species were identified. Figure 2 shows the composition of the rumen microbiota in different level. The results showed that the top 7 sequences were from *Bacteroidota* (42%), *Firmicutes* (42%), *Spirochaetota* (7%), *Fibrobacterota* (7%), *Proteobacteria* (2%), *Euryarchaeota* (1%), *Actinobacteriota* (1%) *Actinobacteriota* (1%), and *Patescibacteria* (0%), at the phylum level, as shown in Figure 2A. at the phylum level, among the major classified taxa *Patescibacteria* was differentially abundant in the LM group (Supplementary Table 2). At the class level, the top 8 sequences were from *Bacteroidia* (41%), *Clostridia* (36%), *Spirochaetia* (7%), *Fibrobacteria* (7%), *Negativicutes* (3%), *Bacilli* (3%), *Gammaproteobacteria* (2%), *Methanobacteria* (1%), *Saccharimonadia* (0%) as shown in Figure 2B. At the class level, among the major classified

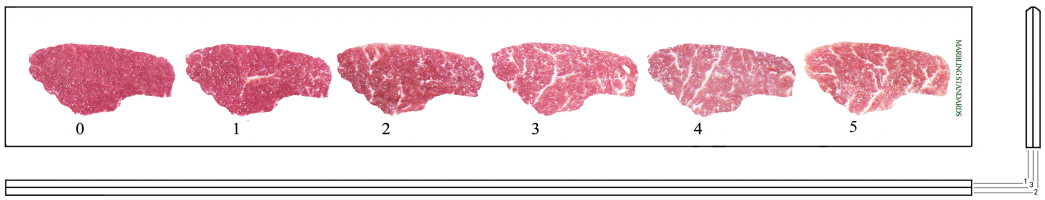


FIGURE 1
Representative images used for marble grading scale.

TABLE 2 The different analysis with the muscle chemical composition of meat and marbling grade.

| Item | Marbling grade groups | | | SEM | *P*-value |
|---|---|---|---|---|---|
| | LM | MM | HM | | |
| Fat (%) | 3.69$^{b}$ | 3.56$^{b}$ | 4.40$^{a}$ | 0.17 | 0.003 |
| Moisture (%) | 71.35 | 71.31 | 71.08 | 0.29 | 0.837 |
| Salt (%) | 0.53 | 0.49 | 0.54 | 0.03 | 0.434 |
| Protein (%) | 22.28 | 22.26 | 21.80 | 0.21 | 0.328 |
| Collagen (%) | 1.27 | 1.29 | 1.33 | 0.06 | 0.804 |

LM, marbling grade $\leq$ 1; MM, 1 $<$ marbling grade $\leq$ 3; and HM, 3 $<$ marbling grade $\leq$ 5. *P*-value showed the significant differences among the three groups by used one-way ANOVA analysis. Different letters indicate significant differences (LSD, $P < 0.05$). The same letter indicates no significant difference.

taxa *Saccharimonadia* was differentially abundant in the LM group (Supplementary Table 3). At the order level, the top 10 sequences were from *Bacteroidales* (42%), *Lachnospirales* (13%), *Oscillospirales* (10%), *Spirochaetales* (7%), *Fibrobacterales* (7%), *Clostridia* (6%), *Christensenellales* (4%), *Erysipelotrichales* (3%), *Veillonellales_Selenomonadales* (2%), *Aeromonadales* (1%), as shown in Figure 2C. At the order level, among the major classified taxa *Lachnospirales* was differentially abundant in the HM group (Supplementary Table 4). At the family level, the top 10 sequences were *Prevotellaceae* (27%), *Lachnospiraceae* (13%), *Spirochaetaceae* (7%), *Fibrobacteraceae* (7%), *Rikenellaceae* (6%), *Hungateiclostridiaceae* (6%), *F082* (6%), *Ruminococcaceae* (6%), *Christensenellaceae* (4%), *Oscillospiraceae* (4%), as shown in Figure 2D. At the family level, among the major classified taxa *Lachnospiraceae* was differentially abundant in the HM group. However, *Prevotellaceae* was differentially abundant in the MM group (Supplementary Table 5). At the genus level, the top 10 sequences were from *Prevotella* (20%), *Treponema* (7%), *Fibrobacter* (7%), *Saccharofermentans* (6%), *Rikenellaceae_RC9_gut_group* (6%), *F082* (6%), *V12* (6%), *Ruminococcus* (5%), *Christensenellaceae_R_7_group* (4%), *Lachnospiraceae_NK3A20_group* (3%), *NK4A214_group* (2%), *Clostridia_UCG-014* (1%) as shown in Figure 2E. At the genus level, among the major classified taxa *Clostridia_UCG-014* was differentially abundant in the HM group, but *NK4A214_group* was differentially abundant in the LM group (Supplementary Table 6). At the species level, the top 10 sequences were from unclassified *species* (59%), V4 (21%), *Fibrobacte* (4%), *Treponema_bryantii* (2%),*Clostridiales_bacterium* (2%), *Fibrobacter_succinogenes* (2%), *Prevotella_ruminicola* (1%), *Ruminococcus_flavefaciens* (1%), *rumen_bacterium* (1%), *bacterium_YRD2003* (1%), as shown in Figure 2F.

This study identified differential abundant bacteria composition among the three groups by using LEfSe analysis (linear discriminant analysis score $>$ 2), at the genus level. Meanwhile, 10 different microorganisms were identified when using random forest analysis. Figure 3A shows that *Kandleria, Pseudobutyrivibrio, Turicibacter* and *Monoglobus* were identified as important microbial biomarkers, where only *Kandleria* was enriched in the HM group. Figure 3B shows that probable genus 10, *Acidaminococcus*, *spiraceae ND3007 group*, *Kandleria* and *Butyrivibrio* were enriched in the HM group. *Pseudobutyrivibrio*, *Monoglobus*, *Treponema* and *Turicibacter* were enriched in the MM group. Only *Bacteroidales UCG001* was enriched in the LM group.

## Bacterial function prediction in the rumen of sheep

Rumen microbial functions were predicted by the KEGG pathways analysis. Brite_Hierarchies, Cellular_Processes, Environmental_Information_Processing, Genetic_Information_Processing, Human_Diseases, Metabolism, Not_Included_in_Pathway_or_Brite and Organismal_Systems comprises the predominant level 1 KEGG pathways. More detailed functions of microorganisms are shown in Figure 4,

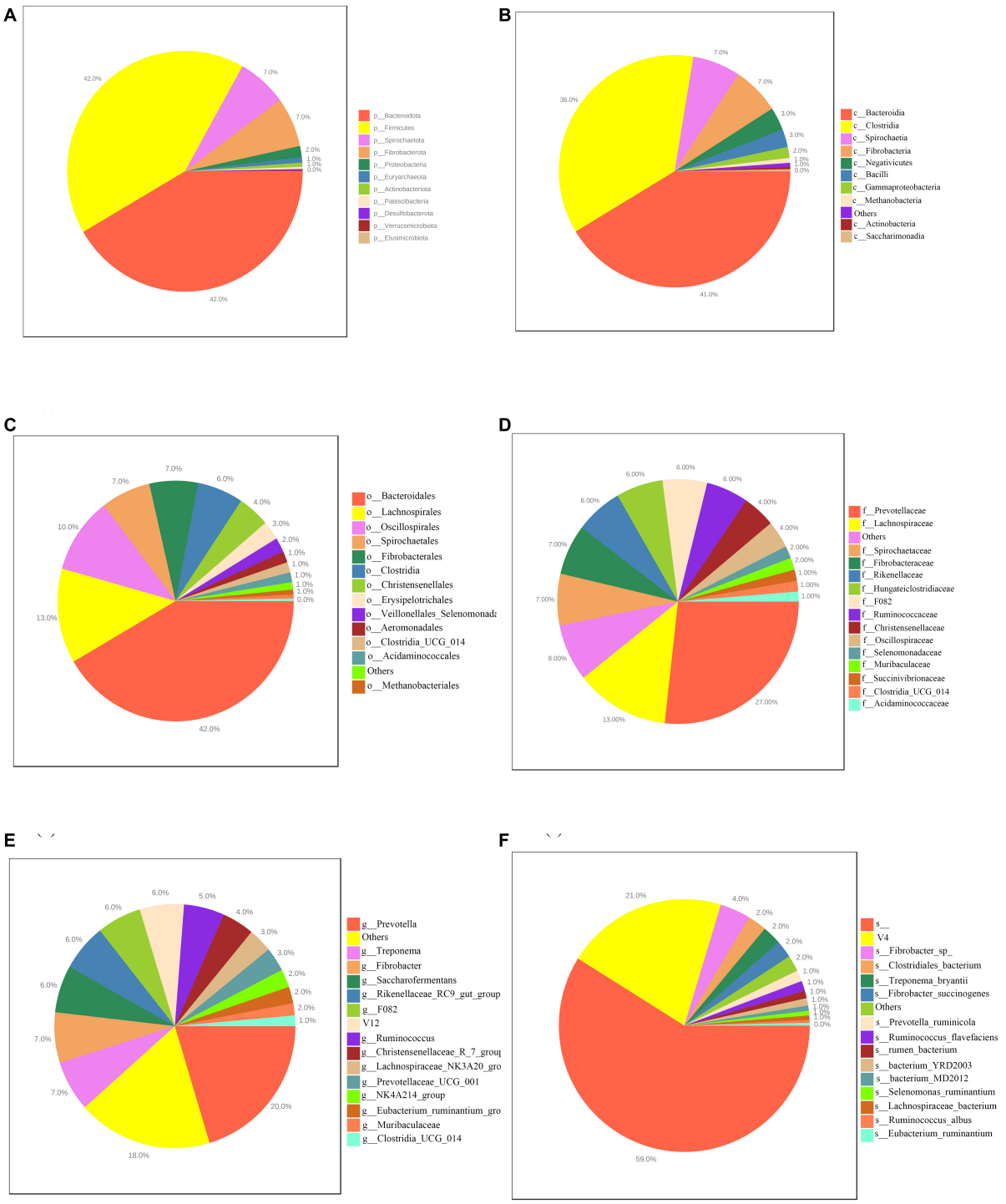


FIGURE 2
Pie charts showing the average relative abundance of the dominant bacterial phylum **(A)**, class **(B)**, order **(C)**, family **(D)**, genera **(E)** and species **(F)** in the rumen of Hu sheep.

where genetic information processing, signaling and cellular processes and metabolism in the protein families had the higher proportion, followed by carbohydrate metabolism, amino acid metabolism, energy metabolism, metabolism of cofactors, and vitamins, nucleotide metabolism, translation, and replication and repair.

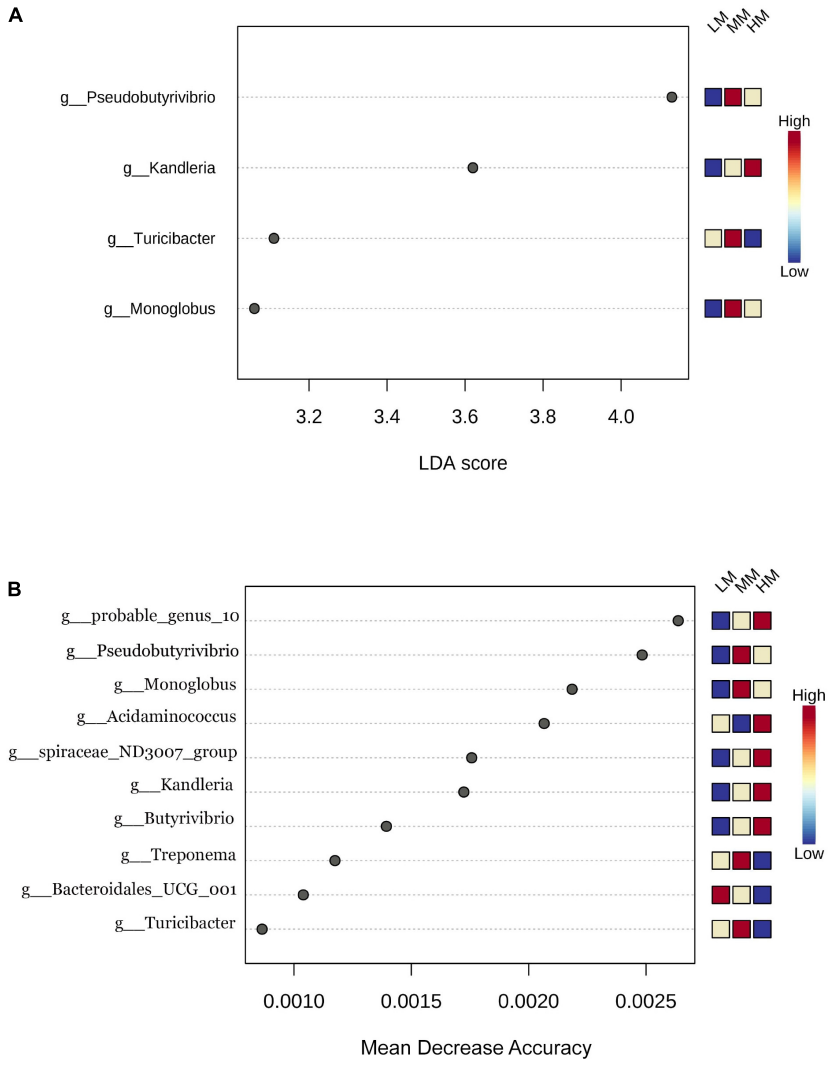


FIGURE 3
Linear discriminant analysis effect size **(A)**; differentially abundant bacterial taxa defined by LEfSe analysis) computed among low marbling grade (LM), middle marbling grade (MM) and high marbling grade (HM). (LDA > 2, FDR < 0.1) **(B)** the biomarker bacteria classes were identified by applying Random Forest regression analysis of the relative abundance of rumen bacteria.

## Potential relationship between significant biomarkers and marbling grade

There is a positive correlation between significant biomarkers of rumen digesta and marbling grade. *Pseudobutyrivibrio* have positive correlation with marbling grade, whereas *Kandleria*, *Monoglobus* and *Turicibacter* do not have a direct impact on marbling grade. *Kandleria* and *Pseudobutyrivibrio* were closely related to VFAs (Figure 5A).

The correlation between muscle chemical composition and VFAs was further studied. The results showed that there was a positive correlation between fat and butyrate, acetate, whereas propionate and isobutyrate were negatively correlated with fat. In addition, there was a negative correlation between butyrate, acetate and protein. Protein had a positive correlation with valeric acid and isovaleric acid (Figure 5B).

Finally, the correlation between rumen epithelial histomorphology and VFAs was tested. As shown in Figure 5C, there was a significant correlation between rumen morphological development and VFAs. Acetate was positively correlated with ruminal papillae length. Valeric acid and isovaleric acid were significantly negatively correlated with ruminal papillae length. Isovaleric acid, acetate and butyrate were positively correlated with ruminal papillae width. Valeric acid, propionate and isobutyrate were negatively correlated with ruminal papillae width. Isovaleric acid was positively correlated with ruminal muscle thickness.

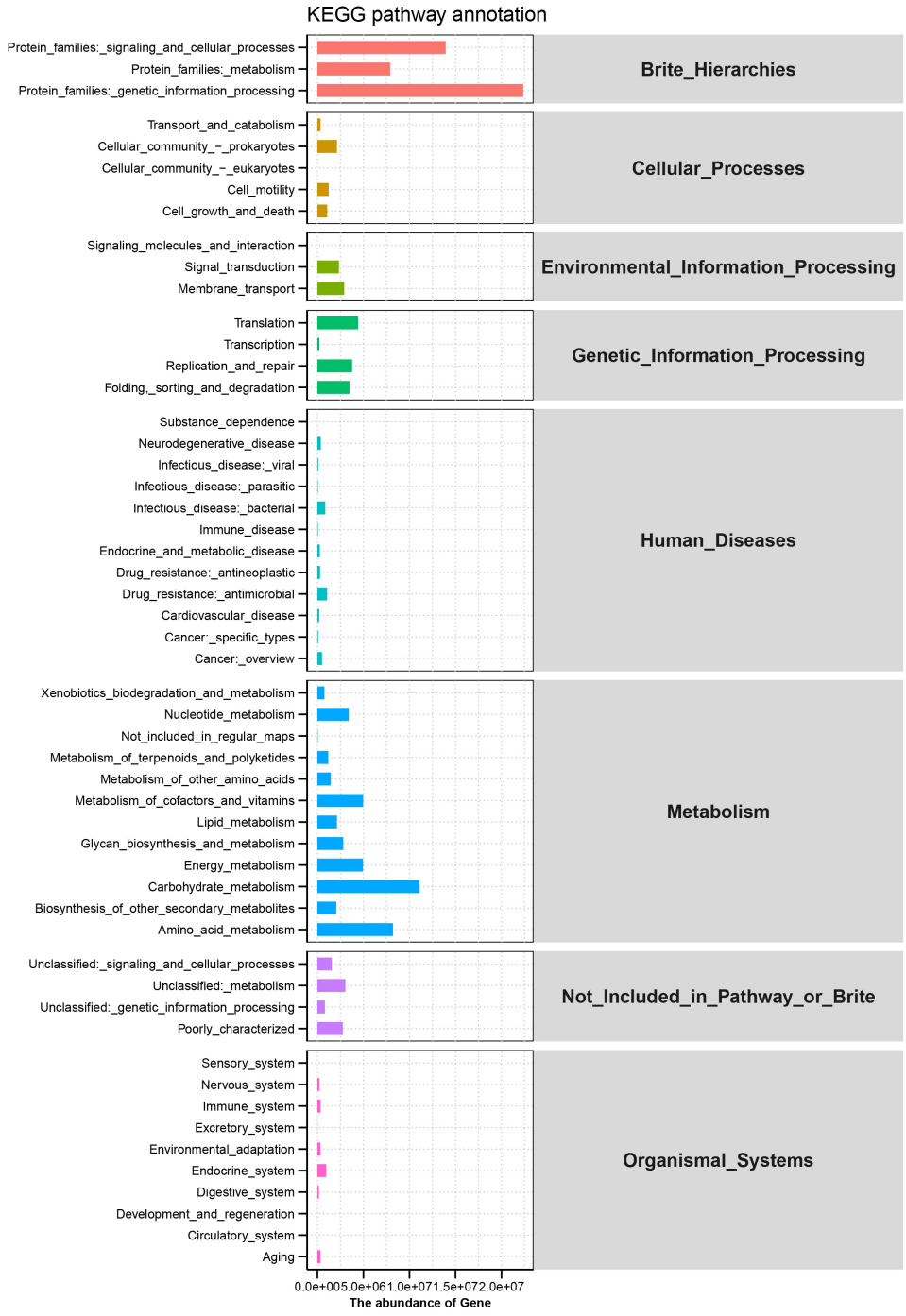


FIGURE 4
Predicted microbiome function and Kyoto Encyclopedia of Genes (KEGG) pathways in the rumen of Hu sheep.

## Validation of target genes

As shown in Figure 6, acyl-CoA dehydrogenase long chain (*ACADL*), aldehyde dehydrogenase 2 family member (*ALDH2*), fibroblast growth factor receptor like 1 (*FGFRL1*), SIX homeobox 1 (*SIX1*), phospholipase C beta 1 (*PLCB1*) expression was significantly higher in the rumen tissue of the LM group than the HM group ($P > 0.05$). There was no significant difference in the expression of solute carrier family 9 member A1 (*NHE1*) or propionyl-CoA carboxylase subunit alpha (*PCCA*) in the rumen tissue of HM and LM groups ($P > 0.05$).

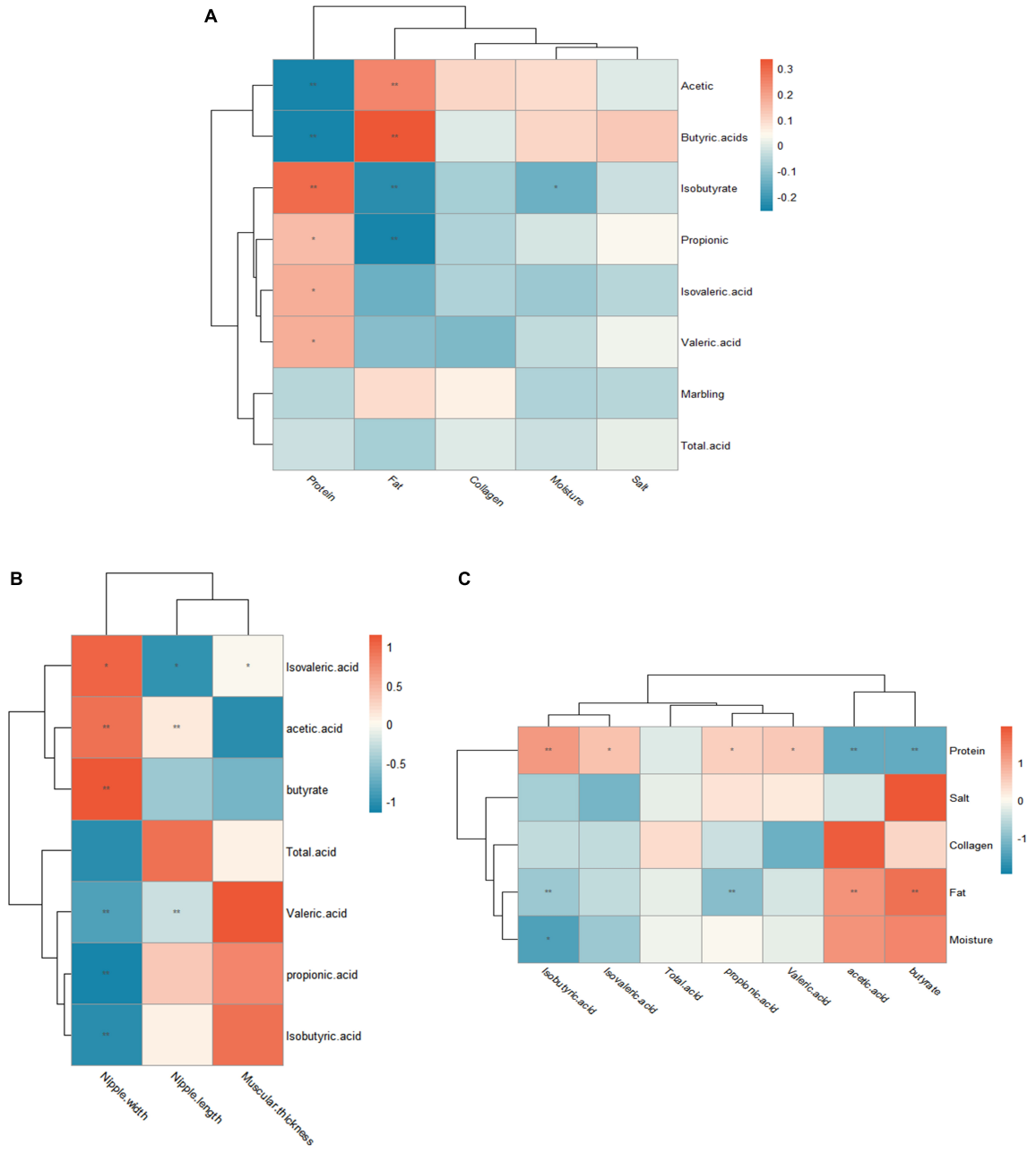


FIGURE 5
Correlation analysis between significant biomarkers and marbling grade. **(A)** Heat map showing the Spearman's correlation coefficients among significant biomarkers and marbling grade, VFA. **(B)** Heat map showing the Spearman's correlation coefficients among muscle chemical composition and VFA. **(C)** Heat map showing the Spearman's correlation coefficients between VFA and rumen epithelial histomorphology.

## Discussion

Ruminants use the VFAs and bacterial protein from rumen microorganisms to provide nutrition for themselves. Previous studies have shown that rumen microorganisms are closely related to fat deposition (Zhang Y. et al., 2021). This study found that the marbling grade was significantly associated with IMF content by the different analysis. In addition, this view was

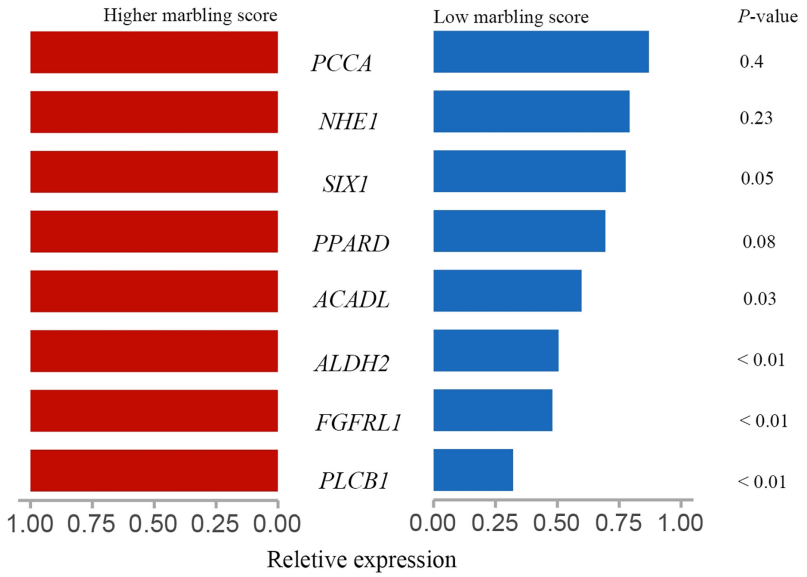


FIGURE 6
Validation of the target genes in the rumen of Hu sheep. The red indicate the relative expression of target genes in rumen tissue of high marbling grade group; blue indicate the relative expression of target genes in rumen tissue of low marbling grade group. Statistical difference was declared at $P < 0.05$ (*T*-test).

verified by linear Fitting ($R^2 = 0.1229$,$P < 0.01$) (**Supplementary Figure 1**). This study found a significant correlation between VFAs and IMF content ($P < 0.05$). Therefore, we hypothesized that rumen microorganisms have a potential relationship with marbling grade.

In beef, research has found that the observed ASVs, Chao1 estimates of the HM group were higher significantly than the LM group (Kim et al., 2020). However, in this study, Alpha-Diversity measurements showed no significant difference among the three groups (**Supplementary Figure 2**), which may be due to the species, grouping and the number of samples. In addition, other studies observed a difference in marbling grades across different residual feed intake (RFI) groups of beef cattle (Ahola et al., 2011). Previous studies have shown that the abundance of *Bacteroidetes* and *Firmicutes* is related to fat deposition (Magne et al., 2020). Our study found that *Firmicutes* were rich in the HM group, while *Bacteroidetes* were rich in the LM and MM groups (**Supplementary Table 2**). Thus, we are confident that the abundance of *Bacteroidetes* and *Firmicutes* is closely related to IMF.

Among the differentially abundant taxa listed for the HM group, *Kandleria* is considered a lactate producer, where lactate is converted to butyrate by *Megasphaera* spp. (Kumar et al., 2018). The abundance of *Kandleria* increased with the marbling grade. Previous studies have shown that the *Monolobus* is related to host immunity, where the abundance of *Monolobus* is positively correlated with CD4 + T cell counts and cytokine levels and negatively correlated with the relative numbers of regulatory T cells (Tregs) and T helper (Th17)/Treg ratio (Wang et al., 2022). Our study showed that *Monolobus* can affect IMF deposition, but further research is needed to understand how to affect fat deposition. MM-enriched *Pseudobutyrivibrio* participated in carbohydrate metabolism to produce butyrate (Pidcock et al., 2021). In addition, Correlation analysis shows that *Pseudobutyrivibrio* has made great contributions to the production of VFAs, *Kandleria* plays an important role in the production of Butyrate. Butyrate regulates energy metabolism and increases leptin gene expression (Soliman et al., 2011). Propionate inhibits appetite (Soliman et al., 2011) and is involved in hepatic gluconeogenesis and reduces the expression of enzymes participating in the *de novo* synthesis of fatty acids and cholesterol (Demigné et al., 1995; Chambers et al., 2015). Acetate was also absorbed and reached the systemic circulation and peripheral organs, stimulated the hepatic synthesis of lipids (Gao et al., 2016), and promoted the pancreas to secrete insulin and the gastric mucosa to secrete ghrelin (Perry et al., 2016). The results of our correlation analysis are consistent with the above conclusions (**Figure 2**), which suggests that butyrate and acetate promote meat with higher marbling formation, while propionate inhibits marbling formation. In summary, it is possible to increase marbling in meat by adjusting the composition of the rumen microbiota.

Prediction of microbiota function indicated that the functions of rumen microorganisms are very complex. Notably, carbohydrate metabolism and amino acid metabolism occupy an important position in the rumen. Carbohydrates are one of the carbon sources of rumen bacteria, especially *Bacteroides* and *Firmicutes*, which can disintegrate complex carbohydrates with

the help of digestive enzymes. Amino acid metabolism is one of the nitrogen sources of rumen bacteria. In addition, rumen microorganisms are also involved in lipid metabolism, but the relationship between microbial function and IMF deposition needs to be further explored.

Previous studies indicated that acetate was a substrate for fat differentiation and synthesis, and acetate enhanced the activation of peroxisome proliferator-activated receptor gamma (PPARγ) (Fu et al., 2018). Peroxisome proliferator-activated receptors (PPARs) include three subtypes: PPARα, PPARγ, and PPARβ/δ, where PPARα affects energy homeostasis (Wagner and Wagner, 2020). Some genes are down-stream targets of PPARα and participate in lipid oxidation and metabolism, such as fatty acid degradation and lipoprotein metabolism (Mao et al., 2021). PPARβ/δ regulates fatty acid metabolism (Wagner and Wagner, 2020), and PPARγ enhances fat and glucose metabolism (Wagner and Wagner, 2020). In conclusion, gene regulation plays a key role in lipid deposition.

Butyrate promotes the growth and proliferation of rumen epithelial cells (Luo et al., 2019). However, excessive butyrate can enhance the apoptosis of rumen epithelial cells (Luo et al., 2019). Previous studies have shown that *ACADL*, *ALDH2*, *FGFRL1*, *SIX1* and *PLCB1* are involved in fatty acid degradation, fatty acid metabolism and the PPAR signaling pathway (Bedford et al., 2020; Fang et al., 2021; Shi et al., 2021). In addition, *NHE1* and *PCCA* participate in butyric acid metabolism, phenylalanine metabolism and fatty acid metabolism (Fu et al., 2022; Zhen et al., 2022). Our research found that acetate was used to form IMF lipid by the PPAR signaling pathway. Butyrate and propionate may mainly contribute to the development of the rumen.

## Conclusion

The current study predicted the potential effect of the rumen microbiota to the marbling of lamb. In addition, our research further showed that microbial metabolites (VFAs) regulate IMF deposition through the PPAR signaling pathway and fatty acid metabolism through correlation analysis and qPCR verification. The preliminary study shows that it is possible to develop a strategy for regulating the marbling of meat by microorganisms. However, fat deposition is a complex metabolic process, where host genetic and cell experiments need to be performed to further explore the relationship between IMF deposition and marbling grade.

## Data availability statement

The data presented in the study is deposited in the Sequence Read Archive of NCBI, accession number PRJNA867677.

## Ethics statement

All experiments in this study were ratified by the Animal Welfare and Ethics Committee of Gansu Agricultural University and implemented in compliance with the Food and Drug Supervision and Administration Regulations of the People's Republic of China (permit number for conducting animal experiments: No. 2012-2-159).

## Author contributions

WW and FL conceived and designed the study. XXZ and XJW revised the manuscript. JW wrote the manuscript. LZ, DX, JC, WL, CL, XY, XWZ, and RZ participated in RNA and DNA extraction. DZ, XL, YKZ, and YZ contributed to the feeding experiment and sample collection. PC, JL, and ZM participated in the measurement of VFA and muscle chemical composition. 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/fmicb.2022.978263/full#supplementary-material

# References


Ahola, J. K., Skow, T. A., Hunt, C. W., and Hill, R. A. (2011). Relationship between residual feed intake and end product palatability in longissimus steaks from steers sired by Angus bulls divergent for intramuscular fat expected progeny difference. *Prof. Anim. Sci.* 27, 109–115.

Ban, Y., and Guan, L. L. (2021). Implication and challenges of direct-fed microbial supplementation to improve ruminant production and health. *J. Anim. Sci. Biotechnol.* 12:109. doi: 10.1186/s40104-021-00630-x

Bedford, A., Beckett, L., Harthan, L., Wang, C., Jiang, N., Schramm, H., et al. (2020). Ruminal volatile fatty acid absorption is affected by elevated ambient temperature. *Sci. Rep.* 10:13092. doi: 10.1038/s41598-020-69915-x

Berlutti, F., Pantanella, F., Natalizi, T., Frioni, A., Paesano, R., Polimeni, A., et al. (2011). Antiviral properties of lactoferrin–a natural immunity molecule. *Molecules* 16, 6992–7018. doi: 10.3390/molecules16086992

Chambers, E. S., Viardot, A., Psichas, A., Morrison, D. J., Murphy, K. G., Zac-Varghese, S. E., et al. (2015). Effects of targeted delivery of propionate to the human colon on appetite regulation, body weight maintenance and adiposity in overweight adults. *Gut* 64, 1744–1754. doi: 10.1136/gutjnl-2014-307913

Costa, T. C., Gionbelli, M. P., and Duarte, M. S. (2021). Fetal programming in ruminant animals: Understanding the skeletal muscle development to improve meat quality. *Anim. Front.* 11, 66–73. doi: 10.1093/af/vfab061

Demigné, C., Morand, C., Levrat, M. A., Besson, C., Moundras, C., and Rémésy, C. (1995). Effect of propionate on fatty acid and cholesterol synthesis and on acetate metabolism in isolated rat hepatocytes. *Br. J. Nutr.* 74, 209–219. doi: 10.1079/bjn19950124

Fang, C., Guo, F., Zhao, X., Zhang, Z., Lu, J., Pan, H., et al. (2021). Biological mechanisms of growth performance and meat quality in porcine muscle tissue. *Anim. Biotechnol.* 1–9. [Epub ahead of print]. doi: 10.1080/10495398.2021.1886939

Fu, C., Liu, L., and Li, F. (2018). Acetate alters the process of lipid metabolism in rabbits. *Animal* 12, 1895–1902. doi: 10.1017/s1751731117003275

Fu, Y., Wang, Y., Wang, X., Sun, Y., Ren, J., and Fang, B. (2022). Responses of human gut microbiota abundance and amino acid metabolism in vitro to berberine. *Food Funct.* 13, 6329–6337. doi: 10.1039/d1fo04003k

Gao, X., Lin, S. H., Ren, F., Li, J. T., Chen, J. J., Yao, C. B., et al. (2016). Acetate functions as an epigenetic metabolite to promote lipid synthesis under hypoxia. *Nat. Commun.* 7:11960. doi: 10.1038/ncomms11960

Gorocica-Buenfil, M. A., Fluharty, F. L., Bohn, T., Schwartz, S. J., and Loerch, S. C. (2007). Effect of low vitamin A diets with high-moisture or dry corn on marbling and adipose tissue fatty acid composition of beef steers. *J. Anim. Sci.* 85, 3355–3366. doi: 10.2527/jas.2007-0172

Harris, C. L., Wang, B., Deavila, J. M., Busboom, J. R., Maquivar, M., Parish, S. M., et al. (2018). Vitamin A administration at birth promotes calf growth and intramuscular fat development in Angus beef cattle. *J. Anim. Sci. Biotechnol.* 9:55. doi: 10.1186/s40104-018-0268-7

Helrich, K., and AOAC (1991). Official methods of analysis of the association of official analytical chemist. *Anal. Chim. Acta* 242:302.

Kim, M., Park, T., Jeong, J. Y., Baek, Y., and Lee, H. J. (2020). Association between rumen microbiota and marbling score in Korean native beef cattle. *Animals* 10:712. doi: 10.3390/ani10040712

Kumar, S., Treloar, B. P., Teh, K. H., McKenzie, C. M., Henderson, G., Attwood, G. T., et al. (2018). Sharpea and Kandleria are lactic acid producing rumen bacteria that do not change their fermentation products when co-cultured with a methanogen. *Anaerobe* 54, 31–38. doi: 10.1016/j.anaerobe.2018.07.008

Kwon, A., Srikanth, K., Lee, E., Kim, S., and Chung, H. (2016). Confirmation of genotypic effects for the bovine APM1 gene on marbling in Hanwoo cattle. *J. Anim. Sci. Technol.* 58:15. doi: 10.1186/s40781-016-0096-2

Li, X., Fu, X., Yang, G., and Du, M. (2020). Review: Enhancing intramuscular fat development via targeting fibro-adipogenic progenitor cells in meat animals. *Animal* 14, 312–321. doi: 10.1017/s175173111900209x

Luo, D., Peng, Z., Yang, L., Qu, M., Xiong, X., Xu, L., et al. (2019). Niacin protects against butyrate-induced apoptosis in rumen epithelial cells. *Oxid. Med. Cell Longev.* 2019:2179738. doi: 10.1155/2019/2179738

Magne, F., Gotteland, M., Gauthier, L., Zazueta, A., Pesoa, S., Navarrete, P., et al. (2020). The firmicutes/bacteroidetes ratio: A relevant marker of gut dysbiosis in obese patients? *Nutrients* 12:1474. doi: 10.3390/nu12051474

Mao, Z., Feng, M., Li, Z., Zhou, M., Xu, L., Pan, K., et al. (2021). ETV5 regulates hepatic fatty acid metabolism through PPAR signaling pathway. *Diabetes* 70, 214–226. doi: 10.2337/db20-0619

Nguyen, D. V., Flakemore, A. R., Otto, J. R., Ives, S. W., Smith, R. W., Nichols, P. D., et al. (2017). Nutritional value and sensory characteristics of meat eating quality of Australian prime lambs supplemented with pelleted canola and flaxseed oils: Fatty acid profiles of muscle and adipose tissues. *Intern. Med. Rev.* 3, 1–21.

Nguyen, D. V., Nguyen, O. C., and Malau-Aduli, A. E. O. (2021). Main regulatory factors of marbling level in beef cattle. *Vet. Anim. Sci.* 14:100219. doi: 10.1016/j.vas.2021.100219

Perry, R. J., Peng, L., Barry, N. A., Cline, G. W., Zhang, D., Cardone, R. L., et al. (2016). Acetate mediates a microbiome-brain-β-cell axis to promote metabolic syndrome. *Nature* 534, 213–217. doi: 10.1038/nature18309

Pickworth, C. L., Loerch, S. C., and Fluharty, F. L. (2012). Restriction of vitamin A and D in beef cattle finishing diets on feedlot performance and adipose accretion. *J. Anim. Sci.* 90, 1866–1878. doi: 10.2527/jas.2010-3590

Pidcock, S. E., Skvortsov, T., Santos, F. G., Courtney, S. J., Sui-Ting, K., Creevey, C. J., et al. (2021). Phylogenetic systematics of *Butyrivibrio* and *Pseudobutyrivibrio* genomes illustrate vast taxonomic diversity, open genomes and an abundance of carbohydrate-active enzyme family isoforms. *Microb. Genom.* 7:000638. doi: 10.1099/mgen.0.000638

Sasaki, S., Yamada, T., Sukegawa, S., Miyake, T., Fujita, T., Morita, M., et al. (2009). Association of a single nucleotide polymorphism in akirin 2 gene with marbling in Japanese Black beef cattle. *BMC Res. Notes* 2:131. doi: 10.1186/1756-0500-2-131

Schloss, P. D., Westcott, S. L., Ryabin, T., Hall, J. R., Hartmann, M., Hollister, E. B., et al. (2009). Introducing mothur: Open-source, platform-independent, community-supported software for describing and comparing microbial communities. *Appl. Environ. Microbiol.* 75, 7537–7541. doi: 10.1128/aem.01541-09

Schmid, A., Collomb, M., Sieber, R., and Bee, G. (2006). Conjugated linoleic acid in meat and meat products: A review. *Meat Sci.* 73, 29–41. doi: 10.1016/j.meatsci.2005.10.010

Shi, J., Wang, X., Song, Y., Liu, T., Cheng, S., and Zhang, Q. (2021). Excavation of genes related to the mining of growth, development, and meat quality of two crossbred sheep populations based on comparative transcriptomes. *Animals* 11:1492. doi: 10.3390/ani11061492

Smith, S. B., and Crouse, J. D. (1984). Relative contributions of acetate, lactate and glucose to lipogenesis in bovine intramuscular and subcutaneous adipose tissue. *J. Nutr.* 114, 792–800. doi: 10.1093/jn/114.4.792

Soliman, M. M., Ahmed, M. M., Salah-Eldin, A. E., and Abdel-Aal, A. A. (2011). Butyrate regulates leptin expression through different signaling pathways in adipocytes. *J. Vet. Sci.* 12, 319–323. doi: 10.4142/jvs.2011.12.4.319

Stewart, S. M., Gardner, G. E., McGilchrist, P., Pethick, D. W., Polkinghorne, R., Thompson, J. M., et al. (2021). Prediction of consumer palatability in beef using visual marbling scores and chemical intramuscular fat percentage. *Meat Sci.* 181:108322. doi: 10.1016/j.meatsci.2020.108322

Wagner, N., and Wagner, K. D. (2020). The role of PPARs in disease. *Cells* 9:2367. doi: 10.3390/cells9112367

Wang, J., Zhang, X., Wang, X., Li, F., Zhang, D., Li, X., et al. (2021). Polymorphism and expression of the HMGA1 gene and association with tail fat deposition in Hu sheep. *Anim. Biotechnol.* 1–9. [Epub ahead of print]. doi: 10.1080/10495398.2021.1998093

Wang, Q., Zhang, S. X., Chang, M. J., Qiao, J., Wang, C. H., Li, X. F., et al. (2022). Characteristics of the gut microbiome and its relationship with peripheral CD4(+) T cell subpopulations and cytokines in rheumatoid arthritis. *Front. Microbiol.* 13:799602. doi: 10.3389/fmicb.2022.799602

Yoshiko, A., Hioki, M., Kanehira, N., Shimaoka, K., Koike, T., Sakakibara, H., et al. (2017). Three-dimensional comparison of intramuscular fat content between young and old adults. *BMC Med. Imaging* 17:12. doi: 10.1186/s12880-017-0185-9

Zhang, D., Zhang, X., Li, F., Li, C., La, Y., Mo, F., et al. (2019). Transcriptome analysis identifies candidate genes and pathways associated with feed efficiency in hu sheep. *Front. Genet.* 10:1183. doi: 10.3389/fgene.2019.01183

Zhang, Y. K., Zhang, X. X., Li, F. D., Li, C., Li, G. Z., Zhang, D. Y., et al. (2021). Characterization of the rumen microbiota and its relationship with residual feed intake in sheep. *Animal* 15:100161. doi: 10.1016/j.animal.2020.100161

Zhang, Y., Zhang, X., Li, F., Li, C., Zhang, D., Li, X., et al. (2021). Exploring the ruminal microbial community associated with fat deposition in lambs. *Animals* 11:3584. doi: 10.3390/ani11123584

Zhen, Y., Ge, L., Xu, Q., Hu, L., Wei, W., Huang, J., et al. (2022). Normal light-dark and short-light cycles regulate intestinal inflammation, circulating short-chain fatty acids and gut microbiota in period2 gene knockout mice. *Front. Immunol.* 13:848248. doi: 10.3389/fimmu.2022.848248

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# Effects of high concentrate rations on ruminal fermentation and microbiota of yaks

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Ruminal microflora is closely correlated with the ruminant's diet. However, information regarding the effect of high concentrate diets on rumen microflora in yaks is lacking. In the current study, 24 healthy male yaks were randomly assigned to two groups, each fed with different diets: less concentrate (LC; concentrate: coarse=40: 60) and high concentrate (HC; concentrate: coarse=80: 20) diets. Subsequently, a 21-day feeding trial was performed with the yaks, and rumen fluid samples were collected and compared using 16s rRNA sequencing. The results showed that $NH_3$-N, total VFA, acetate, butyrate, isobutyrate, and isovalerate were significantly higher in the HC group than that in the LC group ($p<0.05$), while microbial diversity and richness were significantly lower in the HC group ($p<0.05$). Principal coordinate analysis indicated that rumen microflora was significantly different in LC and HC groups ($p<0.05$). In the rumen, phyla Firmicutes and Bacteroidota were the most abundant bacteria, with Firmicutes being more abundant, and Bacteroidota being less abundant in the HC group than those found in the LC group. *Christensenellaceae_R-7_group* and *Prevotella* are the highest abundant ones at the genus level. The relative abundance of *Acetitomaculum*, *Ruminococcus*, and *Candidatus_Saccharimonas* were significantly higher in the HC group than that in the LC group ($p<0.05$), while the relative abundance of *Olsenella* was significantly lower in the HC group than in the LC group ($p<0.05$). Compared to the LC group, the relative abundance of *Prevotella*, *Ruminococcus*, and *Candidatus_Saccharimonas* was significantly higher in the HC group. The relative abundances of *Prevotella*, *Prevotellaceae_UCG-003*, *Olsenella*, *Ruminococcus*, *Acetitomaculum*, *Candidatus_Saccharimonas*, and *NK4A214_group* were correlated with ruminal fermentation parameters ($p<0.05$). Furthermore, PICRUSt 2 estimation indicated that microbial genes associated with valine, leucine, and isoleucine biosynthesis were overexpressed in the rumen microflora of yaks in the HC group ($p<0.05$). Conclusively, our results suggest that high concentrate diets affect the microflora composition and fermentation function in yak rumen. The present findings would provide new insights into the health of yaks under high concentrate feeding conditions and serve as a potent reference for the short-term fattening processes of yaks.

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KEYWORDS

high concentrate, rations, yak, rumen fermentation, rumen microorganisms

## Introduction

Yak (*Bos grunniens*)，an endemic livestock species living on the Qinghai-Tibetan Plateau (QTP), is an important means of production and livelihood for local herders (Long et al., 2018). In recent years, yak breeding has gradually developed from traditional grazing to a large-scale short-term fattening process, where high concentrate feeding is a common practice. The proportion of concentrate in the diet is an important factor affecting ruminant nutrition, and an appropriate proportion of concentrate can improve rumen microbiota (Petrir et al., 2012; Nugroho et al., 2013). When high concentrate diets are fed for a long period, the concentrate will be rapidly fermented in the rumen, producing large amounts of volatile fatty acids and lactic acid causing rumen acidosis as well as nutritional metabolic diseases. Severe metabolic diseases can induce mortality in ruminants, resulting in reduced economic efficiency (Malekkhahi et al., 2016; Nagata et al., 2018).

The rumen is the main site of digestion and metabolism in ruminants, and it contains microorganisms such as protozoa, bacteria, and fungi (Qiu et al., 2020). These microflora degrade diet fibers, produce volatile fatty acids, and use nitrogenous substances to synthesize microbial proteins that provide protein and energy to animals and are essential for the growth and reproduction of ruminant animals (Shabat et al., 2016). It is known that diet structure is an important factor affecting the composition of the rumen microbiota (Petri et al., 2013; Yáñez-Ruiz et al., 2015). The response of the dietary structure to ruminal microbiota has been a research focus in recent years. For example, the study by Fernando et al. (2010) used multiple molecular approaches to provide a more comprehensive illustration of the structure of the ruminal microbial community in beef cattle during adaptation from a high-grain diet to a forage diet. Mao et al. (2013) found that the effect of subacute rumen acidosis (SARA) adaptation on the rumen microbiota of dairy cows was studied by feeding a high-concentrate ration. However, to date, there is limited understanding of the effects of high concentrate diets on ruminal fermentation and ruminal microbiota, and their interactions. The current comprehensive analysis could provide valuable information to the short-term yak fattening industry.

We hypothesized that high concentrate feeds would affect ruminal fermentation, rumen fiber-degrading bacteria species, and rumen metabolic function of yaks, which in turn would affect the rumen health of yaks. As comprehensive analyses of the effects of high concentrate diets on yak microbiota are absent, in this study, we have used 16 s rRNA sequencing technology to analyze the effects of high concentrate rations on ruminal fermentation and rumen microbiota of yaks extensively and discussed the possible correlation of these two factors.

## Materials and methods

### Animals, diets, and experimental design

The feeding trial was conducted in September 2019 at the Lao Zhaxi breeding base, Guinan County, Qinghai Province, China. Twenty-four healthy male yaks at 3 weeks of age, with uniform health conditions (weight: 164.46 ± 31.18 kg), were randomly divided into two groups and each group was fed a full mixed ration of different diets: low concentrate (LC; concentrate: coarse = 40: 60) and high concentrate (HC; concentrate: coarse = 80: 20) diets. The diets were formulated according to the Chinese Beef Cattle Feeding Standard (NY/T815-2004), and the composition and nutrient contents of the two diets are shown in Table 1. Yaks were selected from grazing pastures. All yaks were uniformly numbered and fed alone. The rations were fed daily from 8: 00–9: 00 and 17:00–18:00 with free access to water. The

TABLE 1 Ingredients and nutritional composition of each diet.

| Ingredients (%) | Group | |
|---|---|---|
| | LC | HC |
| Oat hay | 60.00 | 20.00 |
| Corn | 3.75 | 44.09 |
| Wheat bran | 12.95 | 11.96 |
| Rapeseed meal | 11.00 | 11.92 |
| Soybean meal | 6.65 | 6.38 |
| Palm oil powder[2)] | 0.86 | 0.86 |
| Rapeseed oil | 0.80 | 0.80 |
| NaCI | 0.79 | 0.79 |
| Premix[1] | 3.20 | 3.20 |
| Nutrient composition (%) | | |
| DM | 86.18 | 85.38 |
| CP | 15.43 | 15.82 |
| EE | 4.62 | 5.46 |
| ME MJ/kg | 9.40 | 11.53 |
| NDF | 45.58 | 25.60 |
| ADF | 27.24 | 13.80 |
| Ca | 0.29 | 0.23 |
| P | 0.44 | 0.48 |

The premix provided the following per kg of the diet: VA 3500 IU，VD 1000 IU，VE 40 IU，Mn 40 mg，Fe 50 mg，Cu 10 mg，Zn 40 mg，Se 0.3 mg; others were measured values.

pretest period was 7 days, and the experimental period was 21 days.

## Sample collection and measurements

At the end of the experiment, before feeding in the morning, rumen fluid was collected using a bendable oral gastric tube with a metal filter, which was pre-cleaned by rinsing with clean warm water. The first 100 ml of rumen fluid was discarded to eliminate saliva contamination. Finally, a 50 ml rumen fluid sample from each yak was collected and filtered through four layers of gauze before measuring rumen pH with a pH meter (Model HI221, HANNA, Italy). The samples were divided into 15 ml sterile centrifuge tubes and stored in liquid nitrogen for the determination of ruminal fermentation parameters and microbiota analysis.

The filtered rumen fluid was centrifuged (at 17,000 *g* for 30 min at 4°C) to obtain the supernatant, which was further analyzed for $NH_3$-N using phenol hypochlorite analysis (Broderick and Kang, 1980). Freshly prepared metaphosphoric acid (25% w/v, 2 ml) was added to filtered rumen fluid (8 ml) and then centrifuged (at 17,000 *g* for 10 min at 4°C). Volatile Fatty Acids (VFAs) concentrations were determined using gas chromatography (GC-2014; Shimadzu Corporation, Japan) as described by Cao et al. (2008).

## 16s rRNA gene amplification and MiSeq sequencing

Microbial DNA was extracted using the CTAB (Sigma-Aldrich, Milan, Italy) method according to the instructions provided by the manufacturer. The purity and concentration of DNA were checked using 1% agarose gel electrophoresis. An appropriate amount of DNA sample was taken in a centrifuge tube and the sample was diluted to 1 ng/μL with sterile water. Specific primers with barcodes were synthesized for the V3-V4 variable region of the bacterial 16 s rRNA gene. Common primer sequences were as follows: 515F (5′-GTGCCAGCMGCCGCGG-3′) and 806R (5′-GGACTACHVGGGTWTCTAAT-3′). PCR (polymerase chain reaction) was performed using a 25 μl amplification system; 1 μl each of 5 μmol/l upstream and downstream primers and 5 ng of template DNA. Equal amounts of purified amplicons were pooled together to construct paired-end sequencing libraries, which were sequenced by Beijing Ovison Gene Technology Co., Ltd. (Beijing, China) using a platform (Mixed PE 300) according to a standard protocol.

### Sequence and rumen microflora processing

The raw sequencing data were processed and filtered for quality using Trimmomatic (Version 0.36; Bolger et al., 2014) software, and valid sequences were obtained by removing chimeras through VSearch software and species databases. In addition, sequences with ≥97% similarity were categorized as operational taxonomic units (OTUs) using UPARSE software (*Uparse* v7.0.1001; Edgar, 2013).[1] To obtain species classification information of each out, representative sequences were compared and analyzed using the RDP classifier algorithm version 2.2 (Wang, 2007) and Silvadatabase1 (Quast et al., 2012.), allowing community annotation at the kingdom, phylum, class, order, family, and genus levels. Alpha Diversity (Chao1, Shannon, PD-whole-tree, and Observed-species) was calculated by QIIME 2 (version 1.9.0), and the richness of the community was analyzed using Chao1 richness The Chao1 richness index (Chao1), the Shannon index, the coverage index of PD-whole-tree were applied to analyze the diversity of the community. The number of OTUs was analyzed using the observed-species index. Beta diversity was calculated based on the unweighted UniFracdistance and visualized by principal coordinate analysis (PCoA). Linear discriminant analysis effect sizes (LEfSe, LDA > 3) were used to identify important bacteria in both groups (Miller et al., 2016). To predict microbiota function and explore differences between the two groups, PICRUSt 2 software was used (Douglas et al., 2019).

## Statistical analysis

Independent samples t-test based on SAS (SAS, version 9.2) was applied to compare ruminal fermentation parameters between LC and HC groups, and the differences were considered statistically significant at $p < 0.05$. Microbial networks were generated using Gephi software (version 0.9.2)[2] to calculate correlations between dominant taxa. Pearson correlation coefficients between the relative abundances of rumen bacteria (genera) and ruminal fermentation parameters were calculated using the heat map package in R software (version 4.0.2). Functional prediction of rumen microflora in yaks of LC and HC groups was studied with PICRUSt 2 and differences between the two groups in levels 1, 2, and 3 of the KEGG (Kyoto Encyclopedia of Genes and Genomes) pathway were determined.

# Results

## Ruminal fermentation parameters

The effect of high concentrate diets on ruminal fermentation parameters in yaks is shown in Table 2. $NH_3$-N ($p = 0.014$), total VFA ($p < 0.001$), butyrate ($p < 0.001$), isobutyrate ($p = 0.007$), isovalerate ($p < 0.001$) were significantly higher in the HC group than those in the LC group ($p < 0.05$). The proportion of Acetate is higher in the LC group and the Propionate higher in the HC group.

1 http://drive5.com/*uparse*/

2 https://gephi.org/

## Richness, diversity estimates, and rumen bacteria composition

According to the Venn diagram, 1,352 OTUs were present in the rumen of yaks in LC and HC groups, with 448 and 138 unique OTUs, respectively (Figure 1A). According to PCoA (Figure 1B), significant differences were observed between the rumen microflora of the LC and HC groups. Alpha diversity calculations (Figure 2) showed significant differences in Chao1, Shannon, PD-whole-tree, and Observed-species between the two groups, indicating that Chao1, Shannon, PD-whole-tree, and Observed-species were significantly lower in the HC group than that in the LC group ($p < 0.05$).

TABLE 2 Effect of high concentrate diets on ruminal fermentation parameters in yaks.

| Parameters | Treatment groups | | SEM | *p*-Value |
|---|---|---|---|---|
| | LC | HC | | |
| pH | 6.42 | 6.13 | 0.06 | 0.074 |
| MCP | 2.21 | 2.85 | 0.43 | 0.273 |
| $NH_3$-N, mg/dL | 16.76$^b$ | 21.39$^a$ | 0.86 | 0.014 |
| TVFA, mM | 60.93$^b$ | 74.96$^a$ | 1.95 | <0.001 |
| VFAs, molar % of TVFA | | | | |
| Acetate | 63.53$^a$ | 61.18$^b$ | 0.59 | 0.048 |
| Propionate | 22.69 | 22.78 | 1.09 | 0.928 |
| Butyrate | 10.68$^b$ | 12.73$^a$ | 0.40 | <0.001 |
| Isobutyrate | 0.78$^b$ | 0.91$^a$ | 0.05 | 0.128 |
| Valerate | 1.52 | 1.23 | 0.11 | 0.077 |
| Isovalerate | 0.81$^b$ | 1.17$^a$ | 0.04 | <0.001 |
| Acetate: Propionate | 2.88 | 2.72 | 0.09 | 0.366 |

MCP, microbial protein; $NH_3$-N, ammonia nitrogen; TVFA, total volatile fatty acids; SEM, standard error of the mean. LC, low-concentrate group; HC, high-concentrate group.
$^{a,b}$The values in same row with differenct superscript letter differ significantly ($p < 0.05$).

## Bacterial compositions among treatment groups

We performed a taxonomic analysis of the 10 bacterial phyla identified. At the phylum level, among them, Firmicutes and Bacteroidota represented 61.55 and 30.69% of the total reads, respectively, followed by Actinobacteriota (3.65%), Patescibacteria (2.33%) and Verrucomicrobiota (0.46%; Figure 3A). The relative abundances of Patescibacteria and Firmicutes were higher in the HC group than that in the LC group, while a lower relative abundances of Bacteroidota, Actinobacteriota and Verrucomicrobiota were detected in the HC group (Figure 3B). A total of 167 genera was identified from rumen samples. *Christensenellaceae_R-7_group* (19.06%) and *Prevotella* (13.35%) were the most dominant genera, followed by *NK4A214_group* (10.17%), *Rikenellaceae_RC9_gut_group* (4.97%), *Ruminococcus* (4.30%), *Lachnospiraceae_NK3A20_group* (4.11%), *Acetitomaculum* (3.60%), *Candidatus_Saccharimonas* (2.33%), *Olsenella* (2.18%), and *Prevotellaceae_UCG-003* (1.11%; Figure 3C). Among them, the relative abundance of *Acetitomaculum*, *Ruminococcus*, and *Candidatus_Saccharimonas* were significantly higher in the HC group than that in the LC group ($p < 0.05$), whereas the relative abundance of *Olsenella* was significantly lower in the HC group than that in the LC group ($p < 0.05$; Figure 3D).

To better understand the dominance of specific bacteria in HC and LC groups, we used the LEfSe method (Figure 4).

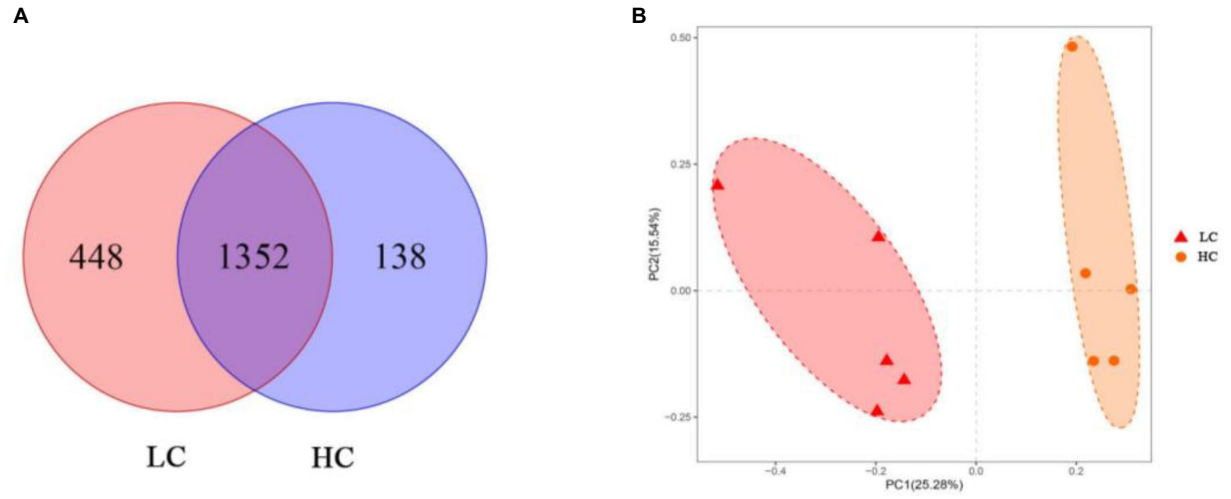


FIGURE 1
Differences in ruminal microbial communities and operational taxonomic units (OTUs) of yaks fed low concentrate (LC) and high concentrate diets (HC). Venn diagrams **(A)** show the specific and shared OTU between the two groups. Differences in rumen microflora between the two groups were calculated using weighted UniFrac distances **(B)** and coordinates were calculated using principal coordinate analysis (PCoA).

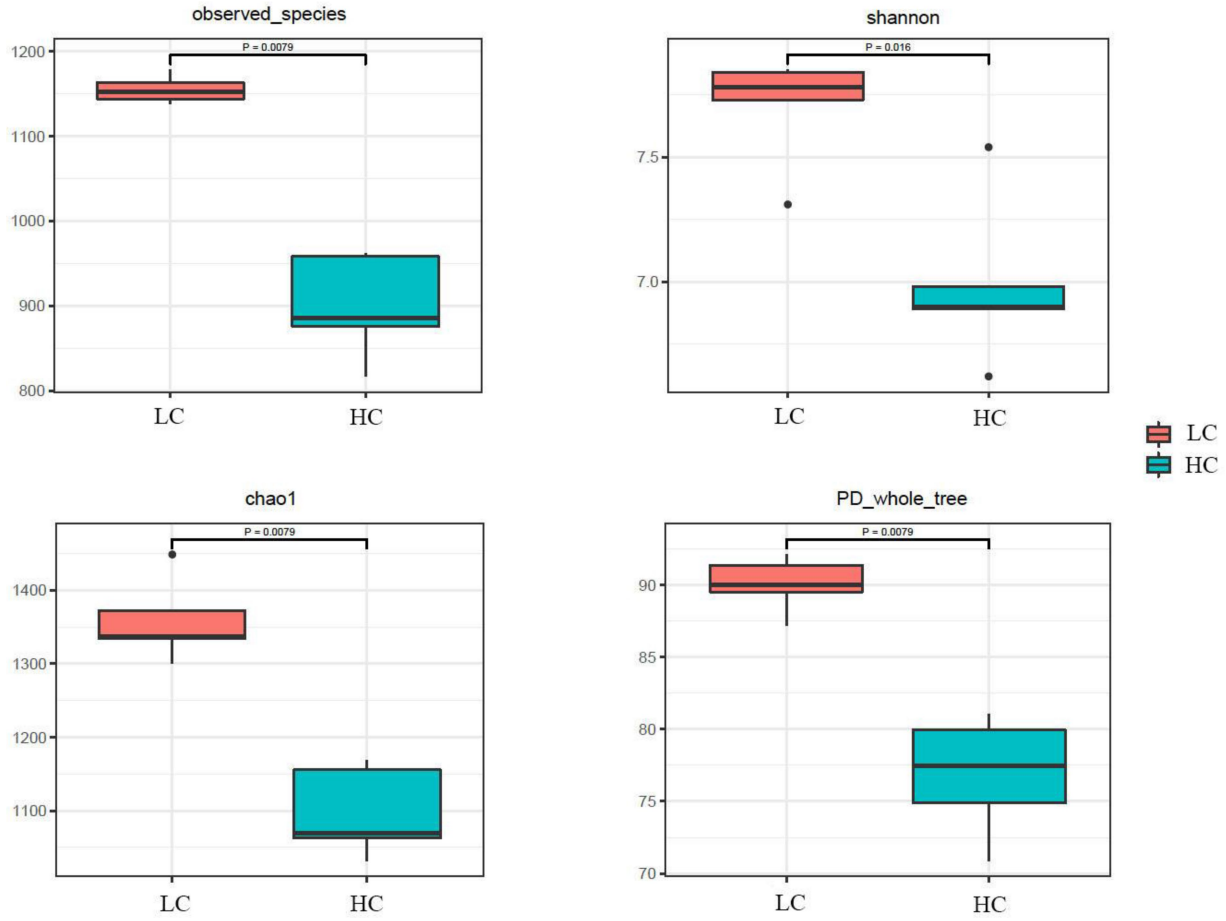


FIGURE 2
Diversity in the ruminal microbial community of yaks fed low concentrate (LC) and high concentrate diets (HC). $p<0.05$ indicates a significant difference.

Figure 4A depicts a representative cladogram of the predominant microbiome structure, showing the most remarkable differences in taxa in the two groups. The data comparing the two groups indicated that 11 clades were more abundant in the LC group and seven clades were more abundant in the HC group. Prevotellaceae, *Prevotella*, Atopobiaceae, Coriobacteriia, Coriobacteriales, *Olsenella*, *Prevotellaceae _UCG-003*, and *Prevotella_ruminicola* were abundant in the LC group. *Clostridia_UCG_014*, Patescibacteria, *Candidatus_Saccharimonas*, Saccharimonadia, Saccharimonadaceae, Saccharimonadales, *Acetitomaculum*, *Ruminococcus*, *Ruminococcaceae*, *NK4A214_group*, Oscillospirales, and *Clostridia* were in the HC group in an Overrepresentation (Figure 4B).

## Network analysis of bacterial communities

Microbial interactions between rumen bacterial communities in yaks were analyzed using microbial networks. The results showed that the high concentrate diet altered the correlation within the microbiota (Figure 5), and we verified that the negative correlation was stronger in the HC group than that observed in the LC group.

## Correlations between rumen bacteria and ruminal fermentation parameters

Correlation analyses between rumen bacteria and ruminal fermentation parameters were based on Spearman's correlation coefficients, and significantly influential rumen microflora (genus level) were significantly correlated with ruminal fermentation parameters (Figure 6). *Prevotella* was negatively correlated with $NH_3$-N and isovalerate concentrations; *Prevotellaceae_UCG-003* was negatively correlated with isovalerate; *Olsenella* was negatively correlated with Isobutyrate, isovalerate, and butyrate concentrations; *Ruminococcus* was positively correlated with Butyrate concentration; *Acetitomaculum* was positively correlated with acetate and butyrate concentrations. Positively correlation

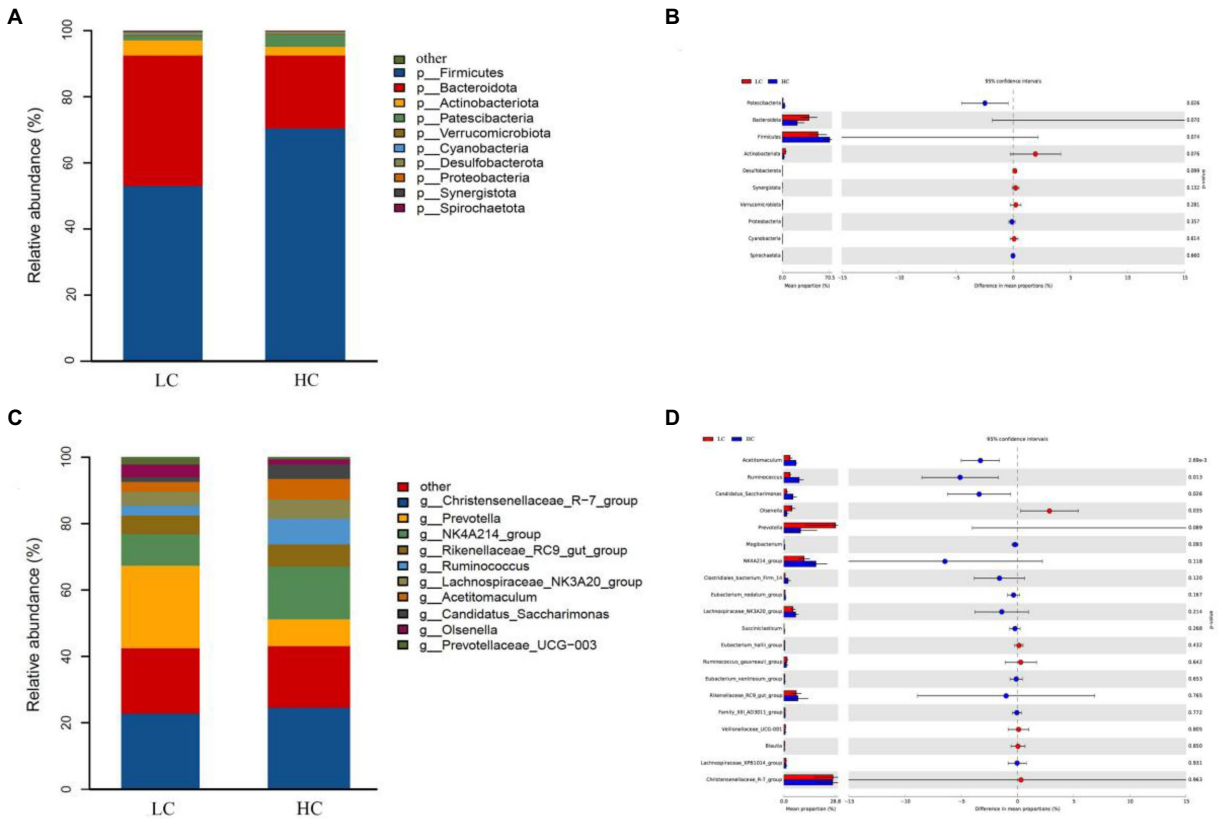


FIGURE 3
Classification of bacterial community composition in yaks fed low concentrate (LC) and high concentrate (HC) diets. **(A)** Phylum level. **(C)** Extended error bars showing the bacteria that differed between the two groups at the phylum level. **(B)** Genus level. **(D)** Extended error bars showing the two groups of bacteria that differed at the genus level.

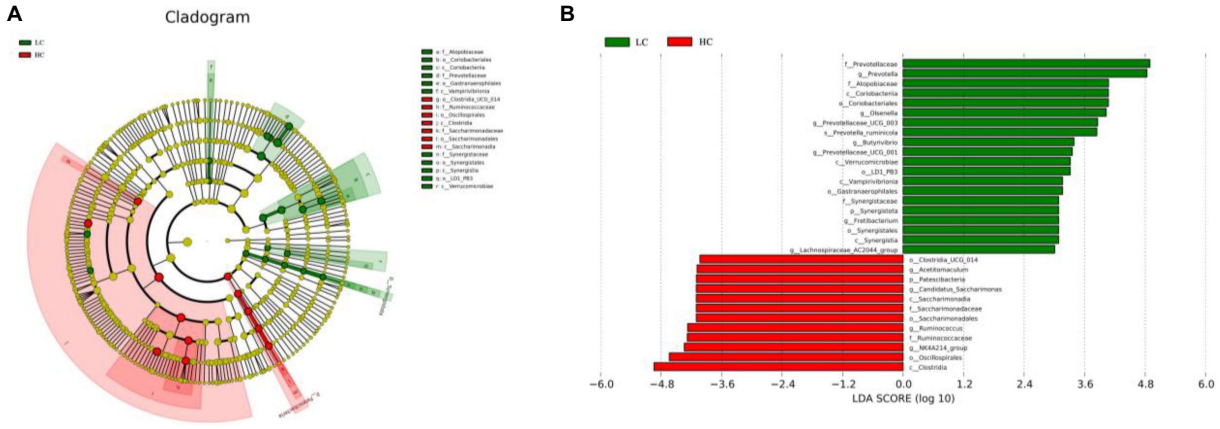


FIGURE 4
LEfSe analysis of rumen microflora of yaks fed low concentrate (LC) and high concentrate diets (HC). **(A)** Histogram of linear discriminant analysis scores based on categorical information. **(B)** Linear discriminant analysis effect size classification plot based on categorical information.

was observed for *Candidatus_Saccharimonas* with concentrations of sovalerate, MCP, propionate, acetate, and butyrate, and for *NK4A214_group* with isobutyrate, isovalerate, and acetate concentrations.

## PICRUSt2 function prediction

PICRUSt 2 gene function assessment was used to predict the function of rumen microflora in LC and HC groups of yaks. The

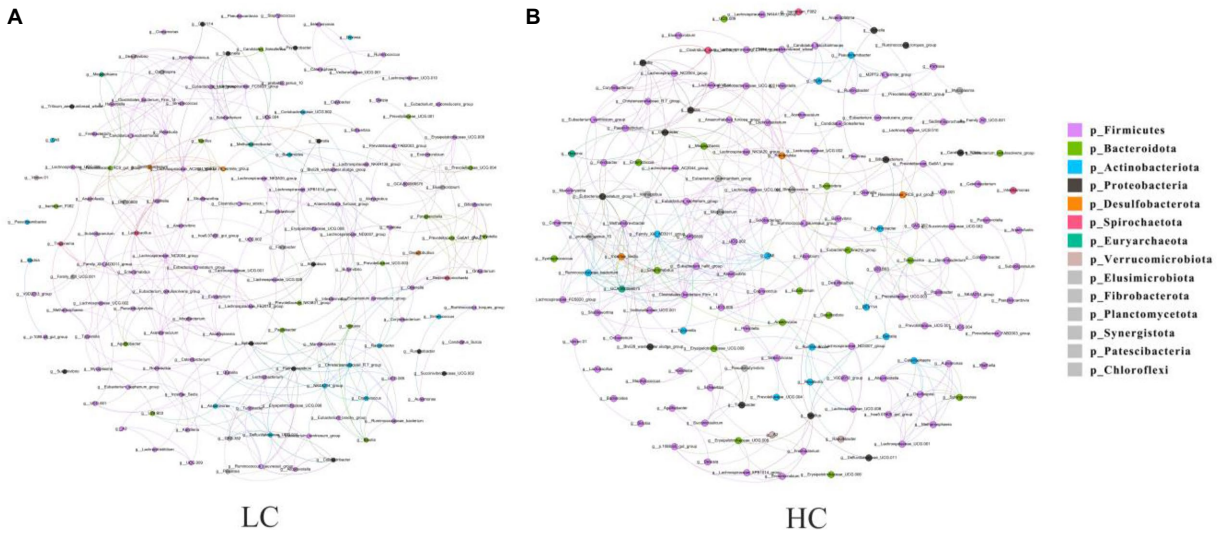


FIGURE 5
Interaction network of ruminal microflora. The ruminal microflora correlation network based on 16S rRNA genes showed statistically significant interactions with absolute values of correlation coefficients >0.6. The size of the nodes is scaled according to the abundance of each taxon in the microflora. The red line indicates a positive correlation and the green line indicates a negative correlation.

highest abundance of valine, leucine, and isoleucine biosyntheses (26.58%) was observed, followed by lysine biosynthesis (18.74%). PICRUSt 2 prediction software enriched 40 major pathways (relative abundance >1%) in the 3-level KEGG pathway, 19 of which showed significant differences between the LC and HC groups ($p < 0.05$; Figure 7). Notably, the relative abundance of metabolism of other amino acids and xenobiotics biodegradation were significantly increased in the HC group ($p < 0.05$).

## Discussion

In this study, we investigated the effects of high concentrate diet on microflora and fermentation in rumen of yaks and interactions between these two factors. A stable intra-rumen environment is particularly important for ruminants; ruminal pH, $NH_3$-N and VFA molar concentrations are important indicators of a stable intra-rumen environment and reflect the status of ruminal fermentation (Tomczak et al., 2019). A stable ruminal pH is a prerequisite for its normal function, and it is determined by volatile fatty acid and lactic acid accumulation (Tomkins et al., 2015). The present results showed that the pH decreasing tendency in the HC group. The study conducted by Ogata et al. (2019) found that ruminal pH was significantly lower in Japanese black wagyu cattle that were fed a high grain diet. Similar results were obtained in other studies (Kmicikewycz et al., 2015; Ramos et al., 2021). The reduction in pH may have resulted from the significant change in the ruminal bacterial community as a result of feeding high concentrates. The increased frequency of lactic acid producing bacteria and lactic acid utilizing bacteria in the rumen and an imbalance in the bacterial flora leads to the accumulation of lactic acid in the rumen, further reducing ruminal pH. Moreover, the number of fiber degrading bacteria also decreases drastically due to the low fiber content in the diet and the accumulation of lactic acid in the rumen (Mackie and Gilchrist, 1979; Tajima et al., 2001; Lee et al., 2019).

$NH_3$-N is endogenous ruminal nitrogen, a product of fermentation and decomposition of diet proteins, and a major component for microbial synthesis of bacteriophage proteins in the rumen (Garcia-Gonzalez et al., 2010; Thao et al., 2014; Lv et al., 2020). Carlos et al., 2021 reported that the $NH_3$-N concentration in the rumen of dairy goats that were fed high concentrate diets was significantly higher than that of the low concentrate diet group. $NH_3$-N concentrations in the current HC group were comparable to the above results and with those of Jahan et al. (2018); a large amount of nitrogenous substances in the high concentrate diet may be the possible reason for this outcome. The more nutrients in the rumen are decomposed by ruminal microorganisms to produce a larger amount of $NH_3$-N, increasing $NH_3$-N concentration and even ammonia toxicity in severe cases (Vcdsa et al. 2020).

Volatile fatty acids are derived from the fermentation of carbohydrates and proteins in the diet by rumen microorganisms and are an important source of energy for ruminants (Li et al., 2016). Acetic acid, propionic acid, and butyric acid in the rumen are the main components of volatile fatty acids (Guilloteau et al., 2010). Acetic and propionic acids enter the portal circulation and are metabolized in the liver (Minamoto et al., 2019), acetic acid is the main synthetic precursor of fat in ruminants (Hou et al., 2020), and propionic acid is converted to glucose by gluconeogenesis or enters the tricarboxylic acid cycle for oxidative energy supply (Young, 1977; Jia et al., 2018), and butyric acid is an important

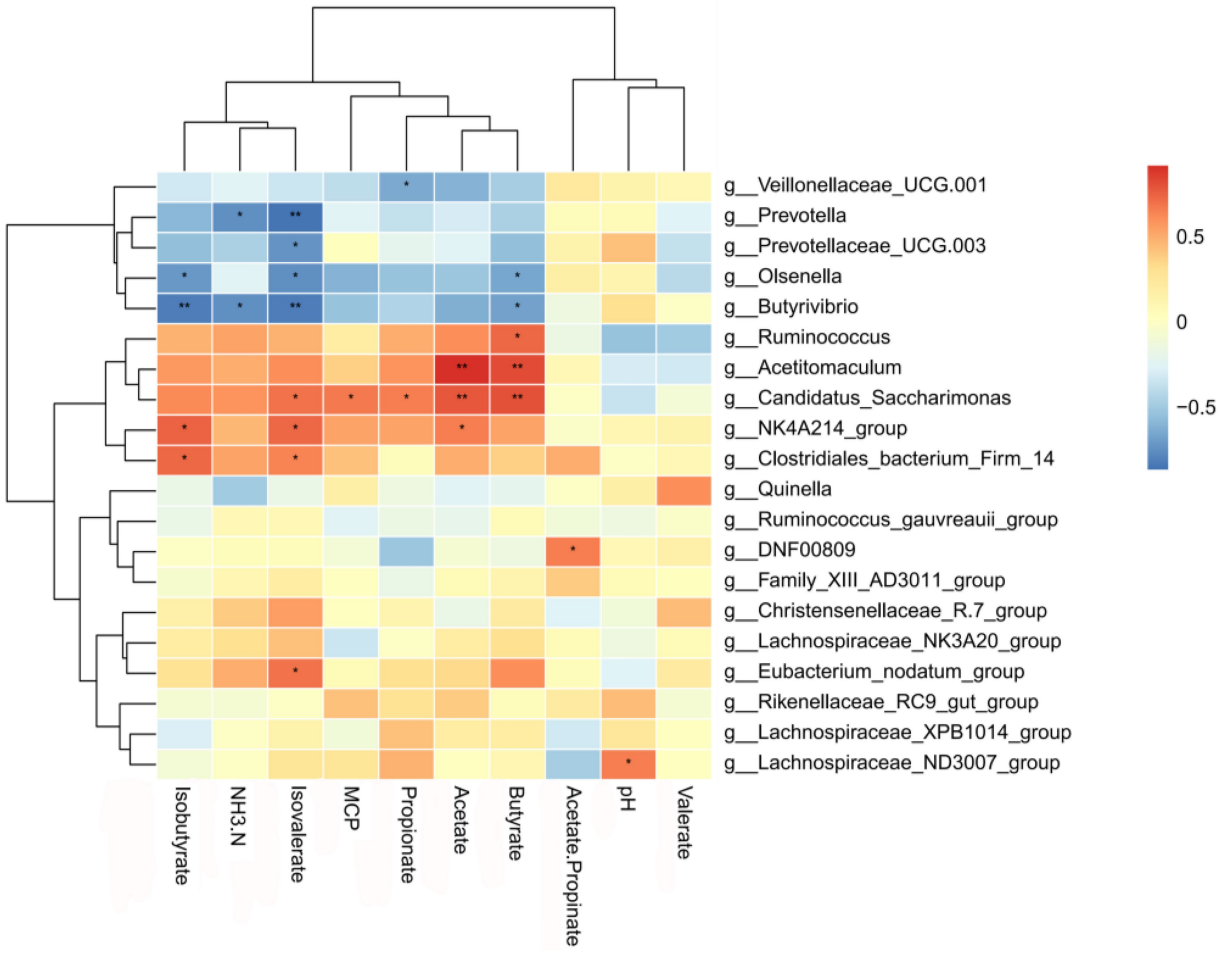


FIGURE 6
Correlation between bacteria and fermentation parameters in the rumen. Each row in the figure represents a genus, each column represents a metabolite, and each grid represents the Pearson correlation coefficient between a component and a metabolite. The red color represents a positive correlation, while the blue color represents a negative correlation. * and ** indicate significant levels of 0.05 and 0.01, respectively.

source of energy for animals (Gorka et al., 2009); in this study, the concentrations of Total VFA, Acetate, Propionate and Butyrate were higher in the HC group than in the LC group, which is similar to Bevans et al. (2005), Sato (2016), and Nagata et al. (2018) studies were similar. Due to the high percentage of concentrate in the diet, the diet enters the rumen to produce a large amount of VFA (including acetic acid, propionic acid and butyric acid) by rapid fermentation, resulting in a lower absorption rate of VFA and excessive accumulation of VFA in the rumen (Goad et al., 1998), which affects ruminal fermentation in yaks, which in turn can lead to reduced feed intake as well as digestive problems affecting the health of yaks.

We further investigated the effect of high concentrate diets on rumen microflora of yaks using 16S rDNA high-throughput sequencing technology. In the present study, both alpha and beta diversity indices of microflora were significantly different between the two groups, indicating that the diversity of rumen microflora in yaks was closely related to the diet concentrate ratio. Our results showed that intra-ruminal bacterial diversity and richness were reduced in yaks fed a high-concentrate diet, which was comparable with the results obtained by Petri et al. (2013) and Zened et al. (2013). This suggests that feeding high concentrate diets to yaks causes a decrease in rumen pH and inhibition of cellulose-degrading bacteria, leading to a decrease in the diversity and abundance of ruminal bacteria. PCoA analysis showed that rumen microbial communities aggregated according to the concentrate ratio in diets, and significant differences between intra-ruminal microbial communities were observed, which were consistent with previous reports (Hu et al., 2019; Islam et al., 2021).

In the current study, as similar to previous studies, Bacteroidota and Firmicutes were the dominant phyla among ruminal microorganisms in yaks (Zhou et al., 2017; Bi et al., 2018; Liu C. et al., 2019a; Fan et al., 2020), indicating that these bacteria play an important role in the yak rumen. Previous studies have shown that Bacteroidota is mainly responsible for energy conversion and acquisition; while Firmicutes play an important role in the degradation of non-fibrous material (Evans et al., 2011; Reigstad and Kashyap, 2013; Ya-Bing et al., 2016). Among the two

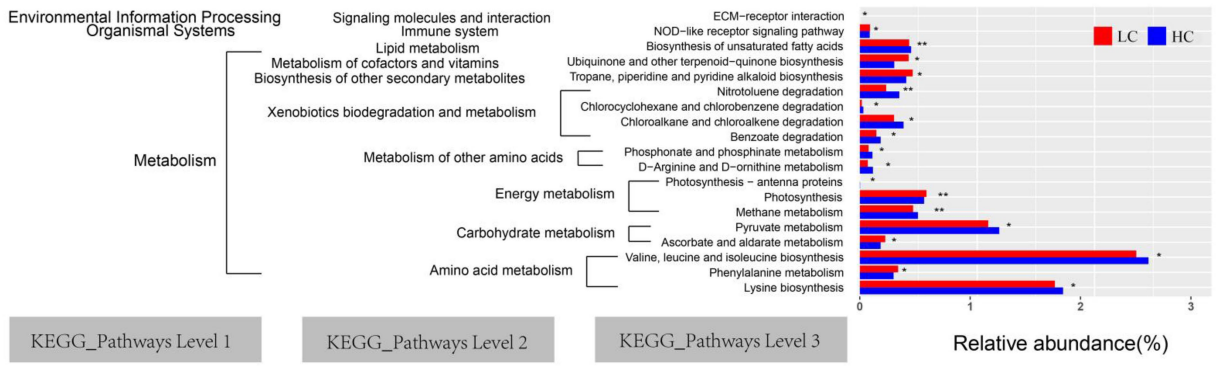


FIGURE 7
Yaks, which were fed low concentrate (LC) and high concentrate diets (HC), had significantly different functional predictions of the rumen microbiota of the KEGG pathway ($p < 0.05$). The graph shows the KEGG pathways for levels 1, 2, and 3. "*" and "**" indicate significance levels of 0.05 and 0.01, respectively.

groups considered in this study, Bacteroidota was more abundant in the LC group, while Firmicutes were more abundant in the HC group, indicating that the number of bacteria involved in ruminal starch digestion and metabolism was significantly increased in yaks fed with high concentrate.

At the genus level, *Christensenellaceae_R-7_group*, *Prevotella* and *NK4A214_group* were the dominant bacteria in the rumen in this experiment. *Christensenellaceae_R-7_group* belongs to the phylum of thick-walled bacteria (Waters and Ley, 2019), which mainly catabolizes fibrous material (Evans et al., 2011). *Prevotella* is a protein-degrading bacterium of the rumen and gastrointestinal tract of ruminants; it mainly degrades the hemicellulose component of the food and promotes the degradation of non-fibrous polysaccharides and pectins (Purushe et al., 2010). In the present study, *Christensenellaceae_R-7_group* and *Prevotella* were more abundant in the LC group, which may be related to the low level of concentrate fed to the yaks. The *NK4A214_group* belongs to the family Rumenococcaceae, rumen bacteria are rich in endo-1, 4-beta-xylanase and Cellulase genes, these genes play an important role in the degradation of cellulose and hemicellulose, which are degraded to produce short-chain fatty acids that are available for use by the host. Thus, its relative abundance is related to the diet concentrate ratio (Koike and Kobayashi, 2009; Biddle et al., 2013). The relative abundance of NK4A214_group was found to be associated with isobutyl acid and isovaleric acid concentrations were positively correlated (Liu C. et al., 2019a). In the present study, *NK4A214_group* was more abundant in the HC group, similar to a previously report (Chen et al., 2021), this could explain the higher isobutyl acid and isovaleric acid concentration in the HC group. Some bacteria, including *Ruminococcus, Acetitomaculum*, *Candidatus_Saccharimonas* and *NK4A214_group*, were positively correlated with acetate, butyrate, isovalerate, propionate and isobutyrate concentrations, respectively, indicating that these bacteria may favor VFA production. For instance, *Ruminococcus* can ferment cellobiose or cellulose to produce butyric acid (Henderson et al., 2015). *Acetitomaculum*, which is mainly found in ruminants fed high concentrate diet, can produce acetic acid from monosaccharides (Hua et al., 2017). Due to the complex interactions between bacteria (Olotu et al., 2019), it is difficult for us to understand the bacterial activities that directly produce VFA (Mahowald et al., 2009).

Microbial flora has an important role in ecosystem function, and the relationships among microorganisms involved in ruminal fermentation ecosystems are very complex (Liu H. et al., 2019b). In this study, key microbial taxa with topological properties in the rumen of yaks were identified for the first time. The results showed that high concentrate diet altered the correlation between components of microbial flora. Network analysis can reveal interactions between species in both positive and negative ways (Liu et al., 2020). Negative interactions may weaken competitive relationships, while positive interactions may strengthen them back (Fan et al., 2018). In our study, compare to the low concentrate diet group, the high concentrate diet group revealed stronger negative correlations. We hypothesized that the cellulose-degrading bacteria in the rumen were inhibited due to the low cellulose content of the high concentrate feeds, which resulted in fewer bacteria. This further suggests that high concentrate rations can regulate the microbial dynamics of the rumen in yaks.

In the current study, we used PICRUSt 2 to predict the function of the ruminal microbial community in yaks. The data suggest that the assessment of gene functions of the ruminal microbiota is significantly influenced by the ration concentrate ratio. In most cases, genes involved in the metabolism of amino acids in the level 2 KEGG pathway were enhanced in yaks fed high concentrate diets. This suggests that the ruminal microflora of yaks fed high concentrates produce large amounts of protein, which provide the host with raw materials such as protein, which in turn sustains life and normal metabolism. It is worth noting that valine, leucine and isoleucine biosyntheses are the most expressed pathways, directly involved in amino acid metabolisms. In this study, valine, leucine and isoleucine biosyntheses were

active at significantly higher levels in HC group, indicating that the increase in the percentage of concentrates increased the protein content of the rations, creating a more favorable environment for fermentation and the growth of cytolytic bacteria, which promote the participation of rumen microorganisms in the digestion and metabolism of nutrients. However, our results are based on predicted macrogenomics only and may not be representative of the actual function of ruminal bacteria. Further macrogenomic analyses are needed to explore the mechanism of these gene functions in feeding high concentrate diet to yaks.

## Conclusion

In this study, ruminal fermentation parameters and microflora of yaks that were fed high concentrate diets were analyzed. In fact, high concentrate diets altered the ruminal fermentation pattern, and the structure and composition of the microflora of yaks, which in turn affected their functions. The promotion of *Ruminococcus* and *Acetitomaculum* growth led to increased acetic acid and butyric acid contents in the rumen of yaks fed high concentrate diets, and the fermentation produced large amounts of VFA, tending to decrease ruminal pH and affecting yak ruminal health.

## Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found at: NCBI, PRJNA843719.

## Ethics statement

The animal study was reviewed and approved by Institutional Animal Care and Use Committee of the Qinghai University. Written informed consent was obtained from the owners for the participation of their animals in this study.

## Author contributions

SW, SC, and KP contributed to the conception and design of the study. KP, SW, and XW collected the samples. SW, KP, YY, HW, XB, and SC conducted relevant experiments. KP and SW organized the database. XW and SL performed the statistical analysis. KP wrote the first draft of the manuscript. XW, YY, SL, HW, and SC wrote sections of 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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## References

Bevans, D. W., Beauchemin, K. A., Schwartzkopf-Genswein, K. S., McKinnon, J. J., and McAllister, T. A. (2005). Effect of rapid or gradual grain adaptation on subacute acidosis and feed intake by feedlot cattle1,2. *J. Anim. Sci.* 83, 1116–1132. doi: 10.2527/2005.8351116x

Bi, Y. L., Zeng, S. Q., Zhang, R., Diao, Q. Y., and Tu, Y. (2018). Effects of dietary energy levels on rumen bacterial community composition in Holstein heifers under the same forage to concentrate ratio condition. *BMC Microbiol.* 18:69. doi: 10.1186/s12866-018-1213-9

Biddle, A., Stewart, L., Blanchard, J., and Leschine, S. (2013). Untangling the genetic basis of fibrinolytic specialization by lachnospiraceae and ruminococcaceae in diverse gut communities. *Diversity* 5, 627–389640. doi: 10.3390/d5030627

Bolger, A. M., Lohse, M., and Usadel, B. (2014). Trimmomatic: a flexible trimmer for Illumina sequence data. *Bioinformatics* 30, 2114–2120. doi: 10.1093/bioinformatics/btu170

Broderick, G. A., and Kang, J. H. (1980). Automated simultaneous determination of ammonia and total amino acids in ruminal fluid and in vitro medial. *Dairy Sci.* 63, 64–75. doi: 10.3168/jds.S0022-0302(80)82888-8

Cao, Z. J., Li, S. L., Xing, J. J., Ma, M., and Wang, L. L. (2008). Effects of maize grain and lucerne particle size on ruminal fermentation, digestibility and performance of cows in midlactation. *Anim. Physiol. Anim. Nutr. (Berl).* 92, 157–167. doi: 10.1111/j.1439-0396.2007.00721.x

Carlos, F., Alberto, H., Julio, G. T., and Loor, J. J. (2021). Changes in nutrient balance, methane emissions, physiologic biomarkers and production performance in goats fed different forage-to-concentrate ratios during lactation. *J. Anim. Sci.* 99:7. doi: 10.1093/jas/skab114

Chen, H., Wang, C., Huasai, S., and Chen, A. (2021). Effects of dietary forage to concentrate ratio on nutrient digestibility, ruminal fermentation and rumen bacterial composition in angus cows. *Sci. Rep.* 11:17023. doi: 10.1038/s41598-021-96580-5

Douglas, G. M., Maffei, V. J., Zaneveld, J., Yurgel, S. N., and Langille, M. (2019). PICRUSt2: an improved and extensible approach for metagenome inference. *Cold Spring Harbor Laboratory.* 38, 685. doi: 10.1101/672295

Edgar, R. C. (2013). UPARSE: highly accurate OTU sequences from microbial amplicon reads. *Nat. Methods* 10, 996–998. doi: 10.1038/nmeth.2604

Evans, N. J., Brown, J. M., Murray, R. D., Getty, B., Birtles, R. J., Hart, C. A., et al. (2011). Characterization of novel bovine gastrointestinal tract Treponema isolates and comparison with bovine digital dermatitis treponemes. *Appl. Environ. Microbiol.* 77, 138–147. doi: 10.1128/AEM.00993-10

Fan, Q., Wanapat, M., Yan, T., and Hou, F. (2020). Altitude influences microbial diversity and herbage fermentation in the rumen of yaks. *BMC Microbiol.* 20:370. doi: 10.1186/s12866-020-02054-5

Fan, K., Weisenhorn, P., Gilbert, J. A., Shi, Y., Bai, Y., and Chu, H. (2018). Soil pH correlates with the co-occurrence and assemblage process of diazotrophic communities in rhizosphere and bulk soils of wheat fields. Elds. *Soil Biol. Biochem.* 121, 185–192. doi: 10.1016/j.soilbio.2018.03.017

Fernando, S. C., Purvis, H. T., Najar, F. Z., Sukharnikov, L. O., Krehbiel, C. R., Nagaraja, T. G., et al. (2010). Rumen microbial population dynamics during adaptation to a high-grain diet. *Appl. Environ. Microbiol.* 76, 7482–7490. doi: 10.1128/AEM.00388-10

Garcia-Gonzalez, , Gonzalez, J. S., and Lopez, (2010). Decrease of ruminal methane production in Rusitec fermenters through the addition of plant material from rhubarb (rheum spp.) and alder buckthorn (Frangula alnus). *J. Dairy Sci.* 93, 3755–3763. doi: 10.3168/jds.2010-3107

Goad, D. W., Goad, C. L., and Nagaraja, T. G. (1998). Ruminal microbial and fermentative changes associated with experimentally induced subacute acidosis in steers. *J. Anim. Sci.* 76, 234–241. doi: 10.2527/1998.761234x

Gorka, P., Kowalski, Z. M., Pietrzak, P., Kotunia, A., and Zabielski, R. (2009). Effect of sodium butyrate supplementation in milk replacer and starter diet on rumen development in calves. *J. Physiol. Pharmacol.* 102, 2563–2575. doi: 10.1152/jn.00345.2009

Guilloteau, P., Martin, L., Eeckhaut, V., Ducatelle, R., Zabielski, R., and Immerseel, F. V. (2010). From the gut to the peripheral tissues: the multiple effects of butyrate. *Nutr. Res. Rev.* 23, 366–384. doi: 10.1017/S0954422410000247

Henderson, G., Cox, F., Ganesh, S., Jonker, A., Young, W., and Janssen, P. H. (2015). Rumen microbial community composition varies with diet and host, but a core microbiome is found across a wide geographical range. *Sci. Report.* 5:14567. doi: 10.1038/srep14567

Hou, K., Tong, J., Zhang, H., Gao, S., Guo, Y., Niu, H., et al. (2020). Microbiome and metabolic changes in milk in response to artemisinin supplementation in dairy cows. *AMB Express* 10:154. doi: 10.1186/s13568-020-01080-w

Hu, R., Zou, H., Wang, Z., Cao, B., Peng, Q., Jing, X., et al. (2019). Nutritional interventions improved rumen functions and promoted compensatory growth of growth-retarded yaks as revealed by integrated transcripts and microbiome analyses. *Front. Microbiol.* 10:318. doi: 10.3389/fmicb.2019.00318

Hua, C., Tian, J., Tian, P., Cong, R., Luo, Y., Geng, Y., et al. (2017). Feeding a high concentration diet induces unhealthy alterations in the composition and metabolism of ruminal microbiota and host response in a goat model. *Front. Microbiol.* 8:138. doi: 10.3389/fmicb.2017.00138

Islam, M., Kim, S., Ramos, S. C., Mamuad, L. L., Son, A.-R., and Zhong, T. Y. (2021). Holstein and Jersey steers differ in rumen microbiota and enteric methane emissions even fed the same total mixed ration. *Front. Microbiol.* 12:601061. doi: 10.3389/fmicb.2021.601061

Jahan, R., Amin, M. R., Sarker, N. R., and Kamal, M. T. (2018). Feeding effects of total mixed ration on rumen metabolic profile in cattle. *Bangladesh J. Animal Sci.* 47, 35–39. doi: 10.3329/bjas.v47i1.39401

Jia, P., Cui, K., Ma, T., Wan, F., Wang, W., Yang, D., et al. (2018). Influence of dietary supplementation with bacillus licheniformis and Saccharomyces cerevisiae as alternatives to monensin on growth performance, antioxidant, immunity, ruminal fermentation and microbial diversity of fattening lambs. *Sci. Rep.* 8:16712. doi: 10.1038/s41598-018-35081-4

Kmicikewycz, A. D., Harvatine, K. J., and Heinrichs, A. J. (2015). Effects of corn silage particle size, supplemental hay, and forage-to-concentrate ratio on rumen pH, feed preference, and milk fat profile of dairy cattle. *J. Dairy Sci.* 98, 4850–4868. doi: 10.3168/jds.2014-9249

Koike, S., and Kobayashi, Y. (2009). Fibrolytic rumen bacteria: their ecology and functions. *Asian Australasian J. Animal Sci.* 22, 131–138. doi: 10.5713/ajas.2009.r.01

Lee, M., Jeong, S., Seo, J., and Seo, S. (2019). Changes in the ruminal fermentation and bacterial community structure by a sudden change to a high-concentrate diet in Korean domestic ruminants. *Asian-Australas J Anim Sci.* 32, 92–102. doi: 10.5713/ajas.18.0262

Li, D., Zhang, Y., Cui, Z., He, L., Chen, W., Meng, Q., et al. (2016). Effects of Phytoecdysteroids (PEDS) extracted from Cyanotis arachnoidea on rumen fermentation, enzyme activity and microbial efficiency in a continuous-culture system. *PLoS One* 11:e0153584. doi: 10.1371/journal.pone.0153584

Liu, H., Hu, L., Han, X., Zhao, N., and Xu, S. (2020). Tibetan sheep adapt to plant phenology in alpine meadows by changing rumen microbial community structure and function. *Front. Microbiol.* 11:587558. doi: 10.3389/fmicb.2020.5875583

Liu, C., Wu, H., Liu, S., Chai, S., and Zhou, Z. (2019a). Dynamic alterations in yak rumen bacteria community and metabolome characteristics in response to feed type. *Front. Microbiol.* 10:1116. doi: 10.3389/fmicb.2019.01116

Liu, H., Xu, T., Xu, S., Ma, L., and Zhao, X. (2019b). Effect of dietary concentrate to forage ratio on growth performance, rumen fermentation and bacterial diversity of Tibetan sheep under barn feeding on the Qinghai-Tibetan plateau. *PeerJ.* 7:e7462. doi: 10.7717/peerj.7462

Long, R., Ding, L., and Shang, Z. (2018). The yak grazing system on the Qinghai-Tibetan plateau and its status. *Rangel. J.* 30, 241–246. doi: 10.1071/RJ08012

Lv, F., Wang, X., Pang, X., and Liu, G. (2020). Effects of supplementary feeding on the rumen morphology and bacterial diversity in lambs. *PeerJ.* 8:e9353. doi: 10.7717/peerj.9353

Mackie, R. I., and Gilchrist, F. M. C. (1979). Changes in lactate-producing and lactate-utilizing bacteria in relation to pH in the rumen of sheep during stepwise adaptation to a high-concentrate diet. *Appl. Environ. Microbiol.* 38, 422–430. doi: 10.1007/BF00403678

Mahowald, M. A., Rey, F. E., Seedorf, H., Turnbaugh, P. J., Fulton, R. S., Wollam, A., et al. (2009). Characterizing a model human gut microbiota composed of members of its two dominant bacterial phyla. *Proc. Natl. Acad. Sci. U. S. A.* 106, 5859–5864. doi: 10.1073/pnas.0901529106

Malekkhahi, M., Tahmasbi, A. M., Naserian, A. A., Naserian, M., Danesh-Mesgaran, J. L., Kleen, O., et al. (2016). Effects of supplementation of active dried yeast and malate during sub-acute ruminal acidosis on rumen fermentation, microbial population, selected blood metabolites and milk production in dairy cows. *Anim. Feed Sci. Technol.* 213, 29–43. doi: 10.1016/j.anifeedsci.2015.12.018

Mao, S. Y., Zhang, R. Y., Wang, D. S., and Zhu, W. Y. (2013). Impact of subacute ruminal acidosis (SARA) adaptation on rumen microbiota in dairy cattle using pyrosequencing. *Anaerobe* 24, 12–19. doi: 10.1016/j.anaerobe.2013.08.003

Miller, G. E., Engen, P. A., Gillevet, P. M., Maliha, S., Masoumeh, S., Forsyth, C. B., et al. (2016). Lower neighborhood socioeconomic status associated with reduced diversity of the colonic microbiota in healthy adults. *PLoS One* 11:e148952. doi: 10.1371/journal.pone.0148952

Minamoto, Y., Minamoto, T., Isaiah, A., Sattasathuchana, P., Buono, A., Rangachari, V. R., et al. (2019). Fecal short-chain fatty acid concentrations and dysbiosis in dogs with chronic enteropathy. *J. Vet. Intern. Med.* 33, 1608–1618. doi: 10.1111/jvim.15520

Nagata, R., Kim, Y. H., Ohkubo, A., Kushibiki, S., Ichijo, T., and Sato, S. (2018). Effects of repeated subacute ruminal acidosis challenges on the adaptation of the rumen bacterial community in Holstein bulls. *J. Dairy Sci.* 101, 4424–4436. doi: 10.3168/jds.2017-13859

Nugroho, D., Sevilla, C. C., Angeles, A. A., and Sunarso, S. (2013). The effects of dietary neutral detergent fiber ratio from forage and concentrate on the dietary rumen degradability and growth performance of Philippine native goats (Capra hircus Linn.). *Intern. J. Sci. Engineering.* 6, 75–80. doi: 10.14334/jitv.v18i4.336

Ogata, T., Makino, H., Ishizuka, N., Iwamoto, E., Masaki, T., Ikuta, K., et al. (2019). Long-term high-grain diet altered the ruminal pH, fermentation, and composition and functions of the rumen bacterial community, leading to enhanced lactic acid production in Japanese black beef cattle during fattening. *PLoS One* 14:e0225448. doi: 10.1371/JOURNAL.PONE.0225448

Olotu, C., Lehmensiek, F., Koch, B., Kiefmann, M., Riegel, A. K., Hammerschmidt, S., et al. (2019). Streptococcus pneumoniae inhibits purinergic signaling and promotes purinergic receptor P2Y2internalization in alveolar epithelial cells. *J. Biol. Chem.* 294, 12795–12806. doi: 10.1074/jbc.RA118.007236

Petri, R. M., Tyler, S., Penner, G. B., Beauchemin, K. A., Forster, R. J., McKinnon, J. J., et al. (2013). Characterization of the core rumen microbiome in cattle during transition from forage to concentrate as well as during and after an acidotic challenge. *PLoS One* 8:e83424.8. doi: 10.1371/journal.pone.0083424

Petrir, M.， Forster, R. J., Yang, W., Mckinnon, J. J., and Mcallister, T. A. (2012). Characterization of rumen bacterial diversity and fermentation parameters in concentrate fed cattle with and without forage. *J. Appl. Microbiol.* 112:1152–1162. doi:10.1111/j.1365-2672.2012.05295.x

Purushe, J., Fouts, D. E., Morrison, M., White, B. A., and Mackie, R. I. (2010). Comparative genome analysis of Prevotella ruminicola and Prevotella bryantii: insights into their environmental niche. *Microb. Ecol.* 60, 721–729. doi: 10.1007/s00248-010-9692-8

Qiu, Q., Gao, C., Rahman, M., Cao, B., and Su, H. (2020). Digestive ability,physiological characteristics,and rumen bacterial community of Holstein finishing steers in response to three nutrient density diets as fattening phases advanced. *Microorganisms.* 8:335. doi: 10.3390/microorganisms8030335

Quast, C., Pruesse, E., Yilmaz, P., Gerken, J., and Glckner, F. O. (2012). The SILVA ribosomal RNA gene database project: improved data processing and web-based tools. *Nucleic Acids Res.* 41, D590–D596. doi: 10.1093/nar/gks1219

Ramos, S. C., Chang, D. J., Mamuad, L. L., Ho, S., and Sang, S. L. (2021). Diet transition from high-forage to high-concentrate alters rumen bacterial community composition, epithelial transcriptomes and ruminal fermentation parameters in dairy cows. *Animals: an open access journal from MDPI.* 11:838. doi: 10.3390/ani11030838

Reigstad, C. S., and Kashyap, P. C. (2013). Beyond phylotyping: understanding the impact of gut microbiota on host biology. *Neurogastroenterol. Motil.* 25, 358–372. doi: 10.1111/nmo.12134

Sato, S. (2016). Pathophysiological evaluation of subacute ruminal acidosis (SARA) by continuous ruminal pH monitoring. *Anim. Sci. J.* 87, 168–177. doi: 10.1111/asj.12415

Shabat, S. K., Sasson, G., Doron-Faigenboim, A., Durman, T., Yaacoby, S., Berg Miller, M. E., et al. (2016). Specific microbiome-dependent mechanisms underlie the energy harvest efficiency of ruminants. *ISME J.* 10, 2958–2972. doi: 10.1038/ismej.2016.62

Tajima, K., Aminov, R. I., Nagamine, T., Matsui, H., Nakamura, M., and Benno, Y. (2001). Diet-dependent shifts in the bacterial population of the rumen revealed with real-time PCR. *Appl. Environ. Microbiol.* 67, 2766–2774. doi: 10.1128/AEM.67.6.2766-2774.2001

Thao, N. T., Wanapat, M. A., et al. (2014). Effects of eucalyptus crude oils supplementation on rumen fermentation, microorganism and nutrient digestibility in swamp buffaloes. *Asian Australasian J. Animal Sci.* 27, 46–54. doi: 10.5713/ajas.2013.13301

Tomczak, D. J., Samuelson, K. L., Jennings, J. S., and Richeson, J. T. (2019). Oral hydration therapy with water and bovine respiratory disease incidence affects rumination behavior, rumen pH, and rumen temperature in high-risk, newly received beef calves. *J. Anim. Sci.* 97, 2015–2024. doi: 10.1093/jas/skz102

Tomkins, N. W., Denman, S. E., Pilajun, R., Wanapat, M., Mcsweeney, C. S., and Elliott, R. (2015). Manipulating rumen fermentation and methanogenesis using an essential oil and monensin in beef cattle fed a tropical grass hay. *Anim. Feed Sci. Technol.* 200, 25–34. doi: 10.1016/j.anifeedsci.2014.11.013

Vcdsa, C., Jdm, A., Edb, B., Klgca, A., Avld, A., Lmc, C., et al. (2020). Assessing amino acid utilization in young Nellore steers fed high-concentrate diets with different sources and levels of nitrogen. *Anim. Feed Sci. Technol.* 269:114642. doi: 10.1016/j.anifeedsci.2020.114642

Wang, Q. (2007). Naive bayesian classifier for rapid assignment of rRNA sequences into the new bacterial taxonomy. *Appl. Environ. Microbiol.* 73, 5261–5267. doi: 10.1128/AEM.00062-07

Waters, J. L., and Ley, R. E. (2019). The human gut bacteria Christensenellaceae are widespread, heritable, and associated with health. *BMC Biol.* 17:83. doi: 10.1186/s12915-019-0699-4

Ya-Bing, C., Dao-Liang, L., Cheng, T., Xiao-Nong, Y., and Jian, L. (2016). Effect of DNA extraction methods on the apparent structure of yak rumen microbial communities as revealed by 16S rDNA sequencing. *Pol. J. Microbiol.* 64, 29–36. doi: 10.33073/pjm-2015-004

Yáñez-Ruiz, D. R., Leticia, A., and Newbold, C. J. (2015). Manipulating rumen microbiome and fermentation through interventions during early life:a review. *Front. Microbiol.* 6:1133. doi: 10.3389/fmicb.2015.01133

Young, J. W. (1977). Gluconeogenesis in cattle: significance and methodology. *J. Dairy Sci.* 60, 1–15. doi: 10.3168/jds.S0022-0302(77)83821-6

Zened, A., Combes, S., Cauquil, L., Mariette, J., Klopp, C., Bouchez, O., et al. (2013). Microbial ecology of the rumen evaluated by 454 GS FLX pyrosequencing is affected by starch and oil supplementation of diets. *FEMS Microbiol. Ecol.* 83, 504–514. doi: 10.1111/1574-6941.12011

Zhou, Z. M., Fang, L., Meng, Q. X., Li, S. L., Chai, S. T., Liu, S. J., et al. (2017). Assessment of ruminal bacterial and archaeal community structure in yak (Bos grunniens). *Front. Microbiol.* 8:179. doi: 10.3389/fmicb.2017.00179

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# The effects of *Clostridium butyricum* on Ira rabbit growth performance, cecal microbiota and plasma metabolome

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Clostridium butyricum (C. butyricum) can provide many benefits for animals' growth performance and gut health. In this study, we investigated the effects of *C. butyricum* on the growth performance, cecal microbiota, and plasma metabolome in Ira rabbits. A total of 216 Ira rabbits at 32 days of age were randomly assigned to four treatments supplemented with basal diets containing 0 (CG), 200 (LC), 400 (MC), and 600 mg/kg (HC) *C. butyricum* for 35 days, respectively. In comparison with the CG group, *C. butyricum* supplementation significantly improved the average daily gain (ADG) and feed conversion rate (FCR) at 53 and 67 days of age ($P < 0.05$) and digestibilities of crude protein (CP) and crude fiber (CF) at 67 days of age ($P < 0.05$). The cellulase activity in the HC group was higher respectively by 50.14 and 90.13% at 53 and 67 days of age, than those in the CG groups ($P < 0.05$). Moreover, at 67 days of age, the diet supplemented with *C. butyricum* significantly increased the relative abundance of Verrucomicrobia at the phylum level ($P < 0.05$). Meanwhile, the concentrations of different metabolites, such as amino acids and purine, were significantly altered by *C. butyricum* ($P < 0.05$). In addition, 10 different genera were highly correlated with 52 different metabolites at 53-day-old and 6 different genera were highly correlated with 18 different metabolites at 67-day-old Ira rabbits. These findings indicated that the *C. butyricum* supplementation could significantly improve the growth performance by modifying the cecal microbiota structure and plasma metabolome of weaned Ira rabbits.

KEYWORDS

***C. butyricum*, Ira rabbits, growth performance, gut microbiota, plasma metabolites**

# Introduction

Ira Rabbit is an excellent breed of meat rabbit, which has the characteristics of a high feed utilization rate and fast growth rate (Chipo and Tapiwa, 2019). In rabbit production, antibiotics as feed additives can relieve weaning stress, which can reduce huge economic losses (Hu et al., 2018). However, the long-term abuse of antibiotics in production has caused negative effects such as dysbacteriosis in animals. Therefore, it is imperative to find safe and effective alternatives to antibiotics (Alayande et al., 2020).

*Clostridium butyricum* is an acid-resisting, high temperature-resisting probiotic and can tolerate the tough intestinal environment (Chen et al., 2020; Gao et al., 2021), and non-toxigenic strains are currently used as probiotics in Asia (Cato, 1986). Butyric acid is one of the most important metabolites of *C. butyricum*, which can not only promote development but also maintain the energy source of intestinal epithelial cells (Zhang et al., 2018; Han et al., 2020). Other metabolites such as teichoic acid can promote the colonization of *C. butyricum* due to its highly adhesive properties (D'Elia et al., 2009). Meanwhile, teichoic acid can also inhibit the adhesion of Escherichia coli and protect the stability of the gut microbial structure (Gao et al., 2012). *C. butyricum* can secrete various enzymatic active substances such as cellulase, pectinase, amylase, lipase, and protease, thereby degrading the nutrients in the feed and promoting the digestion and absorption of nutrients in the intestinal tract (Zhang et al., 2016). *C. butyricum* has been proved to promote growth performance, and gut health (Hsiao et al., 2021; Liang et al., 2021) for aquatic animals (Tran et al., 2020; Luo et al., 2021), poultry (Molnár et al., 2020), and livestock (Han et al., 2020; Lopez et al., 2021). A previous study on weaning Rex rabbits has indicated that dietary supplementation with *C. butyricum* in the quantity of spore state of $1.0 \times 10^5$ CFU/g increased the average daily gain and small intestinal digestive enzyme activity (Liu et al., 2019b). A basal diet supplemented with a dosage of $2.5 \times 10^9$ CFU/kg *C. butyricum* can increase apparent nutrient digestibilities, and promote the proliferation of beneficial bacteria in weaned pigs, such as *Streptococcus* and *Bifidobacterium* (Han et al., 2020). Furthermore, studies have shown that *C. butyricum* MIYAIRI 588 reduced host diarrhea rate by enhancing gut colonization resistance to *Clostridioides difficile* (Hagihara et al., 2021). The intestinal microbiome is extremely important for the digestion and absorption of nutrients in animals (Turnbaugh et al., 2006; Anhê et al., 2015). However, the effect of *C. butyricum* on the growth performance and intestinal flora of the Ira rabbit is still unclear.

This study evaluated the effect of dietary supplementation of *C. butyricum* on growth performance, nutrient digestibilities of Ira rabbits. Meanwhile, the effect of *C. butyricum* on the cecal microbiota and plasma metabolites in Ira rabbits was determined. Furthermore, correlation analysis among growth performance, gut microbiome, and plasma metabolites was performed to verify the effect of *C. butyricum* on the intrinsic relationship between host and microbial metabolisms. The purpose of this study was to provide a scientific basis for the application of *C. butyricum* to partly replace antibiotics in the production of meat rabbits.

# Materials and methods

## Animal feeding experiment

A total of 216 healthy male Ira rabbits at 32 days of age with similar weight mass were randomly assigned to different single cages and raised to 67 days of age. All rabbits were randomly assigned into four groups and each group has fifty-four rabbits. The control group (CG) was fed a basal diet without the addition of *C. butyricum*, while the low dose group (LC), the medium dose group (MC), and the high dose group (HC) were fed a test diet supplemented with 200, 400 and 600 mg/kg *C. butyricum* in the basal diet, respectively. The number of viable *C. butyricum* is $2 \times 10^8$ CFU/g (Hubei Lvxue Biotechnology Co., Ltd.). The feeding amount is based on the feeding test data in the internship stage, and the feeding amount is different on different days. The additional amount of Clostridium butyrate is based on the conclusion of the product description and previous experiments. The experiment was maintained for five weeks, while a 4-day pre-trail was conducted for dietary adaptation of rabbits. The basal diet met the Nutrition Research Council (NRC) for weaned rabbits and was chemically analyzed (Cunniff et al., 1995), shown in **Supplementary Table 1** (Nutrition Research Council, 1930). Every cage was cleaned and disinfected by a high-temperature spray gun 3 days before rabbits were placed in them. The inner temperature was maintained at 28°C during the whole experimental period. Rabbits were exposed to continuous light from 7 a.m. to 7 p.m. per day. The rabbits in all cages were vaccinated with maternal immunity.

## Growth performance, nutrient digestibilities, and cellulase activity assay

The body weight (BW) was recorded, and the average daily gain (ADG) and feed conversion rate (FCR) were calculated at 32, 39, 46, 53, 60, and 67 days of age in Ira rabbits, respectively. The formula for FCR is:

$$FCR = \frac{\text{Food intake(g)}}{\text{Gain weight(g)}}$$

Using the total feces collection method, feces of each rabbit at 51, 52, 53 days, and 65, 66, and 67 days of age were collected, weighed, and mixed in groups. 10% of the total amount of feces,

soaked in 10% sulfuric acid overnight, air-dried at 65°C for 24 h, regained moisture for 24 h, and passed through a sieve (60 mesh screen) for digestibility testing. The content of crude fiber (CF) and crude protein (CP) in diets and feces is strictly carried out following the analysis methods in "Feed Analysis and Feed Quality Testing Technology" (Zhang, 2007). The calculation formula of apparent digestibility is:

$$Apparentdigestibility = (1 - \frac{\text{the content of a nutrient in feces}}{\text{the content of a nutrient in feed}}) \times 100\%$$

At 53 and 67 days of age, 6 rabbits in every group were randomly selected before feeding, separately. The cecum aseptically and collected 3–5 g of cecal contents in the EP tube, stored at −80°C for 16s rDNA sequencing and cellulase activity detection. According to the manufacturer's instructions, the kit (Nanjing Jiancheng Institute of Bioengineering) was used to determine cellulase activity. Blood was collected from the ear vein, centrifuged at 3500 r/min for 10 min, and the supernatant was taken and stored at −80°C for plasma metabolomics test.

## 16S rDNA sequencing analysis

According to the manufacturer's instructions, microbial community genomic DNA was extracted from cecal content samples using the E.Z.N.A.® soil DNA Kit (Omega Bio-Tek, Norcross, GA, USA). The hypervariable region V3-V4 of the bacterial 16S rDNA was amplified with primer pairs 338F (5′-ACTCCTACGGGAGGCAGCAG-3′) and 806R (5′-GGACTACHVGGGTWTCTAAT-3′) by an ABI GeneAmp® 9700 PCR thermocycler (ABI, CA, USA). The purified products of amplification were sequenced on the Hiseq-2500 platform (Illumina, USA). The quality control of the original data was completed by QIIME (QIIME 1.9.1, USA). The raw 16S rDNA sequencing reads were demultiplexed and quality-filtered by fastp version 0.20.0 (Chen et al., 2018) and merged by FLASH version 1.2.7 (Magoč and Salzberg, 2011) with the following criteria: (i) the 300 bp reads were truncated at any site receiving an average quality score of < 20 over a 50 bp sliding window, and the truncated reads shorter than 50 bp were discarded, reads containing ambiguous characters were also discarded; (ii) only overlapping sequences longer than 10 bp were assembled according to their overlapped sequence. The maximum mismatch ratio of the overlap region is 0.2. Reads that could not be assembled were discarded; (iii) Samples were distinguished according to the barcode and primers, and the sequence direction was adjusted, exact barcode matching, 2 nucleotide mismatch in primer matching. Operational taxonomic units (OTUs) with 97% similarity cutoff were clustered using UPARSE version 7.1 (Stackebrandt and Goebel, 1994; Edgar, 2013), and chimeric sequences were identified and removed. The taxonomy of each OTU representative sequence was analyzed by RDP Classifier version 2.2 (Wang et al., 2007) against the 16S rDNA database (e.g., Silva v138) using a confidence threshold of 0.7. The alpha and beta diversity indices were calculated using QIIME (v.1.9.1). Beta diversity was evaluated by principal component analysis (PCA) plots based on Euclidean distances using the R. Bar plot was constructed to analyze the microbial composition at the phylum and genus levels based on OTU abundance using the R package (animalcules).

## LC-MS analysis

A 100 μL plasma sample was transferred to an E.P. tube, and 400 μL extract solution (acetonitrile: methanol = 1: 1) containing internal standard (L-2-Chlorophenylalanine, 2 μg/mL) was added. After 30 s vortex, the samples were sonicated for 10 min in the ice-water bath. Then the samples were incubated at −40°C for 1 h and centrifuged at 10,000 rpm for 15 min at 4°C. 400 μL supernatant was transferred to a fresh tube and dried in a vacuum concentrator at 37°C. Then, the dried samples were reconstituted in 200 μL 50% acetonitrile by sonication on ice for 10 min. The solution was centrifuged at 13000 rpm for 15 min at 4°C, and 75 μL of supernatant was transferred to a fresh glass vial for LC/MS analysis. The quality control (QC) sample was prepared by mixing an equal aliquot of the supernatants from all samples. The UHPLC separation was carried out using a 1290 Infinity series UHPLC System (Agilent Technologies Co., Ltd., Los Angeles, USA), equipped with a UPLC BEH Amide column (2.1 * 100 mm, 1.7 μm, Waters, Shanghai, China). The mobile phase consisted of 25 mmol/L ammonium acetate and 25 mmol/L ammonia hydroxide in water (pH = 9.75) (A) and acetonitrile (B). The analysis was carried with elution gradient as follows: 0∼0.5 min, 95% B; 0.5∼7.0 min, 95∼65% B; 7.0∼8.0 min, 65∼40% B; 8.0∼9.0 min, 40% B; 9.0∼9.1 min, 40∼95% B; 9.1∼12.0 min, 95% B. The column temperature was 25°C. The auto-sampler temperature was 4°C, and the injection volumes were always 2 μL (positive ion mode) or 2 μL (negative ion mode) (Wang et al., 2016), respectively. The TripleTOF 6600 mass spectrometry (AB Sciex) was used for its ability to acquire MS/MS spectra on an information-dependent basis (IDA) during an LC/MS experiment. In this mode, the acquisition software (Analyst TF 1.7, AB Sciex) continuously evaluates the full scan survey M.S. data as it collects and triggers the acquisition of MS/MS spectra depending on preselected criteria. The most intensive 12 precursor ions (intensity > 100) were chosen for MS/MS at collision energy (CE) of 30 eV in each cycle. The cycle time was 0.56 s. The ESI source conditions were set as follows: Gas 1 as 60 psi, Gas 2 as 60 psi, Curtain Gas as 35 psi, Source Temperature as 600°C, Declustering potential as

60 V, Ion Spray Voltage Floating (ISVF) as 5000 V or –4000 V in positive or negative modes, respectively.

## Metabolomics data analysis

MS raw data (.wiff) files were converted to the mzXML format by ProteoWizard and processed by the R package XCMS (version 3.2). The process includes peak deconvolution, alignment, and integration. Minfrac and cut-off were set as 0.5 and 0.3, respectively. An in-house MS2 database was applied for metabolite identification (Smith et al., 2006). After obtaining the sorted data, we conduct a series of multivariate pattern recognition analyses on it. The first is principal component analysis. Wilcoxon rank-sum test with false discovery rate (FDR) correction was performed to detect differences in microbial diversity indices and relative abundances of microbes at different taxonomic levels. Correlations between different metabolites and bacterial communities were assessed by Spearman's correlation analysis using the pheatmap package in R (Kolde, 2015). FDR adjusted *P*-values and the corrected *P*-values below 0.05 were statistically significant.

## Statistical analysis method

The results of this study are presented as the mean ± standard deviation, and a one-way analysis of variance (One-way ANOVA) was performed on the test results using GraphPad Prism8 software, and Turkey multiple comparison was used. $P < 0.05$ means significant difference, $P < 0.01$ means extremely significant difference.

# Results

## Growth performance

The addition of *C. butyricum* significantly affected the BW, ADG, and FCR for Ira rabbits (Table 1). At 53 and 67 days of age, the BW of the HC and MC groups were significantly increased by 4 and 3.35% ($P < 0.05$) compared with CG, and the ADG of HC and LC groups were significantly increased by 7.92 and 4.65% ($P < 0.05$) compared with CG. The MC group performed best in FCR at 53 days of age, which was 8.75% better than the CG group ($P < 0.05$). At 67 days of age, the FCR of the HC group was significantly better than that of the CG group ($P < 0.05$).

## Nutrients digestibilities and cellulase activity in Ira rabbit cecum

The addition of *C. butyricum* significantly affected the digestibility of CP and CF (Table 2). At 53 days of age, the digestibility of CF in the HC group was significantly higher than in the CG group ($P < 0.05$). At 67 days of age, the digestibilities of CP and CF in the HC group were significantly higher than in the CG group ($P < 0.05$). Moreover, at 53 and 67 days of age, the cellulase activity in the HC group was significantly increased by 50.14 and 90.14% compared with the CG group ($P < 0.05$, Table 3).

## Structure and diversity of cecal microbiota in Ira rabbits at different stages

A total of 2,472,383 tags in 24 rabbits (two groups for two sampling times) were obtained according to the quality-controlled 16S rDNA gene sequencing. At the age of 53 days, the ace, chao1, and observed species indexes of the HC1 group showed a significant upward trend compared with the CG1 group, while the goods_coverage showed a significant downward trend. At 67 days of age, there were no significant differences ($P > 0.05$) between the estimates of alpha diversity index (Figure 1). PCA was carried out to study the composition distance relationship using dimensionality reduction. The intestinal bacteria in each group were separated, represented by the group distances (Figure 2).

At the phylum level (Figure 3A), Firmicutes and Bacteroidetes were the top two predominant phyla in each group, containing 59.43% (CG1), 59.15% (CG2), 68.59% (HC1), and 68.22% (HC2), respectively. Meanwhile, the Firmicutes, Bacteroidetes, Verrucomicrobia, and Actinobacteria counted for over 95% of the gut microbiota (Figure 3A). At the genus level, *Ruminococcaceae NK4A214 group*, *Ruminococcaceae UCG-014*, *Akkermansia*, *Ruminococcaceae UCG-013*, *Christensenellaceae R-7 group*, and *Lachnospiraceae NK4A136 group* were the predominant genera (Figure 3B). Furthermore, the differential analysis [Log2(FC)] between each group among phylum and genus levels was analyzed as well. At 53 days of age, the relative abundance of *dgA-11 gut group*, *Ruminococcus UCG-013* significantly decreased, however, the *Ruminococcus UCG-005* increased in the HC1 group ($P < 0.05$, Figure 3C). At 67 days of age, the relative abundances of *Escherichia-Shigella*, *Ruminococcus UCG-005,* and *Ruminococcus 1* significantly increased, while *Ruminococcaceae NK4A214 group* significantly increased in the HG2 group ($P < 0.05$, Figure 3D).

## Plasma metabolomics

Metabolomic analysis was conducted to explore the effect of *C. butyricum* on plasma metabolic profiles. In this study, 1,897 peaks were detected in the positive level, and 1,875 metabolites were left after relative standard deviation de-noising, while 1,850 metabolites were in the negative level (Supplementary Table 2).

The OPLS-DA score plot and permutation test further showed a significant difference between the two stages (Figures 4A,B), suggesting that *C. butyricum* caused metabolic phenotype alterations in rabbits' plasma. The OPLS-DA model was used to determine the differential metabolites between the pairwise comparison groups with the first principal component of variable importance in projection (VIP) values (VIP > 1) combined with $P < 0.05$ (Supplementary Table 3).

Heatmap described the differential metabolites between the CG and HC groups of two stages. Compared with the CG, the HC contained 5 upregulated and 69 downregulated differential metabolites at 53 days of age, and 37 upregulated and 3 downregulated differential metabolites at 67 days of age (Figures 5A,B). To explore the potential metabolic pathways affected by *C. butyricum*, all the differential metabolites were further analyzed by KEGG annotation. At 53 days of age, compared with the CG, the most enriched pathways in the HC were "Alanine, aspartate, and glutamate metabolism," "Aminoacyl-tRNA biosynthesis," "Arginine and proline metabolism," "Tyrosine metabolism," and "phenylalanine, tyrosine, and tryptophan biosynthesis" (Figure 6A). At 67 days of age, compared with the CG, the most enriched pathways in the HC were "Arginine and proline metabolism," "Alanine, aspartate and glutamate metabolism," "Cysteine and methionine metabolism," "Aminoacyl-tRNA biosynthesis," and "Lysine biosynthesis" (Figure 6B).

## Correlation analysis between growth performance and plasma metabolites

According to Spearman's correlation coefficient analysis, there were correlations between BW, ADG, and different metabolites. From Figure 7A, at 53 days of age, L-Valine, L-Methionine, L-Asparagine, L-Threonine, Allantoin, Anthranilic acid (Vitamin L1), Methylthiouracil, Cyclohexylsulfanmate, Uracil, L-Arabinose, Cyclohexylsulfamate, D-glucosamine 6-phosphate, Phenethyl Caffeiate, and L-homocysteic acid showed a positive correlation with BW and ADG. Arachidonic Acid (peroxide-free), Stearic Acid, and D-Biotin were negatively correlated with BW and ADG. From Figure 7B, at 67 days of age, Allantoin, L-Threoine,

TABLE 1 The effects of *C. butyricum* on the growth performance of Ira rabbits.

| Indexes | Days of Age | CG | LC | MC | HC |
|---|---|---|---|---|---|
| Initial BW (kg) | 32 | 0.79 ± 0.10 | 0.91 ± 0.99 | 0.79 ± 0.08 | 0.79 ± 0.09 |
| BW (kg) | 53 | 1.75 ± 0.15$^{c}$ | 1.76 ± 0.02$^{c}$ | 1.79 ± 0.02$^{b}$ | 1.82 ± 0.08$^{a}$ |
| ADG (g) | | 44.2 ± 0.50$^{c}$ | 46.3 ± 2.30$^{b}$ | 45.6 ± 2.20$^{c}$ | 47.7 ± 3.80$^{a}$ |
| Feed intake (g) | | 8.8526 | 9.2661 | 7.3736 | 7.8256 |
| FCR | | 2.97 ± 0.33$^{ab}$ | 3.04 ± 0.42$^{a}$ | 2.71 ± 0.14$^{c}$ | 2.79 ± 0.13$^{bc}$ |
| BW (kg) | 67 | 2.39 ± 0.07$^{b}$ | 2.38 ± 0.11$^{b}$ | 2.31 ± 0.07$^{b}$ | 2.47 ± 0.11$^{a}$ |
| ADG (g) | | 45.2 ± 0.55$^{b}$ | 44.5 ± 5.40$^{b}$ | 43.3 ± 4.20$^{b}$ | 47.3 ± 3.40$^{a}$ |
| Feed intake (kg) | | 6.5305 | 6.3359 | 5.4558 | 4.9665 |
| FCR | | 3.44 ± 0.42$^{a}$ | 3.39 ± 0.44$^{a}$ | 3.00 ± 0.28$^{ab}$ | 2.50 ± 0.14$^{b}$ |

Means with different superscript letters within the same line differ significantly, $P < 0.05$; data are presented as mean ± SEM ($n = 6$). BW, body weight; ADG, average daily gain; FCR, feed conversion ratio.

TABLE 2 The effects of *C. butyricum* on nutrients digestibilities in Ira rabbit cecum.

| Digestibilities, % | Days of Age | CG | LC | MC | HC |
|---|---|---|---|---|---|
| CP | 53 | 80.10 ± 2.58 | 80.79 ± 4.09 | 81.79 ± 4.10 | 84.46 ± 4.21 |
| CF | | 46.96 ± 2.61$^{b}$ | 49.15 ± 1.47$^{b}$ | 53.67 ± 2.62$^{a}$ | 55.26 ± 2.30$^{a}$ |
| CP | 67 | 77.97 ± 2.47$^{b}$ | 78.87 ± 1.41$^{b}$ | 80.43 ± 1.14$^{a}$ | 81.46 ± 2.40$^{a}$ |
| CF | | 43.57 ± 2.55$^{b}$ | 45.13 ± 2.30$^{b}$ | 45.97 ± 1.15$^{b}$ | 50.35 ± 1.40$^{a}$ |

Means with different superscript letters within the same line differ significantly, $P < 0.05$; data are presented as mean ± SEM ($n = 6$). CP, crude protein; CF, crude fiber.

TABLE 3 The effect of *C. butyricum* on cellulase activity in Ira rabbit cecum.

| Index, % | Days of Age | CG | LC | MC | HC |
|---|---|---|---|---|---|
| Cellulase activity | 53 | 17.35 ± 1.21$^{b}$ | 20.95 ± 4.00$^{ab}$ | 24.44 ± 9.60$^{ab}$ | 26.05 ± 3.74$^{a}$ |
| | 67 | 16.02 ± 0.92$^{b}$ | 23.86 ± 3.72$^{ab}$ | 27.65 ± 4.60$^{a}$ | 30.46 ± 1.57$^{a}$ |

Means with different superscript letters within the same line differ significantly, $P < 0.05$; data are presented as mean ± SEM ($n = 6$).

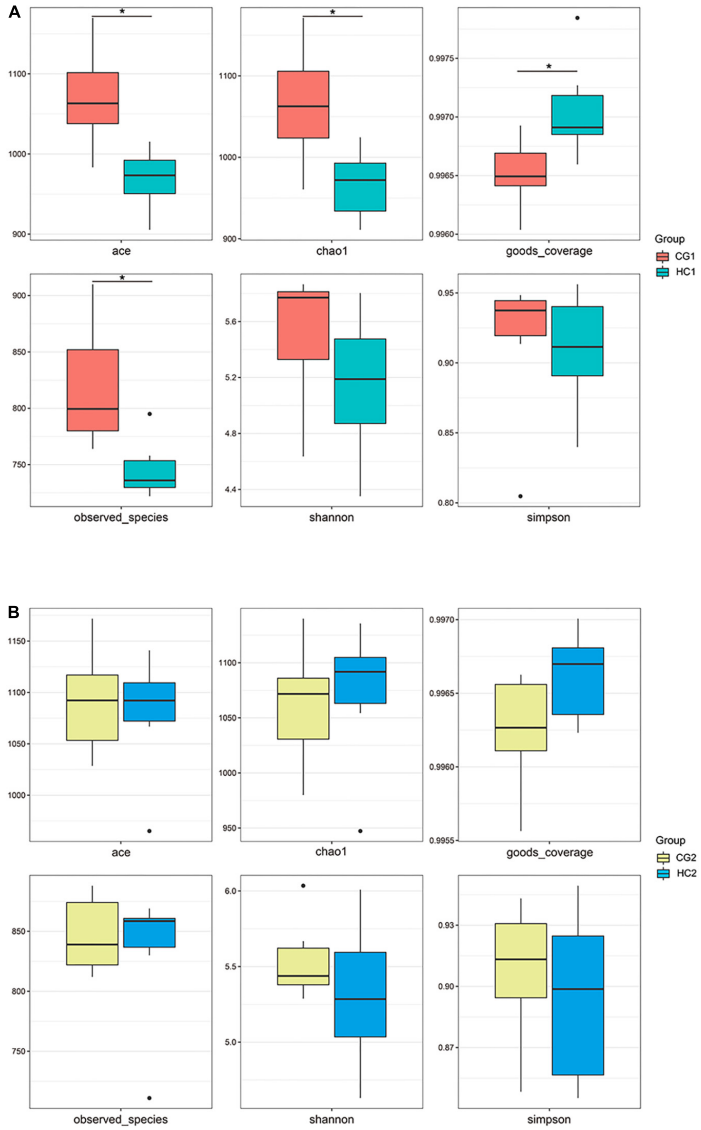


FIGURE 1
Changes in Alpha diversity index of cecal microbiota after *C. butyricum* supplementation at 53 **(A)** and 67 **(B)** days of age. CG1, control group at 53 days of age; CG2, control group at 67 days of age; HC1, the high dose at 53 days of age; HC2, the high dose at 67 days of age. *$P < 0.05$, **$P < 0.01$, ***$P < 0.0001$, and ****$P < 0.0001$.

L-Methionine, Methylthiouracil, and L-Valine were positively correlated with BW and ADG.

## Correlation analysis between gut microbiota and plasma metabolites

To reveal the relationships between gut microbial (at the genera level) and the different metabolites, network Diagram were generated by Spearman correlation analysis (**Figure 8**). Ten critical genera were highly correlated with 52 different metabolites ($|r| > 0.5$, FDR adjusted $P < 0.05$) at 53 days old (**Figure 8A**). Moreover, six important genera were highly correlated with 18 significant metabolites ($|r| > 0.6$, FDR adjusted $P < 0.05$) at 67 days old (**Figure 8B**).

# Discussion

Increasing evidence suggested the opinion that *C. butyricum* can enhance gut health and animal growth (Huang et al., 2021; Li et al., 2021a,b; Yu et al., 2021). Meanwhile, emerging studies of gut microbial and metabolome correlation linked to the growth performance, healthy, and development of

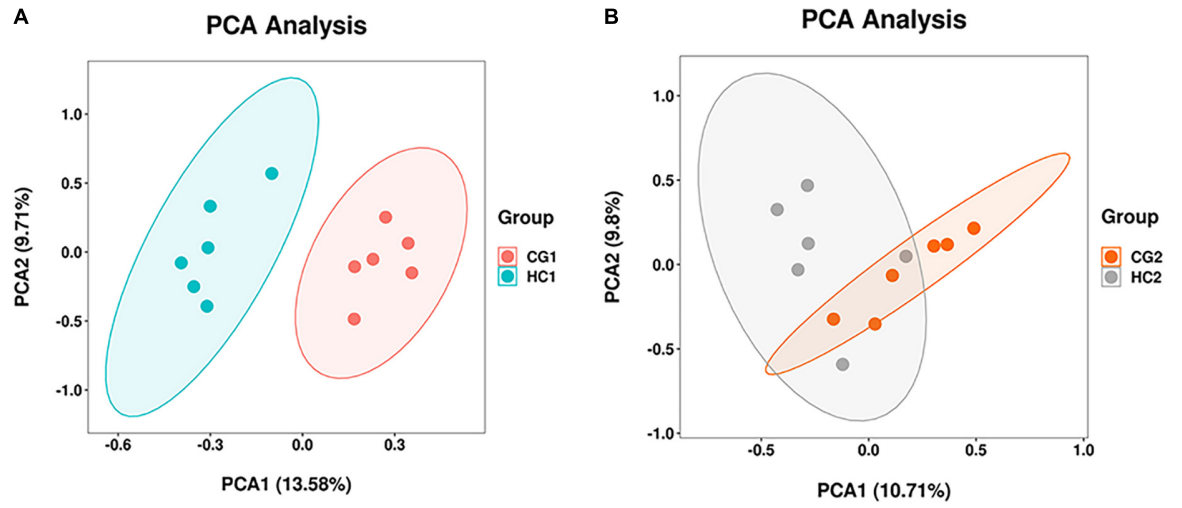


FIGURE 2
PCA of cecal microbiota at 53 **(A)** and 67 **(B)** days of age. CG1, control group at 53 days of age; CG2, control group at 67 days of age; HC1, the high dose at 53 days of age; HC2, the high dose at 67 days of age.

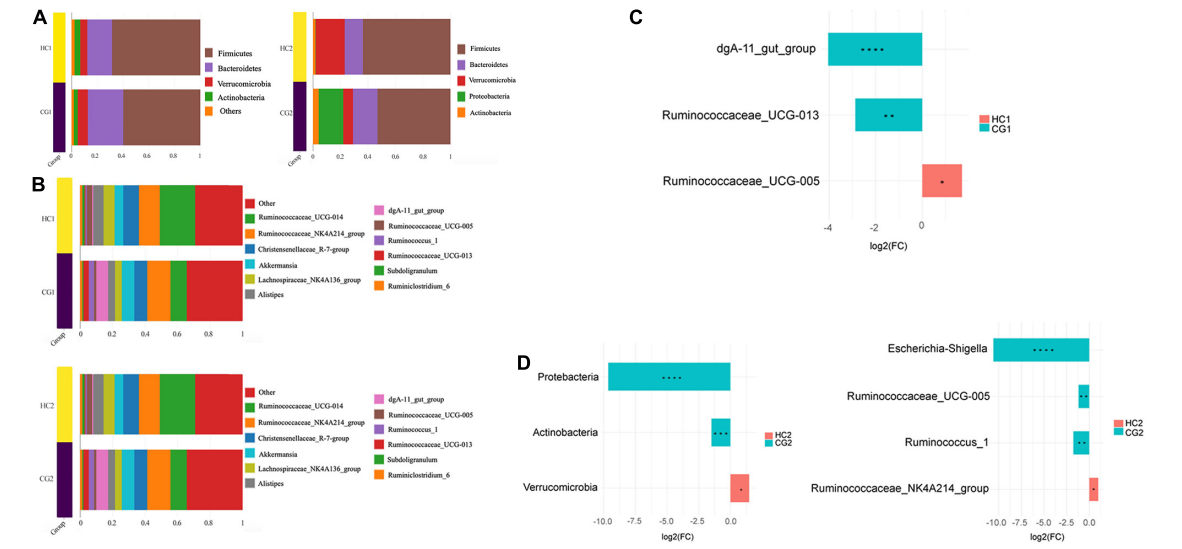


FIGURE 3
The relative abundance of cecal microbiota in Ira rabbits. **(A)** Phylum level. **(B)** Genus level. **(C)** Between-group variance analysis of genus at 53 days of age. **(D)** Between-group variance analysis of phylum and genus at 67 days of age. CG1, control group at 53 days of age; CG2, control group at 67 days of age; HC1, the high dose at 53 days of age; HC2, the high dose at 67 days of age. In the figure, * means $P < 0.05$, ** means $P < 0.01$, *** means $P < 0.0001$, **** means $P < 0.0001$, the same as in the figure below.

livestock (Park et al., 2013; Tian et al., 2015; Huang et al., 2019). Therefore, the understanding of gut microbial and plasma metabolome of Ira rabbits can reveal the possibility of improving the growth performance of rabbits. However, few studies investigated the relationship between microbial and metabolome of Ira rabbits in different growth stages. Thus, we explored the differences in the gut microbial and plasma metabolome of Ira rabbits and their correlations affected by the supplementation of *C. butyricum*.

In this study, the addition of *C. butyricum* significantly promoted ADG and FCR (**Table 1**, $P < 0.05$) of Ira rabbits at different growth stages. Meanwhile, at 67 days of age, *C. butyricum* at a dose of 600 mg/kg in the diet significantly increased the digestibility of CF and CP (**Table 2**, $P < 0.05$). Several reports have shown that *C. butyricum* exhibited a significant positive influence on growth performance and nutrient utilization efficiency in animals (Han et al., 2020; Li et al., 2021a; Liu et al., 2021), consistent with the findings

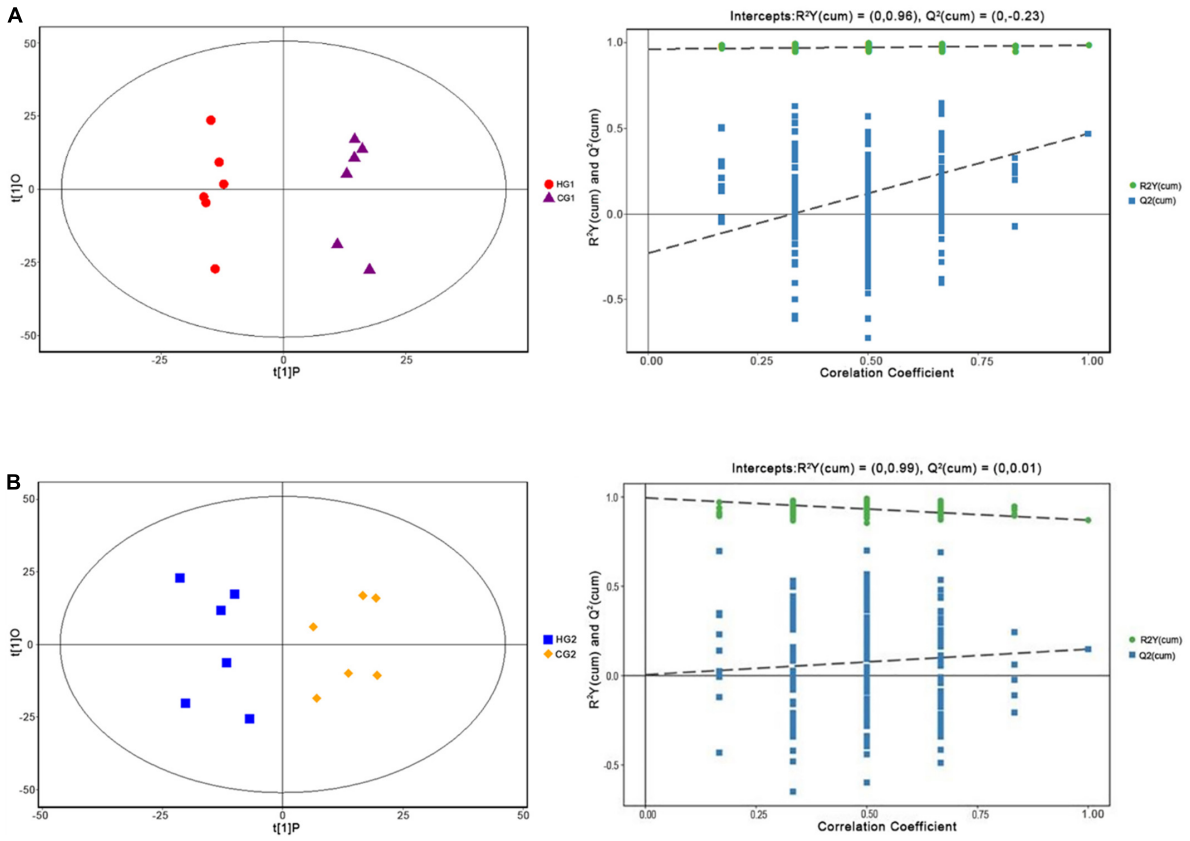


FIGURE 4
Orthogonal partial least squares discriminant analysis (OPLS-DA) plot of plasma metabolites of Ira rabbits supplied with *C. butyricum* at 53 days of age **(A)** and 67 days of age **(B)**.

of this study. It was reported that the supplementation of *C. butyricum* caused an increase in the villus height and enlarged crypt depth, which possibly improve the ADG caused by the increased absorption of intestinal cells (Huang et al., 2021). In this study, cellulase activity was enhanced at different dosages of *C. butyricum*. Thus, it can be demonstrated that *C. butyricum* supplementation has positive effects on the growth performance of Ira rabbits.

As a pivotal part of the intestinal tract, the gut microbial is called the black box of bodies. All the digestibility, development, maintenance, metabolism, and immunity have related to the gut microbial for rabbits (Huang et al., 2021). We evaluated whether the supplement of *C. butyricum* in the diet could alter the microbial community structure, similar to previous results (Huang et al., 2021). We found that the abundance of Firmicutes and *Ruminococcaceae_UGG-014* were changed by the *C. butyricum* supplementation (**Figures 3A,B**). The Ace, Chao1, and Observed species and goods_coverage indexes, were changed by the *C. butyricum*. The results were similar to the earlier studies (Duan et al., 2018; Casas et al., 2020) on rabbits and pigs.

The homeostasis of the gut microbiota has an important influence on maintaining the body's growth and development, nutrient digestion, and absorption (Shen et al., 2006). Our previous study found that 10.42% of the body weight change in weaned Ira rabbits could be explained by the gut microbiome (Fang et al., 2020). Therefore, the effect of *C. butyricum* on the intestinal microbiota of Ira rabbits needs to be considered. The present study showed that Firmicutes, Bacteroides, and Verrucomicrobia were the dominant phyla in weaning and fattening rabbits, consistent with previous findings (Li et al., 2018; Mattioli et al., 2019). Firmicutes play an essential role in degrading diet fiber and cellulose, which are advantageous to cellulose degradation (Zhu et al., 2015). Bacteroides can facilitate the non-digestible polysaccharides metabolism and produce essential vitamins and protein (Avershina et al., 2016; Jin et al., 2018). We found that diets supplemented with *C. butyricum* showed a trend of increasing the ratio of Firmicutes/Bacteroidetes. Meanwhile, *C. butyricum* supplementation significantly increased the relative abundance of Verrucomicrobia for rabbits at 67 days of age. Verrucomicrobia is a relatively newly defined phylum whose functions are largely unknown. However,

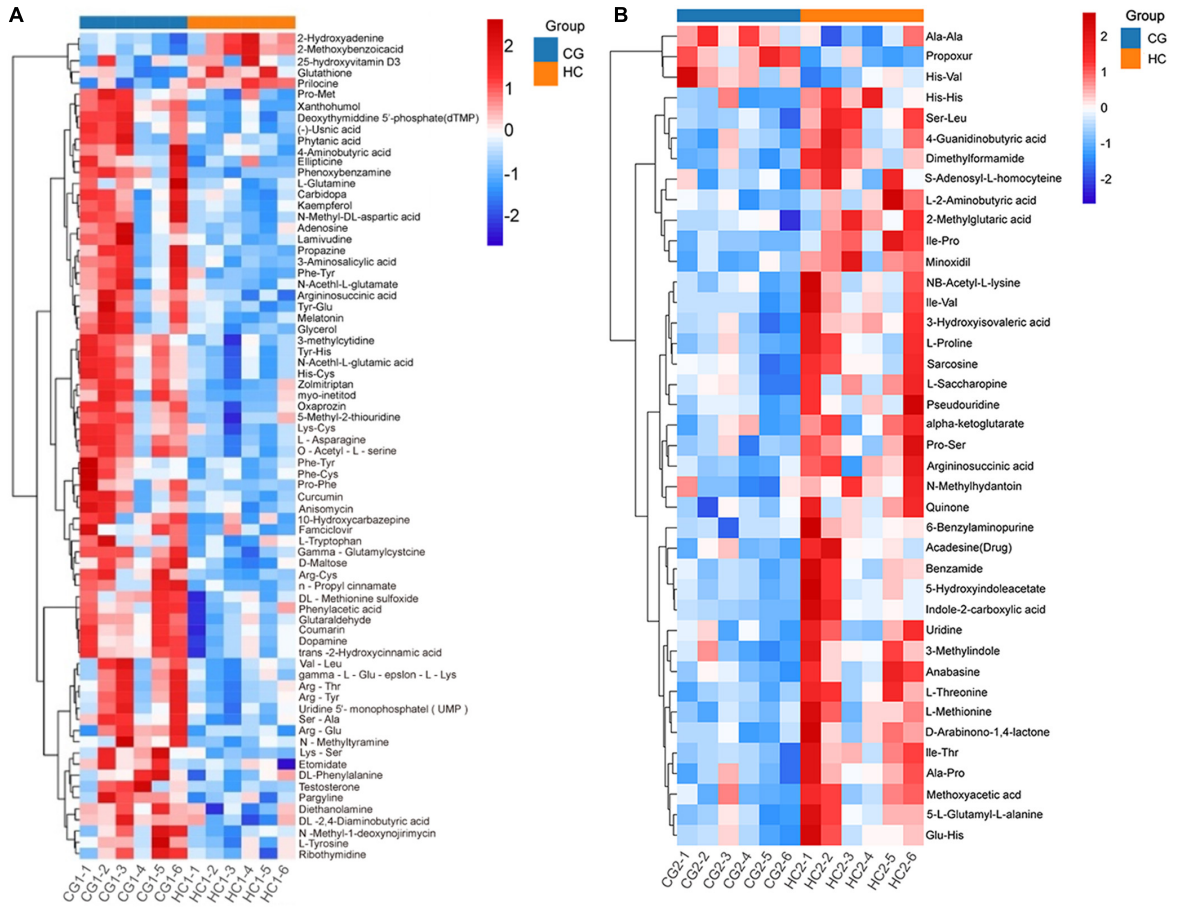


FIGURE 5
Hierarchical clustering analysis for plasma metabolites in Ira rabbits in the high dose at 53 days of age **(A)** and 67 days of age **(B)**. Each column in the figure represents a sample.

the survival rate of fattening rabbits is primarily related to the incidence of epizootic rabbit enteropathy (Davies and Davies, 2003; Jin et al., 2018), which could explain the decrease in the relative abundance of Verrucomicrobia in healthy rabbits.

In this study, *Ruminococcaceae NK4A214 group*, *Ruminococcaceae UGG-014,* and *Akkermansia* were the dominant genera in the growing period of Ira rabbits. The *Ruminococcaceae*, as a member of Firmicutes, such as *Ruminococcaceae NK4A214 group*, *Ruminococcaceae UCG-014*, *Ruminococcaceae V9D2013 group*, and *Ruminococcaceae UCG-013*, have been reported to play a role in the fermentation of dietary fiber and polysaccharides (Louis et al., 2010; Chen et al., 2017; Zhao et al., 2017). Meanwhile, *Ruminococcaceae* one of the most abundant families of the order *Clostridiales* is associated with the maintenance of gut healthy (Biddle et al., 2013). In addition, they primarily produce butyrate in the gut (Ley et al., 2006; Louis and Flint, 2009). Butyrate generated by gut microbiota is a significant energy source for gut microbes and host colonic epithelium, which maintains the gut barrier functions (Bui et al., 2015; Riviére et al., 2016) and plays a critical role in colon health (Louis and Flint, 2009). Importantly, butyrate is recognized by G protein-coupled receptors FFAR3 and GPR109A and is involved in regulating energy and nutrient metabolism and having an anti-inflammatory effect (Le Poul et al., 2003; Ahmed et al., 2009; Kasubuchi et al., 2015). The relative abundances of *Ruminococcaceae*, which degrade solid feed, increase with age and become more dominant during the post-weaning period (Padilha et al., 1995). In this study, we investigated that the increase of the relative abundance of *Ruminococcaceae_NK4A214_group* was affected by the *C. butyricum* supplementation, which enhanced the superior position of Firmicutes in the intestinal microbial. This study found that the relative abundance of family *Lactobacillus* (*Lachnospiraceae NK4A136 group*) increased with the dose of *C. butyricum* and decreased with age, which contributes to the metabolism of breast milk (Jost et al., 2015; de Muinck and Trosvik, 2018).

Metabolomics is a new "omics" including genomics, transcriptomics, and proteomics (Liang et al., 2021). The subtle

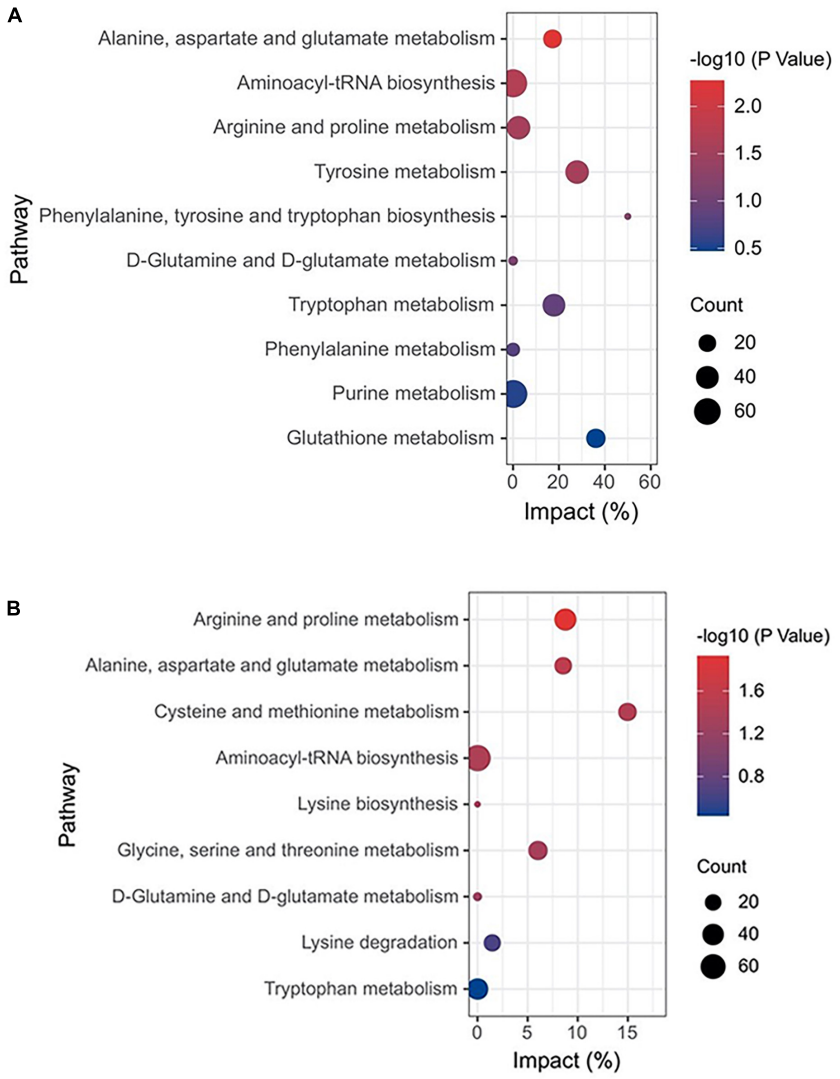


FIGURE 6
KEGG function annotation analysis of plasma metabolites in Ira rabbits. **(A)** The bubble plot of KEGG indicates different enrichments affected by *C. butyricum* of Ira rabbits at 53 days of age. **(B)** The bubble plot of KEGG indicates different enrichments affected by *C. butyricum* of Ira rabbits at 67 days of age.

changes in metabolite content directly correlate with changes in biological phenotype (Nobeli and Thornton, 2006). To identify the *C. butyricum*-based metabolites, we investigated the characteristics of plasma metabolomics. In this study, *C. butyricum* supplementation significantly influenced the dose of metabolites belonging to fatty acids, amino acids, and organic acids (**Figures 6A,B**). The content of L-Valine, L-Methionine, and L-Threonine were upregulated in the HC group of *C. butyricum* (**Figure 6B**). Valine is an important essential amino acid for rabbits, and is cataloged as a branched-chain amino acid. After transamination, oxidative decarboxylation, and dehydrogenation, succinic monoacyl CoA is produced, which enters into the tricarboxylic acid cycle and supplies energy to bodies (Liang et al., 2021). In this study, the association analysis revealed a high correlation between L-Valine and BW, and ADG, which is consistent with these findings (**Figures 7A,B**). Therefore, *C. butyricum* could improve the growth performance of rabbits by promoting amino acid metabolism. L-Methionine is a precursor of other sulfur-containing amino acids and a limiting amino acid for rabbits, and it plays an essential role in protein synthesis of cecal bacteria and regulation of mucosal response to antigens as well (Firkins et al., 2015; Mariz et al., 2018; Liu et al., 2019a). L-Threonine is a major component of mucins (MUCs) and γ-globulins (Wang et al., 2009). In addition, Arginine and glutamine are the substrates to synthesize the functional amino acid L-Proline, upregulated in the experimental group (HC group) in this study. L-Proline is a significant amino acid for maintaining cell

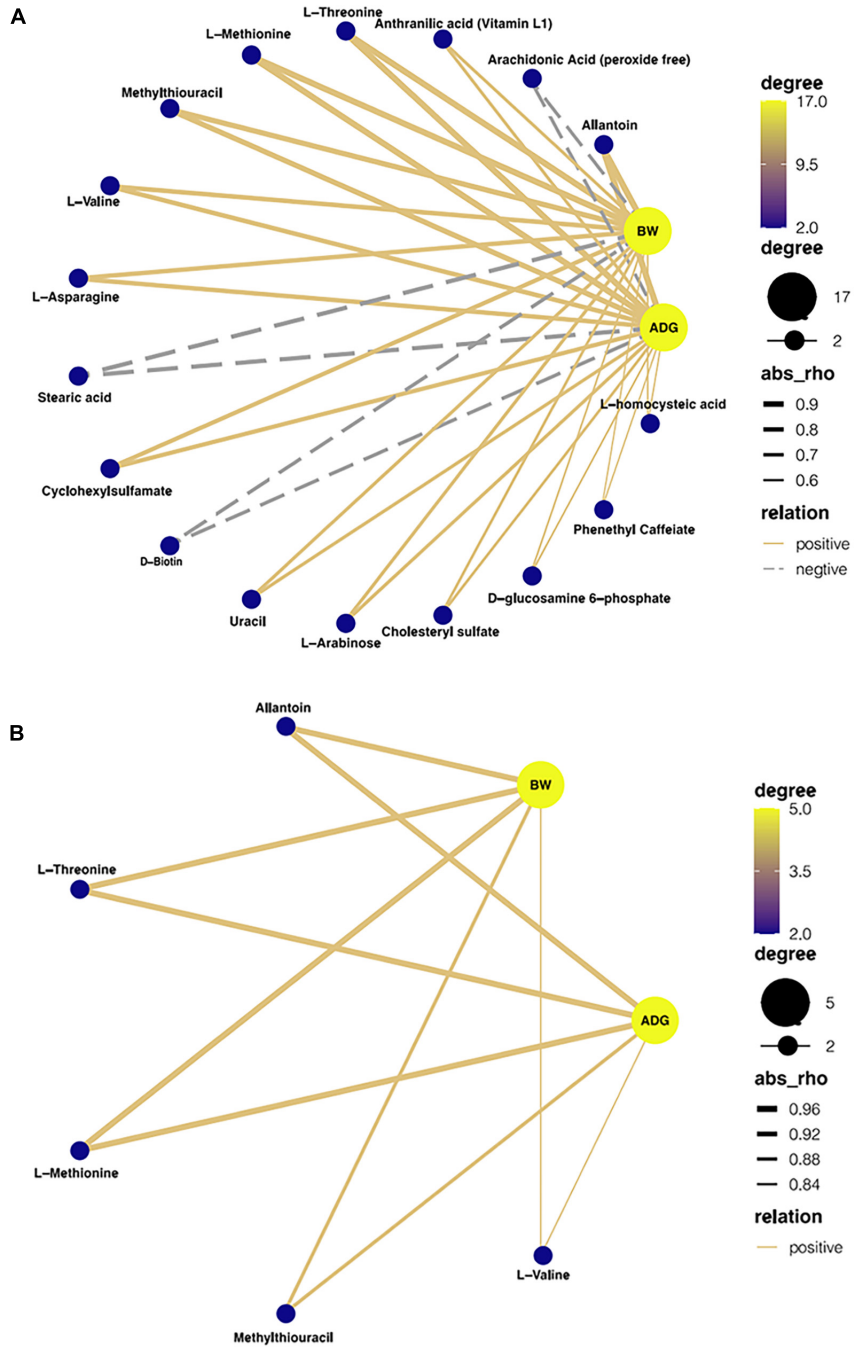


FIGURE 7
Interaction network analysis between growth performance and metabolites in Ira rabbits. **(A)** 53 days old; **(B)** 67 days old. Blue nodes represent the center degree of genera and metabolites. The node size indicates the level of center degree. Lines linked to nodes indicate significant correlations among the species (FDR adjusted $P < 0.05$, $|r| > 0.5$), with orange and gray colors showing positive and negative correlations, respectively.

structure and functions and functioning as an essential regulator of cell metabolism and physiology. Proline is also an essential precursor in protein synthesis and anti-oxidative reactions in wounds and immune responses (McAllan and Smith, 1973; Ametaj et al., 2010). Furthermore, this study discovered increases in the concentrations of metabolites associated with purine metabolism. Purine is catabolized through a few intermediates to hypoxanthine converted to Xanthine; thus, Xanthine is a biomarker of microbial protein synthesis (Duan et al., 2017; Yiwei and Chi, 2019).

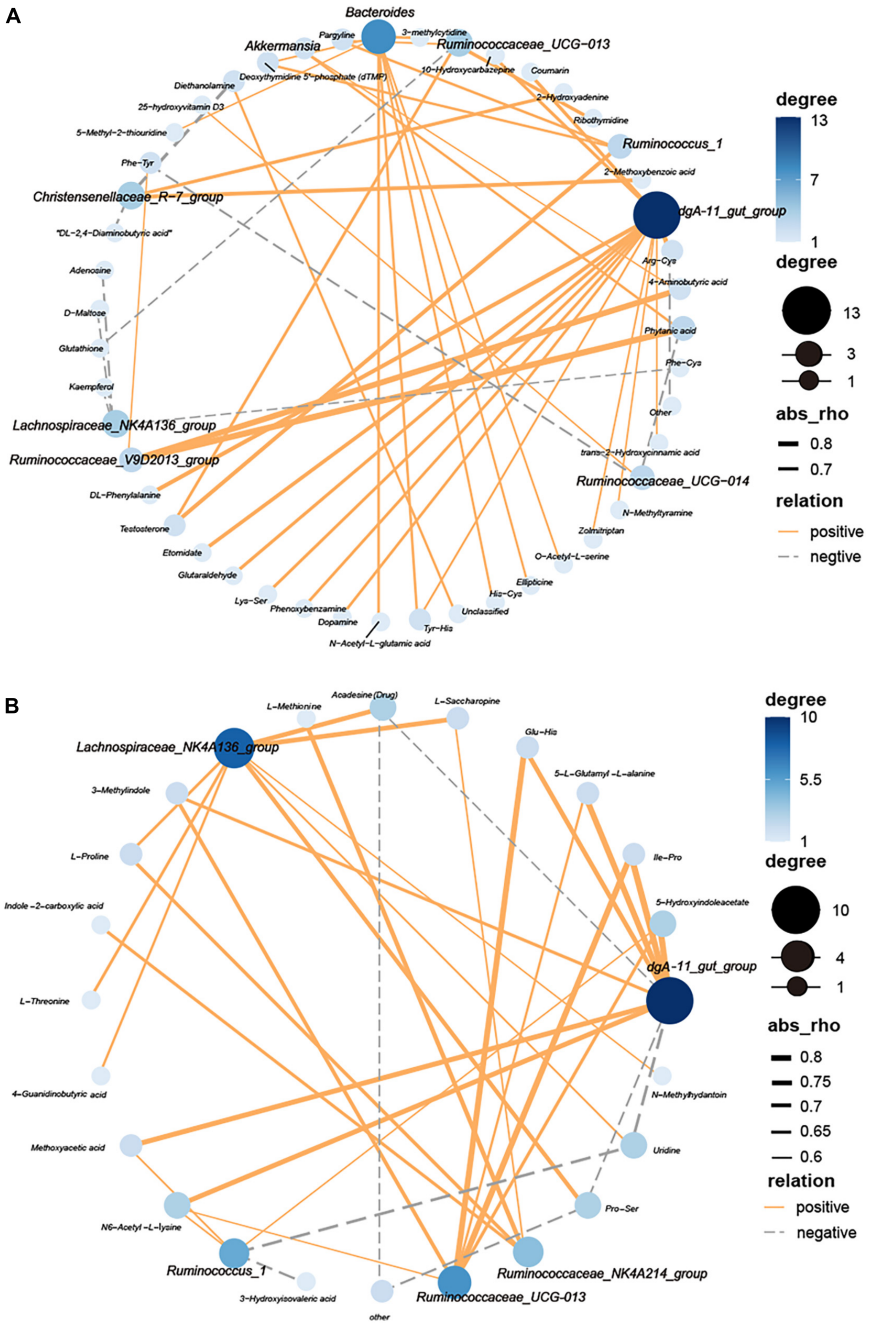


FIGURE 8
Interaction network analysis between cecal bacteria (bold font) and plasma metabolites of Ira rabbits. **(A)** 53 days of age. **(B)** 67 days of age. Blue nodes represent the center degree of genera and metabolites. The node size indicates the level of center degree. Lines linked to nodes indicate significant correlations among the species (FDR adjusted $P < 0.05$, $|r| > 0.5$), with orange and gray colors showing positive and negative correlations, respectively.

An earlier study has revealed that there was a reciprocal relationship between gut microbes and several metabolites that maintains intestinal homeostasis (Vascellari et al., 2020). The altered metabolome profile could influence the differences in the gut microbiome of animals (Coleman et al., 2019; Ye et al., 2021). In our study, several beneficial bacteria, such as families *Ruminococcaceae (Ruminococcaceae NK4A214 group)*and *Lachnospiraceae (Lachnospiraceae NK4A136 group)*were positively correlated with amino acids (e.g., L-Valine, L-Methionine, and L-Threonine), organic acids (e.g., Indole-2-carboxylic acid, 4-Guanidinobutyric acid, 3-Hydroxyisovaleric acid), and fatty acids (e.g., Methoxyacetic

acid). These results suggest that, for Ira rabbits, *C. butyricum* can alter the gut microbial community and plasma metabolome, implying that the interactions among gut microbial-plasma metabolome are important. However, their mechanisms still need to be explored.

Throughout our study, *C. butyricum* was shown to directly influence the gut bacterial community and plasma metabolites in rabbits. Accumulating evidence indicates that phenotypic traits of animals are changed by gut microbes, while the concentrations of plasma metabolites influence the functions of gut bacteria to synthesize and metabolize nutrients (Henry et al., 2020; Tang et al., 2020). Overall, these changes and relationships reveal essential features associated with *C. butyricum* supplementation in rabbit diets and that alteration in the gut microbial composition and metabolic profiles may affect rabbit production efficiency.

## Conclusion

The study results suggested that, based on the different doses of *C. butyricum*, the growth performance was significantly influenced by the *C. butyricum*. We examined the overall comprehension of the patterns of microbial community and metabolite compositions at different growth stages of Ira rabbits. The microbial community was changed by the supplementation of *C. butyricum*, especially prompting the beneficial microbial, which have related to the growth performance of Ira rabbits. Meanwhile, the metabolites were affected by the *C. butyricum*. The control group and the experimental group had different contents of significant metabolites. Furthermore, Spearman's correlation analysis revealed an obvious correlation between the microbiota and metabolites, meanwhile, a strong correlation between the metabolome and growth performance was discovered as well. Our results provide information that could aid future studies in determining the microbiome-metabolome interactions and how it influences the growth performance of Ira 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 below: NCBI, PRJNA861087.

## Author contributions

XX contributed to conception and design of the study, performed the experiments, and revised the manuscript. KL analyzed the data and wrote and revised the manuscript. KY, JN, PT, and HY performed the experiments, analyzed the data, and revised the manuscript. LW, SS, CY, and SM performed the experiments. QF conceived and designed the experiments, supervised the experiment progress, and revised 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.

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## Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2022.974337/full#supplementary-material

# References

Ahmed, K., Tunaru, S., and Offermanns, S. (2009). Gpr109A, Gpr109B and Gpr81, a family of hydroxy-carboxylic acid receptors. *Trends Pharmacol. Sci.* 30, 557–562. doi: 10.1016/j.tips.2009.09.001

Alayande, K. A., Aiyegoro, O. A., and Ateba, C. N. (2020). Probiotics in animal husbandry: Applicability and associated risk factors. *Sustainability* 12:1087. doi: 10.3390/su12031087

Ametaj, B. N., Zebeli, Q., Saleem, F., Psychogios, N., and Wishart, D. S. (2010). Metabolomics reveals unhealthy alterations in rumen metabolism with increased proportion of cereal grain in the diet of dairy cows. *Metabolomics* 6, 583–594. doi: 10.1007/s11306-010-0227-6

Anhê, F. F., Varin, T. V., Le Barz, M., Desjardins, Y., Levy, E., Roy, D., et al. (2015). Gut microbiota dysbiosis in obesity-linked diseases and prebiotic potential of polyphenol-rich extracts. *Curr. Obes. Rep.* 4, 389–400. doi: 10.1007/s13679-015-0172-9

Avershina, E., Lundgård, K., Sekelja, M., Dotterud, C., Storrø, O., Øien, T., et al. (2016). Transition from infant- to adult-like gut microbiota. *Environ. Microbiol.* 18, 2226–2236. doi: 10.1111/1462-2920.13248

Biddle, A., Stewart, L., Blanchard, J., and Leschine, S. (2013). Untangling the genetic basis of fibrolytic specialization by Lachnospiraceae and Ruminococcaceae in diverse gut communities. *Diversity* 5, 627–640. doi: 10.3390/d5030627

Bui, T. P., Ritari, J., Boeren, S., De Waard, P., Plugge, C. M., and De Vos, W. M. (2015). Production of butyrate from lysine and the Amadori product fructoselysine by a human gut commensal. *Nat. Commun.* 6:10062. doi: 10.1038/ncomms10062

Casas, G. A., Blavi, L., Cross, T.-W. L., Lee, A. H., Swanson, K. S., and Stein, H. H. (2020). Inclusion of the direct-fed microbial Clostridium butyricum in diets for weanling pigs increases growth performance and tends to increase villus height and crypt depth, but does not change intestinal microbial abundance. *J. Animal Sci.* 98:skz372. doi: 10.1093/jas/skz372

Cato, P. (1986). Genus Clostridium Prazmowski 1880, 23AL. *Bergeys Manual Syst. Bacteriol.* 2, 1144–1200.

Chen, D., Jin, D., Huang, S., Wu, J., and Cao, H. (2020). Clostridium butyricum, a butyrate-producing probiotic, inhibits intestinal tumor development through modulating Wnt signaling and gut microbiota. *Cancer Lett.* 469, 456–467. doi: 10.1016/j.canlet.2019.11.019

Chen, J., Huang, C., Wang, J., Zhou, H., Lu, Y., Lou, L., et al. (2017). Dysbiosis of intestinal microbiota and decrease in paneth cell antimicrobial peptide level during acute necrotizing pancreatitis in rats. *PLoS One* 12:e0176583. doi: 10.1371/journal.pone.0176583

Chen, S., Zhou, Y., Chen, Y., and Gu, J. (2018). fastp: An ultra-fast all-in-one FASTQ preprocessor. *Bioinformatics* 34, i884–i890. doi: 10.1093/bioinformatics/bty560

Chipo, M. M., and Tapiwa, K. A. (2019). Challenges and opportunities to rabbit (*Oryctolagus cuniculus*) production and marketing. *Int. J. Agric. Agribus.* 5:7.

Coleman, M. C., Whitfield-Cargile, C. M., Madrigal, R. G., and Cohen, N. D. (2019). Comparison of the microbiome, metabolome, and lipidome of obese and non-obese horses. *PLoS One* 14:e0215918. doi: 10.1371/journal.pone.0215918

Nutrition Research Council (1930). *Nutrient requirements of rabbits: 1977*. Washington DC: National Research Council.

Cunniff, P. A., Cunniff, P. A., Cunniff, P., Cunniff, P., Cunniff, P. A., Cunniff, P. A., et al. (1995). Official methods of analysis of aoac international. *AOAC Official Method* 6, 382–382.

Davies, R. R., and Davies, J. A. E. R. (2003). Rabbit gastrointestinal physiology. *Vet. Clin. North Am. Exot. Anim. Pract.* 6, 139–153. doi: 10.1016/S1094-9194(02)00024-5

de Muinck, E. J., and Trosvik, P. (2018). Individuality and convergence of the infant gut microbiota during the first year of life. *Nat. Commun.* 9:2233. doi: 10.1038/s41467-018-04641-7

D'Elia, M. A., Henderson, J. A., Beveridge, T. J., Heinrichs, D. E., and Brown, E. D. (2009). The N-acetylmannosamine transferase catalyzes the first committed step of teichoic acid assembly in *Bacillus subtilis* and *Staphylococcus aureus*. *J. Bacteriol.* 191, 4030–4034. doi: 10.1128/JB.00611-08

Duan, Y., Wang, Y., Dong, H., Ding, X., Liu, Q., Li, H., et al. (2018). Changes in the intestine microbial, digestive, and immune-related genes of Litopenaeus vannamei in response to dietary probiotic Clostridium butyricum supplementation. *Front. Microbiol.* 9:2191. doi: 10.1038/s41598-019-42939-8

Duan, Y., Zhang, Y., Dong, H., Wang, Y., and Zhang, J. (2017). Effect of the dietary probiotic *Clostridium butyricum* on growth, intestine antioxidant capacity and resistance to high temperature stress in kuruma shrimp *Marsupenaeus japonicus*. *J. Therm. Biol.* 66, 93–100. doi: 10.1016/j.jtherbio.2017.04.004

Edgar, R. C. (2013). UPARSE: Highly accurate OTU sequences from microbial amplicon reads. *Nat. Methods* 10, 996–998. doi: 10.1038/nmeth.2604

Fang, S., Chen, X., Pan, J., Chen, Q., Zhou, L., Wang, C., et al. (2020). Dynamic distribution of gut microbiota in meat rabbits at different growth stages and relationship with average daily gain (ADG). *BMC Microbiol.* 20:116. doi: 10.1186/s12866-020-01797-5

Firkins, J. L., Fowler, C. M., Devillard, E., and Bequette, B. J. (2015). Kinetics of microbial methionine metabolism in continuous cultures administered different methionine sources. *J. Dairy Sci.* 98, 1178–1194. doi: 10.3168/jds.2014-8694

Gao, J., Li, X., Zhang, G., Sadiq, F. A., Simal-Gandara, J., Xiao, J., et al. (2021). Probiotics in the dairy industry-advances and opportunities. *Compr. Rev. Food Sci. Food Saf.* 20, 3937–3982. doi: 10.1111/1541-4337.12755

Gao, Q., Qi, L., Wu, T., and Wang, J. (2012). Ability of *Clostridium butyricum* to inhibit *Escherichia coli*-induced apoptosis in chicken embryo intestinal cells. *Vet. Microbiol.* 160, 395–402. doi: 10.1016/j.vetmic.2012.06.009

Hagihara, M., Ariyoshi, T., Kuroki, Y., Eguchi, S., Higashi, S., Mori, T., et al. (2021). Clostridium butyricum enhances colonization resistance against Clostridioides difficile by metabolic and immune modulation. *Sci. Rep.* 11, 1–15. doi: 10.1038/s41598-021-94572-z

Han, Y., Tang, C., Li, Y., Yu, Y., Zhan, T., Zhao, Q., et al. (2020). Effects of dietary supplementation with *Clostridium butyricum* on growth performance, serum immunity, intestinal morphology, and microbiota as an antibiotic alternative in weaned piglets. *Animals (Basel)* 10:2287. doi: 10.3390/ani10122287

Henry, Y., Overgaard, J., and Colinet, H. (2020). Dietary nutrient balance shapes phenotypic traits of *Drosophila melanogaster* in interaction with gut microbiota. *Comp. Biochem. Physiol. A Mol. Integr. Physiol.* 241:110626. doi: 10.1016/j.cbpa.2019.110626

Hsiao, Y. P., Chen, H. L., Tsai, J. N., Lin, M. Y., Liao, J. W., Wei, M. S., et al. (2021). Administration of lactobacillus reuteri combined with *Clostridium butyricum* attenuates cisplatin-induced renal damage by gut microbiota reconstitution, increasing butyric acid production, and suppressing renal inflammation. *Nutrients* 13:2792. doi: 10.3390/nu13082792

Hu, J., Ma, L., Nie, Y., Chen, J., Zheng, W., Wang, X., et al. (2018). A Microbiota-Derived Bacteriocin Targets the Host to Confer Diarrhea Resistance in Early-Weaned Piglets. *Cell Host Microbe* 24, 817.e–832.e. doi: 10.1016/j.chom.2018.11.006

Huang, P., Cui, X., Wang, Z., Xiao, C., Ji, Q., Wei, Q., et al. (2021). Effects of *Clostridium butyricum* and a Bacteriophage Cocktail on growth performance, serum biochemistry, digestive enzyme activities, intestinal morphology, immune responses, and the intestinal microbiota in rabbits. *Antibiotics (Basel)* 10:1347. doi: 10.3390/antibiotics10111347

Huang, X., Gao, J., Zhao, Y., He, M., Ke, S., Wu, J., et al. (2019). Dramatic remodeling of the gut microbiome around parturition and its relationship with host serum metabolic changes in sows. *Front. Microbiol.* 10:2123. doi: 10.3389/fmicb.2019.02123

Jin, D. X., Zou, H. W., Liu, S. Q., Wang, L. Z., Xue, B., Wu, D., et al. (2018). The underlying microbial mechanism of epizootic rabbit enteropathy triggered by a low fiber diet. *Sci. Rep.* 812489. doi: 10.1038/s41598-018-30178-2

Jost, T., Lacroix, C., Braegger, C., and Chassard, C. (2015). Impact of human milk bacteria and oligosaccharides on neonatal gut microbiota establishment and gut health. *Nutr. Rev.* 73, 426–437. doi: 10.1093/nutrit/nuu016

Kasubuchi, M., Hasegawa, S., Hiramatsu, T., Ichimura, A., and Kimura, I. (2015). Dietary gut microbial metabolites, short-chain fatty acids, and host metabolic regulation. *Nutrients* 7, 2839–2849. doi: 10.3390/nu7042839

Kolde, R. (2015). *R package Version 1.0. 8*. Pheatmap: Pretty Heatmaps.

Le Poul, E., Loison, C., Struyf, S., Springael, J. Y., Lannoy, V., Decobecq, M. E., et al. (2003). Functional characterization of human receptors for short chain fatty acids and their role in polymorphonuclear cell activation. *J. Biol. Chem.* 278, 25481–25489. doi: 10.1074/jbc.M301403200

Ley, R. E., Turnbaugh, P. J., Klein, S., and Gordon, J. I. (2006). Microbial ecology: Human gut microbes associated with obesity. *Nature* 444, 1022–1023. doi: 10.1038/4441022a

Li, P., Yang, S., Zhang, X., Huang, S., Wang, N., Wang, M., et al. (2018). Zearalenone changes the diversity and composition of caecum microbiota in weaned rabbit. *Biomed. Res. Int.* 20183623274. doi: 10.1155/2018/3623274

Li, W., Xu, B., Wang, L., Sun, Q., Deng, W., Wei, F., et al. (2021a). Effects of *Clostridium butyricum* on growth performance, gut microbiota and intestinal

barrier function of broilers. *Front. Microbiol.* 12:777456. doi: 10.3389/fmicb.2021.777456

Li, Y., Wang, Y., Lv, J., Dou, X., and Zhang, Y. (2021b). Effects of dietary supplementation With *Clostridium butyricum* on the amelioration of growth performance, rumen fermentation, and rumen microbiota of holstein heifers. *Front. Nutr.* 8:763700. doi: 10.3389/fnut.2021.763700

Liang, J., Kou, S., Chen, C., Raza, S. H. A., Wang, S., Ma, X., et al. (2021). Effects of *Clostridium butyricum* on growth performance, metabonomics and intestinal microbial differences of weaned piglets. *BMC Microbiol.* 21:85. doi: 10.1186/s12866-021-02143-z

Liu, L., Zeng, D., Yang, M., Wen, B., Lai, J., Zhou, Y., et al. (2019b). Probiotic *Clostridium butyricum* improves the growth performance, immune function, and gut microbiota of weaning rex rabbits. *Prob. Antimicrob. Proteins* 11, 1278–1292. doi: 10.1007/s12602-018-9476-x

Liu, C., Wu, H., Liu, S., Chai, S., Meng, Q., and Zhou, Z. (2019a). Dynamic alterations in yak rumen bacteria community and metabolome characteristics in response to feed type. *Front. Microbiol.* 10:1116. doi: 10.3389/fmicb.2019.01116

Liu, Y., Liu, C., An, K., Gong, X., and Xia, Z. (2021). Effect of dietary *Clostridium butyricum* supplementation on growth performance, intestinal barrier function, immune function, and microbiota diversity of pekin ducks. *Animals* 11:2514. doi: 10.3390/ani11092514

Lopez, M., Madrid, J., Hernandez, F., Antonio Ros, M., Carlos Segura, J., Jose Lopez, M., et al. (2021). Effect of feed supplementation with *Clostridium butyricum*, alone or in combination with carob meal or citrus pulp, on digestive and metabolic status of piglets. *Animals* 11:2924. doi: 10.3390/ani11102924

Louis, P., and Flint, H. J. (2009). Diversity, metabolism and microbial ecology of butyrate-producing bacteria from the human large intestine. *FEMS Microbiol. Lett.* 294, 1–8. doi: 10.1111/j.1574-6968.2009.01514.x

Louis, P., Young, P., Holtrop, G., and Flint, H. J. (2010). Diversity of human colonic butyrate-producing bacteria revealed by analysis of the butyryl-CoA:acetate CoA-transferase gene. *Environ. Microbiol.* 12, 304–314. doi: 10.1111/j.1462-2920.2009.02066.x

Luo, K., Tian, X., Wang, B., Wei, C., Wang, L., Zhang, S., et al. (2021). Evaluation of paraprobiotic applicability of *Clostridium butyricum* CBG01 in improving the growth performance, immune responses and disease resistance in Pacific white shrimp, Penaeus vannamei. *Aquaculture* 544:737041.

Magoč, T., and Salzberg, S. L. (2011). FLASH: Fast length adjustment of short reads to improve genome assemblies. *Bioinformatics* 27, 2957–2963. doi: 10.1093/bioinformatics/btr507

Mariz, L. D. S., Amaral, P. M., Valadares Filho, S. C., Santos, S. A., Detmann, E., Marcondes, M. I., et al. (2018). Dietary protein reduction on microbial protein, amino acid digestibility, and body retention in beef cattle: 2. Amino acid intestinal absorption and their efficiency for whole-body deposition. *J. Anim. Sci.* 96, 670–683. doi: 10.1093/jas/sky018

Mattioli, S., Dal Bosco, A., Combes, S., Moscati, L., Crotti, S., Cartoni Mancinelli, A., et al. (2019). Dehydrated alfalfa and fresh grass supply in young rabbits: Effect on performance and caecal microbiota biodiversity. *Animals (Basel)* 9:341. doi: 10.3390/ani9060341

McAllan, A. B., and Smith, R. H. (1973). Degradation of nucleic acids in the rumen. *Br. J. Nutr.* 29, 331–345. doi: 10.1079/BJN19730107

Molnár, A., Such, N., Farkas, V., PáL, L., Menyhárt, L., Wágner, L., et al. (2020). Effects of wheat bran and *Clostridium butyricum* supplementation on cecal microbiota, short-chain fatty acid concentration, ph and histomorphometry in broiler chickens. *Animals (Basel)* 10:2230. doi: 10.3390/ani10122230

Nobeli, I., and Thornton, J. M. (2006). A bioinformatician's view of the metabolome. *Bioessays* 28, 534–545. doi: 10.1002/bies.20414

Padilha, M. T., Licois, D., Gidenne, T., Carré, B., and Fonty, G. (1995). Relationships between microflora and caecal fermentation in rabbits before and after weaning. *Reprod. Nutr. Dev.* 35, 375–386. doi: 10.1051/rnd:19950403

Park, S. H., Hanning, I., Perrota, A., Bench, B. J., Alm, E., and Ricke, S. C. (2013). Modifying the gastrointestinal ecology in alternatively raised poultry and the potential for molecular and metabolomic assessment1 1Presented as part of the Next Generation Sequencing: Applications for Food Safety and Poultry Production Symposium at the Poultry Science Association's annual meeting in Athens, Georgia, July 10, 2012. *Poultry Sci.* 92, 546–561. doi: 10.3382/ps.2012-02734

Riviére, A., Selak, M., Lantin, D., Leroy, F., and De Vuyst, L. (2016). Bifidobacteria and butyrate-producing colon bacteria: Importance and strategies for their stimulation in the human gut. *Front. Microbiol.* 7:979. doi: 10.3389/fmicb.2016.00979

Shen, J., Zhang, B., Wei, G., Pang, X., Wei, H., Li, M., et al. (2006). Molecular profiling of the *Clostridium leptum* subgroup in human fecal microflora by PCR-denaturing gradient gel electrophoresis and clone library analysis. *Appl. Environ. Microbiol.* 72, 5232–5238. doi: 10.1128/AEM.00151-06

Smith, C. A., Want, E. J., O'maille, G., Abagyan, R., and Siuzdak, G. (2006). Xcms: Processing mass spectrometry data for metabolite profiling using nonlinear peak alignment, matching, and identification. *Anal. Chem.* 78, 779–787. doi: 10.1021/ac051437y

Stackebrandt, E., and Goebel, B. M. (1994). Taxonomic note: A place for DNA-DNA reassociation and 16S rRNA sequence analysis in the present species definition in bacteriology. *Int. J. Syst. Evol. Microbiol.* 44, 846–849. doi: 10.1099/00207713-44-4-846

Tang, S., Xin, Y., Ma, Y., Xu, X., Zhao, S., and Cao, J. (2020). Screening of microbes associated with swine growth and fat deposition traits across the intestinal tract. *Front. Microbiol.* 11:586776. doi: 10.3389/fmicb.2020.586776

Tian, H., Wang, W., Zheng, N., Cheng, J., Li, S., Zhang, Y., et al. (2015). Identification of diagnostic biomarkers and metabolic pathway shifts of heat-stressed lactating dairy cows. *J. Proteom.* 125, 17–28. doi: 10.1016/j.jprot.2015.04.014

Tran, N. T., Li, Z., Ma, H., Zhang, Y., Zheng, H., Gong, Y., et al. (2020). *Clostridium butyricum*: A promising probiotic confers positive health benefits in aquatic animals. *Rev. Aquacult.* 12, 2573–2589. doi: 10.1111/raq.12459

Turnbaugh, P. J., Ley, R. E., Mahowald, M. A., Magrini, V., Mardis, E. R., and Gordon, J. I. (2006). An obesity-associated gut microbiome with increased capacity for energy harvest. *Nature* 444, 1027–1031. doi: 10.1038/nature05414

Vascellari, S., Palmas, V., Melis, M., Pisanu, S., Cusano, R., Uva, P., et al. (2020). Gut microbiota and metabolome alterations associated with Parkinson's Disease. *mSystems* 5:e0028321. doi: 10.1128/mSystems.00561-20

Wang, J., Zhang, T., Shen, X., Liu, J., Zhao, D., Sun, Y., et al. (2016). Serum metabolomics for early diagnosis of esophageal squamous cell carcinoma by UHPLC-QTOF/MS. *Metabolomics* 12:116. doi: 10.1007/s11306-016-1050-5

Wang, Q., Garrity, G. M., Tiedje, J. M., and Cole, J. R. (2007). Naive Bayesian classifier for rapid assignment of rRNA sequences into the new bacterial taxonomy. *Appl. Environ. Microbiol.* 73, 5261–5267. doi: 10.1128/AEM.00062-07

Wang, W. W., Qiao, S. Y., and Li, D. F. (2009). Amino acids and gut function. *Amino. Acids* 37, 105–110. doi: 10.1007/s00726-008-0152-4

Ye, X., Zhou, L., Zhang, Y., Xue, S., Gan, Q. F., and Fang, S. (2021). Effect of host breeds on gut microbiome and serum metabolome in meat rabbits. *BMC Vet. Res.* 17:24. doi: 10.1186/s12917-020-02732-6

Yiwei, M., and Chi, C. (2019). Prebiotic functions of mannose oligosaccharides revealed by microbiomic and metabolomic analyses of intestinal digesta (P20-017-19). *Curr. Dev. Nutr.* 3(Suppl 1):nzz040. doi: 10.1093/cdn/nzz040.P20-017-19

Yu, J., Dong, B., Zhao, M., Liu, L., Geng, T., Gong, D., et al. (2021). Dietary *Clostridium butyricum* and bacillus subtilis promote goose growth by improving intestinal structure and function, antioxidative capacity and microbial composition. *Animals (Basel)* 11:3174. doi: 10.3390/ani11113174

Zhang, J., Chen, X., Liu, P., Zhao, J., Sun, J., Guan, W., et al. (2018). Dietary *Clostridium butyricum* induces a phased shift in fecal microbiota structure and increases the acetic acid-producing bacteria in a weaned piglet model. *J. Agric. Food Chem.* 66, 5157–5166. doi: 10.1021/acs.jafc.8b01253

Zhang, L. (2007). *Feed analysis and feed quality testing technology*. Beijing: China Agricultural University Press. .(In Chinese).

Zhang, L., Zhang, L., Zhan, X., Zeng, X., Zhou, L., Cao, G., et al. (2016). Effects of dietary supplementation of probiotic, Clostridium butyricum, on growth performance, immune response, intestinal barrier function, and digestive enzyme activity in broiler chickens challenged with *Escherichia coli* K88. *J. Anim. Sci. Biotechnol.* 73. doi: 10.1186/s40104-016-0061-4

Zhao, L., Zhang, Q., Ma, W., Tian, F., Shen, H., and Zhou, M. (2017). A combination of quercetin and resveratrol reduces obesity in high-fat diet-fed rats by modulation of gut microbiota. *Food Funct.* 8, 4644–4656. doi: 10.1039/C7FO01383C

Zhu, Y., Wang, C., and Li, F. (2015). Impact of dietary fiber/starch ratio in shaping caecal microbiota in rabbits. *Can. J. Microbiol.* 61, 771–784. doi: 10.1139/cjm-2015-0201

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# Alleviation effects of niacin supplementation on beef cattle subjected to heat stress: A metagenomic insight

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The objective of this study was to investigate the alleviation effects of niacin supplementation on beef cattle subjected to heat stress and to provide a theoretical basis for exploring the alleviation methods of heat stress environmental factors on the rumen of beef cattle. In the experiment, 36 Jinjiang bull cattle with a body weight of about 400 ± 20.0 kg were randomly divided into three treatments, each treatment contains four replicates, with three cattle in each replicate. Treatments included thermoneutral treatment (TN; temperature: 24–25°C, humidity: 45–55%), heat stress treatment, exposure to environmental temperature (HS; average THI: 82.74), and heat stress supplemented with niacin treatment (HN; high temperature + 800 mg/kg NA). Measured indicators were body temperature, respiratory rate, production performances, rumen fermentations, and microbial diversity. Results showed that adding niacin reduced the body temperature and respiratory rate ($P < 0.05$) but had no significant effect on the production performances compared with heat-stressed beef cattle. HS treatment significantly increased body temperature and respiratory rate ($P < 0.01$), while decreasing the content of acetic acid, butyric acid, and total volatile fatty acids ($P < 0.05$) compared with the TN treatment. Supplement of niacin did not affect ruminal fermentation parameters ($P > 0.05$) but had a decreased tendency on A/P ($P < 0.1$). Microbial diversity results showed that, at the phylum level, the relative abundance of *Desulfobacterota* in the HS treatment was increased compared with TN and HN treatment ($P < 0.05$). At the genus level, the relative abundance

of *Succiniclasticum* and *Family_XIII_AD3011 group* in the HN treatment significantly proliferated compared with the HS treatment ($P < 0.05$). In conclusion, niacin supplementation may alleviate heat stress by proliferating those bacteria belonging to the phylum *Succiniclasticum*, which may further contribute to the digestion of cellulose and the improvement of the metabolic function of Jinjiang cattle under heat-stress conditions.

KEYWORDS

**Jinjiang bulls, niacin, heat stress, rumen fermentation, rumen microbiome**

## Introduction

Heat stress is one of the most detrimental problems that impact the livestock industry in all subtropical countries during summer, especially in Southern China. The highly humid and hot environment easily induced heat stress on beef cattle resulting in abnormal physiological behavior and decreased immunity and growth performance, which caused serious impacts on the industrial economy. Previous studies have shown that heat stress significantly increased heart rate (HR), respiratory rate, and rectal temperature (RT), which further reduced feed intake, triggered negative energy balance, and lead to reduced performance in beef cattle (O'Brien et al., 2010; Collier et al., 2012; Kim et al., 2018). Under heat stress conditions, ruminant roughage intake decreases, which decreases chewing time and saliva production, and reduced rumination frequencies and rumen peristalsis (Soriani et al., 2013). In addition, heat stress reduced rumen contraction and motility, and altered rumen fermentation patterns (Eslamizad et al., 2020). Simultaneously, changes in the host metabolism caused by heat stress induced disorders in ruminal microbiota and affected the absorption of nutrients (Bergman, 1990; Tajima et al., 2007; Uyeno et al., 2010). Therefore, proper methods in ameliorating the heat stress of beef cattle are seriously needed.

Current strategies for alleviating heat stress mainly included nutritional management, environmental modification, and genetic selection of heat-tolerant cows through selective breeding programs (Sammad et al., 2020). Among these, supplementation with anti-heat stress additives played an effective and economical role. Anti-heat stress additives mainly consist of dietary yeast, chemical supplements, fermentates, betaine, dietary cation anion difference, propionate supplementation, and multivitamins, which play a vital role in enhancing the metabolic status of cattles and improving energy metabolism status and immune function (Renaudeau et al., 2012; Barreras et al., 2013; Bicalho et al., 2014; Wang et al., 2019).

Nicotinic acid (**NA**), also known as niacin, functionally participates in many biochemical processes acted as the precursor of $NAD^+$/NADH and $NADP^+$/NADPH, which included lipid metabolism, tissue oxidation, glycolysis, and respiratory functions (MacKay et al., 2012; Srivastava, 2016). Niacin elicits vasodilatory reactions that may be beneficial for cows under heat stress (Zimbelman et al., 2010). Peripheral and internal vasodilation, caused by therapeutic concentrations of NA (Mitchell, 1965), may enhance heat transfer from core to skin sites and generate a temperature gradient favoring heat loss from skin to environment. Moreover, niacin helps regulate energy metabolism, methylation, DNA rehabilitation, and immune function (Gasperi et al., 2019), this may positively affect heat-stressed animals.

A study found that niacin improves ruminal fermentation and can increase the concentration of rumen bacterial protein, ammonia, and propionic acid (Riddell et al., 1980). Another study showed more efficient use of rumen-degradable N due to improvements in the microbial population, especially the number of protozoa in rumen fluid in the rumen when niacin was supplemented to diets deficient in rumen nitrogen balance (RNB) for lactating dairy cows (Aschemann et al., 2012). The effects of niacin on rumen fermentation may be useful in avoiding ketogenic situations in dairy cows. Niacin has been proven to promote the growth of ruminal microbes, maintain the stability of the microbial community, and avoid lactate accumulation in dairy cows (Doreau and Ottou, 1996; Yang and Qu., 2013). However, few studies focused on the effects of niacin on rumen fermentation and microorganisms in beef cattle under heat stress conditions. Therefore, the purpose of this study is to investigate the alleviation effects of niacin on heat-stressed beef cattle through the measurement of physiological condition, growth performance, nutrient apparent digestibility, fermentation parameters, and rumen bacterial diversity.

## Materials and methods

### Ethical statement

Animal care and procedures followed The Chinese Guidelines for Animal Welfare, which was approved by the Animal Care and Use Committee of Jiangxi

TABLE 1 Composition and nutrient levels of the basal diet (dry matter basis).

| Items | Content (%) |
|---|---|
| **Ingredients, %** | |
| Brewers grains | 20.00 |
| Rice straw | 20.00 |
| Corn | 46.80 |
| Soybean meal | 9.00 |
| Premix[a] | 2.40 |
| $NaHCO_3$ | 1.20 |
| NaCl | 0.60 |
| **Nutrient composition, %** | |
| DM | 95.67 |
| CP | 14.44 |
| EE | 4.03 |
| Ash | 8.31 |
| NDF | 26.99 |
| ADF | 11.62 |
| Ca | 0.69 |
| P | 0.36 |

[a]One kilogram of premix provided the following: Vitamin A 150,000 IU, Vitamin D3 20,000IU, Vitamin E 3,000 IU, Fe 3 200 mg, Mn 1 500 mg, Zn 2 000 mg, Cu 650 mg, I 35 mg, Se 10 mg, Co 10 mg, Ca 130 g, P 30 g.

Agricultural University, with the approval number JXAULL-2021 1237.

## Animals and experimental design

Thirty-six Jinjiang bull beef cattle with an average age of 24-month-old and a mean body weight of 400 ± 20.0 kg were studied in a high ambient temperature environment during the summer months (July to September) in South China (Gaoan, 115.3753, 28.4178). During the trial, a two-factor completely randomized design method was used, and all cattles were randomly divided into thermoneutral treatment (TN, controlling barn temperatures with air conditioning, temperature: 24–25°C, humidity: 45–55%, and feeding basal diet), heat stress control (HS, determination of THI in summer from July to September to ensure that the barn is in a heat-stressed environment and feeding basal diet), and heat stress + niacin treatment (HN, feeding basal diet + 800 mg/kg NA). The ingredient and nutrient compositions of the basal diets are shown in Table 1.

For HN treatment, 800 mg/kg NA was added to the concentrate diet and mixed well. The diets were provided twice per day at 6:00 and 16:00. Freshwater was offered at any time through an automatic drinker. The experimental period lasted for 60 days after a 10-day adaptation period. All experimental protocols were approved by the Committee for the Care and Use of Experimental Animals, Jiangxi Agricultural University, Jiangxi, China.

## Measurement of the temperature-humidity index, body temperature, and respiratory rate

Temperature and relative humidity were measured using a dry bulb hygrometer at 07:30, 13:30, and 19:30 h. The temperature humidity index (THI) was calculated using the following equation:

$(1.8 \times T_{db} + 32) - [(0.55 - 0.0055 \times RH) \times (1.8 \times T_{db} - 26)]$

where $T_{db}$ is the dry-bulb temperature (°C) and RH is the relative humidity (%) (Dikmen and Hansen, 2009; Kim et al., 2018).

In general, the THI classification of heat stress is as follows: No heat stress when THI $\leq$ 72; mild heat stress when 72 <THI $\leq$ 79; high heat stress when 79 < THI$\leq$ 84; and severe heat stress when THI> 84 (Berman, 2005).

Body temperature was measured *via* the rectum using a digital thermometer (Crison model 637, Crison Instruments, Barcelona, Spain) at 08:00 h, and respiratory rate was measured on days 10, 20, 30, 40, 50, 60, through artificially counting of chest fluctuation in 1 min during the experimental period.

## Determination of the growth performance

Body weights of animals on an empty stomach (24 h) were measured at 09:00 h on days 1 and 60 of the experimental period and daily feed intakes were recorded. Based on these data, average daily feed intake (ADFI), average daily gain (ADG), and the feed:gain (F:G) ratio were calculated.

## Nutrient apparent digestibility tests

Samples of concentrate and forage were collected daily during the experiment period at the last 5 days of the experimental period. Total daily feces from each pen were weighed, collected, and stored at −20°C until analysis. Before analysis, the fecal samples from each pen and every single day were thoroughly mixed, and 3% of the wet weight was sampled. Feed and fecal samples were dried in a forced air oven at 65°C for 72 h and then ground by the Wiley mill through a 1 mm screen sieve. These samples were analyzed for dry matter (DM), organic matter (OM), crude proteins (CP), ether extract (EE), acid detergent fiber (ADF), and neutral detergent fiber (NDF), according to the AOAC International (2005) method (AOAC International, 2005). The calcium (Ca) content was estimated by the method of ethylene diaminetetraacetic acid complexometric titration and total phosphorus (P) content was estimated by the method of a visible spectrophotometer, according to GB/T 6436-2002 and GB/T 6437-2002 in China National Standards.

## Rumen fermentation index analysis

At the end of the trial, one cattle from each replicate, a total of 12 beef cattles were randomly selected for slaughtering and rumen fluid was then collected through a four-layer cheesecloth. Slaughter was carried out according to the cattle slaughter operation procedures of China, GB/T19477-2004. The pH value was determined by a portable pH meter immediately (PHS-3C, Shanghai, China). At the same time, storing 1 ml of rumen fluid was preserved at −80°C in sterile cryovials and stored in liquid nitrogen for further DNA extraction; other samples were processed to analyze VFA, microbial protein (MCP), and ammonia–N ($NH_3$-N). The concentrations of VFA were determined by a gas chromatograph (Agilent Technologies 7820A, USA) based on the method reported previously (Zhao et al., 2017); the ruminal MCP concentration was detected using a spectrophotometric method, and the concentration of $NH_3$-N was detected using a uric acid assay kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) according to the manufacturer's instructions.

## DNA Extraction, PCR Amplification, and sequencing of 16S rRNA

Microbial community genomic DNA was extracted from Rumen fluid samples using the E.Z.N.A.® soil DNA Kit (Omega Bio-tek, Norcross, GA, USA) according to the manufacturer's instructions. The DNA extract was checked on 1% agarose gel, and DNA concentration and purity were determined with the NanoDrop 2000 UV-vis spectrophotometer (Thermo Scientific, Wilmington, USA). The hyper-variable region V3-V4 of the bacterial 16S rRNA gene was amplified with primer pairs 338F (5′-ACTCCTACGGGAGGCAGCAG-3′) and 806R (5′-GGACTACHVGGGTWTCTAAT-3′) by an ABI GeneAmp® 9700 PCR thermocycler (ABI, CA, USA). The PCR amplification of the 16S rRNA gene was performed as follows: initial denaturation at 95°C for 3 min, followed by 27 cycles of denaturing at 95°C for 30 s, annealing at 55°C for 30 s, and extension at 72°C for 45 s, and single extension at 72°C for 10 min, and end at 4°C. The PCR mixtures contain 5 × *TransStart* FastPfu buffer 4 μL, 2.5 mM dNTPs 2 μL, forward primer (5 μM) 0.8 μL, reverse primer (5 μM) 0.8 μL, *TransStart* FastPfu DNA Polymerase 0.4 μL, template DNA 10 ng, and finally $ddH_2O$ up to 20 μL. PCR reactions were performed in triplicate. The PCR product was extracted from 2% agarose gel and purified using the AxyPrep DNA Gel Extraction kit (Axygen Biosciences, Union City, CA, USA) according to the manufacturer's instructions and quantified using the Quantus Fluorometer™ (Promega, USA).

## Illumina MiSeq sequencing

Purified amplicons were pooled in equimolar and paired-end sequenced on an Illumina MiSeq PE300 platform/NovaSeq PE250 platform (Illumina, San Diego, USA) according to the standard protocols by Majorbio Bio-Pharm Technology Co., Ltd. (Shanghai, China). The raw reads were deposited into the NCBI Sequence Read Archive (SRA) database (Accession Number: PRJNA732599).

## Bioinformatics analysis

Operational taxonomic units (OTUs) were clustered with a 97% similarity cutoff from the clean FASTQ data, and chimeric sequences were identified and removed using Usearch 7.0.[1] These OTUs were used for diversity (Shannon and Simpson), richness (Ace and Chao), and rarefaction curve analysis using mothur v.1.30.2.[2] Representative sequences of OTUs were aligned to the SILVA database[3] for bacteria taxonomic assignments using QIIMEe.[4]

## Statistical analysis

Production performances, digestibility, ruminal pH, ruminal fermentation variables, were firstly conducted through a normal distribution test using the SAS procedure "proc univariate data = test normal", and subsequently used the SPSS 23.0 software for the independent sample *T*-test between TN treatment and HS treatment, and between HS treatment and HN treatment. Significance would be considered when $P < 0.05$, while a tendency was considered when $0.05 \leq P < 0.10$.

# Results

## Temperature–humidity index and body temperature

The average daily THI values of the HS treatment and the HN treatment during the experimental period were all higher than 72. THI lasted higher than 79 for 53 days. Daily changes in THI values at 08:00, 14:00, and 22:00 h during the experimental period are presented in **Figure 1**. Results of the body temperature are shown in **Table 2**. The body temperature in the HS treatment was significantly higher than that of the TN treatment on days 1, 10, 50, and 60 ($P < 0.01$). HN treatment

1 http://drive5.com/usearch/
2 https://www.mothur.org/wiki/Download_mothur
3 https://www.arb-silva.de
4 http://qiime.org/install/index.html

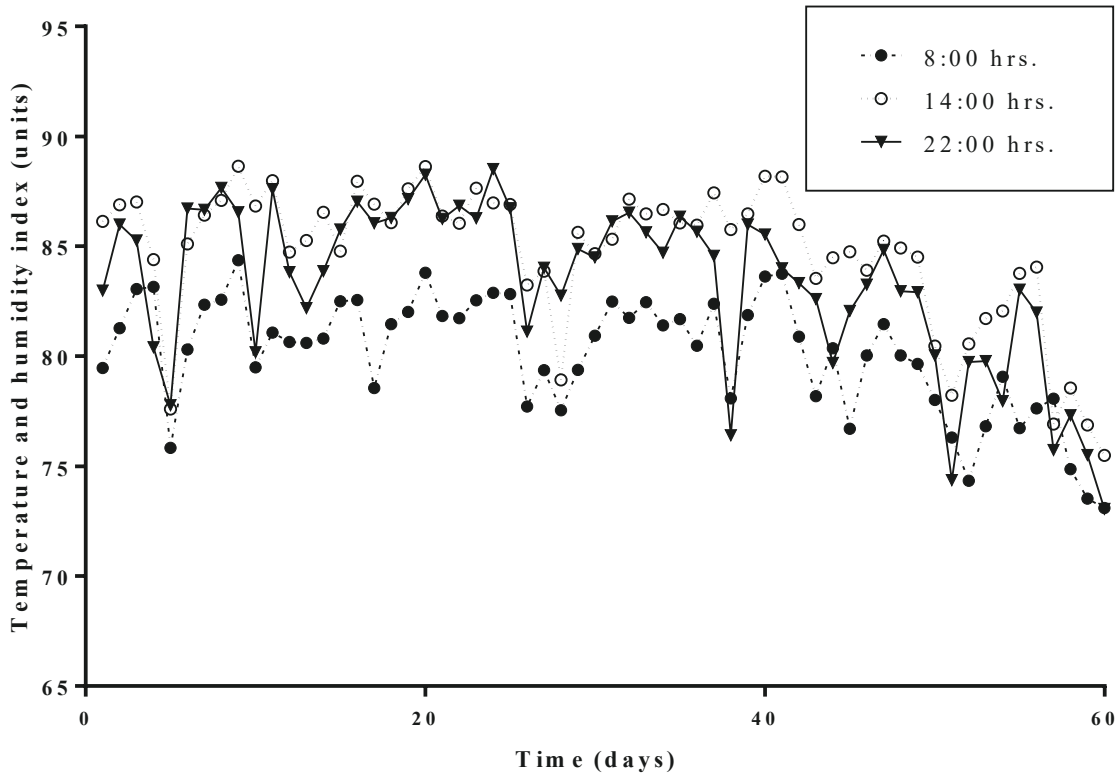


FIGURE 1
Daily changes of the temperature and humidity indexes (THI) at different hours during the trial period.

TABLE 2 Effects of niacin on the body temperature of beef cattle under heat stress (°C).

| Item | TN | HS | HN | *P-value* | |
|---|---|---|---|---|---|
| | | | | TN vs. HS | HS vs. HN |
| 1 day | 38.58 ± 0.05 | 38.89 ± 0.05 | 38.88 ± 0.06 | <0.001 | 0.834 |
| 10 days | 38.53 ± 0.02 | 38.78 ± 0.04 | 38.71 ± 0.03 | <0.001 | 0.210 |
| 20 days | 38.80 ± 0.02 | 38.90 ± 0.05 | 38.77 ± 0.01 | 0.083 | 0.019 |
| 30 days | 38.73 ± 0.03 | 38.83 ± 0.05 | 38.76 ± 0.02 | 0.065 | 0.163 |
| 40 days | 38.79 ± 0.03 | 38.91 ± 0.06 | 38.79 ± 0.02 | 0.076 | 0.066 |
| 50 days | 38.75 ± 0.02 | 38.89 ± 0.03 | 38.81 ± 0.02 | 0.002 | 0.053 |
| 60 days | 38.53 ± 0.03 | 38.68 ± 0.03 | 38.67 ± 0.03 | 0.003 | 0.843 |

The result is presented as the mean values and standard error.
TN, thermoneutral treatment group; HS, heat stress treatment group; HN, heat stress supplemented with niacin treatment group.

showed a significant decline in body temperature on day 20 ($P < 0.05$) but no significant difference in body temperature was recorded on other days, compared with HS treatment.

## Respiratory rate

Table 3 shows that the respiratory frequency of the HS treatment was significantly higher than that of the TN treatment on the 1st, 10th, 40th, 50th, and 60th days. There was no significant difference between the HN and HS treatments most of the time, but the respiratory frequency of the HN treatment was significantly lower than that of the HS treatment on the 50th day.

## Growth performance

As shown in Table 4, the ADG and F/G of the HS treatment showed a decreasing trend compared with the TN treatment

TABLE 3 Effects of niacin on the respiratory rate of beef cattle under heat stress (breaths/min).

| Item | TN | HS | HN | *P-value* | |
|---|---|---|---|---|---|
| | | | | TN vs. HS | HS vs. HN |
| 1 days | 45.25 ± 3.40 | 67.33 ± 6.24 | 65.00 ± 6.47 | 0.006 | 0.798 |
| 10 days | 49.25 ± 2.48 | 64.75 ± 3.97 | 56.69 ± 5.00 | 0.030 | 0.188 |
| 20 days | 55.58 ± 3.30 | 62.00 ± 3.08 | 59.08 ± 4.39 | 0.169 | 0.592 |
| 30 days | 43.50 ± 1.96 | 52.17 ± 4.60 | 43.50 ± 4.90 | 0.097 | 0.211 |
| 40 days | 41.33 ± 3.18 | 69.17 ± 3.96 | 59.08 ± 6.17 | <0.001 | 0.183 |
| 50 days | 36.92 ± 3.40 | 56.25 ± 4.42 | 43.42 ± 3.65 | 0.002 | 0.036 |
| 60 days | 34.50 ± 3.50 | 36.42 ± 2.08 | 38.92 ± 2.78 | 0.642 | 0.479 |

The result is presented as the mean values and standard error.
TN, thermoneutral treatment group; HS, heat stress treatment group; HN, heat stress supplemented with niacin treatment group.

TABLE 4 Effects of dietary niacin on the performance of beef cattle under heat stress.

| Item | TN | HS | HN | *P-value* | |
|---|---|---|---|---|---|
| | | | | TN vs. HS | HS vs. HN |
| Initial BW (kg) | 403.00 ± 13.43 | 409.83 ± 6.31 | 409.00 ± 10.93 | 0.652 | 0.948 |
| Finish BW (kg) | 471.25 ± 15.76 | 466.67 ± 8.34 | 472.67 ± 11.65 | 0.799 | 0.679 |
| ADFI (kg) | 7.12 ± 0.19 | 7.05 ± 0.19 | 7.21 ± 0.11 | 0.81 | 0.488 |
| ADG (kg) | 1.14 ± 0.10 | 0.95 ± 0.05 | 1.06 ± 0.24 | 0.080 | 0.150 |
| F/G (kg/kg) | 6.35 ± 0.13 | 7.47 ± 0.44 | 6.82 ± 0.23 | 0.054 | 0.248 |

The result is presented as the mean values and standard error.
TN, thermoneutral treatment group; HS, heat stress treatment group; HN, heat stress supplemented with niacin treatment group; ADFI, average daily feed intake; ADG, average daily gain; F/G, feed to gain ratio.

($0.05 < P < 0.1$); however, no difference was noticed in the ADFI among the three treatments. The ADG and F/G of the HS treatment have a decreasing trend compared with the TN treatment, no significant difference was noticed in the ADG or the F:G ratio between HS and HN.

## Nutrient apparent digestibility

Table 5 shows the nutrient apparent digestibility of beef cattle during the experimental period. HS decreased the apparent digestibility of CP compared with TN treatment. No difference in nutrient apparent digestibility was observed between HS and HN treatments.

## Rumen fermentation parameters

Volatile fatty acids (VFAs) are the main products of microbial metabolism in the rumen, and the types and quantity of VFAs are regulated by microbial species, diet, and the environment. As shown in Table 6, HS treatment reduced the pH value in the ruminal fluid of beef cattles and there was a trend of decreasing concentration of MCP compared with the TN treatment ($P < 0.05$). The ratio of acetate acid, butyric acid, and TVFA in the HS treatment significantly declined compared with the TN treatment ($P < 0.05$). Moreover, there was a trend of decreasing the acetate/propionate in rumen fluid of HN compared with HS ($P < 0.1$). No difference in the contents of $NH_3$-N among all treatments.

## Diversity of ruminal bacteria

In this experiment, a total of 542,869 valid sequences were generated from 12 samples, and the richness and alpha diversity of the community were analyzed by Sobs, Shannon, Simpson, Ace, Chao, and Coverage indices in Table 7. The Sobs, Shannon, and Ace indices of the HS treatment were significantly lower than those of the TN treatment ($P < 0.05$). The Ace and Chao indices, have a downward trend ($P < 0.1$), and the Simpson index has an upward trend ($P < 0.1$). And the species richness, of the HN treatment was slightly higher than that of the HS treatment ($P < 0.05$). This indicates that the species richness of the HS treatment is lower than that of the TN treatment, while the species richness of the HN treatment is higher than that of the HS treatment.

To measure the extent of similarity between the microbial communities, beta diversity was calculated using a weighted normalized UniFrac, and PCoA was performed. As shown in Figure 2, significant differences were observed in the rumen microorganisms between the HS treatment and the TN treatment ($P = 0.022$).

The relative abundance of rumen microbiota is displayed at the phylum level (Table 8 and Figure 3A) and the genus level (Table 9 and Figure 3B). Phylogenetic analysis identified

TABLE 5 Effects of dietary niacin on nutrient apparent digestibility of beef cattle under heat stress (%).

| Item | TN | HS | HN | *P-value* | |
|---|---|---|---|---|---|
| | | | | TN vs. HS | HS vs. HN |
| EE | 87.80 ± 0.57 | 85.19 ± 2.07 | 87.01 ± 0.70 | 0.27 | 0.44 |
| CP | 74.88 ± 0.33 | 71.83 ± 1.53 | 71.46 ± 0.88 | 0.10 | 0.84 |
| NDF | 67.28 ± 1.48 | 63.25 ± 1.98 | 65.71 ± 0.59 | 0.55 | 0.31 |
| ADF | 62.62 ± 1.70 | 60.58 ± 2.92 | 61.67 ± 0.85 | 0.79 | 0.74 |
| Ca | 45.02 ± 2.59 | 37.59 ± 4.59 | 38.67 ± 4.58 | 0.21 | 0.87 |
| P | 46.77 ± 5.83 | 44.05 ± 10.19 | 40.47 ± 7.14 | 0.82 | 0.62 |

The result is presented as the mean values and standard error.
TN, thermoneutral treatment group; HS, heat stress treatment group; HN, heat stress supplemented with niacin treatment group; EE, ether extract; CP, crude proteins; ADF, acid detergent fiber; NDF, neutral detergent fiber; Ca, calcium; P, phosphorus.

TABLE 6 Effects of dietary niacin on rumen fermentation parameters of beef cattle under heat stress.

| Item | TN | HS | HN | *P-value* | |
|---|---|---|---|---|---|
| | | | | TN vs. HS | HS vs. HN |
| pH | 6.73 ± 0.11 | 6.09 ± 0.12 | 6.31 ± 0.08 | 0.024 | 0.189 |
| Acetic acidmmol/L | 71.26 ± 2.92 | 50.27 ± 4.07 | 54.18 ± 3.52 | 0.006 | 0.278 |
| Propionic acidmmol/L | 18.30 ± 2.88 | 12.57 ± 1.99 | 17.38 ± 1.99 | 0.154 | 0.139 |
| Isobutyric acidmmol/L | 0.82 ± 0.08 | 0.83 ± 0.11 | 0.81 ± 0.08 | 0.956 | 0.899 |
| Butyric acidmmol/L | 16.95 ± 1.66 | 9.06 ± 1.65 | 10.76 ± 3.91 | 0.015 | 0.703 |
| Isovaleric acidmmol/L | 1.74 ± 0.08 | 1.95 ± 0.31 | 2.04 ± 0.24 | 0.550 | 0.827 |
| Valeric acidmmol/L | 1.84 ± 0.04 | 1.50 ± 0.19 | 1.71 ± 0.32 | 0.177 | 0.598 |
| TVFAmmol/L | 110.91 ± 5.18 | 76.18 ± 4.29 | 86.88 ± 22.34 | 0.002 | 0.111 |
| Acetic acid/Propionic acid | 4.19 ± 0.63 | 4.04 ± 0.20 | 3.32 ± 0.29 | 0.822 | 0.085 |
| MCP (μg/ml) | 266.91 ± 12.17 | 211.11 ± 25.31 | 263.27 ± 15.80 | 0.094 | 0.131 |
| $NH_3$-N (mg/dL) | 34.05 ± 2.26 | 30.72 ± 7.21 | 37.93 ± 7.26 | 0.690 | 0.510 |

The result is presented as the mean values and standard error.
TN, thermoneutral treatment group; HS, heat stress treatment group; HN, heat stress supplemented with niacin treatment group; MCP, microbial crude protein; $NH_3$-N, ammonia nitrogen; VFA, volatile fatty acid.

TABLE 7 Effects of dietary niacin on the diversity of ruminal bacteria of beef cattle under heat stress.

| Item | TN | HS | HN | *P-value* | |
|---|---|---|---|---|---|
| | | | | TN vs. HS | HS vs. HN |
| Sobs | 900.5 ± 43.46 | 740.5 ± 31.40 | 867 ± 31.18 | 0.025 | 0.029 |
| Shannon | 4.95 ± 0.13 | 4.26 ± 0.23 | 5.03 ± 0.06 | 0.040 | 0.019 |
| Simpson | 0.02 ± 0.004 | 0.08 ± 0.027 | 0.02 ± 0.002 | 0.088 | 0.066 |
| Ace | 1095.42 ± 47.12 | 918.47 ± 55.06 | 1056.31 ± 45.33 | 0.050 | 0.080 |
| Chao | 1121.16 ± 54.84 | 943.51 ± 57.16 | 1070.23 ± 47.92 | 0.086 | 0.175 |
| Coverage | 0.991 ± 0.0005 | 0.992 ± 0.0008 | 0.991 ± 0.004 | 0.207 | 0.362 |

The result is presented as the mean values and standard error.
TN, thermoneutral treatment group; HS, heat stress treatment group; HN, heat stress supplemented with niacin treatment group.

five phyla from the rumen fluid, four of which had a relative abundance >0.3% of the total community, and the most abundant phyla were *Bacteroidetes, Firmicutes, Actinobacteria, Desulfobacterota,* and *Proteobacteria*. However, there were no differences in these indicators. Results in Table 8 indicate that HS increased the relative abundance of phylum *Desulfobacterota* compared with the TN treatment ($P < 0.05$), while HN reduced the relative abundance of phylum *Desulfobacterota* compared with the HS treatment ($P < 0.05$). *Proteobacteria* in HS treatment shows a decreasing trend compared with the TN treatment ($P < 0.05$). No differences were found in other phyla among treatments. At the genus level in Table 9, the relative abundance of *Succiniclasticum, norank_f__UCG-011*, *Lachnospiraceae_NK3A20_group,* and *UCG-005* in the HS treatment showed a decreasing trend ($P < 0.01$), and the relative abundance of *norank_f__Bifidobacteriaceae* performed an increasing trend compared with the TN treatment ($P < 0.1$). In addition, the relative abundance of *Succiniclasticum* and *Family_XIII_AD3011_group* in the HN group was proliferated compared with the HS group ($P < 0.05$). *Christensenellaceae_R-7_group* showed an increased trend compared with HS group ($P < 0.05$), while *norank_f__F082*

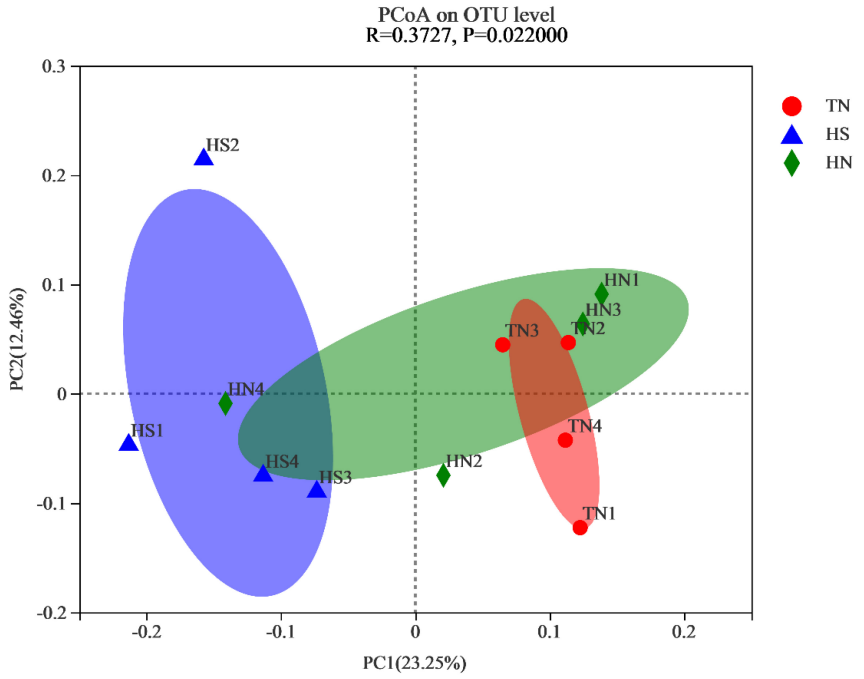


FIGURE 2
Principal coordinate analysis (PCoA) plot based on OTU abundance. TN: the basal diet, thermoneutral condition ($n$ = 4); HS: the basal diet, heat stress environment ($n$ = 4); HN: the basal diet supplemented with 800 mg/kg NA ($n$ = 4).

TABLE 8 Effects of niacin on rumen bacterial flora structure (phylum level)%.

| Item | TN | HS | HN | *P-value* | |
|---|---|---|---|---|---|
| | | | | TN vs. HS | HS vs. HN |
| *Bacteroidota* | 50.45 ± 3.39 | 47.89 ± 6.72 | 44.34 ± 6.30 | 0.745 | 0.714 |
| *Firmicutes* | 45.04 ± 3.39 | 38.91 ± 5.52 | 47.93 ± 4.42 | 0.380 | 0.249 |
| *Actinobacteriota* | 1.18 ± 0.45 | 3.81 ± 1.45 | 3.14 ± 1.69 | 0.105 | 0.334 |
| *Desulfobacterota* | 0.60 ± 0.13 | 1.10 ± 0.10 | 0.61 ± 0.13 | 0.020 | 0.021 |
| *unclassified_k__norank_d__Bacteria* | 0.94 ± 0.48 | 0.27 ± 0.06 | 0.99 ± 0.80 | 0.219 | 0.432 |
| *Patescibacteria* | 0.33 ± 0.05 | 0.71 ± 0.32 | 0.84 ± 0.54 | 0.288 | 0.846 |
| *Spirochaetota* | 0.40 ± 0.11 | 0.67 ± 0.25 | 0.69 ± 0.10 | 0.353 | 0.957 |
| *Proteobacteria* | 0.20 ± 0.06 | 1.07 ± 0.38 | 0.12 ± 0.01 | 0.105 | 0.092 |

The result is presented as the mean values and standard error.
TN, thermoneutral treatment group; HS, heat stress treatment group; HN, heat stress supplemented with niacin treatment group.

*showed* a decreased trend compared with the HS group ($P < 0.1$).

# Discussion

## Effects of niacin supplement on heat dissipation and productive performances

Temperature humidity index is widely used as an indicator to estimate the degree of HS in livestock animals. In this study, 53 days were detected with THI values higher than 79 (beef cattle were in high or severe stress state) in HS and HN treatments during the experimental period, which indicated the experimental beef cattle were raised under heat stress conditions. In this experiment, the body temperature and respiratory rate of beef cattle in the heat stress treatment were significantly higher than those in the thermoneutral treatment during the test period, while effectiveness decreased after the addition of niacin. This may attribute to the induction of vasodilation of niacin supplement under heat stress conditions, which increases the fast blood flow to the skin surface (Khan et al., 2012). Previous studies revealed varying vasomotor and skin temperature responses of heat-stressed dairy cows

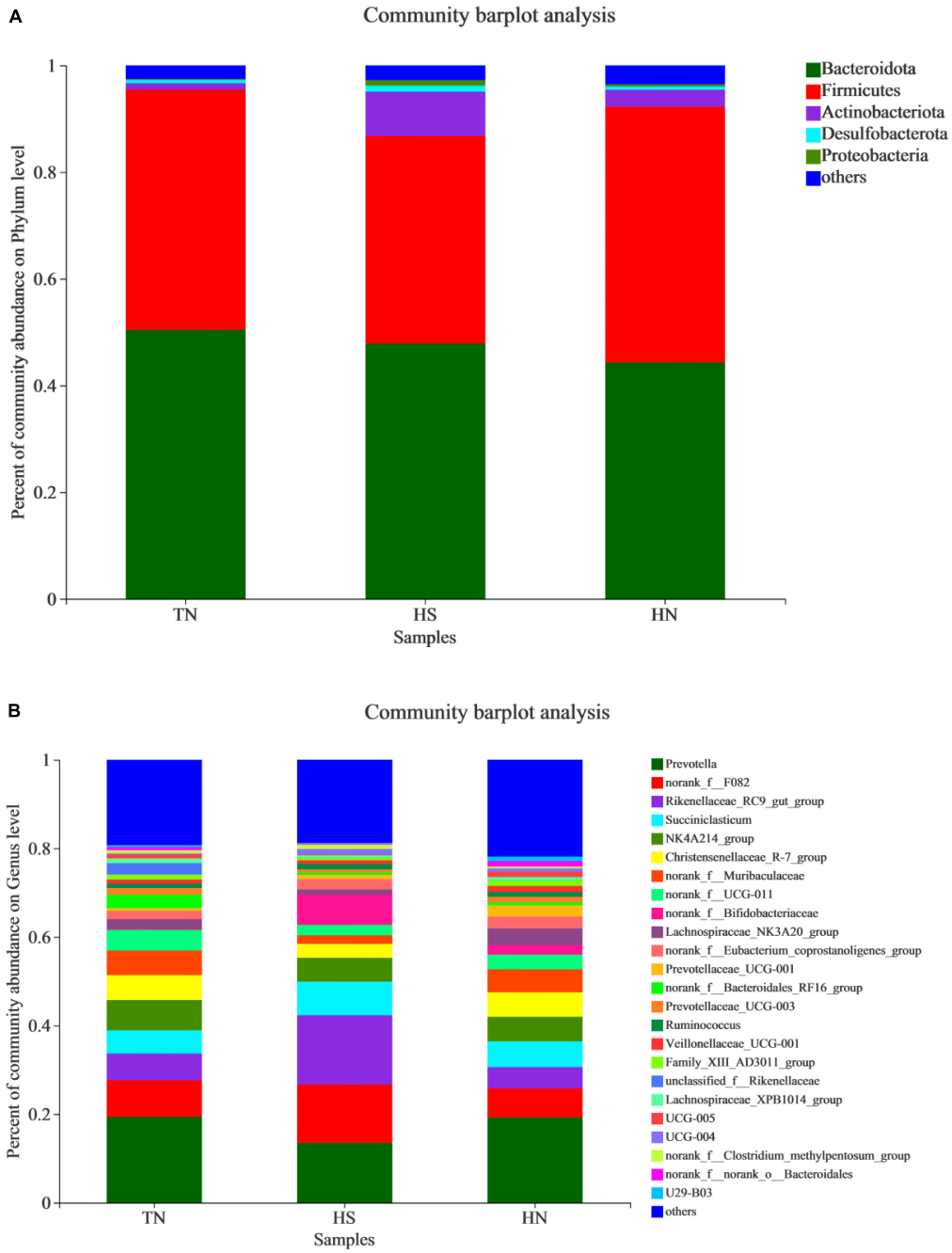


FIGURE 3
Relative abundance distribution of rumen flora at **(A)** phylum and **(B)** genus levels. TN: the basal diet, thermoneutral condition ($n$ = 4); HS: the basal diet, heat stress environment ($n$ = 4); HN: the basal diet supplemented with 800 mg/kg NA ($n$ = 4).

to dietary niacin supplementation, which partially included reductions in body temperature (Di Costanzo et al., 1997). Free nicotinic acid supplementation may therefore alleviate severe heat stress through decreased skin temperatures and the improvement of heat dissipation.

Heat stress could decrease dry matter intake and nutrient digestibility, by impairing the intestinal peristalsis, and reducing the activity and content of digestive enzymes (West, 2003). Previous studies demonstrated that heat stress lowers average daily feed intake (ADFI) and average daily gain (ADG) (Polsky and von Keyserlingk, 2017; Herbut et al., 2018), the gastrointestinal tract is considered one of the main target organs affected by heat stress. In our study, the HN group slightly increased ADFI and ADG of heat-stressed beef cattle,

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TABLE 9 Effects of niacin on rumen bacterial flora structure (genus level)%.

| Item | TN | HS | HN | *P-value* | |
|---|---|---|---|---|---|
| | | | | TN vs. HS | HS vs. HN |
| Prevotella | 22.86 ± 5.85 | 16.27 ± 4.01 | 19.28 ± 4.86 | 0.406 | 0.670 |
| norank_f__F082 | 8.22 ± 2.07 | 13.13 ± 2.40 | 6.55 ± 2.38 | 0.172 | 0.099 |
| Rikenellaceae_RC9_gut_group | 6.07 ± 0.58 | 4.97 ± 1.62 | 4.80 ± 0.28 | 0.502 | 0.906 |
| Succiniclasticum | 6.89 ± 1.75 | 0.86 ± 0.18 | 7.58 ± 1.64 | 0.073 | 0.015 |
| NK4A214_group | 6.84 ± 2.31 | 5.38 ± 1.50 | 5.55 ± 0.46 | 0.586 | 0.89 |
| Christensenellaceae_R-7_group | 5.62 ± 1.38 | 3.14 ± 0.48 | 5.51 ± 0.85 | 0.141 | 0.051 |
| norank_f__Muribaculaceae | 5.57 ± 2.21 | 1.96 ± 0.79 | 5.19 ± 2.28 | 0.205 | 0.257 |
| norank_f__UCG-011 | 4.66 ± 0.99 | 2.35 ± 0.38 | 3.33 ± 0.76 | 0.072 | 0.291 |
| norank_f__Bifidobacteriaceae | 0.19 ± 0.07 | 2.67 ± 1.17 | 2.15 ± 1.31 | 0.052 | 0.785 |
| Lachnospiraceae_NK3A20_group | 2.23 ± 0.48 | 1.11 ± 1.19 | 3.75 ± 1.55 | 0.073 | 0.142 |
| norank_f__Eubacterium_coprostanoligenes_group | 1.84 ± 0.76 | 2.34 ± 0.49 | 2.66 ± 1.47 | 0.597 | 0.842 |
| Prevotellaceae_UCG-001 | 0.67 ± 0.20 | 0.97 ± 0.40 | 2.60 ± 1.07 | 0.533 | 0.204 |
| norank_f__Bacteroidales_RF16_group | 0.97 ± 0.23 | 0.54 ± 0.28 | 0.65 ± 0.31 | 0.771 | 0.795 |
| Prevotellaceae_UCG-003 | 1.55 ± 0.58 | 0.71 ± 0.37 | 1.21 ± 0.44 | 0.276 | 0.414 |
| Ruminococcus | 0.97 ± 0.30 | 1.22 ± 0.65 | 1.12 ± 0.44 | 0.746 | 0.91 |
| Veillonellaceae_UCG-001 | 0.96 ± 0.40 | 0.84 ± 0.24 | 1.35 ± 0.37 | 0.801 | 0.298 |
| Family_XIII_AD3011_group | 1.11 ± 0.35 | 0.58 ± 0.03 | 1.21 ± 0.19 | 0.225 | 0.016 |
| unclassified_f__Rikenellaceae | 2.57 ± 2.55 | 0.04 ± 0.03 | 0.02 ± 0.01 | 0.394 | 0.554 |
| Lachnospiraceae_XPB1014_group | 1.09 ± 0.29 | 0.48 ± 1.11 | 0.78 ± 0.13 | 0.100 | 0.13 |
| UCG-005 | 0.92 ± 0.27 | 0.16 ± 0.04 | 1.22 ± 0.65 | 0.065 | 0.2 |

The result is presented as the mean values and standard error.
TN, thermoneutral treatment group; HS, heat stress treatment group; HN, heat stress supplemented with niacin treatment group.

which indicated that niacin is capable of elevating growth performance.

## Effects of niacin supplement on ruminal microbiota

For ruminants, rumen played important physiological functions that convert dietary fiber into nutrients, such as volatile fatty acid and ammoniate. Rumen fermentation is an anaerobic process carried out by complex ruminal microbiota, which primarily converts feedstuffs into VFAs, microbial proteins, and vitamins (Wang et al., 2016). The rumen microbiota plays an important role in ruminant digestion of plant material and rumen fermentation, suggesting that optimizing rumen fermentation improved nutrient availability and productivity in cattle (Kim et al., 2011).

In our study, compared with HN treatment, HS treatment significantly decreased rumen pH, butyric acid, and extremely significantly decreased acetic acid and TVFA. Although a decrease in pH is usually accompanied by an increase in TVFA in most studies, this is not the case in conditions of heat stress, Heat stress causes various adverse impacts on ruminants, including lowered rumen pH, decreased production of rumen TVFA (Tajima et al., 2007; Cai et al., 2019) the decrease in rumen TVFA concentrations caused by heat stress may be related to the decreased utilization of dietary nutrients due to decreased rumen microbial abundance and activity under heat stress conditions. The HN treatment increased the content of acetic acid, propionic acid, TVFA, and MCP as compared to the HS treatment. These results might be caused by the following factors. Previous studies found changes in the host metabolism caused by heat stress can induce changes in ruminal microbiota and affect the absorption of nutrients (Bergman, 1990; Tajima et al., 2007; Nonaka et al., 2008). Niacin had no important effect on the concentration of dry matter or lactic acid, or the molar proportions of acetic, butyric, or valeric acids. Niacin increased the concentration of bacterial protein, ammonia, and propionic acid (Riddell et al., 1980). Niacin supplement helps heat dissipation under heat-stressed conditions, which may further regulate microbial diversity. Studies indicated adding niacin in a high-concentrated diet could increase the ruminal pH value and the ruminal microbial composition (Luo et al., 2017); the increased α-diversity helped promoted ruminal N metabolism and ruminal fermentation, and finally increased TVFA. No effect of niacin on the ruminal degradability of N has been previously observed *in vitro* (Hannah and Stern, 1985). Some studies found no significant effect of niacin on VFA fermentation in the rumen.

Besides, a shift in rumen bacterial diversity altered ruminal fermentation. Niacin supplementation may increase propionate concentration and decrease butyrate concentration in rumen liquor (Flachowsky, 1993). A series of *in vitro* studies tested the effects of nicotinic acid on rumen fermentation. Niacin had no effect on gas production but significantly increased the synthesis of microbial protein (Riddell et al., 1980). *Bacteroidetes* and *Firmicutes* are the major bacteria in the rumen. *Bacteroidetes* degrades fibers and cellulose (Evans et al., 2011), while *Firmicutes* digests carbohydrates and ferments organic substances (Spence et al., 2006) and account for the highest proportion of rumen bacteria, which is consistent with the previous studies (Ley et al., 2008; Zhang et al., 2017). The increased *Bacteroidetes* under HN treatment help in increasing the digestibility of cellulose, and therefore increased TVFA.

Furthermore, our results revealed that the addition of niacin had a positive effect on *Succiniclasticum, Christensenellaceae_R-7_group,* and *Family_XIII_AD3011_group*, but had no effect on the main cellulolytic genus (such as *Ruminococcus*, *Butyrivibrio,* and *Fibrobacter*). A high abundance *of Succiniclasticum* has been linked to lower methane emissions, accompanied by improved acetate and hydrogen production (Wallace et al., 2015). Species belonging to the genus *Succiniclasticum* can utilize succinate to produce propionate (Dias et al., 2017). van Gylswyk (1995) detected *Succiniclasticum* fermenting succinic acid to propionate. *Succiniclasticum* is a starch-degrading microbe (Liu et al., 2015). Niacin tended to increase the relative abundance of *Succiniclasticum*, which would suggest that the addition of niacin tended to promote the accumulation of propionic acid in HN treatment. *Desulfovibrio* is one of the major sulfate-reducing bacteria (SRB) in the rumen, SRB is linked to intestinal disease, it accumulated toxins in the rumen epithelium that may cause inflammation (Wu et al., 2021). Niacin supplementation increases and decreases *Desulfobacterota* may help protect beef cattle rumen barrier function under heat stress.

## Conclusion

Niacin supplementation can alleviate the negative effects caused by heat stress, increase the abundance and diversity of rumen microorganisms to a certain extent, and improve rumen fermentation function and production performance. This study provided the theoretical basis for the alleviation effects of niacin supplementation on beef cattle under heat-stressed conditions, and may further provide a directive suggestion for niacin application in beef cattle production. Further research directions are needed: (1) Combined multi-omics analysis was used to deeply study the composition, genome function, and metabolic function of niacin on heat-stressed beef cattle rumen microorganisms. (2) The influence of niacin on the rumen barrier of heat-stressed beef can continue to be explored.

## Data availability statement

The data presented in this study are deposited in the https://www.ncbi.nlm.nih.gov/ repository, accession number PRJNA 732599.

## Ethics statement

The animal study was reviewed and approved by the Animal Care and Use Committee of Jiangxi Agricultural University.

## Author contributions

LX and MQ designed the overall study. BZ, FL, CC, XZ, FX, and MQ performed the experiments. BZ wrote 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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# References


AOAC International (2005). *Official methods of analysis of AOAC*. Arlington, TX: AOAC International.

Aschemann, M., Lebzien, P., Hüther, L., Südekum, K. H., and Dänicke, S. (2012). Effect of niacin supplementation on rumen fermentation characteristics and nutrient flow at the duodenum in lactating dairy cows fed a diet with a negative rumen nitrogen balance. *Arch. Anim. Nutr.* 66, 303–318. doi: 10.1080/1745039x.2012.697353

Barreras, A., Castro-Pérez, B. I., López-Soto, M. A., Torrentera, N. G., Montaño, M. F., Estrada-Angulo, A., et al. (2013). Influence of ionophore supplementation on growth performance, dietary energetics and carcass characteristics in finishing cattle during period of heat stress. *Asian-Australas. J. Anim. Sci.* 26, 1553–1561. doi: 10.5713/ajas.2013.13216

Bergman, E. N. (1990). Energy contributions of volatile fatty acids from the gastrointestinal tract in various species. *Physiol. Rev.* 70, 567–590.doi: 10.1152/physrev.1990.70.2.567

Berman, A. (2005). Estimates of heat stress relief needs for Holstein dairy cows. *J. Anim. Sci.* 83, 1377–1384.

Bicalho, M. L., Lima, F. S., Ganda, E. K., Foditsch, C., Meira, E. B. Jr., Machado, V. S., et al. (2014). Effect of trace mineral supplementation on selected minerals, energy metabolites, oxidative stress, and immune parameters and its association with uterine diseases in dairy cattle. *J. Dairy Sci.* 97, 4281–4295. doi: 10.3168/jds.2013-7832

Cai, L., Yu, J., Hartanto, R., Zhang, J., Yang, A., and Qi, D. (2019). Effects of heat challenge on growth performance, ruminal, blood and physiological parameters of chinese crossbred goats. *Small Ruminant Res.* 174, 125–130.

Collier, R. J., Hall, L. W., Rungruang, S., and Zimbleman, R. B. (2012). Quantifying heat stress and its impact on metabolism and performance. *Paper presented at the 23rd annual ruminant nutrition symposium*, Gainesville, FL, 74–83. doi: 10.1016/j.smallrumres.2019.02.021

Di Costanzo, A., Spain, J. N., and Spiers, D. E. (1997). Supplementation of nicotinic acid for lactating Holstein cows under heat stress conditions. *J. Dairy Sci.* 80, 1200–1206. doi: 10.3168/jds.S0022-0302(97)76048-X

Dias, J., Marcondes, M. I., Noronha, M. F., Resende, R. T., Machado, F. S., Mantovani, H. C., et al. (2017). Effect of pre-weaning diet on the ruminal archaeal, bacterial, and fungal communities of dairy calves. *Front. Microbiol.* 8:1553. doi: 10.3389/fmicb.2017.01553

Dikmen, S., and Hansen, P. J. (2009). Is the temperature-humidity index the best indicator of heat stress in lactating dairy cows in a subtropical environment? *J. Dairy Sci.* 92, 109–116. doi: 10.3168/jds.2008-1370

Doreau, M., and Ottou, J. F. (1996). Influence of niacin supplementation on *in vivo* digestibility and ruminal digestion in dairy cows. *J. Dairy Sci.* 79, 2247–2254. doi: 10.3168/jds.S0022-0302(96)76601-8

Eslamizad, M., Albrecht, D., and Kuhla, B. (2020). The effect of chronic, mild heat stress on metabolic changes of nutrition and adaptations in rumen papillae of lactating dairy cows. *J. Dairy Sci.* 103, 8601–8614. doi:10.3168/jds.2020-18417

Evans, N. J., Brown, J. M., Murray, R. D., Getty, B., Birtles, R. J., Hart, C. A., et al. (2011). Characterization of novel bovine gastrointestinal tract Treponema isolates and comparison with bovine digital dermatitis treponemes. *Appl. Environ. Microbiol.* 77, 138–147. doi: 10.1128/AEM.00993-10

Flachowsky, G. (1993). Niacin in dairy and beef cattle nutrition. *Arch. Tierernahr.* 43, 195–213.doi: 10.1080/17450399309386036

Gasperi, V., Sibilano, M., Savini, I., and Catani, M. V. (2019). Niacin in the central nervous system: An update of biological aspects and clinical applications. *Int. J. Mol. Sci.* 20:974. doi: 10.3390/ijms20040974

Hannah, S. M., and Stern, M. D. (1985). Effect of supplemental niacin or niacinamide and soybean source on ruminal bacterial fermentation in continuous culture. *J. Anim. Sci.* 61, 1253–1263.doi: 10.2527/jas1985.6151253x

Herbut, P., Angrecka, S., and Walczak, J. (2018). Environmental parameters to assessing of heat stress in dairy cattle-a review. *Int. J. Biometeorol.* 62, 2089–2097. doi: 10.1007/s00484-018-1629-9

Khan, N., Kewalramani, N., and Kant, K. (2012). Niacin supplementation for lactating cows under heat stress conditions - a review. *Agric. Rev.* 33, 143–149.

Kim, M., Morrison, M., and Yu, Z. (2011). Status of the phylogenetic diversity census of ruminal microbiomes. *FEMS Microbiol. Ecol.* 76, 49–63. doi: 10.1111/j.1574-6941.2010.01029.x

Kim, W. S., Lee, J. S., Jeon, S. W., Peng, D. Q., Kim, Y. S., Bae, M. H., et al. (2018). Correlation between blood, physiological and behavioral parameters in beef calves under heat stress. *Asian-Australas. J. Anim. Sci.* 31, 919–925. doi: 10.5713/ajas.17.0545

Ley, R. E., Lozupone, C. A., Hamady, M., Knight, R., and Gordon, J. I. (2008). Worlds within worlds: Evolution of the vertebrate gut microbiota. *Nat. Rev. Microbiol.* 6, 776–788.doi: 10.1038/nrmicro1978

Liu, J. H., Bian, G. R., Zhu, W. Y., and Mao, S. Y. (2015). High-grain feeding causes strong shifts in ruminal epithelial bacterial community and expression of Toll-like receptor genes in goats. *Front. Microbiol.* 6:167. doi: 10.3389/fmicb.2015.00167

Luo, D., Gao, Y., Lu, Y., Qu, M., Xiong, X., Xu, L., et al. (2017). Niacin alters the ruminal microbial composition of cattle under high-concentrate condition. *Anim. Nutr.* 3, 180–185. doi: 10.1016/j.aninu.2017.04.005

MacKay, D., Hathcock, J., and Guarneri, E. (2012). Niacin: Chemical forms, bioavailability, and health effects. *Nutr. Rev.* 70, 357–366.doi: 10.1111/j.1753-4887.2012.00479.x

Mitchell, J. (1965). Niacin in vascular disorders and hyperlipemia. *Proc. R. Soc. Med. London* 58:387.

Nonaka, I., Takusari, N., Tajima, K., Suzuki, T., Higuchi, K., and Kurihara, M. (2008). Effects of high environmental temperatures on physiological and nutritional status of prepubertal holstein heifers. *Livestock Sci.* 113, 14–23.

O'Brien, M. D., Rhoads, R. P., Sanders, S. R., Duff, G. C., and Baumgard, L. H. (2010). Metabolic adaptations to heat stress in growing cattle. *Domest. Anim. Endocrinol.* 38, 86–94.

Polsky, L., and von Keyserlingk, M. (2017). Invited review: Effects of heat stress on dairy cattle welfare. *J. Dairy Sci.* 100, 8645–8657.

Renaudeau, D., Collin, A., Yahav, S., de Basilio, V., Gourdine, J. L., and Collier, R. J. (2012). Adaptation to hot climate and strategies to alleviate heat stress in livestock production. *Animal* 6, 707–728.

Riddell, D. O., Bartley, E. E., and Dayton, A. D. (1980). Effect of nicotinic acid on rumen fermentation *in vitro* and *in vivo*. *J. Dairy Sci.* 63, 1429–1436. doi: 10.3168/jds.S0022-0302(80)83100-6

Sammad, A., Wang, Y. J., Umer, S., Lirong, H., Khan, I., Khan, A., et al. (2020). Nutritional physiology and biochemistry of dairy cattle under the influence of heat stress: Consequences and opportunities. *Animals* 10:793. doi: 10.3390/ani10050793

Soriani, N., Panella, G., and Calamari, L. (2013). Rumination time during the summer season and its relationships with metabolic conditions and milk production. *J. Dairy Sci.* 96, 5082–5094. doi: 10.3168/jds.2013-6620

Spence, C., Wells, W. G., and Smith, C. J. (2006). Characterization of the primary starch utilization operon in the obligate anaerobe *Bacteroides* fragilis: Regulation by carbon source and oxygen. *J. Bacteriol.* 188, 4663–4672. doi: 10.1128/JB.00125-06

Srivastava, S. (2016). Emerging therapeutic roles for NAD(+) metabolism in mitochondrial and age-related disorders. *Clin. Transl. Med.* 5:25.

Tajima, K., Nonaka, I., Higuchi, K., Takusari, N., Kurihara, M., Takenaka, A., et al. (2007). Influence of high temperature and humidity on rumen bacterial diversity in Holstein heifers. *Anaerobe* 13, 57–64. doi: 10.1016/j.anaerobe.2006.12.001

Uyeno, Y., Sekiguchi, Y., Tajima, K., Takenaka, A., Kurihara, M., and Kamagata, Y. (2010). An rRNA-based analysis for evaluating the effect of heat stress on the rumen microbial composition of Holstein heifers. *Anaerobe* 16, 27–33. doi: 10.1016/j.anaerobe.2009.04.006

van Gylswyk, N. O. (1995). Succiniclasticum ruminis gen. nov., sp. nov., a ruminal bacterium converting succinate to propionate as the sole energy-yielding mechanism. *Int. J. Syst. Bacteriol.* 45, 297–300. doi: 10.1099/00207713-45-2-297

Wallace, R. J., Rooke, J. A., McKain, N., Duthie, C. A., Hyslop, J. J., Ross, D. W., et al. (2015). The rumen microbial metagenome associated with high methane production in cattle. *BMC Genomics* 16:839. doi: 10.1186/s12864-015-2032-0

Wang, B., Wang, C., Guan, R., Shi, K., Wei, Z., Liu, J., et al. (2019). Effects of dietary rumen-protected betaine supplementation on performance of postpartum dairy cows and immunity of newborn calves. *Animals* 9:167. doi: 10.3390/ani9040167

Wang, W., Li, C., Li, F., Wang, X., Zhang, X., Liu, T., et al. (2016). Effects of early feeding on the host rumen transcriptome and bacterial diversity in lambs. *Sci. Rep.* 6:32479. doi: 10.1038/srep32479

West, J. W. (2003). Effects of heat-stress on production in dairy cattle. *J. Dairy Sci.* 86, 2131–2144.doi: 10.3168/jds.S0022-0302(03)73803-X

Wu, H., Li, Y., Meng, Q., and Zhou, Z. (2021). Effect of high sulfur diet on rumen fermentation, microflora, and epithelial barrier function in steers. *Animals* 11:2545. doi: 10.3390/ani11092545

Yang, Y., and Qu. (2013). Effects of nicotinic acid on lactate and volatile fatty acid concentrations,and related enzyme activities in rumen of jinjiang cattle. *Chin. J. Anim. Nutr.* 25:1610.

Zhang, R., Ye, H., Liu, J., and Mao, S. (2017). High-grain diets altered rumen fermentation and epithelial bacterial community and resulted in rumen epithelial injuries of goats. *Appl. Microbiol. Biotechnol.* 101, 6981–6992.

Zhao, X. H., Zhou, S., Bao, L. B., Song, X. Z., and Qu, M. R. (2017). Response of rumen bacterial diversity and fermentation parameters in beef cattle to diets containing supplemental daidzein. *Ital. J. Anim. Sci.* 17, 1–7.doi: 10.1080/1828051X.2017.1404943

Zimbelman, R. B., Baumgard, L. H., and Collier, R. J. (2010). Effects of encapsulated niacin on evaporative heat loss and body temperature in moderately heat-stressed lactating Holstein cows. *J. Dairy Sci.* 93, 2387–2394.doi: doi.org/10.3168/jds.2009-2557

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# Fermented grape seed meal promotes broiler growth and reduces abdominal fat deposition through intestinal microorganisms

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The fermentation of grape seed meal, a non-conventional feed resource, improves its conventional nutritional composition, promotes the growth and development of livestock and fat metabolism by influencing the structure and diversity of intestinal bacteria. In this study, the nutritional components of Fermented grape seed meal (FGSM) and their effects on the growth performance, carcass quality, serum biochemistry, and intestinal bacteria of yellow feather broilers were investigated. A total of 240 male 14-day-old yellow-feathered broilers were randomly selected and divided into four groups, with three replicates of 20 chickens each. Animals were fed diets containing 0% (Group I), 2% (Group II), 4% (Group III), or 6% (Group IV) FGSM until they were 56days old. The results showed that Acid soluble protein (ASP) and Crude protein (CP) contents increased, Acid detergent fiber (ADF) and Neutral detergent fiber (NDF) contents decreased, and free amino acid content increased in the FGSM group. The non-targeted metabolome identified 29 differential metabolites in FGSM, including organic acids, polyunsaturated fatty acids, and monosaccharides. During the entire trial period, Average daily gain (ADG) increased and Feed conversion ratio (FCR) decreased in response to dietary FGSM supplementation ($p < 0.05$). TP content in the serum increased and BUN content decreased in groups III and IV ($p < 0.05$). Simultaneously, the serum TG content in group III and the abdominal fat rate in group IV were significantly reduced ($p < 0.05$). The results of gut microbiota analysis showed that FGSM could significantly increase the Shannon and Simpson indices of broilers (35 days). Reducing the relative abundance of *Bacteroidetes* significantly altered cecal microbiota composition by increasing the relative abundance of *Firmicutes* ($p < 0.05$). By day 56, butyric acid content increased in the cecal samples from Group III ($p < 0.05$). In addition, Spearman's correlation analysis revealed a strong correlation between broiler growth performance, abdominal fat percentage, SCFAs, and gut microbes. In summary, the addition of appropriate levels of FGSM to rations improved broiler growth performance and reduced fat deposition by regulating gut microbes through differential metabolites and affecting the microbiota structure and SCFA content of the gut.

KEYWORDS

fermented grape seed meal, yellow feather broilers, gut microbiota, growth performance, abdominal fat deposition

## Introduction

Grapes are the largest fruit crop in the world, and 80% of grape production is used for wine making (Yang and Xiao, 2013). A large amount of grape seed by-products are formed during the winemaking process that are not properly exploited and utilized. Recently, there has been increasing concern about reducing the impact of agro-industrial waste through its use as a new feed product (Câmara et al., 2020). Grape seeds have been found to be rich in fatty acids, amino acids, dietary fiber, polysaccharides and other nutrients (Zhu et al., 2015). It contains a large number of bioactive substances (such as proanthocyanidins, resveratrol and tannins, etc.) and is a feed material with good functional properties and biological activity (Chikwanha et al., 2018; Tang et al., 2018). Therefore, the development of grape seed meal resources combined with modern biotechnology not only strengthens the contribution to the implementation of circular economy, but also alleviates the current situation of feed resource shortage.

Feed biological pre-digestion techniques mainly include fermentation and enzymatic hydrolysis. Through microbial metabolic activities, feeds are enriched with more probiotics, metabolites, organic acids and other small molecules (Yang et al., 2021). After the livestock consumes the fermented feed, the number of probiotic bacteria in the feed increases and the pH value in the intestine decreases. Thus, this inhibits the growth of harmful bacteria and achieves a balance of bacteria in animal intestines (Heres et al., 2003; Chen and Yu, 2020). Consumption of fermented feed can also improve immunity (Abu-Elala et al., 2020), reduce antioxidative stress (Wu et al., 2015), increase feed intake and improve growth performance among other effects (Zhu et al., 2020). In addition, enzymatic hydrolysis was used to degrade anti- nutritional factors and increase small peptides in raw materials (Tie et al., 2020). Following feeding, animals are deficient in endogenous enzyme supplementation (Engberg et al., 2004; Alagawany et al., 2018), improving intestinal villus growth (Ritz et al., 1995; Berwanger et al., 2017) and improving nutrient uptake capacity (Córdova-Noboa et al., 2020).

Fermented Feed by Bacteria Coupled Fermentation with Enzymes has the advantages of both of these, rich in a large number of probiotics, exogenous enzymes and metabolites. Because the cecum of chickens has a large number of microorganisms and is abundant in species, they interact closely and densely with the host and ingested feed. Moreover, there is growing evidence that manipulating the composition of the gut microbiota through diet can alter the metabolic pathways and metabolite production (e.g., SCFAs) of the host, ultimately having a significant impact on the themself (Sugiharto and Ranjitkar, 2019). Therefore, according to its key functions, we hypothesized that the nutritional composition of grape seed meal would change after fermentation, producing metabolites that may have beneficial effects on broiler growth performance and intestinal health. In this study, based on untargeted metabolomics to investigate the effect of feed predigestion technology on the nutritional composition of grape seed meal, and studied the effects of different levels of FGSM added to the basal diet on the growth performance, slaughter performance, serum biochemistry, intestinal bacteria, and SCFAs content of broilers.

## Materials and methods

All experimental procedures were performed in strict accordance with the guidelines and were reviewed and approved by the Institutional Animal Bioethics Committee of Shihezi University (Xinjiang, China).

### Materials

Grape seed meal was provided by Western Animal Husbandry Co., Ltd., *Saccharomyces cerevisiae* was preserved by the Feed Biotechnology Laboratory of Shihezi University, and acid protease was purchased from Shanghai Yuanye Biotechnology Co., Ltd. Other feed ingredients, such as soybean meal and corn, were purchased from the Shihezi Farmers' Market in Xinjiang. FGSM was fermented using *Saccharomyces cerevisiae* and acid protease enzymes in the Feed Biotechnology Laboratory of Shihezi University. UGSM was used as the control group (CON) without protease and probiotics were added for fermentation.

### Preparation of FGSM

The fermentation process of grape seed meal was as follows: the mixture ratio of grape seed meal, corn flour, and bran was 90:5:5, and the ratio of substrate to water was 1:0.8. The mixed substrate was then autoclaved at 121°C for 15 min. After high-pressure cooling, 60 ml (bacterial solution concentration of $2.97 \times 10^9$ CFU/ml) of *Saccharomyces cerevisiae* liquid and 120 μg/g of acid protease (dissolved in double-distilled water before use and filtered with a 0.22 μm filter membrane for sterilization) were inoculated into each kg of fermentation substrate. Consistent mixing was performed followed by 48 h of fermentation in an incubator at 30°C. At the end of fermentation, the samples were dried in an oven at 45°C and pulverized through a 60-mesh sieve

for storage. Finally, the dried samples were ground and stored at room temperature until mixture into experimental rations.

## Routine nutrient testing of FGSM

Dry matter (DM), crude protein (CP), ether extract (EE), Ca, P, ASH, and acid-soluble protein (ASP) content in FGSM were determined according to the AOAC (Association of Official Analytical Chemists) (2000) method. The neutral detergent fiber (NDF) and acid detergent fiber (ADF) contents were determined using the thermostable amylase method and its residual ash method (Van Soest et al., 1991). Free amino acid profile analysis was performed according to a previously reported method (Liu et al., 2018).

## Untargeted metabolomics analysis of FGSM

### Metabolite extraction

100 mg of samples were weighed, precooled extract (methanol:acetonitrile:water volume ratio: 2:2:1) was added, and the solution was vortexed. Low-temperature sonication was performed on the samples for 30 min, then samples were incubated at −20°C for 10 min. Samples were centrifuged at 14,000× *g* for 20 min at 4°C, and the supernatant was collected for vacuum drying. During mass spectrometry, 100 µl of acetonitrile aqueous solution (acetonitrile:water volume ratio of 1:1) was added for rehydration. Samples were vortexed then centrifuged at 14,000× *g* for 15 min at 4°C, and the supernatant was analyzed.

### Chromatographic conditions

An Agilent 1290 Infinity LC Ultra High-Performance Liquid Chromatography (UHPLC) HILIC column was used to separate samples; column temperature was 25°C; flow rate was 0.5 ml/min; mobile phase A was comprised of water +25 mm ammonium acetate +25 mm ammonia, and mobile phase B was acetonitrile with gradient elution. The procedure was as follows: 0 ~ 0.5 min, 95% B; 0.5 ~ 7 min, B varied linearly from 95 to 65%; 7 ~ 8 min, B varied linearly from 65 to 40%; 8 ~ 9 min, B was maintained at 40%; 9 ~ 9.1 min, B varied linearly from 40 to 95%; 9.1 ~ 12 min, B was maintained at 95%; the sample was placed in the autosampler at 4°C during the whole analysis. 4°C autosampler. The samples were also analyzed in random order to minimize the effect of fluctuations in the instrument signal. QC samples were inserted in the sample queue to monitor and evaluate the stability of the system and the reliability of the experimental data.

### Q-TOF mass spectrometry conditions

Primary and secondary spectra of the samples were collected using an AB Triple TOF 6600 mass spectrometer. The electrospray source conditions after HILIC chromatographic separation were as follows: ion source gas 1 (Gas 1): 60, ion source gas 2 (Gas 2): 60, curtain gas (CUR): 30, source temperature: 600°C, ion capacitor voltage float (ISVF): ± 5500 V (positive and negative two modes); TOF MS scanning range: 60 ~ 1,000 m/z, product ion scanning range: 25 ~ 1,000 m/z, TOF MS scanning cumulative time 0.20 s, and product ion scanning cumulative time 0.05 s. Secondary mass spectra were acquired with information-dependent acquisition (IDA) using a high sensitivity mode, depolymerization potential (DP): ± 60 V (both positive and negative modes), collision energy within 35 ± 15 eV.

### Metabolomics data processing

Raw data in Wiff format were converted to the mzXML format using the ProteoWizard software, followed by peak alignment, retention time correction, and extraction of peak areas using the XCMS software. The data obtained from XCMS extraction were first subjected to metabolite structure identification, data preprocessing, and then chemometric principles such as PCA, OPLS-DA, and multivariate statistical analysis using SIMCA 14.1 software. Metabolomics data were analyzed by Shanghai Zhongke New Life Biotechnology Co., Ltd.

## Experimental design and animal management

The experiments were performed at the experimental station of the College of Animal Science and Technology, Shihezi University. 240 male yellow-feathered broilers (average initial weight: 189.7 ± 5.9 g) at 14 days were randomly divided into four groups with three replicates of 20 birds each. Every group's basal diet was supplemented with 0% (group I), 2% (group II), 4% (group III), and 6% (group IV) FGSM. Broilers were maintained in an online flattening model with regular disinfection and cleaning. The initial temperature of the chicken house was 32°C, decreased by 2 ~ 3°C per week to 26°C, with a 24 h light cycle. The experimental period was 42 days and divided into 2 stages, with 14 to 35 days of age in stage 1 and 35 to 56 days of age in stage 2. Chickens were fed and watered freely during the experiment, and immunized according to standard immunization procedures. The chickens used in the experiment were medium-speed yellow-feathered broilers purchased from the Changji Prefecture Green Base Breeding Co. The basal diet consisted of a corn-soybean meal diet, formulated according to the Chinese agricultural industry standard "Nutritional requirements for yellow-feathered broiler chicks" (NY/T 33–2004). The test diet was crushed and turned into powder form. The composition and nutritional levels are shown in Table 1.

## Assessing the growth performance of broilers

The daily feed intake of each group was recorded in detail during the experiment, and the growth performance was

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TABLE 1 Composition and nutrient levels of diets (%).

| Items | 14 ~ 35 day | | | | 35 ~ 56 day | | | |
|---|---|---|---|---|---|---|---|---|
| | I | II | III | IV | I | II | III | IV |
| Ingredient (%) | | | | | | | | |
| Corn | 59.70 | 59.80 | 59.90 | 60.00 | 63.50 | 63.60 | 63.70 | 63.80 |
| Soybean meal | 27.80 | 27.70 | 27.60 | 27.50 | 23.00 | 22.90 | 22.80 | 22.70 |
| FGSM | 0.00 | 2.00 | 4.00 | 6.00 | 0.00 | 2.00 | 4.00 | 6.00 |
| UGSM(CON) | 6.00 | 4.00 | 2.00 | 0.00 | 6.00 | 4.00 | 2.00 | 0.00 |
| Vegetable oil | 1.50 | 1.50 | 1.50 | 1.50 | 2.50 | 2.50 | 2.50 | 2.50 |
| Premix | 5.00 | 5.00 | 5.00 | 5.00 | 5.00 | 5.00 | 5.00 | 5.00 |
| Total | 100.00 | 100.00 | 100.00 | 100.00 | 100.00 | 100.00 | 100.00 | 100.00 |
| Nutrient content (%) | | | | | | | | |
| ME (MJ/kg) | 11.77 | 11.78 | 11.78 | 11.78 | 12.16 | 12.16 | 12.17 | 12.17 |
| Crude protein | 19.07 | 19.07 | 19.07 | 19.07 | 17.10 | 17.10 | 17.10 | 17.10 |
| Calcium | 0.90 | 0.90 | 0.90 | 0.90 | 0.80 | 0.80 | 0.80 | 0.80 |
| Available phosphorous | 0.40 | 0.40 | 0.40 | 0.40 | 0.35 | 0.35 | 0.35 | 0.35 |
| Lys | 1.13 | 1.12 | 1.12 | 1.12 | 0.99 | 0.99 | 0.99 | 0.98 |
| Met | 0.40 | 0.40 | 0.40 | 0.40 | 0.34 | 0.34 | 0.34 | 0.34 |
| Thr | 0.81 | 0.80 | 0.80 | 0.80 | 0.73 | 0.73 | 0.73 | 0.72 |
| Ser | 0.22 | 0.22 | 0.22 | 0.22 | 0.19 | 0.19 | 0.19 | 0.19 |

1. The premix provided the following per kg of diets: Nacl 2.940 g, Lys 0.234 g, Met 1.791 g, VA 8800 IU, VD3 3,000 IU, VE 30 mg, VK2 1.65 mg, VB1 2.5 mg, VB2 6.6 mg, VB3 11 mg, VB4 500 mg, nicotinic acid 60 mg, VB6 4.0 mg, biotin 0.2 mg, folic acid 1.0 mg, VB12 0.02 mg, VC 50 mg, Fe (as ferrous sulfate) 80.0 mg, Cu (as copper sulfate) 8.0 mg, Zn (as zinc sulfate) 60.0 mg, Mn (as manganesesulfate) 70.0 mg, I (as potassium iodide) 0.5 mg, Se (as sodium selenite) 0.3 mg, (14~56 d) Ca 7.415 g, P 2.64 g. 2. The nutritional components were calculated.

calculated in replicates. At days 14, 35, and 56, the broilers were weighed 12 h after fasting, and the leftovers were recovered. The average daily feed intake (ADFI), average daily gain (ADG), and feed conversion ratio (FCR) of the yellow-feathered broilers were calculated.

## Sample collection for broilers

On days 35 and 56 of the experiment, six birds approaching the average weight of each group were weighed and slaughtered individually. Carcass traits, including carcass and abdominal fat, were collected, weighed, and expressed as a percentage of live weight. Blood samples were collected from euthanized birds, placed in non-heparinized tubes, and stored at −20°C until serum biochemistry analysis. Digests from the cecum were collected and divided into two subsamples, stored in RNAse and DNAse-free tubes, placed in liquid nitrogen, and stored at −80°C. One subsample was used for the analysis of intestinal bacteria, and the other was used for the determination of SCFAs.

## Biochemical analysis of broiler serum

Serum biochemical parameters, including total protein (TP), albumin (ALB), blood urea nitrogen (BUN), triglycerides (TG), and total cholesterol (TC) were measured using commercially available kits (Jiancheng Bioengineering Institute, Nanjing, China).

## Analysis of broilers cecal microorganism 16S rRNA

DNA was extracted from the samples using a stool panel DNA extraction kit. After passing the test by 1% agarose gel electrophoresis, the V3 – V4 region of the 16S rDNA of the samples was PCR-amplified using the universal primers 338F (5′-ACTCCTACGGGAGGCAGCAG-3′) and 806R (5′-GGAC TACHVGGGTWTCTAAT-3′). The amplification program consisted of: 27 cycles (denaturation at 95°C for 30 s, annealing at 55°C for 30 s, and extension at 72°C for 30 s), and a final extension at 72°C for 10 min. Purified amplified fragments were sequenced using an Illumina MiSeq PE 2500 platform. The double-end sequencing data obtained by MiSeq sequencing removed barcodes and primer splicing, and further removed chimeras and short sequences to obtain clean tags. Under the condition of 97% similarity, the QIIME (v1.8.0) software was used for operational taxonomic unit (OTUs) clustering and species annotation. The mothur software package (version 1.31.2) was used for data processing and analysis.

## Analysis of broilers cecal contents for SCFAs

To analyze SCFA, 0.30 g of the cecal contents was added to 1.5 ml of ultrapure water, vortexed for 30 s, and centrifuged at 5,000× *g* for 4 min. Five hundred μl of supernatant was added to a new sterile Eppendorf tube. Then, 100 μl of 25% metaphosphoric

acid solution was added to the tube. The tube was vortexed for 30 s, and centrifuged at 1,500×*g* for 15 min through a 0.22 μm water system. The supernatant was collected into a vial for gas chromatography (Agilent 7890 B) to analyze SCFAs in the sample; the chromatographic column was DB-WAX (30 m × 0.25 mm × 0.50 μm).

## Statistical analysis

One-way analysis of variance (one-way ANOVA) was performed using SPSS 22.0, and significant difference analysis was performed using Duncan's method ($p < 0.05$). All data are expressed as mean ± SD. A correlation analysis was performed between growth performance, SCFAs, and intestinal microorganisms. Data were entered into SPSS version 22.0, and correlation coefficients were calculated based on Spearman correlation distances. Heat maps were constructed using ORIGIN 2021 to assess the bivariate relationships between variables.

# Results

## The nutritional components of FGSM

After fermentation, the contents of ASP and CP in FGSM reached 0.93 and 11.33%, respectively. It increased 365 and 24.45% with the CON group. The EE content increased to 12.31%. NDF and ADF decreased by 6.82 and 8.23%, compared with those before fermentation. The contents of Ca and P did not change significantly. The free amino acids in FGSM increased compared with CON group, except lysine. The total free amino acids were 823.56 mg/g and 903.84 mg/g in CON and FGSM group, respectively. Among them, glutamate content was the highest, accounting for 22.76 and 22.69% of the total free amino acids (Table 2).

TABLE 2 Chemical composition and amino acid profile of FGSM and CON.

| Item | CON | FGSM |
|---|---|---|
| Chemical Composition, % of DM | | |
| ASP | 0.20 | 0.93 |
| CP | 9.11 | 11.33 |
| DM | 98.30 | 98.34 |
| ASH | 5.09 | 4.58 |
| EE | 10.91 | 12.31 |
| NDF | 60.85 | 56.70 |
| ADF | 48.58 | 44.58 |
| Ca | 0.42 | 0.47 |
| *P* | 0.21 | 0.20 |
| Amino Acid, % | | |
| Aspartic acid | 8.49 | 7.63 |
| Glutamate | 22.76 | 22.69 |
| Serine | 5.15 | 5.00 |
| Glycine | 9.47 | 9.27 |
| Histidine | 2.84 | 2.92 |
| Arginine | 6.24 | 6.32 |
| Threonine | 4.09 | 4.12 |
| Alanine | 5.42 | 5.67 |
| Proline | 6.45 | 6.79 |
| Tyrosine | 1.86 | 1.98 |
| Valine | 5.80 | 5.93 |
| Methionine | 0.38 | 0.45 |
| Cystine | <0.01 | <0.01 |
| Isoleucine | 5.00 | 5.10 |
| Leucine | 7.85 | 8.20 |
| Phenylalanine | 4.25 | 4.36 |
| Lysine | 3.96 | 3.56 |
| Total (mg/g) | 823.56 | 903.84 |

## Metabolite identification in FGSM

To reveal the changes more intuitively in metabolites after fermentation of grape seed meal, high-resolution LC–MS-based untargeted metabolomics analysis was performed. In the ESI (+) and ESI (−) modes, 240 and 169 ionic features were detected, respectively. Both the ESI positive (Figure 1A, $R^2X = 0.805$) and ESI negative (Figure 1B, $R^2X = 0.820$) models clearly indicated a clear separation trend between FGSM and CON, which reflects the difference in metabolites between the two sample groups. The differentially significant metabolites were identified and screened. A total of 29 differential metabolites (indexed by VIP > 3, $p < 0.05$) were detected in the fermented and unfermented grape seed meal samples. It mainly included 1 metabolite as a nucleic acid, 7 metabolites as lipids, 8 metabolites as carbohydrates, and 5 metabolites as organic acids. In addition, nine unclassified metabolites were identified (Table 3).

## Analysis of differentially expressed metabolite pathways and enrichment analysis

In order to observe the significance of differences between metabolic pathways and enrichment of metabolites more intuitively, differential metabolites in the two groups of samples under positive and negative ion mode were combined in this study for enrichment analysis by KEGG pathway. The top 20 metabolic pathways with the highest significance were selected based on *p* values (Figure 2). The pathway enrichment analysis showed that the enriched pathways were mainly involved in Metabolic pathways. The differentially expressed metabolites detected by non-targeted metabolomics were mainly involved in ABC transporters, Biosynthesis of amino acids and Galactose metabolism. Specifically, these metabolic pathways may be critical to identify differences between FGSM and CON, whereas important differentially expressed metabolites play crucial roles in critical pathways.

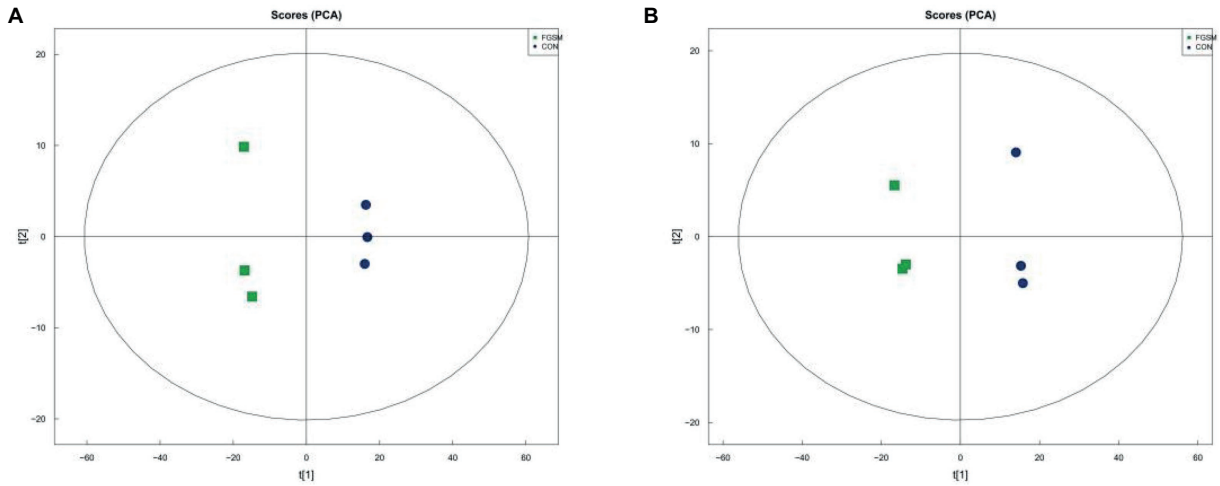


FIGURE 1
The principal component score plot analysis (PCA) of FGSM. **(A)** PCA plot in positive-ion mode; **(B)** PCA plot in negative-ion mode.

TABLE 3 Main difference metabolites between FGSM group and CON group.

| Number | Metabolite | Qualification mode | Vip | FC | m/z | rt(S) | *p*-value | Classification |
|---|---|---|---|---|---|---|---|---|
| 1 | Adenosine | Positive | 6.07 | 0.01 | 268.1 | 174.04 | <0.01 | Nucleic acids |
| 2 | 16-Hydroxypalmitc acid | Positive | 5.11 | 8.86 | 295.22 | 214.77 | <0.01 | Lipids |
| 3 | alpha-Linolenic acid | Positive | 6.24 | 1.64 | 279.23 | 57.81 | 0.031 | Lipids |
| 4 | 3-Isopropylmalate | Negative | 4.37 | 0.03 | 175.06 | 132.45 | <0.01 | Lipids |
| 5 | Caproic acid | Negative | 7.98 | 0.55 | 115.08 | 82.76 | <0.01 | Lipids |
| 6 | Azelaic acid | Negative | 10 | 0.34 | 187.1 | 346.41 | <0.01 | Lipids |
| 7 | Linoleic acid | Negative | 7.08 | 0.39 | 559.47 | 45.14 | 0.003 | Lipids |
| 8 | D-Lactose | Positive | 4.09 | 2.81 | 360.15 | 419.43 | <0.01 | Carbohydrates |
| 9 | Maltotriose | Positive | 5.83 | 3.89 | 522.201 | 450.16 | 0.025 | Carbohydrates |
| 10 | D-Mannose | Negative | 5.99 | 38.05 | 239.08 | 308.76 | <0.01 | Carbohydrates |
| 11 | L-Fucose | Negative | 4.76 | 49.49 | 223.08 | 253.97 | <0.01 | Carbohydrates |
| 12 | Sucrose | Negative | 7.91 | 0.04 | 341.11 | 368.73 | 0.003 | Carbohydrates |
| 13 | Tartaric acid | Negative | 3.12 | 0.65 | 149.01 | 435.79 | 0.005 | Carbohydrates |
| 14 | D-Glucarate | Negative | 4.34 | 1.56 | 191.02 | 285.42 | 0.007 | Carbohydrates |
| 15 | Ribitol | Negative | 4.22 | 1.41 | 133.05 | 195.755 | 0.049 | Carbohydrates |
| 16 | L-Phenylalanine | Positive | 4.6 | 5.96 | 166.08 | 262.59 | <0.01 | Organic acids |
| 17 | 4-Guanidinobutyric acid | Positive | 4.59 | 0.67 | 146.09 | 359.45 | 0.001 | Organic acids |
| 18 | L-Pyroglutamic acid | Negative | 7.32 | 1.63 | 128.04 | 303.51 | 0.001 | Organic acids |
| 19 | L-Proline | Negative | 3.73 | 2.07 | 114.06 | 315.3 | 0.001 | Organic acids |
| 20 | DL-lactate | Negative | 4.69 | 1.75 | 89.02 | 233.12 | 0.007 | Organic acids |
| 21 | Adenine | Positive/negative | 11.89/7.13 | 4.92/4.81 | 136.06/134.05 | 164.87/166.39 | <0.01 | Unclassified |
| 22 | Limonene-1,2-epoxide | Positive | 9.27 | 0.27 | 213.15 | 158.73 | <0.01 | Unclassified |
| 23 | Stearidonic Acid | Positive | 5.71 | 0.41 | 277.22 | 164.12 | <0.01 | Unclassified |
| 24 | 3.alpha.-Mannobiose | Positive | 7.41 | 5.09 | 325.11 | 393.60 | 0.003 | Unclassified |
| 25 | Scytalone | Negative | 6.27 | 44.88 | 193.05 | 160.62 | <0.01 | Unclassified |
| 26 | D-Lyxose | Negative | 4.54 | 5.63 | 149.05 | 220.54 | <0.01 | Unclassified |
| 27 | 9R,10S-EpOME | Negative | 13.02 | 0.63 | 295.23 | 57.60 | 0.001 | Unclassified |
| 28 | 9-OxoODE | Negative | 3.69 | 0.77 | 293.21 | 52.834 | 0.002 | Unclassified |
| 29 | 9(S)-HODE | Negative | 6.00 | 0.52 | 295.23 | 38.738 | 0.039 | Unclassified |

m/z is the mass-to-charge ratio; RT is the residence time; VIP means variable importance projection; FC means variable difference multiple, the average value of the peak area obtained by the FGSM group/the average value of the peak area obtained by the UGSM group, the FC value less than 1 indicates that the FGSM metabolite is lower than the UGSM group.

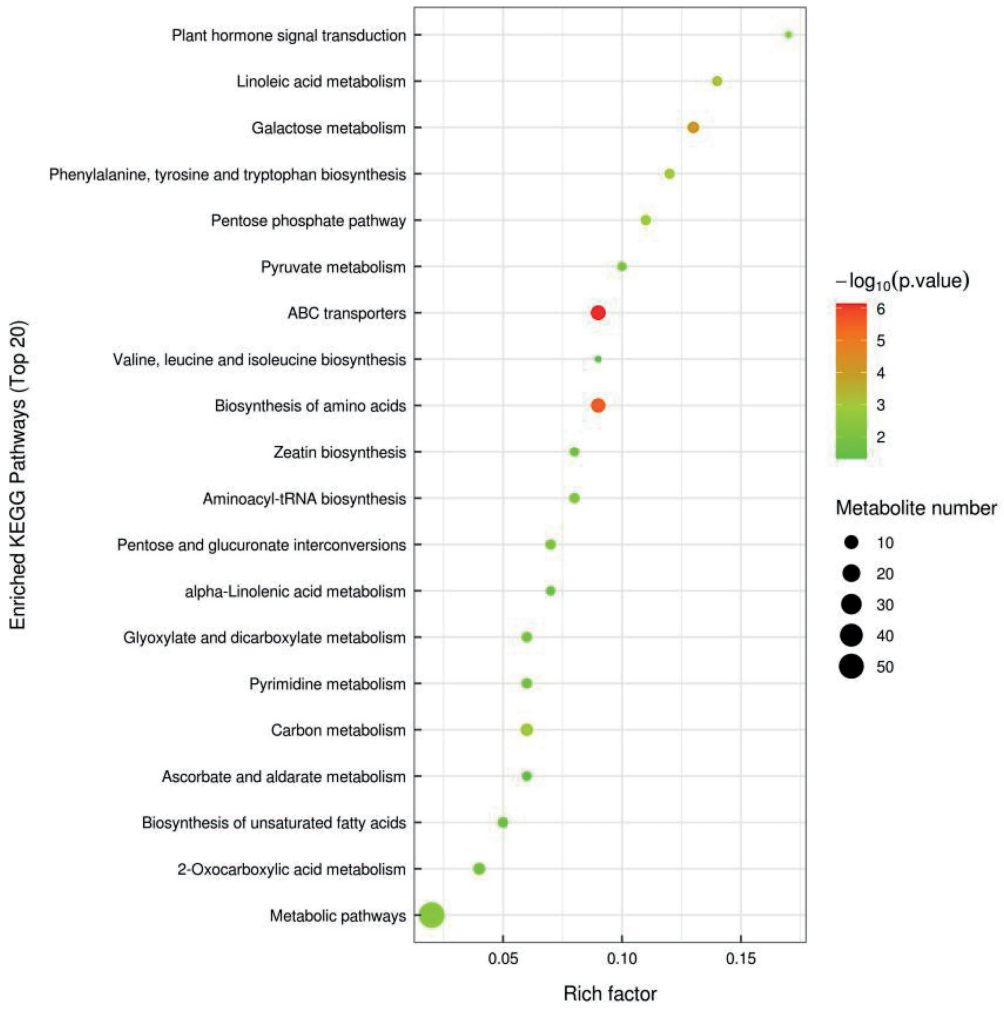


FIGURE 2
Differentially expressed metabolic pathway maps of FGSM and CON.

## Growth performance of broilers

The results showed that different levels of FGSM could increase ADG and ADFI in broilers, and different doses of FGSM had different effects on weight gain in broilers. Compared with group I, ADG was significantly increased and FCR was significantly decreased in groups III and IV ($p < 0.05$). FGSM had a significant effect on ADFI in broilers at the early stage of the experiment. Compared with group I, ADFI was significantly increased in group III, while ADFI was significantly decreased in group IV ($p < 0.05$). We found that the highest ADG and ADFI were observed in group III with 4% FGSM addition. The lowest FCR values were obtained in group IV. These data could all indicate that FGSM improved the growth performance of broilers (Table 4).

## Carcass attributes of broilers

By day 35, compared with group I, the leg muscle rates of groups II, III, and IV were significantly increased by 5.89, 7.94, and 6.70%, respectively ($p < 0.05$). The abdominal fat rate in group IV was significantly lower than that in the other treatment groups ($p < 0.05$). By day 56, the pectoral muscle rate of Group II was significantly higher than other three groups, which were significantly increased by 15.49, 9.35, and 14.06%, respectively ($p < 0.05$). The abdominal fat rate in Group IV was significantly lower than that in Group I by 29.63% ($p < 0.05$) (Table 5).

## Serum biochemistry of broilers

By day 35, The serum TP level of Group IV was significantly higher than that of Groups I and II ($p < 0.05$). Although Group III was higher than these, it was not significant ($p > 0.05$). By day 56, Groups III and IV were significantly higher than Groups I and II ($p < 0.05$). At 35 and 56 days, the serum TC content of group III was significantly lower than other three groups ($p < 0.05$), and the serum BUN levels of Group I were significantly higher than other three groups ($p < 0.05$). There were no significant differences in the other indicators among the groups ($p > 0.05$) (Table 6).

TABLE 4 Effects of different levels of FGSM on growth performance.

| Items | Group I | Group II | Group III | Group IV |
|---|---|---|---|---|
| 14–35 day | | | | |
| ADFI/(g/d) | 69.47 ± 1.29$^{b}$ | 68.35 ± 1.78$^{b}$ | 74.02 ± 3.05$^{a}$ | 61.99 ± 2.65$^{c}$ |
| ADG/(g/d) | 28.40 ± 0.93$^{b}$ | 31.86 ± 1.45$^{a}$ | 32.39 ± 1.60$^{a}$ | 31.48 ± 1.93$^{a}$ |
| FCR | 2.45 ± 0.12$^{a}$ | 2.15 ± 0.14$^{b}$ | 2.22 ± 0.10$^{ab}$ | 1.98 ± 0.21$^{b}$ |
| 35–56 day | | | | |
| ADFI/(g/d) | 137.04 ± 4.52 | 131.51 ± 2.62 | 139.15 ± 8.26 | 134.60 ± 3.00 |
| ADG/(g/d) | 44.46 ± 1.09$^{b}$ | 44.76 ± 0.56$^{b}$ | 52.78 ± 1.58$^{a}$ | 52.39 ± 2.25$^{a}$ |
| FCR | 3.08 ± 0.09$^{a}$ | 2.94 ± 0.05$^{a}$ | 2.64 ± 0.21$^{b}$ | 2.57 ± 0.11$^{b}$ |
| 14–56 day | | | | |
| ADFI(g/d) | 103.26 ± 1.70$^{ab}$ | 99.93 ± 1.23$^{bc}$ | 106.58 ± 2.78$^{a}$ | 98.30 ± 1.95$^{c}$ |
| ADG/(g/d) | 36.43 ± 0.87$^{c}$ | 38.31 ± 0.97$^{b}$ | 42.59 ± 0.30$^{a}$ | 41.93 ± 0.26$^{a}$ |
| FCR | 2.83 ± 0.04$^{a}$ | 2.61 ± 0.07$^{b}$ | 2.50 ± 0.05$^{c}$ | 2.34 ± 0.04$^{d}$ |

In the same line, values with different letter superscripts mean significant differences ($p < 0.05$), while values with the same letter superscripts mean insignificant differences ($p > 0.05$).

TABLE 5 Effects of different levels of FGSM on carcass attributes (%).

| Items | Group I | Group II | Group III | Group IV |
|---|---|---|---|---|
| 35 day | | | | |
| Dressed carcass | 92.29 ± 1.27 | 91.51 ± 1.76 | 92.91 ± 1.44 | 92.30 ± 0.83 |
| Eviscerated | 64.10 ± 0.58 | 63.62 ± 2.61 | 63.64 ± 1.97 | 64.71 ± 1.63 |
| Semi-eviscerate | 82.59 ± 0.73 | 82.41 ± 1.93 | 82.29 ± 1.65 | 82.74 ± 1.30 |
| Breast muscle | 13.44 ± 1.29 | 13.64 ± 0.86 | 13.09 ± 0.99 | 13.93 ± 0.56 |
| Leg muscle | 18.51 ± 0.60$^{b}$ | 19.60 ± 0.72$^{a}$ | 19.98 ± 1.07$^{a}$ | 19.75 ± 1.06$^{a}$ |
| Liver weight | 3.71 ± 0.34 | 3.59 ± 0.30 | 3.40 ± 0.31 | 3.58 ± 0.26 |
| Abdominal fat | 1.99 ± 0.34$^{a}$ | 1.98 ± 0.53$^{a}$ | 2.31 ± 0.40$^{a}$ | 1.53 ± 0.29$^{b}$ |
| 56 day | | | | |
| Dressed carcass | 93.86 ± 1.21 | 93.52 ± 0.90 | 93.15 ± 0.65 | 93.49 ± 0.98 |
| Eviscerated | 68.67 ± 1.04 | 69.28 ± 1.67 | 69.47 ± 1.55 | 69.29 ± 1.20 |
| Semi-eviscerate | 86.30 ± 1.89 | 87.48 ± 0.95 | 86.79 ± 1.14 | 86.92 ± 1.15 |
| Breast muscle | 14.73 ± 1.09$^{b}$ | 17.43 ± 1.86$^{a}$ | 15.80 ± 1.00$^{b}$ | 14.98 ± 1.17$^{b}$ |
| Leg muscle | 18.32 ± 0.87 | 17.76 ± 0.83 | 18.07 ± 0.63 | 17.85 ± 0.90 |
| Liver weight | 2.62 ± 0.24 | 2.46 ± 0.36 | 2.66 ± 0.43 | 2.77 ± 0.36 |
| Abdominal fat | 3.51 ± 0.95$^{a}$ | 2.96 ± 0.63$^{ab}$ | 2.83 ± 0.57$^{ab}$ | 2.47 ± 0.53$^{b}$ |

In the same line, values with different letter superscripts mean significant differences ($p < 0.05$), while values with the same letter superscripts mean insignificant differences ($p > 0.05$).

TABLE 6 Effects of different levels of FGSM on serum biochemical.

| Items | Group I | Group II | Group III | Group IV |
|---|---|---|---|---|
| 35 day | | | | |
| TC/(mmol/L) | 2.06 ± 0.77 | 2.24 ± 0.58 | 1.98 ± 0.69 | 2.84 ± 1.02 |
| TG/(mmol/L) | 0.67 ± 0.20$^{a}$ | 0.58 ± 0.13$^{a}$ | 0.31 ± 0.12$^{b}$ | 0.79 ± 0.31$^{a}$ |
| TP/(g/L) | 28.82 ± 6.49$^{b}$ | 27.84 ± 2.96$^{b}$ | 31.13 ± 2.20$^{ab}$ | 34.35 ± 6.05$^{a}$ |
| ALB/(g/L) | 11.34 ± 1.01 | 10.99 ± 0.92 | 12.48 ± 2.94 | 12.99 ± 2.03 |
| BUN/(mmol/L) | 1.82 ± 0.71$^{a}$ | 0.69 ± 0.24$^{b}$ | 0.54 ± 0.28$^{b}$ | 0.97 ± 0.48$^{b}$ |
| 56 day | | | | |
| TC/(mmol/L) | 2.85 ± 1.34 | 2.33 ± 0.82 | 2.12 ± 0.65 | 2.92 ± 0.63 |
| TG/(mmol/L) | 0.57 ± 0.24$^{a}$ | 0.58 ± 0.11$^{a}$ | 0.35 ± 0.14$^{b}$ | 0.58 ± 0.27$^{a}$ |
| TP/(g/L) | 23.23 ± 4.30$^{b}$ | 22.04 ± 5.37$^{b}$ | 30.36 ± 5.99$^{a}$ | 29.17 ± 5.87$^{a}$ |
| ALB/(g/L) | 9.98 ± 1.83 | 9.90 ± 1.30 | 9.90 ± 0.95 | 10.71 ± 1.06 |
| BUN/(mmol/L) | 3.46 ± 0.44$^{a}$ | 1.09 ± 0.45$^{c}$ | 1.31 ± 0.74$^{c}$ | 2.07 ± 0.14$^{b}$ |

In the same line, values with different letter superscripts mean significant differences ($p < 0.05$), while values with the same letter superscripts mean insignificant differences ($p > 0.05$).

## Out analysis of cecal microbial community in broilers

From the 48 samples, 6,010,963 valid reads were obtained after filtering low-quality raw tags and removing chimera sequences. An average number of 125,228 high-quality sequences per sample and an average sequence length of 500 bp for subsequent microbial richness and diversity analysis were obtained. A total of 3,285 OTUs were identified in the cecal contents of 35-day-old broilers, including 1,051 OTUs in the four groups. This accounts for 31.99% of the total OTUs (Figure 3A). In Groups I, II, III, and IV, there were 323, 412, 292, and 323 unique OTUs, respectively. In addition, a total of 2409 OTUs were identified in the cecal contents of 56 day broilers, including 777 OTUs in the four groups, accounting for 32.25% of the total OTUs (Figure 3B). There were 181, 121, 269, and 312 characteristic tables unique to groups I, II, III, and IV, respectively. The results showed that the total number of OUT decreased with the extension of the experimental period, and the number of OUT common to the four groups decreased correspondingly. This indicates that the bacterial diversity in the early experimental period was higher than that in the late experimental period.

## Diversity of cecal microbiota

### Alpha diversity

Based on Shannon, Simpson, Chao 1 and ACE, which represent the richness and diversity of microbial communities, alpha diversity of microbial communities in the four groups of samples was detected. By day 35, the Shannon and Simpson indices of groups III and IV were significantly higher than those of group I ($p < 0.05$). There was no significant difference in other indices ($p > 0.05$). By day 56, the observed species indices of Groups III and IV were significantly higher than that of Group I ($p < 0.05$); and the other indices were not significantly different ($p > 0.05$). As feeding time increased, the Shannon, Simpson, Chao1, ACE, observed species, and PD whole-tree indices all decreased. The results showed that the addition of FGSM to daily grains affected the abundance and diversity of the gut microbial communities (Table 7).

### Beta diversity

To facilitate the observation of microbial population differences in the cecal contents between the groups, unweight-based PCoA and NMDS plots were applied to assess beta diversity. By day 35, the contribution rates of principal components 1 and 2

of PCoA were 17.7 and 7.235%, respectively (Figure 4A). By day 56, the contribution rates of principal components 1 and 2 of the PCoA were 10.21 and 8.433%, respectively (Figure 4B). The NMDS plot (Figures 4C,D) shows a clustered distribution of each sample in the four treatment groups, a small difference, similar structure of sample microbiota, and good parallelism. The results showed that the effect of adding FGSM to broiler diets in the early stage was better than that in the late stage, which would lead to a wide distribution of cecal bacteria in broilers in the early stage of the experiment.

## Variation in cecal microbiota composition

To understand the variation in abundance levels within the cecal samples, we constructed bar graphs of the relative abundance of species. Based on the results of species annotation, we selected the top 10 most abundant species in each sample and grouped them at each taxonomic level (phylum, class, order, family, and genus) to generate column cumulative plots of relative abundances of species, allowing the visualization of relative abundances of species in each sample and their proportions at different taxonomic levels.

At the phylum level, *p_Bacteroidetes*, *p_Firmicutes*, and *p_Synergistetes* were the dominant bacteria, with average abundances of 58.98, 34.87 and 3.49%, respectively. At 35 days, the relative abundance of *p_Bacteroidetes* in Groups III and IV was significantly reduced ($p < 0.05$), and the relative abundance of *p_Firmicutes* was significantly increased ($p < 0.05$) compared with Group I. The relative abundances of

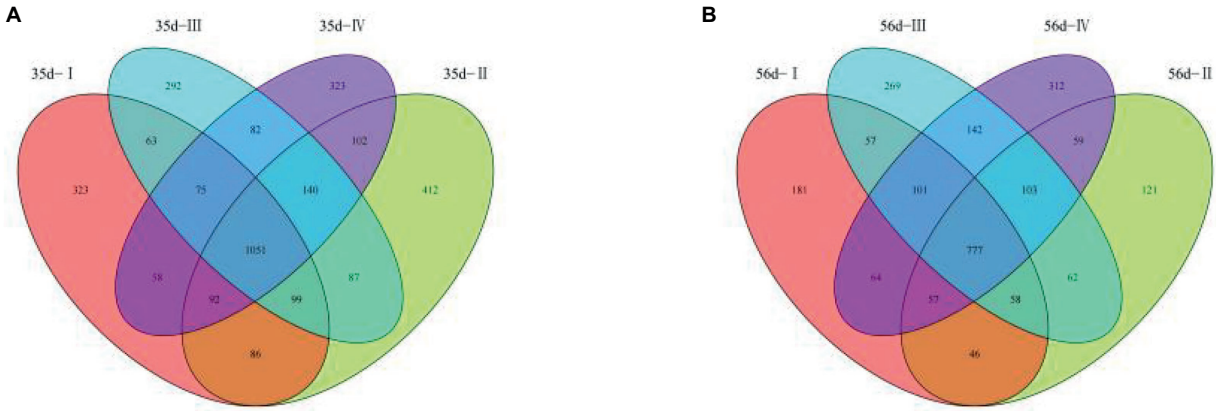


FIGURE 3
Analysis of Venn Diagram and Dilution Curve of Cecal Microorganism in broilers in different treatment groups. **(A)** Venn diagram for 35-day-old broilers; **(B)** Venn diagram for 56-day-old broilers.

TABLE 7 Alpha diversity index of broiler cecal microbiome.

| Items | Group I | Group II | Group III | Group IV |
|---|---|---|---|---|
| 35 day | | | | |
| Observed species | 831.17 ± 106.07 | 931.00 ± 79.96 | 875.50 ± 142.04 | 909.33 ± 78.41 |
| Shannon | 3.85 ± 1.42$^{b}$ | 4.76 ± 0.90$^{ab}$ | 5.16 ± 0.76$^{a}$ | 5.28 ± 0.48$^{a}$ |
| Simpson | 0.71 ± 0.24$^{b}$ | 0.83 ± 0.11$^{ab}$ | 0.89 ± 0.07$^{a}$ | 0.91 ± 0.03$^{a}$ |
| Chao1 | 1010.31 ± 114.44 | 1116.84 ± 61.67 | 1065.36 ± 126.28 | 1093.44 ± 73.22 |
| ACE | 1075.79 ± 128.71 | 1171.34 ± 72.76 | 1088.34 ± 152.59 | 1155.05 ± 82.06 |
| Coverage | 1.00 ± 0.00 | 1.00 ± 0.00 | 1.00 ± 0.00 | 1.00 ± 0.00 |
| PD_whole_tree | 64.63 ± 5.68 | 65.95 ± 2.33 | 64.87 ± 6.33 | 65.58 ± 3.63 |
| 56 day | | | | |
| Observed species | 565.00 ± 152.28$^{ab}$ | 540.17 ± 134.66$^{b}$ | 715.17 ± 148.07$^{a}$ | 725.33 ± 109.41$^{a}$ |
| Shannon | 3.75 ± 0.58 | 3.74 ± 0.61 | 3.82 ± 1.00 | 4.49 ± 0.49 |
| Simpson | 0.80 ± 0.09 | 0.79 ± 0.11 | 0.76 ± 0.17 | 0.86 ± 0.04 |
| Chao1 | 739.75 ± 170.25 | 706.63 ± 171.68 | 903.68 ± 148.77 | 880.80 ± 145.78 |
| ACE | 755.26 ± 169.75 | 730.93 ± 180.33 | 937.79 ± 176.91 | 914.89 ± 156.41 |
| Coverage | 1.00 ± 0.00 | 1.00 ± 0.00 | 1.00 ± 0.00 | 1.00 ± 0.00 |
| PD_whole_tree | 55.22 ± 8.33 | 52.27 ± 7.17 | 60.80 ± 5.17 | 60.49 ± 6.72 |

In the same line, values with different letter superscripts mean significant differences ($p < 0.05$), while values with the same letter superscripts mean insignificant differences ($p > 0.05$).

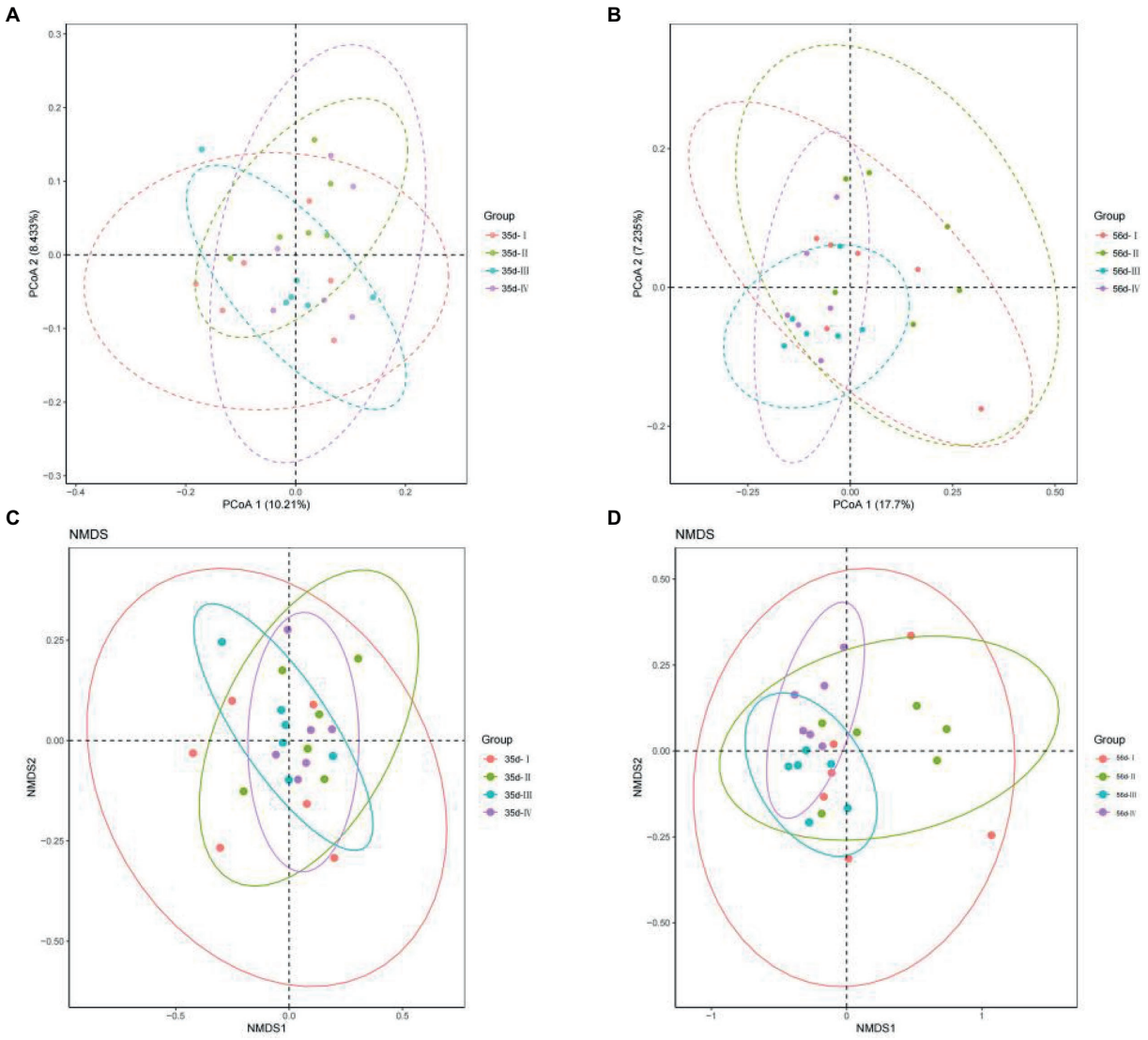


FIGURE 4
The cecal microbial PCoA map and NMDS map of the unweighted UniFrac distance matrix of broilers in different treatment groups. **(A)** 35 day-PCoA plots; **(B)** 56 day-PCoA plots; **(C)** 35 day-NMDS plots; **(D)** 56 day-NMDS plots.

*p_Actinobacteria* and *p_Cyanobacteria* in Group III, were significantly higher than those in Group I ($p < 0.05$; Figure 5A). At 56 days, the relative abundances of *p_Actinobacteria* and *p_Firmicutes* in Group IV increased significantly ($p < 0.05$), and the relative abundance of *p_Bacteroidetes* decreased significantly ($p < 0.05$) compared with Group I. The relative abundances of other phyla were not significantly different among the groups ($p > 0.05$).

In addition, the top ten abundant microbial groups at the genus level were obtained through analysis of bacterial composition. They are *g_Bacteroides, g_Rikenellaceae RC9 gut group* and *g_Alitipes* of *Bacteroides*, *g_[Ruminococcus] torques group*, *g_Phascolarctobacterium*, *g_Faecalibacterium*, *g_Ruminococcaceae UCG-014 of p_Firmicutes*, *g_Synergistes*, *g_Megamonas*, uncultured, and others (Figure 5B). The dominant bacterial groups are *g_Bacteroides*, *g_Rikenellaceae RC9 gut group*, and others. At day 35, the relative abundance of *g_[Ruminococcus] torques group* in Groups II and IV increased significantly ($p < 0.05$). The relative abundance of other was significantly increased in Groups II, III, and IV, which were supplemented with FGSM ($p < 0.05$). At 56 days, the relative abundance of *g_Alistipes* in Group II was higher than that in the other test groups.

## Overview of LEfSe analysis of gut microbiota

Biomarker species with different statistical differences were observed by LEfSe (Linear discriminant analysis Effect Size). Species that met the screening criteria were identified ($|\text{LDAscore}| > 2$), and differential species were colored according to the most abundant group in which the species were located.

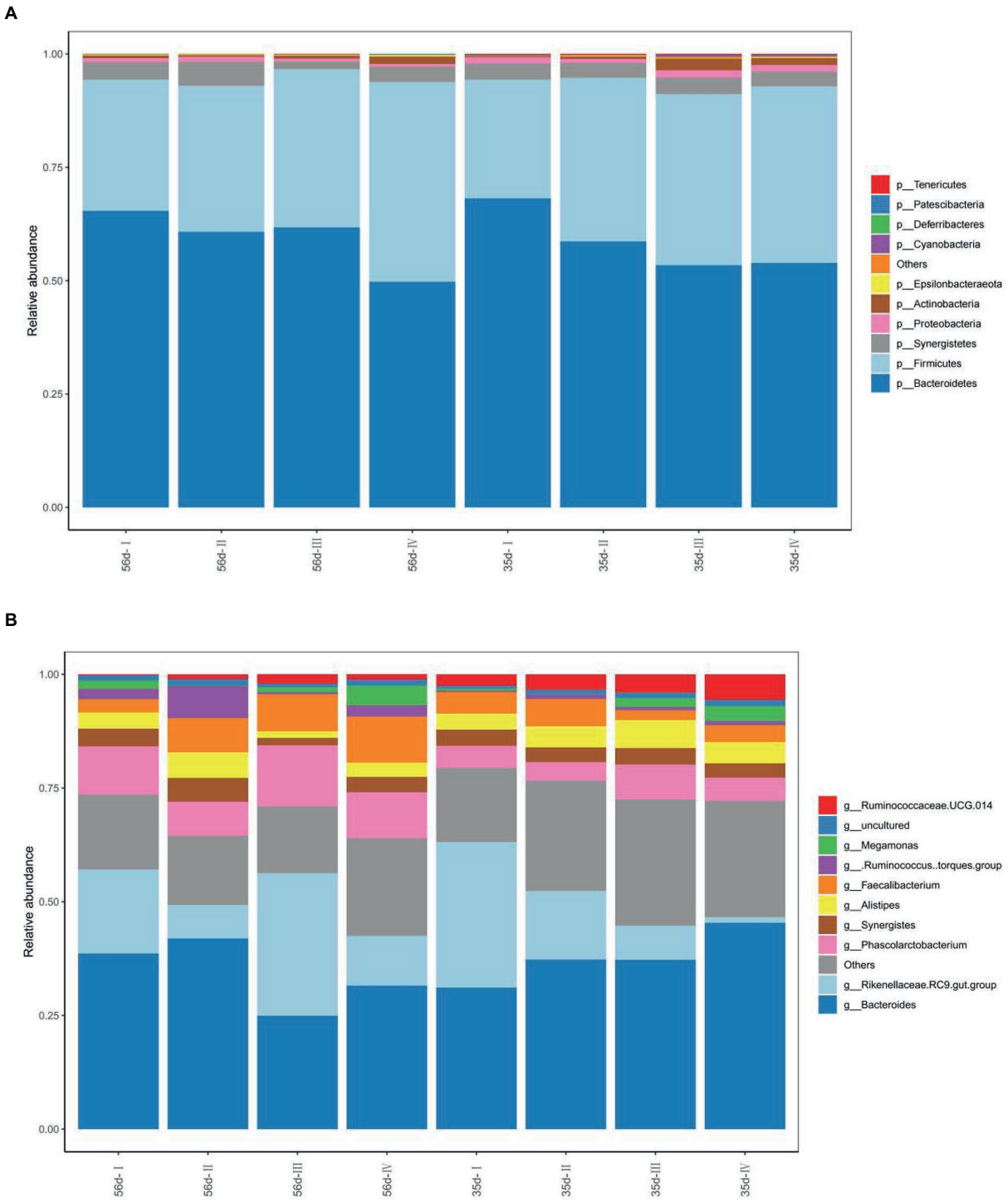


FIGURE 5
The composition of the cecal microflora of broilers in different treatment groups. **(A)** The phylum level composition of the microflora; **(B)** The genus level composition of the microflora.

At 35 days, 21 species showed significant differences in abundance between groups, of which Group III showed the highest abundance (12 spieces). The relative abundances of *g_Paraprevotella* and *g_Flavobacterium* were higher in Group I. The dominant bacteria in Group II were *g_Ruminococcus_torques group* and *g_Flavonifractor*. The species with significant differences in Group III were *p_Actinobacteria*, *g_Ruminiclostridium 5*, *g_Eubacterium hallii group*, *g_Lachnoclo -stridium*, *g_Enorma*, and *g_Parabacteroides*. In Group IV, *g_Megamonas* and *g_Fournierella* were abundant (Figures 6A,C).

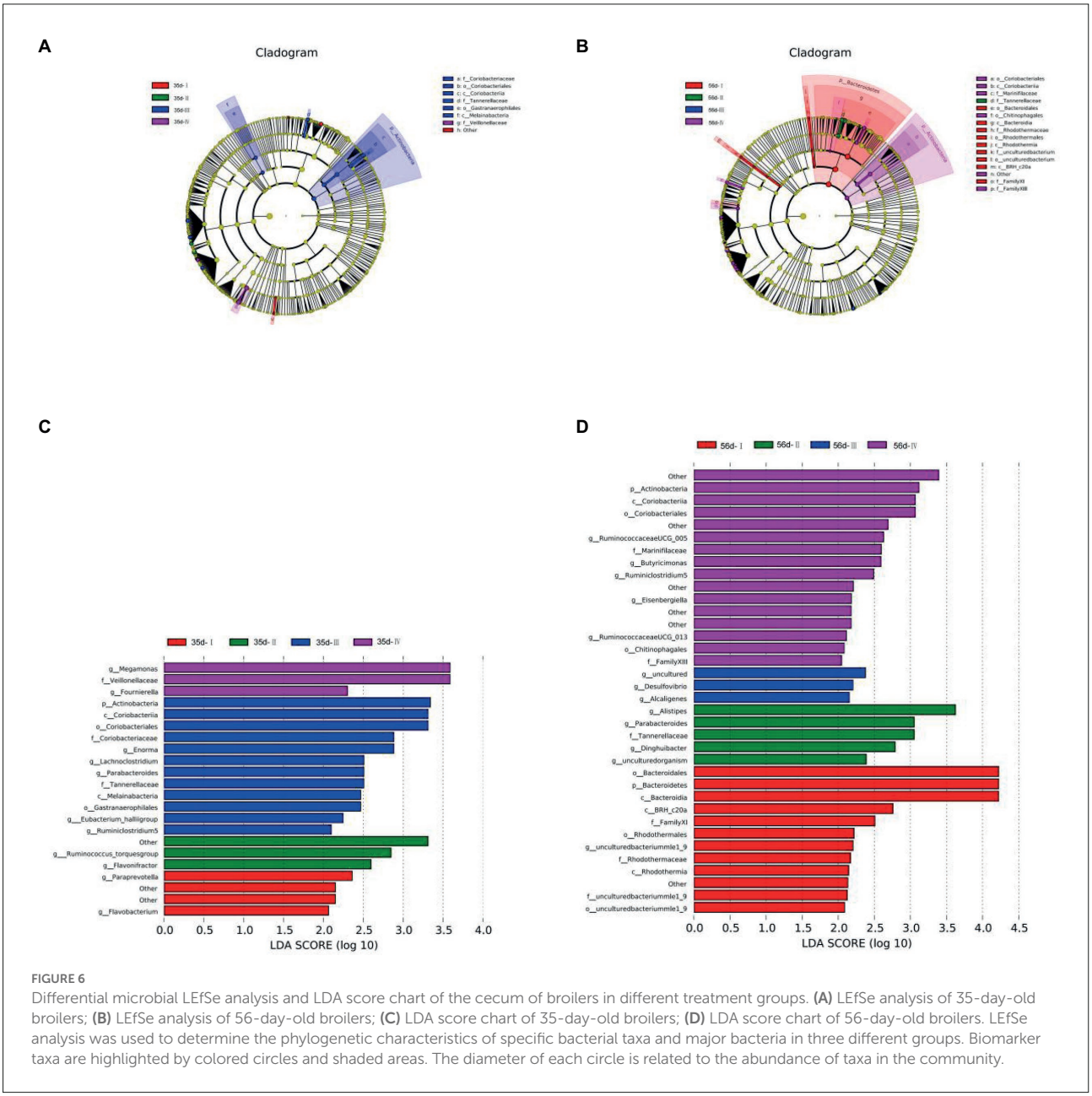


FIGURE 6
Differential microbial LEfSe analysis and LDA score chart of the cecum of broilers in different treatment groups. **(A)** LEfSe analysis of 35-day-old broilers; **(B)** LEfSe analysis of 56-day-old broilers; **(C)** LDA score chart of 35-day-old broilers; **(D)** LDA score chart of 56-day-old broilers. LEfSe analysis was used to determine the phylogenetic characteristics of specific bacterial taxa and major bacteria in three different groups. Biomarker taxa are highlighted by colored circles and shaded areas. The diameter of each circle is related to the abundance of taxa in the community.

At 56 days, 35 species showed significant differences in abundance between groups, with 12 in Group I, 5 in Group II, 3 in Group III, and 16 in Group IV. The relative abundances of *p_Bacteroidetes* and *g_uncultured bacteriummlel_9* in Group I were relatively high. The dominant genera in Group II were *g_Alistipes*, *g_Parabacteroides*, *g_Dinghuibacter*, and *g_uncultured organism*. The species with significant differences in Group III were *g_Desulfovibrio*, *g_Alcaligenes*, and *g_uncultured*. The relative abundances of *p_Actinobacteria*, *g_Ruminococcaceae -UCG005*, *g_Butyricimbergiella*, *g_Eisenbergiella*, *g_Ruminiclostridium 5*, and *g_Ruminococcaceae UCG_013* were high in Group IV (Figures 6B,D).

## SCFAs of cecal contents

Acetic acid, propionic acid, butyric acid, and total acid concentrations did not differ among groups II, III, and IV supplemented with FGSM throughout the test period ($p > 0.05$), but their levels were lower than those of group I. In addition, the yield of Butyric acid was increased in group III by day 56 ($p < 0.05$) (Table 8).

## Spearman correlation analysis

Finally, we assessed the relationships between growth performance, abdominal fat, gut microbes, and SCFAs using

Spearman's correlation analysis. As shown in Figure 7A, at 35 days, FCR of broilers was positively correlated with total acid content and *p_Deferribacteres*, and ADG was positively correlated with *p_Actinobacteria* while showing the opposite trend with FCR. Meanwhile, FCR also showed a negative correlation with *g_[Ruminococcus] torques group*.

TABLE 8 Effects of different levels of FGSM on cecal SCFAs (μg/mL).

| Items | Group I | Group II | Group III | Group IV |
|---|---|---|---|---|
| 35 day | | | | |
| Acetic acid | 7.41 ± 0.81 | 6.25 ± 1.46 | 6.72 ± 1.64 | 7.15 ± 1.11 |
| Propionic acid | 3.83 ± 0.29 | 3.42 ± 0.37 | 3.73 ± 0.62 | 3.82 ± 0.40 |
| Butyric acid | 3.33 ± 0.11 | 3.08 ± 0.20 | 3.11 ± 0.24 | 3.17 ± 0.24 |
| Total acid | 14.56 ± 1.11 | 12.75 ± 1.95 | 13.85 ± 2.73 | 13.87 ± 1.48 |
| 56 day | | | | |
| Acetic acid | 5.46 ± 0.91 | 7.16 ± 1.88 | 6.36 ± 2.37 | 5.32 ± 1.24 |
| Propionic acid | 3.41 ± 0.30 | 3.91 ± 0.57 | 3.48 ± 0.55 | 3.25 ± 0.48 |
| Butyric acid | 2.99 ± 0.15$^{b}$ | 3.11 ± 0.10$^{b}$ | 3.52 ± 0.41$^{a}$ | 3.21 ± 0.24$^{ab}$ |
| Total acid | 11.87 ± 1.21 | 14.18 ± 2.47 | 13.42 ± 3.19 | 11.78 ± 1.61 |

In the same line, values with different letter superscripts mean significant differences ($P < 0.05$), while values with the same letter superscripts mean insignificant differences ($P > 0.05$).

Figure 7B shows the correlation between growth performance, abdominal fat with gut microbes, and SCFAs in broilers at 56 days. ADFI was found to be positively correlated with the *p_Patescibacteria*. Additionally, ADG was positively correlated with butyric acid, *p_Patescibacteria*, *p_Deferribacteres*, and *g_Ruminococcaceae UCG-014* and negatively correlated with *g_Alistipes*. FCR showed a negative correlation with butyric acid, *p_Firmicutes*, *p_Patescibacteria*, *p_Deferribacteres*, and *g_Ruminococcaceae UCG-014* and a positive correlation with *g_Alistipes*. Moreover, abdominal fat was negatively correlated with butyric acid, *p_Actinobacteria*, and *p_Cyanaceae*.

# Discussion

## Improving feed nutrient content to increase broiler feed intake

Fermented grape seed meal by Bacteria coupled fermentation with enzymes has advantages over the individual processes of probiotic fermentation and protease enzymatic hydrolysis. Acid protease can improve the nutritional quality of grape seed meal.

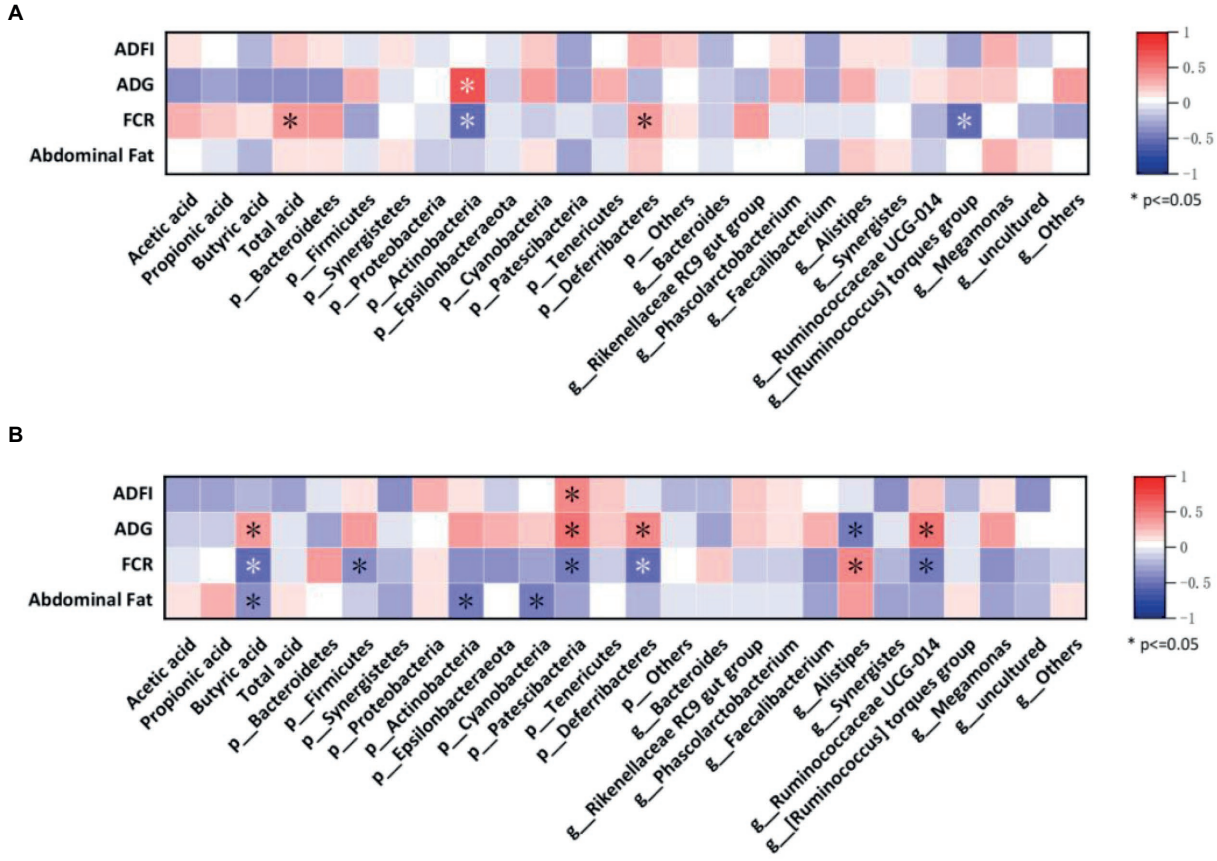


FIGURE 7
Correlation between the most abundant taxa of the cecal microbiota with growth performance and SCFAs of broilers. **(A)** Heatmap of samples from 35-day-old broilers; **(B)** Heatmap of samples from 56-day-old-broilers.

By directly increasing the number of proteases, the reaction rate of protease hydrolysis is increased. The macromolecular protein in feed material is decomposed into peptides and free amino acid, so as to improve the conversion rate of feed in animal organism. When fermenting grape seed meal with *Saccharomyces cerevisiae*, the acid-soluble protein content increases for the following reasons: yeast cells contain a large number of proteins and amino acids. And, yeast can produce protease during the fermentation process. As fermentation proceeds, nutrient-active substances such as organic acids (tartaric, malic, and citric acid) are produced during yeast growth and reproduction. They involved in enzymatic reactions through the tricarboxylic acid cycle, allowing proteases to break down large molecules of proteins at a faster rate. Also, microorganisms can convert non-protein nitrogen into protein nitrogen, which can be absorbed and used by livestock (Tchorbanov and Lazarova, 1988; Zhang, 2012; Wu, 2018). Moreover, the contents of NDF and ADF were reduced. The main reason was that during the fermentation process, the microorganisms produced cellulases that could decompose cellulose, hemicellulose and lignin during growth and reproduction, which reduced the fiber content in the FGSM (Rodríguez Muela et al., 2017). Simultaneously, organic acids produced by *S. cerevisiae* during fermentation could improve feed palatability. The relative concentration of monosaccharides used as sweeteners in feed such as D-mannose and L-fucose increased in FGSM. It has a unique volatile aroma of grapes, fragrant taste, and good palatability. Therefore, the fermented grape seed meal by Bacteria coupled fermentation with enzymes can effectively improve the nutritional composition of feed ingredients and improve animal feed intake.

## FGSM promotes broiler growth and improves carcass quality

In this study, the addition of FGSM significantly increased broiler growth performance. The growth performance of animals is influenced by muscle proteins, which constitute the net balance of protein synthesis and degradation. Amino acids are basic components of proteins and play roles in regulating nutrient transport, gut microbiota, and antioxidant responses (Mott et al., 2008). In the intestine, proteins are broken down into small peptides and free amino acids by enzymes and transported through different nutrient transporters (Wu et al., 2014). By untargeted metabolomics analysis, we found that the relative concentration of adenosine was lower in FGSM. Adenosine plays an important role in cellular metabolism by acting as an intermediate in ATP synthesis. Studies have shown that phosphoribose in yeast cells is degraded into a mixture of adenosine, adenine, ribose, single nucleotide, and polynucleotides by an enzymatic system. Then Adenosine and other substances are phosphorylated through the pathway of glycolysis to produce ATP, which then binds to the protein hydrolysis region of ABC transporters, causing ATP to be hydrolyzed. The energy generated by metabolism changes the conformation of transporters, resulting in transporters transporting various sugars, amino acids, proteins and other cellular metabolites to the extracellular space (Guerrero and Mullet, 1986). It means that the content of small peptides and free amino acids increases through this metabolic pathway. This is supported by the results of the free amino acid content in FGSM. Therefore, enhanced intestinal uptake of amino acids in broilers fed diets supplemented with FGSM would favor protein deposition and ultimately promote growth.

In the early part of the trial, the breast and leg muscle rates increased in broilers that were fed FGSM, but there was no significant difference between the full clearance rate and half clearance rate. This suggests that broiler muscle protein metabolism had the potential to change (Lee et al., 2009). Rodas et al. (1995) found that enhanced synthesis of metabolic proteins can be observed when TP levels increase. The results of this study showed that the addition of 6% FGSM could significantly increase the TP content in the serum of broilers and increase the ALB content. This indicates that FGSM could improve serum TP and ALB concentrations and enhance protein metabolism in broilers. Dhanalakshmi et al. (2007) showed that good nutritional status maintained high levels of total serum protein and albumin, with increased levels of both indicating high metabolic activity. This is similar to (Feng et al., 2007), which demonstrate the positive effect of fermented feed on the metabolic capacity of broilers. BUN is one of the protein metabolites, and its content reflects the status between protein metabolism level and amino acid balance in animals to some extent (Scott et al., 1982). When serum BUN levels are reduced, amino acid catabolism decreases and nitrogen deposition in the body increases, indicating enhanced protein metabolism (Sugiharto et al., 2020). Therefore, adding FGSM to the diet plays an important role in promoting the absorption and utilization of protein in broilers.

At the same time, we found that FCR gradually decreased as the amount of FGSM added increased. In particular, the FCR of group IV was significantly lower than that of the remaining three groups, indicating that the addition of FGSM to the diets had better economic benefits. One study shown that the addition of peas fermented with probiotics or treated with complex enzymes to broiler diets reduces FCR throughout the experimental period (Goodarzi et al., 2017). In another study, the addition of fermented corn gluten flour to daily food improved ADG in broilers (Yan et al., 2018). This is more reflective of the improved nutritional composition of the feed after fermentation or enzymatic digestion, which has the effect of promoting animal growth.

## FGSM regulates lipid metabolism in broilers

Fat deposition in broilers is dominated by three areas: subcutaneous fat, abdominal fat, and intramuscular fat, with abdominal fat receiving more attention than other adipose tissues. Abdominal fat becomes visible at 7 days of age in broilers (Kim and

Voy, 2021), indicating a rapid growth rate. The number of adipocytes gradually increases with age and broiler fat cells increase in size as they absorb and store fatty acids (Bai et al., 2015). Because of ease in dissection and weighing, abdominal fat percentage is widely used to assess the total lipid content in broilers. In the group fed with 6% FGSM, there was a significant reduction in abdominal fat percentage and serum TG levels, suggesting that FGSM regulates body lipid metabolism (Yasar and Yegen, 2017).

After fermentation, the number of probiotic bacteria in FGSM increased. Homma and Shinohara (2004) indicated that probiotics can inhibit lipid synthesis and promote fatty acid catabolism, thereby inhibiting abdominal fat deposition. TC and TG are important components of blood fat, and their levels reflect the absorption and metabolism of fat in the body. Kim and Voy (2021) reported that abdominal fat is the main site of TG storage in mature broilers. When the weight of abdominal fat in broilers decreased, the TG content stored in abdominal fat also decreased and the TG content in the serum increased. Our study found that the addition of appropriate FGSM concentrations resulted in a decrease in serum TG levels. Ooi and Liong (2010) reported that some probiotics are effective at lower levels, whereas others require higher amounts to exert cholesterol-lowering effects. Cholesterol adsorbed in the cell wall of probiotics, or the cells of the bacterium can absorb cholesterol and allow cholesterol removal, thus promoting fat metabolism (Milićević et al., 2014).

The oils in grape seed meal are rich in fatty acids (FAs). Compared with unsaturated fatty acids, saturated fatty acids are more stable. However, they can be converted from saturated fats (palmitoleic and stearic acids) to unsaturated fatty acids (oleic, linolenic, and free fatty acids) by microorganisms with a range of active proteins, such as dehydrogenases and lengthening enzymes (Rhead et al., 1971). The unsaturated fatty acids present in FGSM reduce fat accumulation (Riera-Heredia et al., 2020). Polyunsaturated fatty acids (PUFAs) are classified as n-3 or n-6 depending on the position of the first double bond relative to the methyl end of the molecule. At the cellular level, Fleckenstein-Elsen et al. (2016) found that n-3 PUFAs inhibit lipid production and attenuate lipid accumulation in adipocytes. In contrast, n-6 PUFAs tended to be pro-lipogenic (Massiera et al., 2003). According to the results of non-targeted metabolomics, α-linolenic acid belonging to n-3 PUFAs was upregulated and linoleic acid belonging to n-6 PUFAs was downregulated, resulting in the inhibition of abdominal fat deposition.

## FGSM alters the composition of broilers intestinal bacteria

Intestinal microorganisms play important roles in maintaining animal health, growth, feed absorption, and utilization (Zhang et al., 2018). However, its composition and structure are constrained by various factors such as feed, age, and feeding method (Carrasco et al., 2019). Among these, feed has the greatest effect on intestinal bacteria, which changes their structure and quantity. The health of animals is affected by the production of specific metabolites through biological reactions (Doré and Blottière, 2015). Therefore, changes in feed nutrient composition are very important for the regulation of intestinal bacteria. We found that organic acids, monosaccharides, and unsaturated fatty acids, which are differential metabolites produced by FGSM, have some regulatory effects on broiler gut microbiota.

Our study showed that dietary supplementation of FGSM altered the composition of the cecal microbial community and increased cecal microbial diversity in broilers. At phylum level, Bacteroides, Firmicutes, Synergistetes, and Proteobacteria were the four dominant groups in broiler cecal microorganisms. Similar to the results of Ma et al. (2018), but the third dominant phylum was altered. *Synergistetes* is a newly discovered genus of bacteria that is widely found in anaerobic environments such as the animal intestines and soil (Jumas-Bilak et al., 2009). As a class of Gram-negative, obligate anaerobic bacteria, most of them can grow at room temperature and neutrally and have the ability to degrade amino acids. Simultaneously, *Synergistetes* caused changes in the dominant flora in the gut of broilers, which was responsible for the changes in the structure of the gut community.

When the diet contains high levels of free amino acids, unsaturated fatty acids, and monosaccharides, intestinal bacteria in the body have a higher proportion of *Firmicutes* and a lower proportion of *Bacteroidetes* (De Filippo et al., 2010). Both have a direct relationship in improving the digestion and absorption of nutrients and energy metabolism in animals (Jumpertz et al., 2011), both induce fat deposition in animals. However, *Firmicutes* more effectively extract energy from food, thereby promoting weight gain. Additionally, most studies support an increased (F/B) ratio in obese individuals (Crovesy et al., 2020). In this study, the (F/B) ratio was <1 in all four test groups, and the (F/B) ratio also increased correspondingly with the increase of FGSM addition, but the deposition of abdominal fat decreased. In a Ukrainian study, individuals with an F/B ratio ≥1 were 23% more likely to be overweight than those with an F/B ratio <1 (Koliada et al., 2017). Therefore, an increase in the (F/B) ratio cannot be used as the sole basis for obesity, and the specific ratio has to be discussed. Bervoets et al. (2013) reported that a higher (F/B) indicates a relative abundance of energy-metabolizing microorganisms in the gut microbiota. Similarly, an increased F/B ratio is often associated with growth-promoting performance (Salaheen et al., 2017). In this study, the addition of different concentrations of FGSM to the diet did not change the status of *Bacteroidetes* as the dominant bacterial group. However, with increasing FGSM levels, the relative abundance of *Bacteroidetes* decreased and *Firmicutes* increased. The reduction in abdominal fat percentage, increase in daily weight gain, and reduction in FCR in broilers provided more evidence of efficient energy utilization.

*Actinobacteria* are key players in the maintenance of the intestinal barrier and consist of three major families of anaerobic bacteria (*Bifidobacteria*, *Propionibacterium*, and *Corynebacterium*) and one family of aerobic bacteria (*Streptomyces*). Among these, *Bifidobacteria* are considered

important probiotics in *Actinobacteria*. Studies have reported that most antimicrobials are composed of bioactive substances produced by *Actinobacteria* and show good antibacterial properties in controlling pathogenic microorganisms (Gomes et al., 2017). The results of the non-targeted metabolome showed an increase in the relative concentrations of sugar metabolites such as D-mannose and L-amylose. When animals consume diets containing monosaccharides, they benefit from increased abundance of *Bifidobacteria* and colonization of the intestinal tract, thereby improving broiler intestinal health (Dev et al., 2020). Previous studies have shown that *Actinobacteria* can utilize complex carbohydrates and contribute to improved growth performance and increased feed conversion efficiency in broilers (Courtin et al., 2008). Also, the relative abundance of *Bifidobacteria* has been correlated with fat deposition. In a study comparing fecal samples from lean and obese individuals, it was found that the abundance of *Bifidobacteria* differed between the two, their relative abundance showed a negative correlation with the percentage of body fat (Teixeira et al., 2013).

To further investigate the effect of FGSM on gut microbiota diversity in yellow-feathered broilers, we analyzed differences at the genus level. Among the four groups of samples, *Bacteroides* and *Rikenellaceae RC9* gut *group* under *p_Bacteroides* were the dominant genera. This genus help the host to break down polysaccharides, generate volatile fatty acids and butyrate. In this way, it improves the host immunity and facilitating the regulation of the intestinal immune system (Zhang et al., 2019). In contrast, [*Ruminococcus*] *torques group*, *Phascolarcto -bacterium*, *Faecalibacterium*, and *Ruminococcaceae UCG-014* under *p_Firmicutes* (Louis and Flint, 2017) which can increase the concentration of SCFAs in the intestine. Macfarlane and Macfarlane (2012) pointed out that the content of SCFAs in the gut is closely related to the composition and abundance of gut microbiota. So FGSM increases the concentration of SCFAs in the gut. The main sources of SCFAs in the gut of animals are bacterial metabolites produced by anaerobic fermentation of carbohydrates that are not easily digested (Garron and Henrissat, 2019). It not only acts as an energy source for host cells and gut microbiota, but also improves host lipid and glucose metabolism. Moreover, the production of acetic acid and butyric acid by gut microbiota can cause an increase in broiler feed intake (Chen et al., 2022). Therefore, the pH in the intestine of broilers fed with FGSM decreased, which inhibited the growth of harmful microorganisms. Thereby improving the structure of the intestinal flora and enhancing growth performance, further exerting a potential promotional effect on intestinal health.

In summary, the nutritional composition of grape seeds changed after fermentation, and the concentrations of free amino acids, unsaturated fatty acids, monosaccharides, and organic acids increased. Adding 4% ~ 6% FGSM to broiler diets promotes broiler growth and regulates fat metabolism by altering gut microbiota structure.

## 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/, PRJNA858635.

## Ethics statement

The animal study was reviewed and approved by Animal Ethics Committee of the First Affiliated Hospital of Shihezi University School of Medicine. Written informed consent was obtained from the owners for the participation of their animals in this study.

## Author contributions

SN and CN carried out the experimental design of the study. SN, MY, XZ, and HW contributed to the experimental implementation. SN, MY, HW, and JL contributed to the sampling for this study. SN, MY, and CN contributed to data analysis. SN, MY, XZ, JN, CC, WZ, and CN contributed to the writing of the article. 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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# References


Abu-Elala, N. M., Younis, N. A., AbuBakr, H. O., Ragaa, N. M., Borges, L. L., and Bonato, M. A. (2020). Influence of dietary fermented *Saccharomyces cerevisiae* on growth performance, oxidative stress parameters, and immune response of cultured *Oreochromis niloticus*. *Fish Physiol. Biochem.* 46, 533–545. doi: 10.1007/s10695-019-00711-9

Alagawany, M., Elnesr, S. S., and Farag, M. R. (2018). The role of exogenous enzymes in promoting growth and improving nutrient digestibility in poultry. Iran. *J. Vet. Res.* 19, 157–164.

AOAC (Association of Official Analytical Chemists) (2000). Official Methods of Analysis of Official Analytical Chemists International.17th Edn. Arlington,VA: Association of Official Analytical Chemists.

Bai, S., Wang, G., Zhang, W., Zhang, S., Rice, B. B., Cline, M. A., et al. (2015). Broiler chicken adipose tissue dynamics during the first two weeks post-hatch. *Comp. Biochem. Physiol. A Mol. Integr. Physiol.* 189, 115–123. doi: 10.1016/j.cbpa.2015.08.002

Bervoets, L., Van Hoorenbeeck, K., Kortleven, I., Van Noten, C., Hens, N., Vael, C., et al. (2013). Differences in gut microbiota composition between obese and lean children: a cross-sectional study. *Gut. Pathog.* 5:10. doi: 10.1186/1757-4749-5-10

Berwanger, E., Nunes, R. V., Pasquetti, T. J., Murakami, A. E., de Oliveira, T. M., Bayerle, D. F., et al. (2017). Sunflower cake with or without enzymatic complex for broiler chickens feeding. *Asian-Australas J. Anim. Sci.* 30, 410–416. doi: 10.5713/ajas.15.0644

Câmara, J. S., Lourenço, S., Silva, C., Lopes, A., Andrade, C., and Perestrelo, R. (2020). Exploring the potential of wine industry by-products as source of additives to improve the quality of aquafeed. *Microchem. J.* 155, 104758–104710. doi: 10.1016/j.microc.2020.104758

Carrasco, J. M. D., Casanova, N. A., and Fernández Miyakawa, M. E. (2019). Microbiota, gut health and chicken productivity: what is the connection? *Microorganisms* 7:374. doi: 10.3390/microorganisms7100374

Chen, K., Hu, M., Tang, M., Gao, C., Wang, H., Man, S., et al. (2022). Oligosaccharide and short-chain fatty acid: a double-edged sword in obese mice by regulating food intake and fat synthesis. *Food Res. Int.* 159:111619. doi: 10.1016/j.foodres.2022.111619

Chen, Y. C., and Yu, Y. H. (2020). Bacillus licheniformis-fermented products improve growth performance and the fecal microbiota community in broilers. *Poult. Sci.* 99, 1432–1443. doi: 10.1016/j.psj.2019.10.061

Chikwanha, O. C., Emiliano, R., Opara, U. L., Fawole, O. A., Setati, M. E., Voster, M., et al. (2018). Impact of dehydration on retention of bioactive profile and biological activities of different grape (*Vitis vinifera* L.) pomace varieties. *Anim. Feed Sci. Technol.* 244, 116–127. doi: 10.1016/j.anifeedsci.2018.08.006

Córdova-Noboa, H. A., Oviedo-Rondón, E. O., Ortiz, A., Matta, Y., Hoyos, S., Buitrago, G. D., et al. (2020). Corn drying temperature, particle size, and amylase supplementation influence growth performance, digestive tract development, and nutrient utilization of broilers. *Poult. Sci.* 99, 5681–5696. doi: 10.1016/j.psj.2020.07.010

Courtin, C. M., Broekaert, W. F., Swennen, K., Lescroart, O., Onagbesan, O., Buyse, J., et al. (2008). Dietary inclusion of wheat bran arabinoxylooligosaccharides induces beneficial nutritional effects in chickens. *Cereal Chem. J.* 85, 607–613. doi: 10.1094/CCHEM-85-5-0607

Crovesy, L., Masterson, D., and Rosado, E. L. (2020). Profile of the gut microbiota of adults with obesity: a systematic review. *Eur. J. Clin. Nutr.* 74, 1251–1262. doi: 10.1038/s41430-020-0607-6

De Filippo, C., Cavalieri, D., Di Paola, M., Ramazzotti, M., Poullet, J. B., Massart, S., et al. (2010). Impact of diet in shaping gut microbiota revealed by a comparative study in children from Europe and rural Africa. *Proc. Natl. Acad. Sci. U. S. A.* 107, 14691–14696. doi: 10.1073/pnas.1005963107

Dev, K., Akbar Mir, N., Biswas, A., Kannoujia, J., Begum, J., and Kant, R. (2020). Dietary Mannan-oligosaccharides potentiate the beneficial effects of Bifidobacterium bifidum in broiler chicken. *Lett. Appl. Microbiol.* 71, 520–530. doi: 10.1111/lam.13360

Dhanalakshmi, S., Devi, R. S., Srikumar, R., Manikandan, S., and Thangaraj, R. (2007). Protective effect of Triphala on cold stress-induced behavioral and biochemical abnormalities in rats. *Yakugaku Zasshi* 127, 1863–1867. doi: 10.1248/yakushi.127.1863

Doré, J., and Blottière, H. (2015). The influence of diet on the gut microbiota and its consequences for health. *Curr. Opin. Biotechnol.* 32, 195–199. doi: 10.1016/j.copbio.2015.01.002

Engberg, R. M., Hedemann, M. S., Steenfeldt, S., and Jensen, B. B. (2004). Influence of whole wheat and xylanase on broiler performance and microbial composition and activity in the digestive tract. *Poult. Sci.* 83, 925–938. doi: 10.1093/ps/83.6.925

Feng, J., Liu, X., Xu, Z. R., Liu, Y. Y., and Lu, Y. P. (2007). Effects of aspergillus oryzae 3.042 fermented soybean meal on growth performance and plasma biochemical parameters in broilers. *Anim. Feed Sci. Technol.* 134, 235–242. doi: 10.1016/j.anifeedsci.2006.08.018

Fleckenstein-Elsen, M., Dinnies, D., Jelenik, T., Roden, M., Romacho, T., and Eckel, J. (2016). Eicosapentaenoic acid and arachidonic acid differentially regulate adipogenesis, acquisition of a brite phenotype and mitochondrial function in primary human adipocytes. *Mol. Nutr. Food Res.* 60, 2065–2075. doi: 10.1002/mnfr.201500892

Garron, M. L., and Henrissat, B. (2019). The continuing expansion of CAZymes and their families. *Curr. Opin. Chem. Biol.* 53, 82–87. doi: 10.1016/j.cbpa.2019.08.004

Gomes, K. M., Duarte, R. S., and de Freire Bastos, M. D. C. (2017). Lantibiotics produced by *Actinobacteria* and their potential applications (a review). *Microbiology* 163, 109–121. doi: 10.1099/mic.0.000397

Goodarzi, B. F., Senz, M., Kozłowski, K., Boros, D., Wisniewska, M., Rose, D., et al. (2017). The effects of fermentation and enzymatic treatment of pea on nutrient digestibility and growth performance of broilers. *Animal* 11, 1698–1707. doi: 10.1017/S1751731117000787

Guerrero, F., and Mullet, J. E. (1986). Increased abscisic acid biosynthesis during plant dehydration requires transcription. *Plant Physiol.* 80, 588–591. doi: 10.1104/pp.80.2.588

Heres, L., Engel, B., van Knapen, F., de Jong, M. C., Wagenaar, J. A., and Urlings, H. A. (2003). Fermented liquid feed reduces susceptibility of broilers for *Salmonella enteritidis*. *Poult. Sci.* 82, 603–611. doi: 10.1093/ps/82.4.603

Homma, H., and Shinohara, T. (2004). Effects of probiotic *Bacillus cereus* toyoi on abdominal fat accumulation in the Japanese quail (*Coturnix japonica*). *Anim. Sci. J.* 75, 37–41. doi: 10.1111/j.1740-0929.2004.00152.x

Jumas-Bilak, E., Roudière, L., and Marchandin, H. (2009). Description of 'Synergistetes' phyl. nov. and emended description of the phylum 'Deferribacteres' and of the family Syntrophomonadaceae, phylum 'Firmicutes'. *Int. J. Syst. Evol. Microbiol.* 59, 1028–1035. doi: 10.1099/ijs.0.006718-0

Jumpertz, R., Le, D. S., Turnbaugh, P. J., Trinidad, C., Bogardus, C., Gordon, J. I., et al. (2011). Energy-balance studies reveal associations between gut microbes, caloric load, and nutrient absorption in humans. *Am. J. Clin. Nutr.* 94, 58–65. doi: 10.3945/ajcn.110.010132

Kim, M., and Voy, B. H. (2021). Fighting fat with fat: n-3 polyunsaturated fatty acids and adipose deposition in broiler chickens. *Front. Physiol.* 12:755317. doi: 10.3389/fphys.2021.755317

Koliada, A., Syzenko, G., Moseiko, V., Budovska, L., Puchkov, K., Perederiy, V., et al. (2017). Association between body mass index and Firmicutes/Bacteroidetes ratio in an adult Ukrainian population. *BMC Microbiol.* 17:120. doi: 10.1186/s12866-017-1027-1

Lee, S. D., Kim, H. Y., Jung, H. J., Ji, S. Y., Chowdappa, R., Ha, J. H., et al. (2009). The effect of fermented apple diet supplementation on the growth performance and meat quality in finishing pigs. *Anim. Sci. J.* 80, 79–84. doi: 10.1111/j.1740-0929.2008.00598.x

Liu, J., Yue, S., Yang, Z., Feng, W., Meng, X., Wang, A., et al. (2018). Oral hydroxysafflor yellow A reduces obesity in mice by modulating the gut microbiota and serum metabolism. *Pharmacol. Res.* 134, 40–50. doi: 10.1016/j.phrs.2018.05.012

Louis, P., and Flint, H. J. (2017). Formation of propionate and butyrate by the human colonic microbiota. *Environ. Microbiol.* 19, 29–41. doi: 10.1111/1462-2920.13589

Ma, Y., Wang, W., Zhang, H., Wang, J., Zhang, W., Gao, J., et al. (2018). Supplemental Bacillus subtilis DSM 32315 manipulates intestinal structure and microbial composition in broiler chickens. *Sci. Rep.* 8:15358. doi: 10.1038/s41598-018-33762-8

Macfarlane, G. T., and Macfarlane, S. (2012). Bacteria, colonic fermentation, and gastrointestinal health. *J. AOAC Int.* 95, 50–60. doi: 10.5740/jaoacint.SGE_Macfarlane

Massiera, F., Saint-Marc, P., Seydoux, J., Murata, T., Kobayashi, T., Narumiya, S., et al. (2003). Arachidonic acid and prostacyclin signaling promote adipose tissue development: a human health concern? *J. Lipid Res.* 44, 271–279. doi: 10.1194/jlr.M200346-JLR200

Milićević, D., Vranić, D., Mašić, Z., Parunović, N., Trbović, D., Nedeljković-Trailović, J., et al. (2014). The role of total fats, saturated/unsaturated fatty acids and cholesterol content in chicken meat as cardiovascular risk factors. *Lipids Health Dis.* 13:42. doi: 10.1186/1476-511X-13-42

Mott, C. R., Siegel, P. B., Webb, K. E. Jr., and Wong, E. A. (2008). Gene expression of nutrient transporters in the small intestine of chickens from lines divergently selected for high or low juvenile body weight. *Poult. Sci.* 87, 2215–2224. doi: 10.3382/ps.2008-00101

Ooi, L. G., and Liong, M. T. (2010). Cholesterol-lowering effects of probiotics and prebiotics: a review of in vivo and in vitro findings. *Int. J. Mol. Sci.* 11, 2499–2522. doi: 10.3390/ijms11062499

Rhead, M. M., Eglinton, G., Draffan, G. H., and England, P. J. (1971). Conversion of oleic acid to saturated fatty acids in Severn estuary sediments. *Nature* 232, 327–330.

Riera-Heredia, N., Lutfi, E., Sánchez-Moya, A., Gutiérrez, J., Capilla, E., and Navarro, I. (2020). Short-term responses to fatty acids on lipid metabolism and adipogenesis in rainbow trout (*Oncorhynchus mykiss*). *Int. J. Mol. Sci.* 21:1623. doi: 10.3390/ijms21051623

Ritz, C. W., Hulet, R. M., Self, B. B., and Denbow, D. M. (1995). Growth and intestinal morphology of male turkeys as influenced by dietary supplementation of amylase and xylanase. *Poult. Sci.* 74, 1329–1334. doi: 10.3382/ps.0741329

Rodas, B. Z., Sohn, K. S., Maxwell, C. V., and Spicer, L. J. (1995). Plasma protein for pigs weaned at 19 to 24 days of age: effect on performance and plasma insulin-like growth factor I, growth hormone, insulin, and glucose concentrations. *J. Anim. Sci.* 73, 3657–3665. doi: 10.2527/1995.73123657x

Rodríguez Muela, C., Rodríguez Ramírez, H. E., Díaz Plascencia, D., Bocourt Salabarría, R., and Arzola Álvarez, C. (2017). Concentration of acid detergent fiber, neutral detergent fiber and lignin during solid state fermentation of apple (*Malus domestica*) derivates. *Cuban J. Agric. Sci.* 51, 47–60.

Salaheen, S., Kim, S. W., Haley, B. J., Van Kessel, J. A. S., and Biswas, D. (2017). Alternative growth promoters modulate broiler gut microbiome and enhance body weight gain. *Front. Microbiol.* 8:2088. doi: 10.3389/fmicb.2017.02088

Scott, M. L., Nesheim, M. C., and Young, R. J. (1982). *Nutrition of the Chicken [M]*. New York: Scott and Associates.

Sugiharto, S., and Ranjitkar, S. (2019). Recent advances in fermented feeds towards improved broiler chicken performance, gastrointestinal tract microecology and immune responses: a review. *Anim. Nutr.* 5, 1–10. doi: 10.1016/j.aninu.2018.11.001

Sugiharto, S., Yudiarti, T., Isroli, I., Widiastuti, E., Wahyuni, H. I., and Sartono, T. A. (2020). Growth performance, haematological responses, intestinal microbiology and carcass traits of broiler chickens fed finisher diets containing two-stage fermented banana peel meal. *Trop. Anim. Health Prod.* 52, 1425–1433. doi: 10.1007/s11250-019-02147-y

Tang, G. Y., Zhao, C. N., Liu, Q., Feng, X. L., Xu, X. Y., Cao, S. Y., et al. (2018). Potential of grape wastes as a natural source of bioactive compounds. *Molecules* 23:2598. doi: 10.3390/molecules23102598

Tchorbanov, B., and Lazarova, G. (1988). Evaluation of protein hydrolysates using the fermentation activity of immobilized yeast cells. *Biotechnol. Appl. Biochem.* 10, 301–304. doi: 10.1111/j.1470-8744.1988.tb00021.x

Teixeira, T. F. S., Grześkowiak, L. M., Salminen, S., Laitinen, K., Bressan, J., and Gouveia, P. M. C. (2013). Faecal levels of bifidobacterium and *Clostridium coccoides* but not plasma lipopolysaccharide are inversely related to insulin and HOMA index in women. *Clin. Nutr.* 32, 1017–1022. doi: 10.1016/j.clnu.2013.02.008

Tie, Y., Li, L., Liu, J., Liu, C., Fu, J., Xiao, X., et al. (2020). Two-step biological approach for treatment of rapeseed meal. *J. Food Sci.* 85, 340–348. doi: 10.1111/1750-3841.15011

Van Soest, P. J., Robertson, J. B., and Lewis, B. A. (1991). Methods for dietary fiber, neutral detergent fiber, and nonstarch polysaccharides in relation to animal nutrition. *J. Dairy Sci.* 74, 3583–3597. doi: 10.3168/jds.S0022-0302(91)78551-2

Wu, ZK. (2018). Process optimization of solid-state fermentation of rapeseed meal with multi-strains and its feed value evaluation for broilers[D]. Master's thesis, Beijing: Chinese Academy of Agricultural Sciences.

Wu, G., Bazer, F. W., Dai, Z., Li, D., Wang, J., and Wu, Z. (2014). Amino acid nutrition in animals: protein synthesis and beyond. *Annu. Rev. Anim. Biosci.* 2, 387–417. doi: 10.1146/annurev-animal-022513-114113

Wu, Q. J., Wang, Z. B., Wang, G. Y., Li, Y. X., and Qi, Y. X. (2015). Effects of feed supplemented with fermented pine needles (*Pinus ponderosa*) on growth performance and antioxidant status in broilers. *Poult. Sci.* 94, 1138–1144. doi: 10.3382/ps/pev013

Yan, W., Liu, X., Long, J., Wen, Q., and Zheng, Y. (2018). Effects of fermented corn gluten meal on growth performance, serum parameters, intestinal morphology and immunity performance of three-yellow broilers. *Can. J. Anim. Sci.* 99. doi: 10.1016/j.livsci.2019.103866

Yang, J., and Xiao, Y. Y. (2013). Grape phytochemicals and associated health benefits. *Crit. Rev. Food Sci. Nutr.* 53, 1202–1225. doi: 10.1080/10408398.2012.692408

Yang, L., Zeng, X., and Qiao, S. (2021). Advances in research on solid-state fermented feed and its utilization: the pioneer of private customization for intestinal microorganisms. *Anim. Nutr.* 7, 905–916. doi: 10.1016/j.aninu.2021.06.002

Yasar, S., and Yegen, K. M. (2017). Yeast fermented additive enhances broiler growth. *Rev. Bras. Zootecn.* 46, 814–820. doi: 10.1590/s1806-92902017001000004

Zhang, K. (2012). Study on the production of protein feed with bacterial enzymes and solid-state fermentation of miscellaneous meal[D]. Master's thesis, Wuhan: Wuhan Institute of Technology.

Zhang, W., Ma, C., Xie, P., Zhu, Q., Wang, X., Yin, Y., et al. (2019). Gut microbiota of newborn piglets with intrauterine growth restriction have lower diversity and different taxonomic abundances. *J. Appl. Microbiol.* 127, 354–369. doi: 10.1111/jam.14304

Zhang, L., Wu, W., Lee, Y. K., Xie, J., and Zhang, H. (2018). Spatial heterogeneity and co-occurrence of mucosal and luminal microbiome across swine intestinal tract. *Front. Microbiol.* 26:48. doi: 10.3389/fmicb.2018.00048

Zhu, F., Du, B., Zheng, L., and Li, J. (2015). Advance on the bioactivity and potential applications of dietary fibre from grape pomace. *Food Chem.* 186, 207–212. doi: 10.1016/j.foodchem.2014.07.057

Zhu, F., Zhang, B., Li, J., and Zhu, L. (2020). Effects of fermented feed on growth performance, immune response, and antioxidant capacity in laying hen chicks and the underlying molecular mechanism involving nuclear factor-κB. *Poult. Sci.* 99, 2573–2580. doi: 10.1016/j.psj.2019.12.044

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# Effects of dietary Bopu powder supplementation on intestinal development and microbiota in broiler chickens

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This study aimed to investigate the effect of dietary supplementation with Bopu powder on intestinal development and bacterial community composition in broiler chickens. A total of 486 1-day-old arbor acres broilers were fed a basal diet (CON group), a basal diet supplemented with 50mg/kg aureomycin (AB group), or a basal diet supplemented with 40mg/kg Bopu powder (BP group). The results showed that the BP group had significantly lower serum tumor necrosis factor-α (TNF-α), interleukin (IL)-1β, IL-6, and diamine oxidase concentrations and had significantly higher serum IL-10 concentrations than CON group ($p$<0.05). Groups AB and BP had a significantly higher weight per unit length of the small intestine and villus height than the CON group ($p$<0.05), and BP group had a significantly higher ratio of villus height to crypt depth than groups CON and AB ($p$<0.05). Compared to the CON group, dietary Bopu powder or aureomycin supplementation significantly increased transforming growth factor-α concentration and mRNA expressions of zonula occludens-1 (ZO-1) and occludin, and decreased intestinal mucosal concentrations of TNF-α, IL-6, IL-10, caspase-3, and caspase-8 and mRNA expressions of nuclear factor-kappa-B and *Bax*/*Bcl-2* ratio in the intestinal mucosa ($p$<0.05). Meanwhile, BP group had significantly higher ZO-1, secretory immunoglobulin A, interferon-γ concentrations, and mRNA expressions of glucose transporter type-2 and sirtuin-1, and significantly lower IL-1β concentration than groups CON and AB in intestinal mucosa ($p$<0.05). Dietary Bopu powder supplementation significantly increased the concentration of trefoil factor family member and mRNA expressions of superoxide dismutase-1 and bcl-2 associated X, and significantly reduced casepase-9 concentration and myeloid differentiation primary response-88 expression in the intestinal mucosa of broiler chickens relative to CON group ($p$<0.05). Moreover, results of high-throughput sequencing showed that broilers in the BP group had microbial community structure distinct from that in CON group, and the addition of Bopu powder increased the abundances of *Faecalibacterium* and *Colidextribacter* ($p$<0.05). Therefore, our study suggests a synergic response

of intestinal development and microbiota to the Bopu powder, and provides a theoretical basis as a potential substitute for antibiotics.

KEYWORDS

broiler, Bopu powder, intestinal development, microbiota, plant extract

# Introduction

The intestinal epithelium facilitates selective uptake of nutrients while serving as a barrier against harmful pathogens (Beumer and Clevers, 2021). In poultry production, intestinal mucosal injury is a prevalent disease in broiler chickens (Kogut et al., 2018). When the intestinal barrier is damaged, it will increase the transport of endotoxins and pathogens, resulting in serious damage to the body (Gonzalez-Gonzalez et al., 2018; Beumer and Clevers, 2021). Moreover, the intestinal tract is rich in microorganisms or flora, which is crucial to intestinal development and maintaining intestinal physiological function homeostasis (Diaz Carrasco et al., 2019). Since their discovery and application, antibiotics have played an unparalleled role in livestock and poultry production, and shown a protective effect in development and function of small intestine (Hafeez et al., 2020). However, antibiotic resistance has become a serious threat to human beings and the environment because of the irrational use of antibiotics (Cheng et al., 2014). Nowadays, with the banning of antibiotics, it has become the primary task to find antibiotic substitutes, especially natural feed additives.

An accumulating body of research indicates *Macleaya Cordata* extract (MCE) has antibacterial, anti-inflammatory, insecticidal, anti-tumor and other biological activities (Lin et al., 2018), and is a good substitute for antimicrobial growth promoter (AGP; Zhang et al., 2021). The main active components of MCE include benzophenanthridine alkaloids (sanguinarine and chelerythrine) and isoquinoline alkaloids (proto alkaloids and allocrine alkaloids; Kosina et al., 2010). In 2005, compounds containing sanguinarine and chelerythrine were used as feed additives in the European Union (Xie et al., 2015). Recently, the components made up of protopine and allocryptopine have been registered as veterinary drugs in China, and named Bopu powder (Veterinary Drug No. 180415374; Liu H. et al., 2022). Liu H. et al. (2022) showed that supplementation with 25–50 mg/kg Bopu powder in the diet improved antioxidant capacity and increased the abundances of beneficial bacteria in foregut of laying hens. Our recent study demonstrated that the growth performance and liver health of broiler chickens could be improved by adding protopine and allocryptopine (Bopu powder) to the diet (Liu et al., 2022a). However, there is little literature evaluating the effects of dietary Bopu powder supplemented on intestinal development and microbiota community structures of broiler chickens.

Therefore, the purpose of this study was to explore the effects of dietary supplemented with Bopu powder on intestinal development and microbiota community structures of broiler chickens, and to evaluate the potential applications of Bopu powder as an effective substitute to AGP.

# Meterials and methods

## Experimental design and management

In the 42-day study, 486 newly hatched male Arbor Acres (AA) broilers with an average body weight (BW) of 48.76 ± 0.25 g were selected. The broilers were purchased from Shandong Fengxiang Co. Ltd. (Liaocheng, China) and randomly allocated into three dietary treatments with six replicates per treatment and 27 broilers per replicate. Broilers in each replicate were housed in a three-level cage, and all cages were placed in a temperature- and light-controlled room with continuous light in the experimental farm of Shandong Agricultural University. Three treatment groups were as follows: (1) the control (CON) group, which was fed a basal diet; (2) the AB group, which was fed a basal diet supplemented with 50 mg/kg aureomycin; and (3) the BP group, which was fed a basal diet supplemented with 40 mg/kg Bopu powder (Liu et al., 2022a). The 42-day experiment was conducted with starter phase (day 0–21) and the growth phase (day 21–42). The basal diets (Supplementary Table S1) were formulated to meet the nutritional requirements of the Ministry of agriculture of China (2004). The Bopu powder was provided by the Micolta Bioresource Company Limitid (Changsha 410331, China), and made up of 1% protopine, 0.5% allotypotopine, and 98.5% starch (Liu H. et al., 2022). All management was carried out according to the AA Broiler Management Guide (Acres, 2009), and fresh feed and water were provided every day for broiler chickens during the experiment. The temperature of room was maintained at 35°C for the first week and then gradually reduced by 1°C every 2 days until it reached 21°C.

## Sample collection

After a 12-h fast on the last day of the experiment, a broiler with close to the average body weight was selected in each replicate. Following the collection of blood samples (5 ml) from the wing vein, serum was obtained by centrifugation at 3,000 rpm/min for 15 min at 4°C and stored at −20°C until analysis. After the intestine of each broiler chicken was quickly removed, intestinal

length and weight were measured, and the relative index was calculated. Then a 2-cm length segment was cut from the middle part of small intestine, washed with the 0.9% saline solution, and fixed with 4% paraformaldehyde solution. Subsequently, the mucosal tissue was scraped with sterile glass slide from the same parts of small intestine after being washed with ice-cold saline solution, quick-frozen in liquid nitrogen, and finally stored at −80°C until further analysis. In addition, cecal contents were quickly collected with sterile fecal collection tubes and stored at −80°C for microbiological analysis.

## Intestinal morphology observation

The small intestine tissue was taken out after being fixed with paraformaldehyde for 24 h, and embedded according to the conventional paraffin embedding scheme (Xiao et al., 2021). Then the tissues were sliced into 5-um thickness with automatic slicer (HM355S, Burton International Trading Co., Ltd., China), stained with hematoxylin–eosin, and sealed with neutral resin. The morphology of small intestine was observed and photographed with a Nikon Elipse 80i microscope (Nikon, Tokyo, Japan). A total of 10 well oriented crypt-villus units per sample were chosen to measure villus height (VH) and crypt depth (CD) using image analysis software (JEDA, Nanjing, Jiangsu, China), and the ratio of VH to CD was calculated (Chen et al., 2021a).

## Analysis of serum inflammatory factors

The levels of tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β), interleukin-6 (IL-6), and interleukin-10 (IL-10) in the serum of broiler chickens were assayed with ELISA kits (Jiangsu Meimian Industrial Co., Ltd.) as described in a previous study (Chen et al., 2021a).

## Analysis of serum DAO and D-lactate levels

The serum diamine oxidase (DAO) activity and D-lactate concentrations were analyzed by chicken-specific kits (Jiangsu Meimian) according to the determination steps of ELISA operation described in Li et al. (2022).

## Analysis of intestinal barrier-related function

The zonula occluden-1 (ZO-1), mucin 2 (MUC2), trefoil factor family member (TFF), and transforming growth factor-α (TGF-α) in the intestines were examined using ELISA kits (Jiangsu Meimian) following the protocol described previously (Li et al., 2022).

## Analysis of SIgA and intestinal inflammatory factors

The levels of secretory immunoglobulin A (SIgA), tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β), interleukin-6 (IL-6), interleukin-10 (IL-10), and interferon-γ (IFN-γ) in the intestinal samples of broiler chickens were assayed using ELISA kits (R&D Systems Inc., Minneapolis, MN, United States). The determination steps were followed with the standardized ELISA procedures described in a previous study (Chen et al., 2021a; Liu et al., 2022b).

## Analysis of intestinal caspases activities

The activities of caspase-3, caspase-8, and caspase-9 in intestinal samples of broiler chickens were determined using chicken-specific ELISA kits (Beyotime Biotech) followed with the standardized ELISA procedures described by Chen et al. (2021a).

## Analysis of gene expression

Briefly, 50–100 mg of intestinal tissue samples were taken and placed in a mortar containing liquid nitrogen, and ground to a powder. Then 1 ml of TRIzol-reagent was added to extract total RNA. Subsequently, RNA was reverse transcribed to cDNA by Evo M-MLV RT Kit (Accurate Biotechnology, Hunan, China) according to the kit manual. The mRNA expression levels of zonula occludens-1 (*ZO-1*), occludin (*OCLN*), claudin-2 (*CLND2*), claudin-3 (*CLND3*), glucose transporter type 2 (*GLUT2*), sodium-glucose transporter 1 (*SGLT1*), y + L amino acid transporter-1 (*y + LAT1*), cationic amino acid transporter-1 (*CAT1*), fatty acid binding protein-1 (*FABP1*), Toll-like receptor 4 (*TLR4*), myeloid differentiation factor 88 (*MyD88*), nuclear factor-κB (*NF-κB*), sirtuin1 (*Sirt1*), nuclear factor erythroid 2-related factor 2 (*Nrf2*), heme-oxygenase 1 (*HO-1*), catalase (*CAT*), superoxide dismutase 1 (*SOD1*), superoxide dismutase 2 (*SOD2*), glutathione peroxidase-1 (*GPX1*), NAD(P)H quinone oxidoreductase 1 (*NQO1*), bcl-2 associated X (*Bax*), and b-cell lymphoma-2 (*Bcl-2*) in intestinal samples were assessed using LightCycler 96 (Roch, Switzerland) with SYBR® Green Premix Pro Taq HS qPCR Kit (AG11701, Accurate Biology, Da Lian, China) as described previously (Liu et al., 2022a). *β*-actin was used as an internal reference, and $2^{-\Delta\Delta CT}$ method was used to calculate the relative expressions of target genes. All gene primers are shown in Supplementary Table S2.

## Microbial analysis

Total genomic DNA was extracted from frozen fecal samples using QIAamp DNA·Stool Mini Kits (Qiagen Inc., Hilden, Germany) according to the manufacturer's protocol. Depending on the

concentration, DNA was diluted to 1 ng/μl using sterile solution, and the V4 hypervariable region of 16S rDNA was amplified using 515f and 806r primers (5′-GTGCCAGCMGCCGCGGTAA-3′ and 5′-GGACTACHVGGGTWTCTAAT-3′, respectively; Chen et al., 2021b). Subsequently, TruSeq ® DNA PCR-Free Sample Preparation Kit (Illumina, United States) was used to generate sequencing libraries, and the library quality was assessed on the Qubit@2.0 fluorometer (Thermo Scientific) and Agilent Bioanalyzer 2100 system. At last, the library was sequenced on the Illumina NovaSeq platform and 250 bp paired-end reads were generated. Paired-end reads was assigned to samples based on their unique barcode and truncated by cutting off the barcode and primer sequence. After sequence assembly, data filtration and chimera removal, the final effective sequences were obtained. The sequences with ≥97% similarity were allocated to the same OTU through Uparse software, and then each sequence was labeled with the Silva Database based on the Mothur algorithm to classify it to different classification levels (Quast et al., 2012). Alpha diversity was applied in analyzing complexity of species diversity for a sample through 4 indexes, including Shannon, Simpson, Chao1, and ACE. All these indicators of samples were calculated with QIIME software (Version 1.9.1) and displayed with R software (Version 2.15.3; Lawley and Tannock, 2017). Beta diversity based on bray_curtis distance was calculated using QIIME software. Principal coordinate analysis (PCoA) was selected to calculate and visualize the unifrac distance. The unweighted pair-group method with arithmetic mean (UPGMA) clustering analysis with the bray-curtis distance was also used to visualize the dissimilarity matrices of OTUs (Lozupone and Knight, 2005).

## Statistical analysis

The data were statistically analyzed using one-way ANOVA of SAS 9.4 (Institute Inc., Cary, NC, United States). Multiple comparisons of treatment means were examined using the least significant difference test. Spearman's correlation was used to evaluate the associations between differential bacterial abundances and concentrations of metabolic parameters and immunological markers in serum and intestine. All data were presented as the mean ± standard error. The values of $p < 0.05$ were regarded as statistically significant, and the values of $0.05 \leq p < 0.10$ were considered as a significance trend.

# Results

## Effects of Bopu powder on serum inflammatory factors, DAO, and D-lactate concentrations in broiler chickens

The effects of Bopu powder on serum inflammatory factors, DAO and D-lactate concentrations of broiler chickens are shown in Figure 1. Compared with CON group, serum TNF-α, IL-1β, IL-6, and DAO concentrations were significantly lower in AB group and BP group ($p < 0.05$), and BP group showed significantly lower serum IL-6 concentration than AB group ($p < 0.05$). Serum IL-10 concentration was significantly higher in BP group than in CON group and AB group ($p < 0.05$), and AB group showed significantly lower serum IL-10 concentration than CON group ($p < 0.05$). Serum D-lactate concentration was significantly lower in AB group than in CON group, and the D-lactate concentration of BP group tended to be lower than that of AB group ($p < 0.10$).

## Effect of Bopu powder on intestinal development in broiler chickens

The effect of Bopu powder on intestinal development of broiler chickens is shown in Figure 2. Compared with CON group, weight per unit length and VH of small intestine were significantly higher in AB group and BP group ($p < 0.05$). The VH to CD ratio in BP group was significantly higher than that in AB group and CON group ($p < 0.05$). Compared with AB group, the intestinal CD of BP group tended to be lower ($p < 0.10$). The VH to CD ratio was significantly higher in BP group than in AB group and CON group ($p < 0.05$). There was no significant difference in the ratio of small intestinal weight to body weight among three groups ($p > 0.05$).

## Effect of Bopu powder on intestinal barrier function in broiler chickens

The effect of Bopu powder on intestinal barrier function in broiler chickens is shown in Figure 3. The concentration of intestinal ZO-1 was significantly higher in BP group than in AB group and CON group ($p < 0.05$). Intestinal TFF level in BP group was significantly higher than that in CON group ($p < 0.05$). Compared with CON group, BP group and AB group had significantly higher TGF-α concentration ($p < 0.05$). There was no significant difference in intestinal MUC2 concentration among three groups ($p > 0.05$).

## Effect of Bopu powder on intestinal SIgA and inflammatory factors concentrations in broiler chickens

As shown in Figure 4, the intestinal concentrations of SIgA and IFN-γ in BP group were significantly higher than those in CON group and AB group ($p < 0.05$). The concentrations of TNF-α and IL-6 were significantly lower in AB group and BP group than in CON group ($p < 0.05$). The concentration of intestinal IL-1β was significantly higher in BP group than in AB group and CON group ($p < 0.05$). Compared with the broilers in CON group, broilers in BP group and AB group had

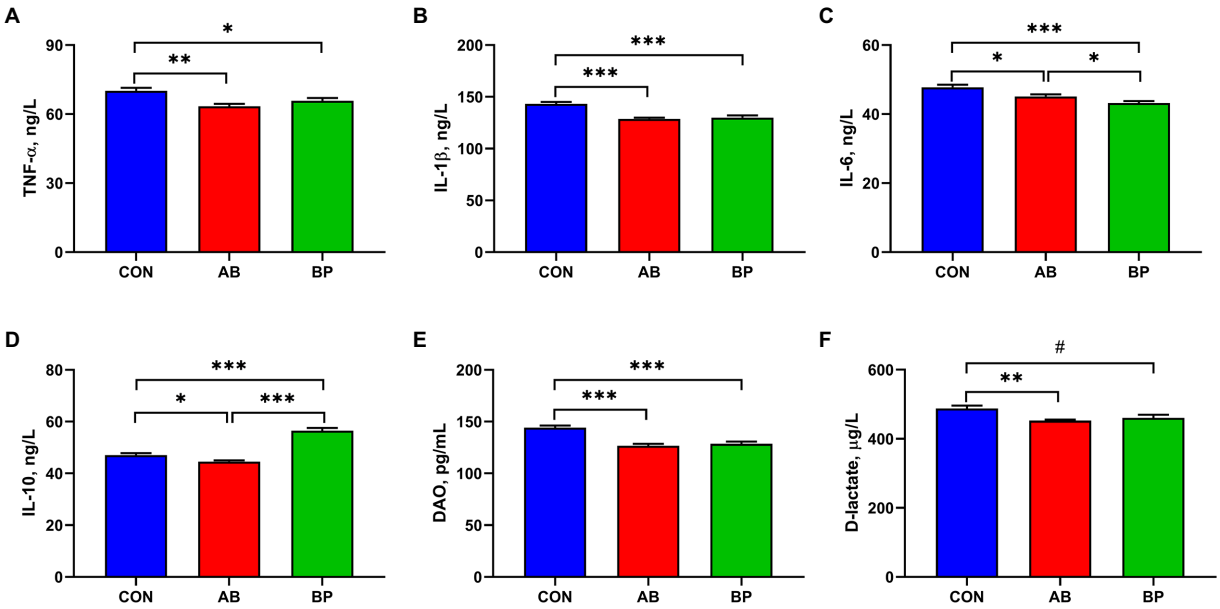


FIGURE 1
Effects of dietary supplemented with Bopu powder on serum inflammatory factors, diamine oxidase (DAO), and D-lactate in broiler chickens. **(A)** Tumor necrosis factor-α (TNF-α); **(B)** Interleukin-1β (IL-1β); **(C)** Interleukin-6 (IL-6); **(D)** Interleukin-10 (IL-10); **(E)** DAO; and **(F)** D-lactate. CON group, broiler chickens fed a basal diet; AB group, broiler chickens fed a basal diet supplemented with 50mg/kg aureomycin; and BP group, broiler chickens fed a basal diet supplemented with 40mg/kg Bopu powder containing protopine and allocryptopine. Values are mean±SE ($n$=6). $^{\#}0.05 \leq p < 0.10$, $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

significantly lower IL-10 concentration ($p < 0.05$), and IL-10 concentration in AB group was significantly lower than that in BP group ($p < 0.05$).

## Effect of Bopu powder on intestinal caspase activity in broiler chickens

The effects of Bopu powder on intestinal caspases activities of broiler chickens are shown in Figure 5. Compared with CON group, BP group and AB group had significantly lower intestinal activities of casepase-3 and casepase-8 ($p < 0.05$). Broilers in BP group had significantly lower casepase-9 activity than broilers in CON group ($p < 0.05$).

## Effect of Bopu powder on gene expression related to intestinal barrier function in broiler chickens

The effects of Bopu powder on genes expressions related to intestinal barrier function in broiler chickens are shown in Figure 6. Compared with CON group, the mRNA expressions of *ZO-1* and *OCLN* were significantly higher in AB group and BP group ($p < 0.05$). Intestinal *CLDN3* mRNA expression in BP group was significantly higher than that in AB group. No significant difference was observed in *CLDN2* mRNA expression among three groups ($p > 0.05$).

## Effect of Bopu powder on genes expressions of nutrient transporters in broiler chickens

As shown in Figure 7, the mRNA expression of *GLUT2* was significantly higher in BP group and AB group than in CON group ($p < 0.05$), and the mRNA expression of *CAT1* in BP group tended to be higher than that in CON group ($p < 0.10$). However, no significant differences were observed in expressions of *SCLT1*, *y + LAT1*, and *FABP1* among the three groups ($p > 0.05$).

## Effect of Bopu powder on intestinal inflammatory gene in broiler chickens

As shown in Figure 8, the mRNA expressions of *MyD88* and *NF-κB* were significantly lower in BP group than in CON group ($p < 0.05$), and the mRNA expression of *NF-κB* in AB group was significantly lower than that in CON group ($p < 0.05$). Meanwhile, the mRNA expression of *TLR4* in BP group tended to be lower than that in CON group ($p < 0.10$).

## Effects of Bopu powder on intestinal antioxidant genes expressions in broiler chickens

As shown in Figure 9, the mRNA expressions of *Sirt1*, *Nrf2*, *HO-1*, and *SOD1* were significantly higher in BP group than in

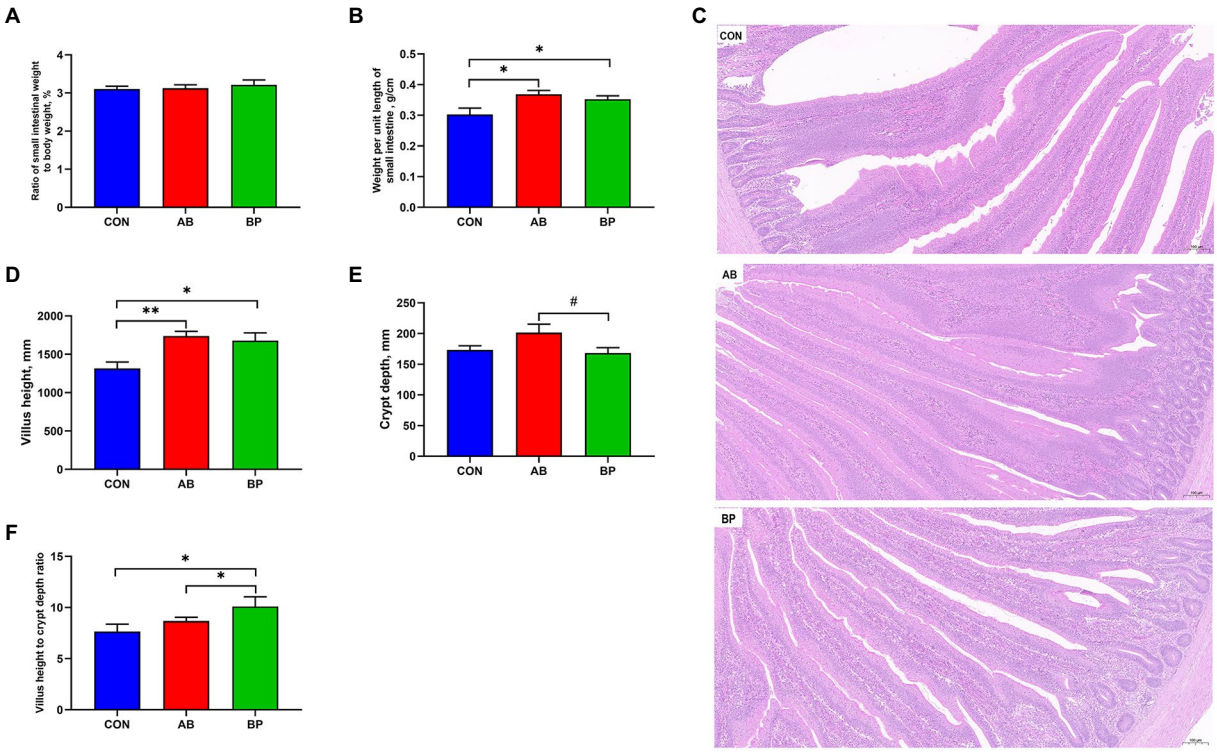


FIGURE 2
Effects of dietary supplemented with Bopu powder on intestinal development in broiler chickens. **(A)** Ratio of small intestinal weight to body weight; **(B)** Weight per until length of small intestine; **(C)** Representative images (×100) were stained with hematoxylin and eosin; **(D)** Villus height; **(E)** Crypt depth; and **(F)** Villus height to crypt depth ratio. CON group, broiler chickens fed a basal diet; AB group, broiler chickens fed a basal diet supplemented with 50mg/kg aureomycin; and BP group, broiler chickens fed a basal diet supplemented with 40mg/kg Bopu powder containing protopine and allocryptopine. Values are mean±standard error ($n=6$). $^{\#}0.05 \leq p < 0.10$, $^{*}p < 0.05$, $^{**}p < 0.01$.

CON group ($p < 0.05$), and *Nrf2* mRNA expression in AB group tended to be higher than that in CON group ($p < 0.10$). The mRNA expression of *HO-1* in BP group tended to be higher than that in AB group ($p < 0.10$), and BP group showed significantly higher *Sirt1*, *CAT*, and *SOD2* mRNA expressions than AB group ($p < 0.05$). Besides, the *GPX1* mRNA expression of BP group tended to be lower than that of CON group ($p < 0.10$), and AB group showed significantly lower *NQO1* mRNA expression than CON group ($p < 0.05$).

## Effects of Bopu powder on intestinal apoptosis genes in broiler chickens

As shown in Figure 10, compared with CON group, BP group had significantly higher *Bcl-2* mRNA expression ($p < 0.05$), and BP group and AB group had significantly lower *Bax/Bcl-2* ratio ($p < 0.05$). Besides, BP group tended to had lower mRNA expression of *Bax* than CON group ($p < 0.10$).

## Effect of Bopu powder on intestinal microbial diversity of broiler chickens

Sequence analysis based on the hypervariable region V4 of the 16S rDNA genes, the three groups obtained 1,097,479 total tags, including 1,024,789 taxon tags, 20 unclassified tags, and 72,670 unique tags. As shown in Figure 11A, the species accumulation curves tended to flatten as the number of analyzed sequences increased up to 18, indicating that our samples were sufficient for OTU testing and could predict the species richness of samples. Based on the OTU of 97% similarity, the rarefaction curve constructed (Figure 11B) tended to approach the asymptote, indicating that the sequence depth was also sufficient to represent most species richness and bacterial community diversity. As shown in Venn diagram (Figure 11C), the number of unique sequences was greatest (1,366) in the BP group and smallest (223) in AB group, for a total of 974 across the three treatment groups. However, there were no significant differences in Shannon (Figure 11D), Simpson (Figure 11E), Chao1 (Figure 11F), and ACE (Figure 11G) indexes among the three treatments ($p > 0.05$).

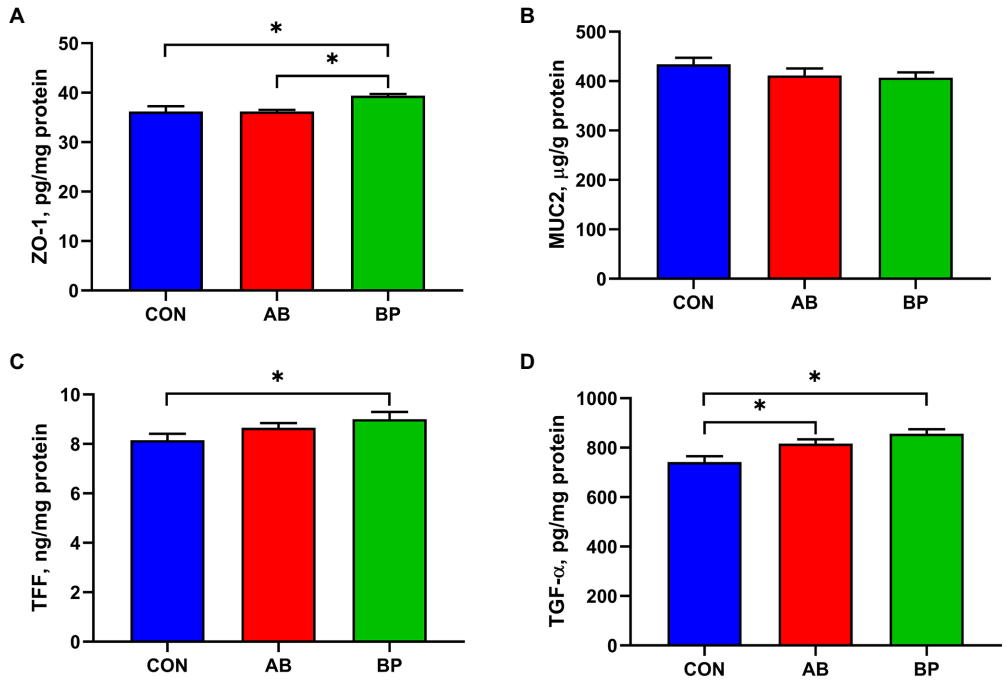


FIGURE 3
Effects of dietary supplemented with Bopu powder on intestinal barrier factors in broiler chickens. **(A)** Zonula occludens-1 (ZO-1); **(B)** Recombinant mucin 2 (MUC2); **(C)** Trefoil peptides (TFF); and **(D)** Transforming growth factor alpha (TGF-α). CON group, broiler chickens fed a basal diet; AB group, broiler chickens fed a basal diet supplemented with 50mg/kg aureomycin; and BP group, broiler chickens fed a basal diet supplemented with 40mg/kg Bopu powder containing protopine and allocryptopine. Values are mean±standard error ($n$=6). *$p$<0.05.

In addition, the heat-map based on the bray_curtis distance matrix (Figure 12A) showed that the AB and BP group pairs had the minimum value, while the CON and the BP group pairs had the maximum value. The PCoA analysis (Figure 12B) revealed the BP samples dispersed far apart with the CON samples, suggesting a clear separation between the BP group and the CON group. Moreover, the UPGMA phylogenetic tree (Figure 12C) also showed that the AB and BP groups were close together and clustered in one group that exhibited the highest similarity, while the CON group was distributed in a separate branch, indicating that the CON group had a clearly different distribution from the other groups. Consistently, the ANOSIM (Figure 12D) showed that there was no significant difference ($p > 0.05$) in the microbial community structure between group CON and AB and group AB and BP pairs, while significant difference was observed in the bacterial community structure between the BP group and CON group ($p < 0.05$).

## Effect of Bopu powder on microbial relative abundance in cecum of broiler chickens

The relative abundances at the phyla level in cecal microbiota (top 10) are shown in Figure 12C. The most abundant phyla were found to be Firmicutes and Bacteroidetes, which accounted for 40.68 and 39.80%, respectively. There were no significant differences observed in relative abundances of the other phyla among the groups ($p > 0.05$, Supplementary Table S3).

Changes in relative abundance at the genus level (top 30) in broiler cecal microbiota are shown in Supplementary Table S4. The relative abundance of Firmicutes was contributed by *Phascolarctobacterium*, *Megamonas*, *Romboutsia*, *Limosilactobacillus*, *Ligilactobacillus*, *Erysipelatoclostridium*, *Butyricicoccus*, *Colidextribacter*, *NK4A214_group*, *UCG-005*, *CHKGl001*, and *Faecalibacterium*; Bacteroidota mainly distributed with *Alistipes*, *Prevotellaceae_UCG-001*, *Barnesiella*, *Bacteroides*, and *Parabacteroides*. Of the top 30 genera, compared with CON group, broiler chickens in the BP group had significantly higher ($p < 0.05$) relative abundances of *Faecalibacterium* and *Colidextribacter*.

## Correlation analysis between differential bacterial abundances and parameters in serum and intestine

As shown in Figure 13, *Faecalibacterium* abundance was significantly positively correlated with intestinal TFF concentration ($r = 0.511$, $p < 0.05$), and negatively correlated with serum IL-6 concentration ($r = -0.478$, $p < 0.05$). *Colidextribacter* abundance had significant negative correlation with serum

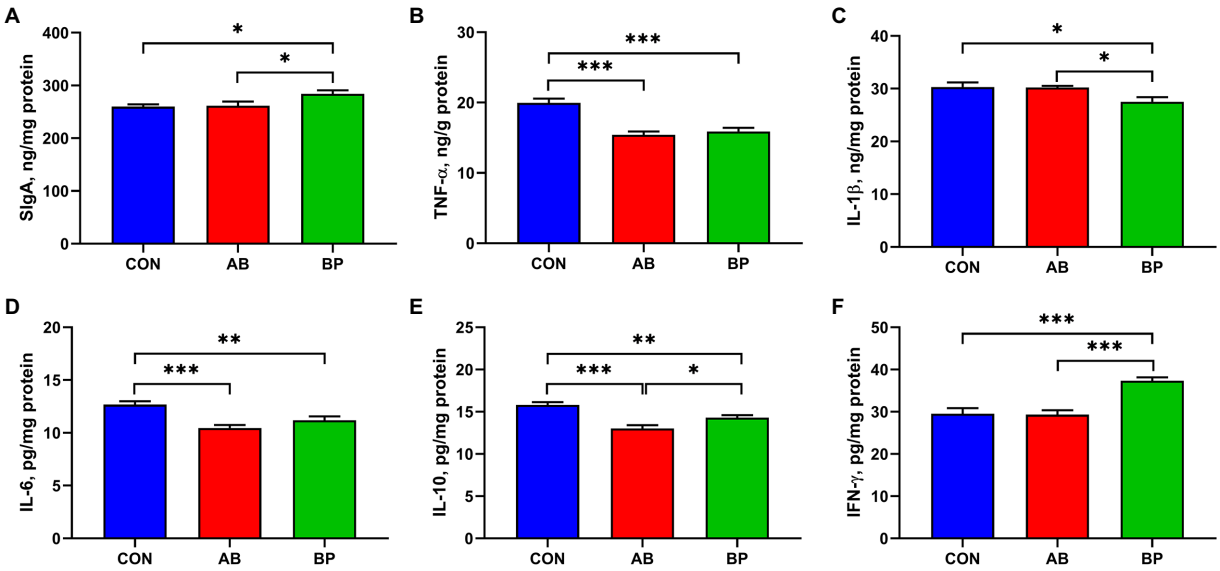


FIGURE 4
Effects of dietary supplemented with Bopu powder on Intestinal SIgA, inflammatory factors in broiler chickens. **(A)** Secretory immunoglobulin A (SIgA); **(B)** Tumor necrosis factor-α (TNF-α); **(C)** Interleukin-1β (IL-1β); **(D)** Interleukin-6 (IL-6); **(E)** Interleukin-10 (IL-10); and **(F)** Interferon-γ (IFN-γ). CON group, broiler chickens fed a basal diet; AB group, broiler chickens fed a basal diet supplemented with 50mg/kg aureomycin; and BP group, broiler chickens fed a basal diet supplemented with 40mg/kg Bopu powder containing protopine and allocryptopine. Values are mean±standard error ($n$=6). *$p$<0.05, **$p$<0.01, ***$p$<0.001.

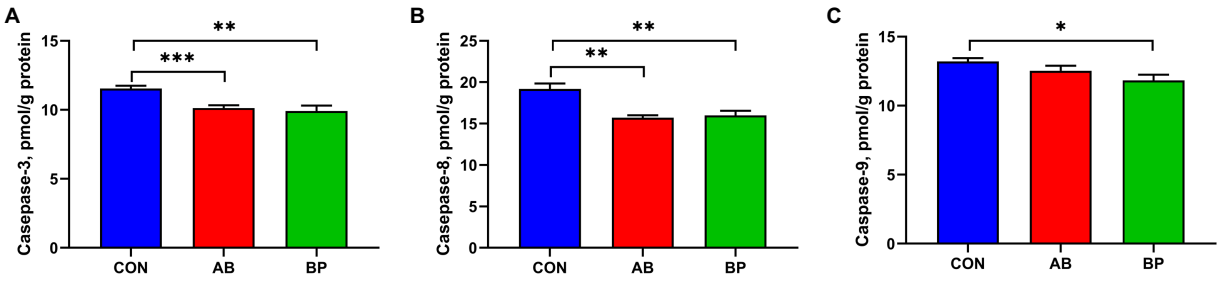


FIGURE 5
Effects of dietary supplemented with Bopu powder on caspases levels of broiler chickens. **(A)** Caspase-3; **(B)** Caspase-8; and **(C)** Caspase-9. CON group, broiler chickens fed a basal diet; AB group, broiler chickens fed a basal diet supplemented with 50mg/kg aureomycin; BP group, broiler chickens fed a basal diet supplemented with 40mg/kg Bopu powder containing protopine and allocryptopine. Values are mean±standard error ($n$=6). *$p$<0.05, **$p$<0.01, ***$p$<0.001.

concentrations of IL-1β ($r$ = −0.478, $p$ < 0.05) and D-lactate ($r$ = −0.478, $p$ < 0.05).

## Discussion

Our current results showed that addition of Bopu powder to the diet increased villus height and VH to CD ratio. Larger villus height and higher VH to CD ratio indicate higher intestinal absorption efficiency of nutrients, which helps to improve growth performance of broilers (Chen et al., 2021a). In weaned piglets, dietary supplementation with 50 mg/kg MCE containing quaternary-benzo(c)phenanthridine alkaloids and protopine alkaloids increases VH and the VH to CD ratio (Chen et al., 2019). In the current study, addition of Bopu powder or aureomycin to the diet reduced serum DAO activity. Aureomycin treatment in rats also reduces intestinal DAO activity (Sato et al., 2016). Serum DAO activity is considered a marker of intestinal permeability which increases upon damage of the intestinal barrier (Luk et al., 1983; Wu et al., 2013). Moreover, we found that Bopu powder supplementation increased mucosal ZO-1, TFF, and TGF-α levels as well as *ZO-1* and *OCLN* mRNA levels in the small intestine of broiler chickens. Trefoil peptides (TFFs) are bioactive peptides, playing an important role in protecting against gastrointestinal

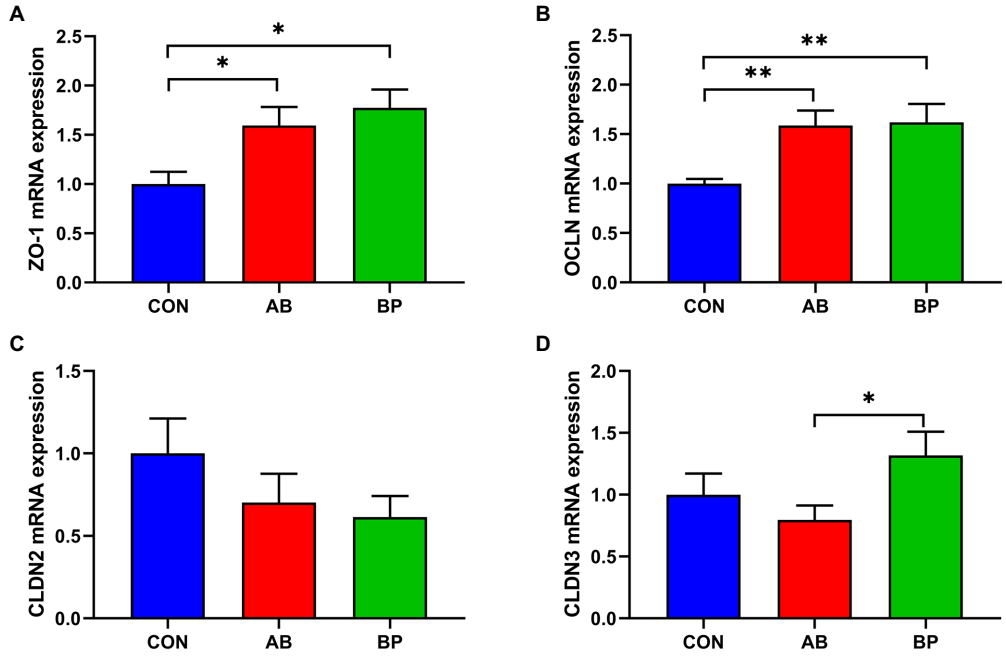


FIGURE 6
Effects of dietary supplemented with Bopu powder on expressions of barrier genes in intestine of broiler chickens. **(A)** zonula occludens-1 (*ZO-1*); **(B)** occludin (*OCLN*); **(C)** claudin-2 (*CLDN2*); and **(D)** claudin-3 (*CLDN3*). CON group, broiler chickens fed a basal diet; AB group, broiler chickens fed a basal diet supplemented with 50mg/kg aureomycin; BP group, broiler chickens fed a basal diet supplemented with 40mg/kg Bopu powder containing protopine and allocryptopine. Values are mean±standard error ($n$=6). *$p$<0.05, **$p$<0.01.

injury and strengthening the intestinal barrier (Buda et al., 2012). Transforming growth factor-α has trophic effects on the intestinal epithelium and is helpful to maintain the integrity of intestinal epithelium cells (Schumacher et al., 2018). Peripheral membrane protein ZO-1 and transmembrane protein OCLN play important roles in maintaining intestinal tight junction integrity and mucosal barrier function (Zhang et al., 2017; Goo et al., 2019). In pigs, supplementation of the diet with isoquinoline alkaloids increases the expression of ZO-1 and enhances intestinal integrity (Ni et al., 2016). Our results indicated that dietary Bopu powder supplementation increased *GLUT2* expression in the small intestinal mucosa of broiler chickens. Elevated *GLUT2* mRNA levels typically contribute to intestinal digestive absorption (Yin et al., 2019).

Dietary Bopu powder supplementation decreased caspase-3, caspase-8, and caspase-9 activities in the small intestine of broiler chickens, and addition of aureomycin to the diet reduced intestinal caspase-3 and caspase-8 activities. Caspases are crucial in inflammation responses and cell death, and caspase-8 and caspase-9 can initiate the apoptotic process by activating the caspase-3, which initiates the process of apoptosis and induces cell death (Thornberry, 1997; Brentnall et al., 2013; D'Arcy, 2019). Further, Bopu powder supplementation increased *Bcl-2* mRNA and decreased the *Bax*/*Bcl-2* ratio in broiler chickens. Anti-apoptotic Bcl-2 and pro-apoptotic Bax play important roles in cell death, and high Bax/Bcl-2 ratio can also lead to the activation of caspase-3 (Reed et al., 1996; Korsmeyer, 1999). Taken together, our results suggest that Bopu powder supplementation can decrease apoptosis through inactivating caspase-3 through suppressing intrinsic or extrinsic pathways, which may in part explain the observed improvement of small intestinal development and mucosal barrier integrity.

Inflammation response is a major factor causing mucosal barrier damage and apoptosis (Sanchez de Medina et al., 2014). In the present study, Bopu powder addition reduced TNF-α, IL-1β, and IL-6 concentrations in the serum and small intestine of broiler chickens. These compounds are established markers of inflammatory responses (Akdis et al., 2016; Ducatelle et al., 2018), and our previous study showed that dietary Bopu powder reduced IL-1β and IL-6 levels in the liver of broilers (Liu et al., 2022a). In the current study, supplementation with aureomycin reduced serum TNF-α, IL-1β, and IL-6 concentrations and intestinal TNF-α and IL-6 concentrations, which is in line with the results of previous study (Song et al., 2022). Furthermore, dietary Bopu powder supplementation increased IL-10 concentrations in the serum and IFN-γ and SIgA concentrations in the intestinal mucosa of broiler chickens in the present study. IL-10 exerts anti-inflammatory and immunomodulatory effects (Kanai et al., 2015). Supplementation with 50 mg/kg MCE increases serum IL-10 concentrations in weaned piglets (Wang et al., 2021). The pleiotropic cytokine IFN-γ exerts antiviral, antitumor, and immunomodulatory functions (Jorgovanovic et al., 2020). Secretory immunoglobulin A is produced by plasma cells of the lamina propria on the surface of the mucosa, and increased

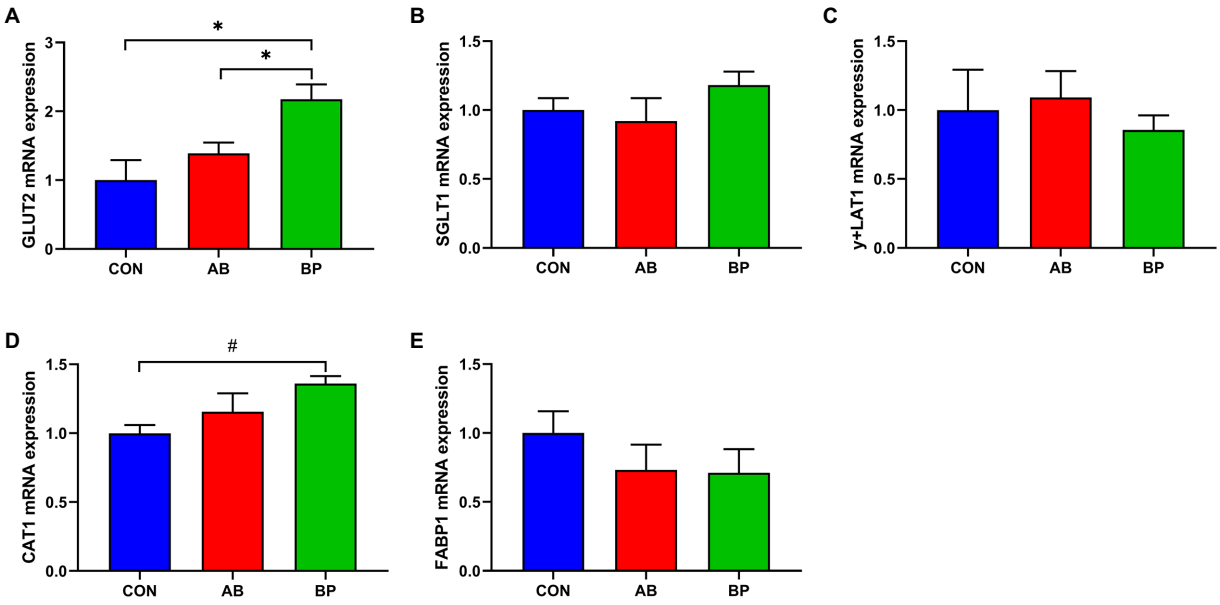


FIGURE 7
Effects of dietary supplemented with Bopu powder on expressions of nutrient transport genes in intestine of broiler chickens. **(A)** Glucose transporter type 2 (*GLUT2*); **(B)** Sodium-glucose transporter 1 (*SGLT1*); **(C)** y+L amino acid transporter-1 (*y+LAT1*); **(D)** Cationic amino acid transporter-1 (*CAT1*); and **(E)** Fatty acid binding protein-1 (*FABP1*). CON group, broiler chickens fed a basal diet; AB group, broiler chickens fed a basal diet supplemented with 50mg/kg aureomycin; BP group, broiler chickens fed a basal diet supplemented with 40mg/kg Bopu powder containing protopine and allocryptopine. Values are mean±standard error ($n$=6). $^{\#}0.05 \leq p < 0.10$, $^{*}p < 0.05$.

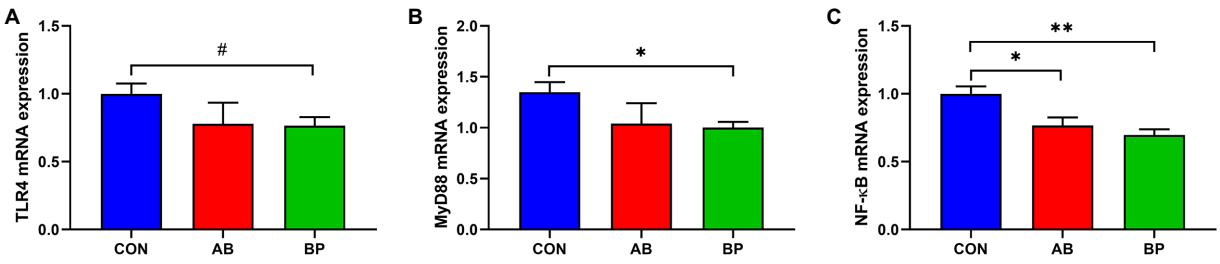


FIGURE 8
Effects of dietary supplemented with Bopu powder on expressions of inflammatory genes in intestine of broiler chickens. **(A)** Toll-like receptor 4 (*TLR4*); **(B)** Myeloid differentiation primary response 88 (*MyD88*); and **(C)** Nuclear factor-kappa B (*NF-κB*). CON group, broiler chickens fed a basal diet; AB group, broiler chickens fed a basal diet supplemented with 50mg/kg aureomycin; BP group, broiler chickens fed a basal diet supplemented with 40mg/kg Bopu powder containing protopine and allocryptopine. Values are mean±standard error ($n$=6). $^{\#}0.05 \leq p < 0.10$, $^{*}p < 0.05$, $^{**}p < 0.01$.

mucosal SIgA is typically associated with increased defense against pathogenic bacteria (Goonatilleke et al., 2019). These results suggest that the protective effect of Bopu powder may be due to reduced pro-inflammatory stimulation and increased anti-inflammatory responses. Interestingly, dietary supplementation with Bopu powder or aureomycin reduced IL-10 concentrations in the small intestine of broiler chickens, which may be related to decreased levels of anti-inflammatory cytokines (Alfen et al., 2018). The TLR4/MyD88/NF-κB signaling pathway was evaluated with regard to inflammatory responses, and dietary supplementation with Bopu powder decreased *MyD88* and *NF-κB* mRNA levels in the intestines of broiler chickens, whereas aureomycin supplementation significantly decreased intestinal *NF-κB* mRNA levels. Previous studies illustrated that TLR4 can induce inflammatory responses through activating the NF-κB signaling pathway *via* the MyD88 protein (Hu et al., 2020). Inhibiting the TLR4/MyD88/NF-κB signaling pathway may be conductive to intestinal protection and inhibition of intestinal inflammation (Li et al., 2020; Yang et al., 2021).

Inflammatory responses are frequently associated with oxidative stress. In the present study, dietary Bopu powder supplementation increased *Sirt1*, *Nrf2*, *HO-1*, and *SOD1* mRNA

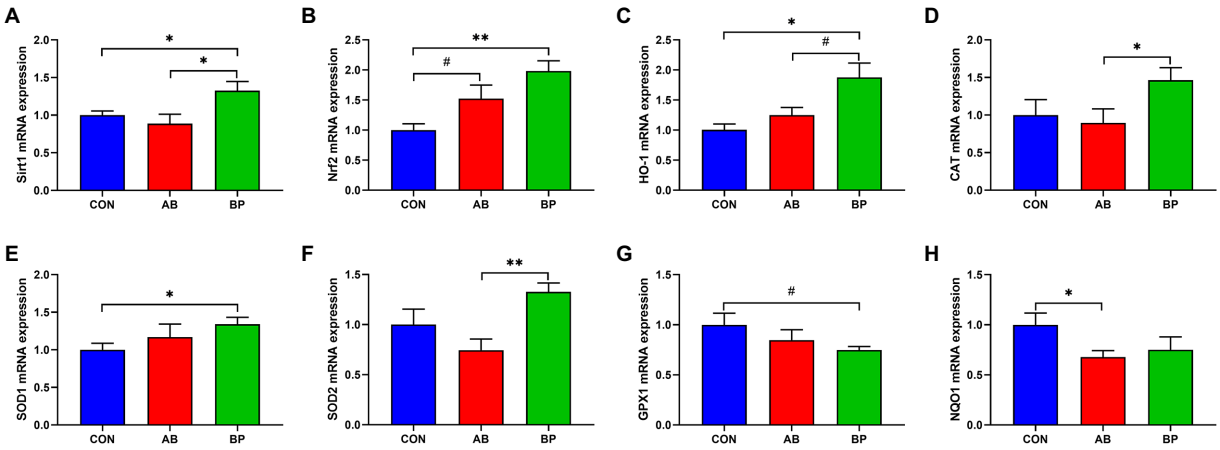


FIGURE 9
Effects of dietary supplemented with Bopu powder on expressions of antioxidant genes in intestine of broiler chickens. **(A)** Sirtuin1 (*Sirt1*); **(B)** Nuclear factor erythroid 2-related factor 2 (*Nrf2*); **(C)** Heme-oxygenase 1 (*HO-1*); **(D)** Catalase (*CAT*); **(E)** Superoxide dismutase 1 (*SOD1*); **(F)** Superoxide dismutase 2 (*SOD2*); **(G)** glutathione peroxidase-1 (*GPX1*); and **(H)** NAD(P)H quinone oxidoreductase 1 (*NQO1*). CON group, broiler chickens fed a basal diet; AB group, broiler chickens fed a basal diet supplemented with 50mg/kg aureomycin; BP group, broiler chickens fed a basal diet supplemented with 40mg/kg Bopu powder containing protopine and allocryptopine. Values are mean±standard error ($n$=6). $^{\#}0.05 \leq p < 0.10$, $^{*}p < 0.05$, $^{**}p < 0.01$.

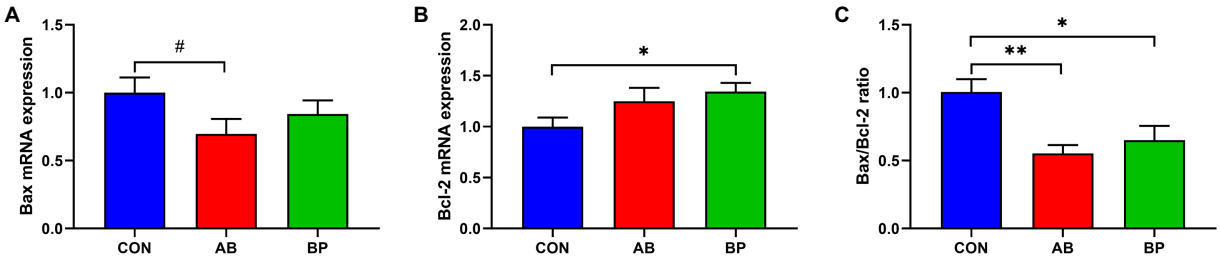


FIGURE 10
Effects of dietary supplemented with Bopu powder on expressions of apoptosis genes in intestine of broiler chickens. **(A)** Bcl-2 associated X (*Bax*); **(B)** B-cell lymphoma-2 (*Bcl-2*); **(C)** Bax/Bcl-2 ratio. CON group, broiler chickens fed a basal diet; AB group, broiler chickens fed a basal diet supplemented with 50mg/kg aureomycin; BP group, broiler chickens fed a basal diet supplemented with 40mg/kg Bopu powder containing protopine and allocryptopine. Values are mean±standard error ($n$=6). $^{\#}0.05 \leq p < 0.10$, $^{*}p < 0.05$, $^{**}p < 0.01$.

levels in the small intestine of broiler chickens. Activation of Sirt1 can protect intestinal epithelium cells from oxidative injury *via* regulating Nrf2-related pathways (Chong et al., 2012). Nrf2 is a key transcription factor in antioxidant defense and can enhance the activity of HO-1, an important antioxidative enzyme regulating the levels of cellular ROS (Loboda et al., 2016; Zhuang et al., 2019). Superoxide dismutase is an important antioxidant enzyme removing free radicals (Chen et al., 2013), and its expression is upregulated through Nrf2 activation (Meng et al., 2018). Therefore, our current results suggest that Bopu powder supplementation can enhance intestinal antioxidant capacity in broiler chickens by activating the Sirt1/Nrf2 signaling pathway.

We observed significant differences in microbial community composition between BP and CON, and addition of Bopu powder in the diet increased cecal abundances of *Faecalibacterium* and *Colidextribacter*. *Faecalibacterium* is one of the major producers of butyrate in the intestine and exerts anti-inflammation functions through maintaining bacterial enzyme activity and deterring pathogens invasion (Miquel et al., 2013). *Faecalibacterium* abundance was positively correlated with intestinal TFF concentrations and negatively correlated with serum IL-6 concentrations in the present study. *Colidextribacter* can promote the production of inosine which helps reduce the secretion of inflammatory factors (Wang et al., 2019; Mager et al., 2020; Guo et al., 2021). In current study, *Colidextribacter* abundance was negatively correlated with serum IL-1β and D-lactate concentrations, suggesting that *Colidextribacter* abundance may help regulate systemic inflammatory responses and maintain integrity of the intestinal mucosa. Bopu powder supplementation thus affected intestinal bacterial communities and increased the abundance of beneficial bacteria, which may also explain the observed improved intestinal development.

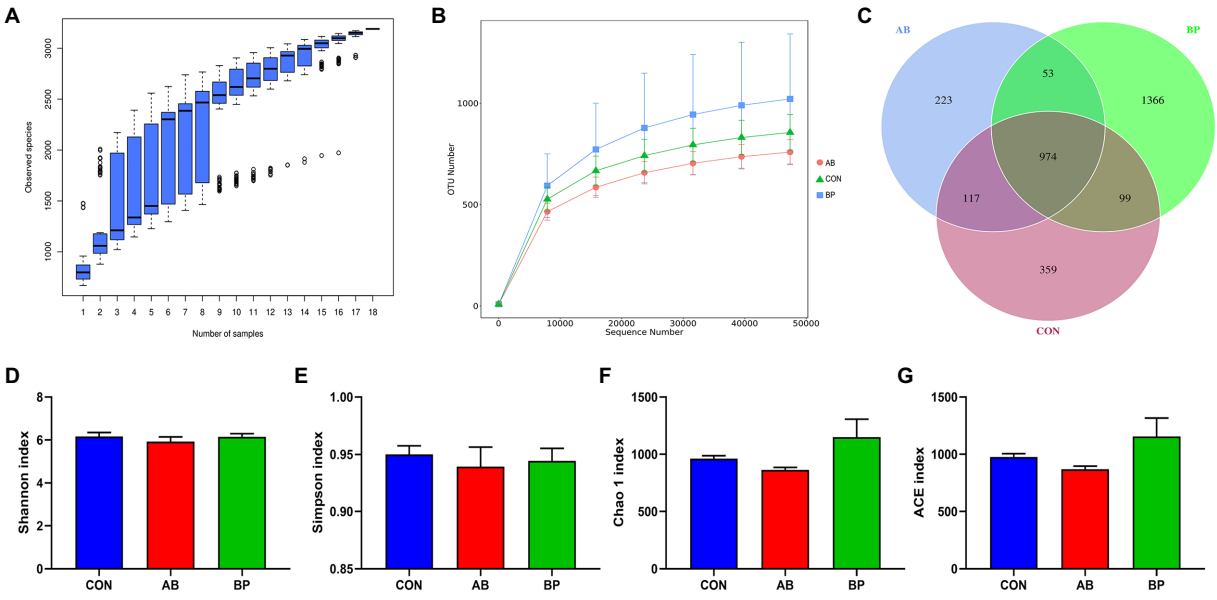


FIGURE 11
Differences in alpha diversity and richness among the three treatments. **(A)** The species accumulation curves; **(B)** The rarefaction curve of OTU; **(C)** Venn diagrams are displayed to describe the shared and unique sequences between processes; **(D)** Shannon index; **(E)** Simpson index; **(F)** Chao 1 index; and **(G)** ACE index. CON group, broiler chickens fed a basal diet; AB group, broiler chickens fed a basal diet supplemented with 50mg/kg aureomycin; BP group, broiler chickens fed a basal diet supplemented with 40mg/kg Bopu powder containing protopine and allocryptopine. $n$=6. Differences between groups were considered significant at $p$<0.05.

## Conclusion

In conclusion, dietary supplementation with 40 mg/kg Bopu powder promoted intestinal development and function in broiler chickens through decreasing inflammatory response, enhancing antioxidant capacity, and increasing beneficial bacteria abundances. Besides, Bopu powder supplementation suppressed intestinal inflammatory response possibly through inhibiting TLR4/MyD88/NF-κB signal pathway and enhanced antioxidant capacity possibly by activating Sirt1/Nrf2 signal pathway in broiler chickens. Our study provides robust support for the application of Bopu powder as a potential substitute to AGP in improving intestinal health in poultry production.

## 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/, PRJNA848931.

## Ethics statement

The animal study was reviewed and approved by the protocol for this experiment involving animals was in accordance with the guidelines of the Care and Use committee of Shandong Agricultural University (Ethics Approval code: SDAUA-2021-019).

## Author contributions

HL, NJ, WY, and YLi: conceptualization. YLiu, QW, and JN: data curation. YLiu, QW, and HL: formal analysis. WY and YLi: funding acquisition. QW, NJ, QG, and WY: investigation. YLiu, JN, NJ, LH, SJ, and YLi: methodology. YLiu, QW, and JN: project administration. JN, HL, and LH: resources. YLiu and QW: software. WY and YLi: supervision and writing—review and editing. SJ and YLi: validation. QW and WY: visualization. YLiu: writing—original draft. All authors contributed to the article and approved the submitted version.

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## Conflict of interest

QW is employed by Shandong Wonong Agro-tech Group Co., Ltd. QG is employed by Shandong Landoff Biotechnology Ltd.

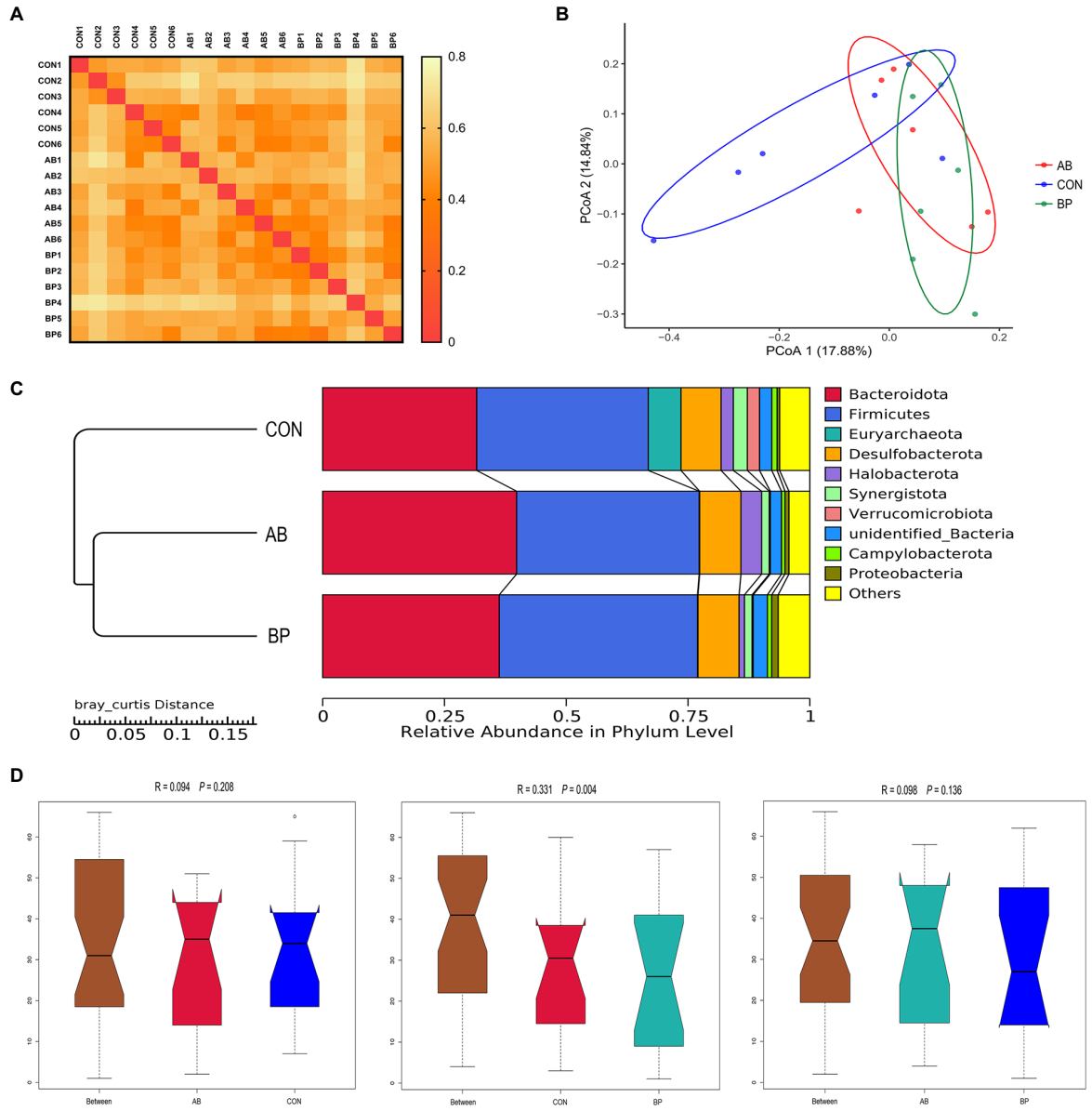


FIGURE 12
Beta diversity analysis of cecal community. **(A)** The results of the heat map drawn using bray_curtis distance matrix; **(B)** The principal coordinate analysis (PCoA); **(C)** Unweighted pair-group method with arithmetic mean (UPGMA) phylogenetic tree; and **(D)** The analysis of ANOSIM. CON group, broiler chickens fed a basal diet; AB group, broiler chickens fed a basal diet supplemented with 50mg/kg aureomycin; BP group, broiler chickens fed a basal diet supplemented with 40mg/kg Bopu powder containing protopine and allocryptopine. $n$=6. Differences between groups were considered significant at $p$<0.05.

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The Supplementary material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2022.1019130/full#supplementary-material

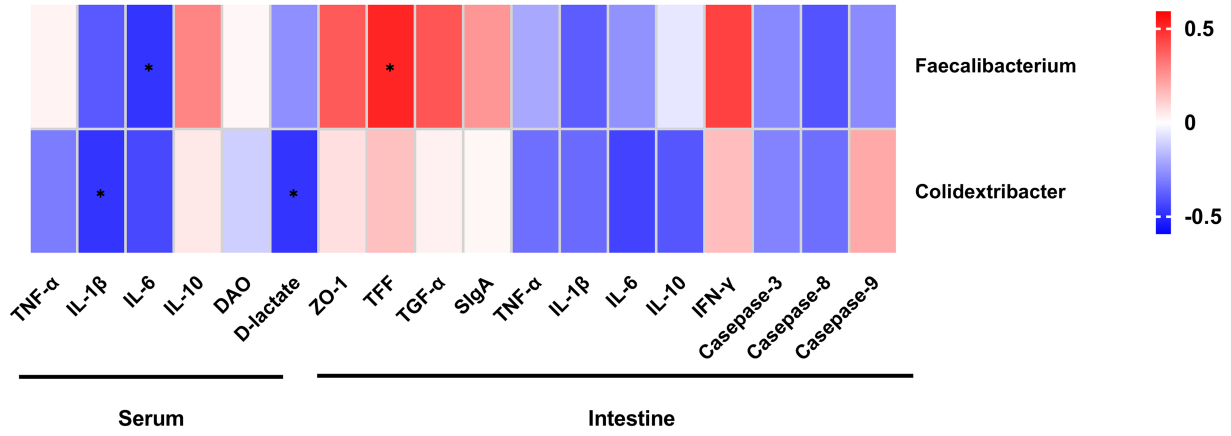


FIGURE 13
Spearman's correlation analysis between differential bacterial abundances and concentrations of metabolic parameters and immunological markers in serum and intestine. Tumor necrosis factor-α (TNF-α); Interleukin-1β (IL-1β); Interleukin-6 (IL-6); Interleukin-10 (IL-10); zonula occludens-1 (ZO-1); Trefoil peptides (TFF); Transforming growth factor alpha (TGF-α); Secretory immunoglobulin A (SIgA); and Interferon-γ (IFN-γ). *The correlation is significant at a level of 0.05.

# References

Acres, A. (2009). *Broiler Management Guide*. Midlothian, UK: Aviagen Limited, 1–59

Akdis, M., Aab, A., Altunbulakli, C., Azkur, K., Costa, R. A., Crameri, R., et al. (2016). Interleukins (from IL-1 to IL-38), interferons, transforming growth factor β, and TNF-α: receptors, functions, and roles in diseases. *J. Allergy Clin. Immunol.* 138, 984–1010. doi: 10.1016/j.jaci.2016.06.033

Alfen, J. S., Larghi, P., Facciotti, F., Gagliani, N., Bosotti, R., Paroni, M., et al. (2018). Intestinal IFN-γ–producing type 1 regulatory T cells coexpress CCR5 and programmed cell death protein 1 and downregulate IL-10 in the inflamed guts of patients with inflammatory bowel disease. *J. Allergy Clin. Immunol.* 142, 1537–1547.e8. doi: 10.1016/j.jaci.2017.12.984

Beumer, J., and Clevers, H. (2021). Cell fate specification and differentiation in the adult mammalian intestine. *Nat. Rev. Mol. Cell Biol.* 22, 39–53. doi: 10.1038/s41580-020-0278-0

Brentnall, M., Rodriguez-Menocal, L., De Guevara, R. L., Enrique, C., and Boise, L. H. (2013). Caspase-9, caspase-3 and caspase-7 have distinct roles during intrinsic apoptosis. *BMC Cell Biol.* 14, 1–9. doi: 10.1186/1471-2121-14-32

Buda, A., Jepson, M. A., and Pignatelli, M. (2012). Regulatory function of trefoil peptides (TFF) on intestinal cell junctional complexes. *Cell Commun. Adhes.* 19, 63–68. doi: 10.3109/15419061.2012.748326

Chen, J., Kang, B., Yao, K., Fu, C., and Zhao, Y. (2019). Effects of dietary Macleaya cordata extract on growth performance, immune responses, antioxidant capacity, and intestinal development in weaned piglets. *J. Appl. Anim. Res.* 47, 349–356. doi: 10.1080/09712119.2019.1636800

Chen, J., Li, F., Yang, W., Jiang, S., and Li, Y. (2021a). Supplementation with exogenous catalase from penicillium notatum in the diet ameliorates lipopolysaccharide-induced intestinal oxidative damage through affecting intestinal antioxidant capacity and microbiota in weaned pigs. *Microbiol. Spectr.* 9, e00654–e00721. doi: 10.1128/Spectrum.00654-21

Chen, J., Li, F., Yang, W., Jiang, S., and Li, Y. (2021b). Comparison of gut microbiota and metabolic status of sows with different litter sizes during pregnancy. *Front. Vet. Sci.* 8:793174. doi: 10.3389/fvets.2021.793174

Chen, X., Zhang, E., Fang, L., Zhang, J., Zhu, J., He, W., et al. (2013). Repair effects of exogenous SOD on Bacillus subtilis against gamma radiation exposure. *J. Environ. Radioact.* 126, 259–263. doi: 10.1016/j.jenvrad.2013.09.005

Cheng, G., Hao, H., Xie, S., Wang, X., Dai, M., Huang, L., et al. (2014). Antibiotic alternatives: the substitution of antibiotics in animal husbandry? *Front. Microbiol.* 5:217. doi: 10.3389/fmicb.2014.00217

Chong, Z. Z., Shang, Y. C., Wang, S., and Maiese, K. (2012). SIRT1: new avenues of discovery for disorders of oxidative stress. *Expert Opin. Ther. Tar.* 16, 167–178. doi: 10.1517/14728222.2012.648926

D'Arcy, M. S. (2019). Cell death: a review of the major forms of apoptosis, necrosis and autophagy. *Cell Biol. Int.* 43, 582–592. doi: 10.1002/cbin.11137

Diaz Carrasco, J. M., Casanova, N. A., and Fernandez Miyakawa, M. E. (2019). Microbiota, gut health and chicken productivity: what is the connection? *Microorganisms* 7:374. doi: 10.3390/microorganisms7100374

Ducatelle, R., Goossens, E., De Meyer, F., Eeckhaut, V., Antonissen, G., Haesebrouck, F., et al. (2018). Biomarkers for monitoring intestinal health in poultry: present status and future perspectives. *Vet. Res.* 49, 43–49. doi: 10.1186/s13567-018-0538-6

Gonzalez-Gonzalez, M., Diaz-Zepeda, C., Eyzaguirre-Velasquez, J., Gonzalez-Arancibia, C., Bravo, J. A., and Julio-Pieper, M. (2018). Investigating gut permeability in animal models of disease. *Front. Physiol.* 9:1962. doi: 10.3389/fphys.2018.01962

Goo, D., Kim, J. H., Choi, H. S., Park, G. H., Han, G. P., and Kil, D. Y. (2019). Effect of stocking density and sex on growth performance, meat quality, and intestinal barrier function in broiler chickens. *Poult. Sci.* 98, 1153–1160. doi: 10.3382/ps/pey491

Goonatilleke, E., Smilowitz, J. T., Marino, K. V., German, B. J., Lebrilla, C. B., and Barboza, M. (2019). Immunoglobulin a N-glycosylation presents important body fluid-specific variations in lactating mothers. *Mol. Cell. Proteomics* 18, 2165–2177. doi: 10.1074/mcp.RA119.001648

Guo, W., Xiang, Q., Mao, B., Tang, X., Cui, S., Li, X., et al. (2021). Protective effects of microbiome-derived inosine on lipopolysaccharide-induced acute liver damage and inflammation in mice via mediating the TLR4/NF-κB pathway. *J. Agric. Food Chem.* 69, 7619–7628. doi: 10.1021/acs.jafc.1c01781

Hafeez, A., Ullah, Z., Khan, R. U., Ullah, Q., and Naz, S. (2020). Effect of diet supplemented with coconut essential oil on performance and villus histomorphology in broiler exposed to avian coccidiosis. *Trop. Anim. Health Prod.* 52, 2499–2504. doi: 10.1007/s11250-020-02279-6

Hu, N., Guo, C., Dai, X., Wang, C., Gong, L., Yu, L., et al. (2020). Forsythiae Fructuse water extract attenuates liver fibrosis via TLR4/MyD88/NF-κB and TGF-β/smads signaling pathways. *J. Ethnopharmacol.* 262:113275. doi: 10.1016/j.jep.2020.113275

Jorgovanovic, D., Song, M., Wang, L., and Zhang, Y. (2020). Roles of IFN-γ in tumor progression and regression: a review. *Biomark Res.* 8, 49–16. doi: 10.1186/s40364-020-00228-x

Kanai, T., Mikami, Y., and Hayashi, A. (2015). A breakthrough in probiotics: clostridium butyricum regulates gut homeostasis and anti-inflammatory response in inflammatory bowel disease. *J. Gastroenterol.* 50, 928–939. doi: 10.1007/s00535-015-1084-x

Kogut, M. H., Genovese, K. J., Swaggerty, C. L., He, H., and Broom, L. (2018). Inflammatory phenotypes in the intestine of poultry: not all inflammation is created equal. *Poult. Sci.* 97, 2339–2346. doi: 10.3382/ps/pey087

Korsmeyer, S. J. (1999). BCL-2 gene family and the regulation of programmed cell death. *Cancer Res.* 59, 1693s–1700s.

Kosina, P., Gregorova, J., Gruz, J., Vacek, J., Kolar, M., Vogel, M., et al. (2010). Phytochemical and antimicrobial characterization of Macleaya cordata herb. *Fitoterapia* 81, 1006–1012. doi: 10.1016/j.fitote.2010.06.020

Lawley, B., and Tannock, G. W. (2017). Analysis of 16S rRNA gene amplicon sequences using the QIIME software package. *Methods Mol. Biol.* 41, D590–D596. doi: 10.1093/nar/gks1219

Li, C., Ai, G., Wang, Y., Lu, Q., Luo, C., Tan, L., et al. (2020). Oxyberberine, a novel gut microbiota-mediated metabolite of berberine, possesses superior anti-colitis effect: impact on intestinal epithelial barrier, gut microbiota profile and TLR4-MyD88-NF-κB pathway. *Pharmacol. Res.* 152:104603. doi: 10.1016/j.phrs.2019.104603

Li, J., Liu, Y., Niu, J., Jing, C., Jiao, N., Huang, L., et al. (2022). Supplementation with paraformic acid in the diet improved intestinal development through modulating intestinal inflammation and microbiota in broiler chickens. *Front. Microbiol.* 13:975056. doi: 10.3389/fmicb.2022.975056

Lin, L., Liu, Y. C., Huang, J. L., Liu, X. B., Qing, Z. X., Zeng, J. G., et al. (2018). Medicinal plants of the genus Macleaya (Macleaya cordata, Macleaya microcarpa): a review of their phytochemistry, pharmacology, and toxicology. *Phytother. Res.* 32, 19–48. doi: 10.1002/ptr.5952

Liu, Y., Azad, M. A. K., Zhao, X., Zhu, Q., and Kong, X. (2022b). Dietary crude protein levels alter diarrhea incidence, immunity, and intestinal barrier function of huanjiang mini-pigs during different growth stages. *Front. Immunol.* 13:908753. doi: 10.3389/fimmu.2022.908753

Liu, Y., Li, Y., Niu, J., Liu, H., Jiao, N., Huang, L., et al. (2022a). Effects of dietary Macleaya Cordata extract containing isoquinoline alkaloids supplementation as an alternative to antibiotics in the diets on growth performance and liver health of broiler chickens. *Front. Vet. Sci.* 9:950174. doi: 10.3389/fvets.2022.950174

Liu, H., Lin, Q., Liu, X., Huang, P., Yang, Z., Cao, M., et al. (2022). Dietary Bopu powder supplementation increased serum antioxidant capacity, improved the egg quality and modulated the intestinal microbiota of laying hens. *Front. Physiol.* 13:902784. doi: 10.3389/fphys.2022.902784

Loboda, A., Damulewicz, M., Pyza, E., Jozkowicz, A., and Dulak, J. (2016). Role of Nrf2/HO-1 system in development, oxidative stress response and diseases: an evolutionarily conserved mechanism. *Cell. Mol. Life Sci.* 73, 3221–3247. doi: 10.1007/s00018-016-2223-0

Lozupone, C., and Knight, R. (2005). UniFrac: a new phylogenetic method for comparing microbial communities. *Appl. Environ. Microbiol.* 71, 8228–8235. doi: 10.1128/AEM.71.12.8228-8235

Luk, G. D., Bayless, T. M., and Baylin, S. B. (1983). Plasma postheparin diamine oxidase. Sensitive provocative test for quantitating length of acute intestinal mucosal injury in the rat. *J. Clin. Invest.* 71, 1308–1315. doi: 10.1172/JCI110881

Mager, L. F., Burkhard, R., Pett, N., Cooke, N. C. A., Brown, K., Ramay, H., et al. (2020). Microbiome-derived inosine modulates response to checkpoint inhibitor immunotherapy. *Science* 369, 1481–1489. doi: 10.1126/science.abc3421

Meng, Q., Guo, T., Li, G., Sun, S., He, S., Cheng, B., et al. (2018). Dietary resveratrol improves antioxidant status of sows and piglets and regulates antioxidant gene expression in placenta by Keap1-Nrf2 pathway and Sirt1. *J. Anim. Sci. Biotechnol.* 9:34. doi: 10.1186/s40104-018-0248-y

Miquel, S., Martin, R., Rossi, O., Bermudez-Humaran, L. G., Chatel, J. M., Sokol, H., et al. (2013). Faecalibacterium prausnitzii and human intestinal health. *Curr. Opin. Microbiol.* 16, 255–261. doi: 10.1016/j.mib.2013.06.003

Ni, H., Martinez, Y., Guan, G., Rodriguez, R., Mas, D., Peng, H., et al. (2016). Analysis of the impact of isoquinoline alkaloids, derived from Macleaya cordata extract, on the development and innate immune response in swine and poultry. *Biomed. Res. Int.* 2016:1352146. doi: 10.1155/2016/1352146

Quast, C., Pruesse, E., Yilmaz, P., Gerken, J., Schweer, T., Yarza, P., et al. (2012). The SILVA ribosomal RNA gene database project: improved data processing and web-based tools. *Nucleic Acids Res.* 41, D590–D596. doi: 10.1093/nar/gks1219

Reed, J. C., Miyashita, T., Takayama, S., Wang, H. G., Sato, T., Krajewski, S., et al. (1996). Bcl-2 family proteins: regulators of cell death involved in the pathogenesis of cancer and resistance to therapy. *J. Cell. Biochem.* 60, 23–32. doi: 10.1002/(SICI)1097-4644(19960101)60:1<23::AID-JCB5>3.0.CO;2-5

Sanchez de Medina, F., Romero-Calvo, I., Mascaraque, C., and Martinez-Augustin, O. (2014). Intestinal inflammation and mucosal barrier function. *Inflamm. Bowel Dis.* 20, 2394–2404. doi: 10.1097/MIB.0000000000000204

Sato, H., Zhang, L. S., Martinez, K., Chang, E. B., Yang, Q., Wang, F., et al. (2016). Antibiotics suppress activation of intestinal mucosal mast cells and reduce dietary lipid absorption in Sprague-Dawley rats. *Gastroenterology* 151, 923–932. doi: 10.1053/j.gastro.2016.07.009

Schumacher, M. A., Danopoulos, S., Al Alam, D., and Frey, M. R. (2018). Growth factors in the intestinal tract. *Physiol. Gastrointest. Tract.* 71–101. doi: 10.1016/B978-0-12-809954-4.00003-7

Song, D., Li, A., Wang, Y., Song, G., Cheng, J., Wang, L., et al. (2022). Effects of synbiotic on growth, digestibility, immune and antioxidant performance in broilers. *Animal* 16:100497. doi: 10.1016/j.animal.2022.100497

Thornberry, N. A. (1997). The caspase family of cysteine proteases. *Br. Med. Bull.* 53, 478–490. doi: 10.1093/oxfordjournals.bmb.a011625

Wang, H., Mei, L., Deng, Y., Liu, Y., Wei, X., Liu, M., et al. (2019). Lactobacillus brevis DM9218 ameliorates fructose-induced hyperuricemia through inosine degradation and manipulation of intestinal dysbiosis. *Nutrition* 62, 63–73. doi: 10.1016/j.nut.2018.11.018

Wang, F., Yin, Y., Yang, M., Chen, J., Fu, C., and Huang, K. (2021). Effects of combined supplementation of Macleaya cordata extract and benzoic acid on the growth performance, immune responses, antioxidant capacity, intestinal morphology, and microbial composition in weaned piglets. *Front. Vet. Sci.* 8:708597. doi: 10.3389/fvets.2021.708597

Wu, Q. J., Zhou, Y. M., Wu, Y. N., Zhang, L. L., and Wang, T. (2013). The effects of natural and modified clinoptilolite on intestinal barrier function and immune response to LPS in broiler chickens. *Vet. Immunol. Immunopathol.* 153, 70–76. doi: 10.1016/j.vetimm.2013.02.006

Xiao, Y., Zhou, Y., Sun, S., Wang, H., Wu, S., and Bao, W. (2021). Effect of promoter methylation on the expression of porcine MUC2 gene and resistance to PEDV infection. *Front. Vet. Sci.* 8:646408. doi: 10.3389/fvets.2021.646408

Xie, H., Yang, J., Feng, S., Cheng, P., Zeng, J., and Xiong, X. (2015). Simultaneous quantitative determination of sanguinarine, chelerythrine, dihydrosanguinarine and dihydrochelerythrine in chicken by HPLC–MS/MS method and its applications to drug residue and pharmacokinetic study. *J. Chromatogr. B* 985, 124–130. doi: 10.1016/j.jchromb.2015.01.001

Yang, J., Wu, Y., Xu, Y., Jia, J., Xi, W., Deng, H., et al. (2021). Dexmedetomidine resists intestinal ischemia-reperfusion injury by inhibiting TLR4/MyD88/NF-κB signaling. *J. Surg. Res.* 260, 350–358. doi: 10.1016/j.jss.2020.11.041

Yin, F., Lan, R., Wu, Z., Wang, Z., Wu, H., Li, Z., et al. (2019). Yupingfeng polysaccharides enhances growth performance in Qingyuan partridge chicken by up-regulating the mRNA expression of SGLT1, GLUT2 and GLUT5. *Vet. Med. Sci.* 5, 451–461. doi: 10.1002/vms3.167

Zhang, Q., Zhang, Z., Zhou, S., Jin, M., Lu, T., Cui, L., et al. (2021). Macleaya cordata extract, an antibiotic alternative, does not contribute to antibiotic resistance gene dissemination. *J. Hazard. Mater.* 412:125272. doi: 10.1016/j.jhazmat.2021.125272

Zhang, C., Zhao, X. H., Yang, L., Chen, X. Y., Jiang, R. S., Jin, S. H., et al. (2017). Resveratrol alleviates heat stress-induced impairment of intestinal morphology, microflora, and barrier integrity in broilers. *Poult. Sci.* 96, 4325–4332. doi: 10.3382/ps/pex266

Zhuang, Y., Wu, H., Wang, X., He, J., He, S., and Yin, Y. (2019). Resveratrol attenuates oxidative stress-induced intestinal barrier injury through PI3K/Akt-mediated Nrf2 signaling pathway. *Oxidative Med. Cell. Longev.* 2019:7591840. doi: 10.1155/2019/7591840

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# Cecum microbiome and metabolism characteristics of Silky Fowl and White Leghorn chicken in late laying stages

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Cecal microflora plays a key role in the production performance and immune function of chickens. White Leghorn (WL) is a well-known commercial layer line chicken with high egg production rate. In contrast, Silky Fowl (SF), a Chinese native chicken variety, has a low egg production rate, but good immune performance. This study analyzed the composition of cecal microbiota, metabolism, and gene expression in intestinal tissue of these varieties and the correlations among them. Significant differences were observed in the cecal microbes: *Bacteroides* was significantly enriched in WL, whereas *Veillonellaceae* and *Parabacteroides* were significantly enriched in SF. Carbohydrate biosynthesis and metabolism pathways were significantly upregulated in WL cecum, which might provide more energy to the host, leading to persistently high levels of egg production. The higher *Parabacteroides* abundance in SF increased volicitin content, enhanced α-linolenic acid metabolism, and significantly negatively correlated with metabolites of propanoate metabolism and carbohydrate metabolism. Genes related to lipid metabolism, immunity, and melanogenesis were significantly upregulated in the SF cecum, regulating lipid metabolism, and participating in the immune response, while genes related to glucose metabolism and bile acid metabolism were expressed at higher levels in WL, benefiting energy support. This study provided a mechanism for intestinal microorganisms and metabolic pathways to regulate chicken egg-laying performance and immunity.

KEYWORDS

**White Leghorn, Silky Fowl, cecal microbiome, cecal metabolome, correlation analysis**

## Introduction

Gut microbes have been shown to play a key role in physiological activities, such as obesity (Maruvada et al., 2017), immune coordination (Zheng et al., 2020), metabolism (Postler and Ghosh, 2017), and host gene expression regulation (Collins and Patterson, 2020). Intestinal microorganisms directly stimulate or indirectly (metabolites) affect the host's intestinal function, which in turn affects the host's absorption and utilization of dietary nutrients and regulates production performance and immune function (Hill and Round, 2021). The small intestine is the main site of nutrient digestion and absorption. The cecum is a major part of the large intestine, where microbial fermentation produces volatile fatty acids, which provide energy for the host (Binek et al., 2017). Fermentation of cecal microbiota contributes to the health and productivity of chickens and helps resist colonization by invading pathogens (Bjerrum et al., 2006; Saxena et al., 2016).

The gut microbiota is closely related to poultry productivity (Diaz Carrasco et al., 2019). Chen et al. (2019) showed that the cecal microbiome and metabolites contribute more efficiently to a higher growth performance in chickens. Yan et al. (2017) showed that *Lactobacillus* and *Akkermansia* had significantly higher abundance in the cecal contents of hens with better feed efficiency and enriched functions related to carbohydrate and amino acid metabolism, suggesting that *Lactobacillus* can improve the feed efficiency of hosts. In addition to determining the role of the gut microbiome in growth (Rubio et al., 2015), studies have found that probiotic supplementation can improve laying performance, egg quality, and hatching in laying hens (Mikulski et al., 2020). Therefore, intestinal microbes may be potential targets for regulating chicken production performance.

Regarding the effects of intestinal microbes on immune regulation, preliminary studies on germ-free chickens suggest that microbial exposure is necessary for proper development and maturation of the intestinal immune system (Dibner et al., 1998). Yang et al. (2014) showed that *Lactobacillus* strains could significantly reduce the expression of *Salmonella* virulence genes and protect body health. Probiotics supplementation can change intestinal flora, stimulate the immune system, reduce inflammation, and prevent colonization by pathogenic bacteria (Jha et al., 2020).

Microbial colonization may be host dependent and species specific. Waite and Taylor showed that although sampling location, diet, and captivity status play a role, the host is the most important factor determining microbial community composition (Waite and Taylor, 2014). Studies have shown the presence of *Bacteroidetes* in the ileal contents of 20-day-old Cobb broilers but not in Ross broilers. However, *Actinomycetes* was present in the ileal contents of Ross broilers, but not in Cobb broilers (Nakphaichit et al., 2011; Kim J. E. et al., 2015). When maintaining the same growth environment, there are differences in the composition of intestinal microbes among different breeds or strains of chickens. Pandit et al. (2018) found significant differences in the cecal microbial community structure between Ross and Cobb broilers and Indian native breeds of the same age. Bidirectional selection for specific economic traits also has significant effects on intestinal flora (Schokker et al., 2015). Yang et al. (2017) observed significant differences in fecal flora composition between strains using chickens from the 40th generation of a bidirectional antibody titer breeding line at Virginia Tech. Some researchers have also observed microbial differences in the feces of high-fat and low-fat hens (Ding et al., 2016). Kundu et al. (2016) evaluated the immune competence of native and White Leghorn (WL). Native chickens showed a higher haemagglutinin test, while WL showed the lowest response and highest mortality.

In this study, we compared the cecal microbiome, metabolome, and tissue transcriptome between WL and Silky Fowl (SF) chickens. WL is a well-known commercial layer line with early maturity, high egg productivity, and low feed consumption (Allonby and Wilson, 2018). SF is a local breed with lower egg productivity and strong immunity, with widely distributed melanocytes. Melanocytes play an important role in innate immunity during viral infection (Han et al., 2021). Chickens were raised in the same chicken house, fed the same feed, and had similar growth curves. We investigated the effects of cecal microorganisms and host genes on egg-laying and immune differences between the two varieties. This may provide a way to improve egg productivity or immunity through transplantation of dominant flora.

## Materials and methods

### Sample collection and index determination

WL and SF hens of the same age in separate conservation populations were raised in the Experimental Unit for Poultry Genetic Resource and Breeding, with the same feeding, management, and environmental conditions; single cage feeding; and free access to feed and water. Laboratory Animal Welfare Experiment License from China Agricultural University was obtained (permit number: SKLAB-2012-04-07). All experiments were approved by the Committee on the Animal Experimental Ethical Inspection of China Agricultural University (issue number: AW32802202-1-1).

Eight SF and eight WL hens (48-weeks old) were sacrificed by severing the jugular veins after anesthesia after weighing, bled for 3–5 min, and then dissected. The luminal contents of cecum samples were collected after slaughter. Liver weight and cecum length were determined, and the liver index was calculated as: liver index = (liver weight/body weight) × 100%.

Cecum tissue were cut into two along the sagittal plane; one half was fixed in 4% paraformaldehyde solution (Beijing Solarbio Life Science and Technology Co., Ltd., Beijing, China), and the other half was stored in liquid nitrogen and then transferred into −80°C freezer until use.

## Oil red O staining

After fixation in 4% paraformaldehyde solution for a minimum period of 24 h before use, the caeca were trimmed and dehydrated by 30 and 50% sucrose solution, and then embedded in OCT (Opti-mum Cutting Temperature compound, Leica, Shanghai, China) to prepare 15 mm frozen sections. Sections were washed with distilled water and incubated in oil red O for 10 min. After being rinsed with isopropanol for 2 s and distilled water for 1 s, the sections were rinsed with Hematoxylin solution for 5 min, and then incubated in distilled water for 10 min. And then the sections were mounted with neutral balsam for observation under light microscope. The histopathological changes were observed and pictured using a Zeiss camera system (Carl Zeiss Optics Co., Ltd., Guangzhou, China).

## 16S rRNA gene amplicon sequencing

Total genomic DNA samples of cecum contents were extracted using the OMEGA Soil DNA Kit (D5625-01) (Omega Bio-Tek, Norcross, GA, USA), following the manufacturer's instructions, and stored at −20°C prior to further analysis. PCR amplification of the bacterial 16S rRNA genes V4-V5 region was performed using the forward primer 515F (5′-GTGCCAGCMGCCGCGGTAA-3′) and the reverse primer 907R (5′- CCGTCAATTCMTTTRAGTTT-3′). After the individual quantification step, amplicons were pooled in equal amounts, and pair-end 2,250 bp sequencing was performed using the Illumina MiSeq platform with MiSeq Reagent Kit v3 at Shanghai Personal Biotechnology Co., Ltd. (Shanghai, China).

Microbiome bioinformatics were performed with QIIME2 2019.4 (Bolyen et al., 2019) with slight modification according to the official tutorials.[1] Briefly, raw sequence data were demultiplexed using the demux plugin, followed by primer cutting with cutadapt plugin (Martin, 2011). Sequences were then quality filtered, denoised, merged, and chimera removed using the DADA2 plugin (Callahan et al., 2016). Non-singleton amplicon sequence variants (ASVs) were aligned with mafft (Katoh et al., 2002) and used to construct a phylogeny using fasttree2 (Price et al., 2010). Sequence data analyses were mainly performed using QIIME2 (Caporaso, 2019) and R packages (v3.2.0). ASV-level alpha diversity indices, such as Chao1 richness estimator, Observed species, Shannon diversity index, Simpson index, Faith's PD, Pielou's evenness and Good's coverage were calculated using the ASV table in QIIME2, and visualized as box plots. The number under the label of diversity index is the *P*-value tested by Kruskal-Wallis test.

Beta diversity analysis was performed to investigate the structural variation of microbial communities across samples using Bray-Curtis metrics and visualized *via* principal coordinate analysis (PCoA). The taxonomy compositions and abundances were visualized using MEGAN and GraPhlAn. Linear discriminant analysis (LDA) effect size (LEfSe) method[2] was used to perform cecal microbiota features differentiating. LEfSe uses the Kruskal-Wallis rank sum test to detect features with significantly different abundances between assigned taxa and an effect size threshold of 3.5 were used for all biomarkers discussed in this study. Microbial functions were predicted by PICRUSt2 (Phylogenetic investigation of communities by reconstruction of unobserved states) using ASVs sequence and abundance, upon MetaCyc Metabolic Pathway Database (MetaCyc),[3] Kyoto Encyclopedia of Genes and Genomes Database (KEGG)[4] and Cluster of Orthologous Groups of proteins Database (COG)[5] databases. All sequences were deposited in the National Center for Biotechnology Information (NCBI) and can be accessed in the Short Read Archive (SRA) under the accession number PRJNA848375.

## Untargeted metabolomics

In this experiment, HILIC UHPLC-Q-EXACTIVE MS technology combined with data-dependent acquisition method was used to analyze the full spectrum of the sample, and the primary and secondary mass spectrometry data were obtained at the same time, and then Compound Discoverer 3.0 (Thermo Fisher Scientific) was used to perform peak extraction and metabolite identification of the data.

To monitor the stability and repeatability of instrument analysis, quality control (QC) samples were prepared by pooling 10 μl of each sample and analyzed together with the other samples. The ACQUITY UPLC BEH C18 column (100 mm*2.1 mm, 1.7 μm, Waters, USA) was used for chromatographic separation. The mobile phase A was water and 0.1% formic acid, B mobile phase is acetonitrile. The loading volume for each sample is 5 μl. The sample was placed in the 4°C autosampler during the entire analysis. In order to avoid the influence caused by the fluctuation of the detection signal of the instrument, a random order is adopted for continuous analysis of samples. QC samples are inserted after each group of samples

1 https://docs.qiime2.org/2019.4/tutorials/

2 http://huttenhower.sph.harvard.edu/lefse/

3 https://metacyc.org/

4 https://www.kegg.jp/

5 https://www.ncbi.nlm.nih.gov/COG/

in the sample queue to monitor and evaluate the stability of the system and the reliability of experimental data.

Electrospray ionization (ESI) positive ion and negative ion modes were used for detection. The samples were separated by UHPLC and analyzed by Q-Exactive quadrupole-electrostatic field orbitrap high-resolution mass spectrometer (Thermo Fisher Scientific). In the extracted ion features, only the variables having more than 50% of the non-zero measurement values in at least one group were kept. Compound identification of metabolites was performed by comparing of accuracy m/z value (0<25 ppm), and MS/MS spectra with an in-house database established with available authentic standards. After normalized to total peak intensity, the processed data were uploaded, then imported into SIMCA-P (version 14.1, Umetrics, Umea, Sweden), where they were subjected to multivariate data analysis, including Pareto-scaled principal component analysis (PCA) and orthogonal partial least-squares discriminant analysis (OPLS-DA). The variable importance in the projection (VIP) value of each variable in the OPLS-DA model was calculated to indicate its contribution to the classification. Metabolites with the VIP value > 1 was further applied to Student' s *t*-test at univariate level to measure the significance of each metabolite, the *p*-values less than 0.05 were considered as statistically significant. The significant difference metabolites were screened, and then cluster analysis and KEGG metabolic pathway analysis were performed on the difference metabolites.

## Transcriptome sequencing

Cecum tissues were collected and the total RNA was extracted by using TRIZOL reagent (Invitrogen, USA) according to the manufacturer's protocol. RNA purity and quantification were evaluated using the NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, USA). RNA integrity was assessed using the Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA). Libraries were constructed using the TruSeq$^{TM}$ RNA Sample Prep kit (Illumina, San Diego, CA, USA) according to the manufacturer's instructions. Sequencing of the libraries was performed on an Illumina HiSeq2000 instrument by Shanghai Personal Biotechnology Co., Ltd. (Shanghai, China). The sequencing data contained a few connectors and low-quality Reads, and thus Cutadapt (v1.15) software was used to filter the sequencing data to get high-quality sequence (Clean Data) for further analysis. Reads with number of expected errors higher than [default: 2.0] were discarded. The clean reads were mapped to the chicken genome (GRCg6a/galGal6) using HISAT2. FPKM of each gene was calculated using Cufflinks, and the read counts of each gene were obtained by HTSeq-count. Differential expression analysis was performed using the DESeq (2012) R package. Padjust value < 0.05 [Benjamini-Hochberg (BH) multiple test correction], and fold change > 2 was set as the threshold for significant differential expression. Hierarchical cluster analysis of differentially expressed genes (DEGs) was performed to demonstrate the expression pattern of genes in different groups and samples. Top GO (2.40.0) was used for Gene Ontology (GO) enrichment analysis of the DEGs, and ClusterProfiler (3.16.1) software was used for KEGG pathway enrichment analysis to understand the high-level functions and utilities of the biological system. All RNA sequences were deposited in the NCBI and can be accessed in the SRA under the accession number PRJNA848673.

## Statistical analysis

All statistical analyses were performed using Prism 6.0 (GraphPad Software, San Diego, CA). Data were expressed as means ± standard error (M ± SE). Statistical significance was evaluated using Student's *t*-test. The redundancy analysis (RDA) was performed by the genescloud tools, a free online platform for data analysis.[6] MetOrigin[7] was used to integrate the statistical correlations and biological relationships between microbiome and metabolomics (Yu et al., 2022). Statistically, Spearman correlation analysis was chosen in MetOrigin. Biologically, each metabolite was searched against the KEGG database to identify bacterial species that could participate in a metabolic reaction. Sankey network diagrams were used to integrate and demonstrate statistically and biologically significant associations between microorganisms and metabolites. Differential metabolites from host, microbiota, and co-metabolic sources, and related bacteria were integrated, respectively, to obtain microbial and metabolite interaction networks.

# Results

## Phenotypic characteristics of White Leghorn and Silky Fowl

There was no significant difference in body weight between SF and WL, but the liver index and cecum length of SF were significantly lower than those of WL (**Figure 1A**). After 45 weeks of age, the SF egg-laying rate showed an obvious downward trend, while that of WL remained relatively stable at a high level (**Figure 1B**). Oil Red O staining showed that positive sites were mainly located in the submucosa of the cecum, and the lipid content of SF was significantly higher than that of WL (**Figure 1C**).

6 https://www.genescloud.cn/login

7 http://metorigin.met-bioinformatics.cn/

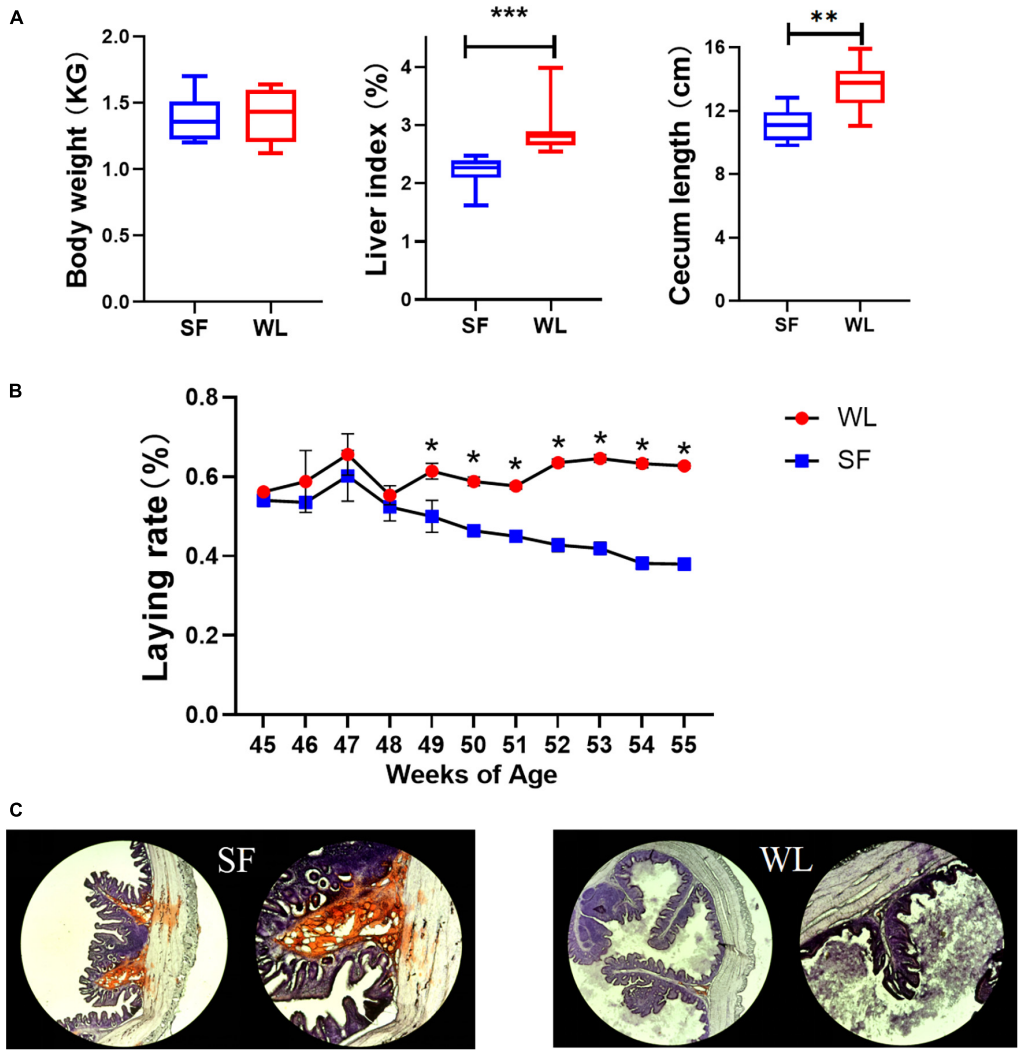


FIGURE 1
Phenotypic index and oil red O staining. **(A)** Comparison of WL and SF in weight, liver index and cecum length. **(B)** Laying rate of SF and WL flock from 45 to 55-week-old. **(C)** Histological observation of WL and SF cecum after oil red O staining. Asterisk coding is indicated in the $*P < 0.05$; $**P < 0.01$; $***P < 0.001$.

SF and WL had similar body size, but SF deposited more fat in the intestines and WL produced more eggs, that is, formed more yolks, which may indicate different mechanisms of fat formation and distribution between the two breeds.

## Differential microbiome composition and potentially functional prediction in the cecum of White Leghorn and Silky Fowl

A total of 1,512,400 raw reads (2 × 250 bp) were obtained by 16S rRNA gene sequencing, and 1,352,777 reads passed the filtering, with an average value of 84,548 reads/sample (SD: 15,686) and a median sequence length of 441 bp. To avoid potential biases due to different sequencing depths, all samples were rarefied at 3,000 reads after raw read quality filtering. Rarefaction analysis and Good's coverage indicated satisfactory coverage for all samples (average Good's coverage of 97.76%).

Compared to WL, SF had higher Chao1 and Faith pd indices but lower Good's coverage index (Figure 2A). The results showed that microbial community richness of the SF cecum was higher than that of WL chickens ($P < 0.05$), and the proportion of unclassified microbial species in SF cecal microbe samples was higher ($P < 0.05$). β-diversity analysis was used to compare the overall microbial profiles of all the groups as displayed in Figure 2B. PCoA was performed to present a holistic perception of the microbiota. The results of PCoA showed that the groups were mainly organized into two clusters, which illustrated that

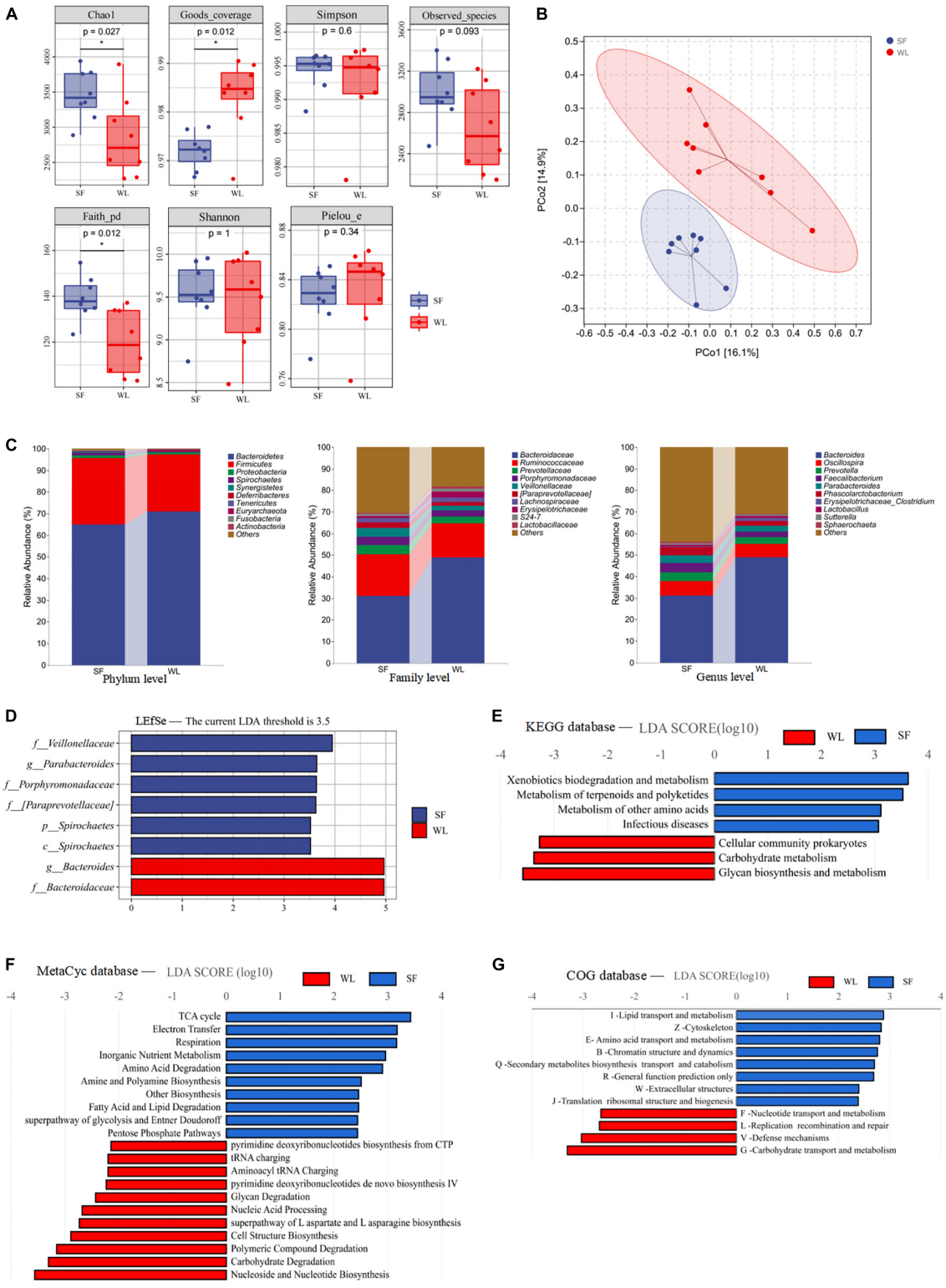


FIGURE 2

Cecal microbiome description of WL and SF. **(A)** Cecal Microbial Alpha Diversity Index Diagram of WL and SF. **(B)** Principal coordinate analysis (PCoA) based on Bray-Curtis distance, with 95% ellipse confidence. **(C)** Average relative abundances of dominant bacterial phylum, family and genus. **(D)** LEfSe analysis with LDA threshold 3.5, and metabolism pathways prediction of the cecal microbiota based on KEGG **(E)**, MetaCyc **(F)**, and COG **(G)** databases. Statistical significance is given as $^{***}P < 0.001$; $^{**}P < 0.01$; $^{*}P < 0.05$. Brackets are used to emphasize that this is the official Greengenes database recommended taxonomic information, there may be corrections or improvements to this taxonomic information.

the microbiota composition in each group was dissimilar. The samples from each group were fully aggregated; moreover, the intestinal microbial flora between the SF individuals was more uniform and similar.

Sequence alignment and annotation showed that most microbiota taxa belonged to 15 phyla, among which the dominant phyla were Bacteroides (67.97%) and Firmicutes (28.61%). The high proportion of Bacteroides in both groups indicated that this phylum was consistent as the dominant phylum, especially in WL (71.05%), where Bacteroides exhibited a higher dominance than that in SF (64.89%). Although Bacteroides was the dominant phylum in SF, the abundances of Firmicutes and Spirochetes ($P < 0.05$) in SF were higher than those in WL (**Figure 2C**). At the family level, the relative abundance of *Bacteroidaceae* was significantly lower in SF (31.18%) than in WL (49.00%), but the abundances of *Porphyromonadaceae*, *Veillonellaceae* ($P < 0.05$), and *[paraprevotellaceae]* ($P < 0.05$) were higher in SF than in WL. At the genus level, these microbes belonged to > 150 genera. Differences were also observed at the genus level: *Bacteroides* was significantly higher in WL (48.89%) than in SF (31.05%), while *Faecalibacterium*, *Parabacteroides*, and *Phascolarctobacterium* ($P < 0.05$) were higher in SF.

LEfSe was used to analyze the differential abundances of bacterial taxa. Specifically, *Bacteroides* and *Bacteroidaceae* were enriched in WL, *Veillonellaceae*, *Parabacteroides*, *Prophyromonadaceae*, *[Paraprevotellaceae]*, and Spirochetes were enriched in SF (**Figure 2D**). In the KEGG (**Figure 2E**) database, the enriched pathways of WL were glycan biosynthesis and metabolism, and carbohydrate metabolism, while those of SF were xenobiotics biodegradation and metabolism. Using the MetaCyc (**Figure 2F**) database, the enriched pathways of WL were carbohydrate degradation and glycan degradation, while in SF, the enriched pathways were the TCA cycle and amino acid, fatty acid and lipid degradation. In the COG (**Figure 2G**) database, the enriched pathways of WL were carbohydrate transport and metabolism, while those in SF were lipid transport and metabolism as well as amino acid transport and metabolism.

## Metabolomic difference of cecal contents in White Leghorn and Silky Fowl

To characterize the metabolite changes induced in cecal contents, we performed LC-MS/MS-based metabolomic analysis of WL and SF. PCA analysis showed that all QC samples were close and well gathered near the ordinate origin, indicating that the detection platform was stable, and the instrument precision was good. SF and WL were clearly distinguished, indicating that there were notable differences in cecal metabolites (**Figure 3A**). In Hotelling T2 Ellipse, the cumulative values of R2Y and Q2 were both greater than 0.6, indicating that the OPLS-DA model could explain the difference between the two groups of samples well, and further confirming that there was a significant difference between SF and WL cecal content-related metabolic components (**Figure 3B**). The OPLS-DA model R2Y was very close to 1, which showed that the established model conformed to the real situation of the sample data (**Supplementary Figures 1A,B**).

The differential metabolites were screened using a *t*-test where $P < 0.05$ indicated a significant difference (**Supplementary Figures 1C,D**). In total, 271 potential chicken cecal metabolic markers that could distinguish the SF and WL groups were screened. SF significantly upregulated 103 metabolites and downregulated 168 differential metabolites, which may have important biological functions in the cecum of SF and WL (**Supplementary Table 1**).

We submitted the differential metabolites (including positive and negative ion model results) obtained in the sample group to the KEGG website for relevant pathway analysis. The most significantly different metabolic pathways were α-linolenic acid metabolism, nicotinate, and nicotinamide metabolism, phenylalanine metabolism, glycosaminoglycan biosynthesis-heparan sulfate/heparin, propanoate metabolism, and ABC transporters (**Figure 3C** and **Table 1**).

Acetate is produced in the modification stage of heparan sulfate biosynthesis, and its content in WL cecal content was 1.56 times that in SF, providing a substrate for WL energy metabolism. Propanoate, as an important product of propanoate metabolism and nicotinate and nicotinamide metabolism, that could provide ample energy to the body and basic substances for acetic acid metabolism, was significantly enriched in WL. Deoxyuridine, L-Proline, and spermidine are ABC transporters that are mainly responsible for transporting minerals, organic ions, and phosphate. Their concentrations increased significantly in WL, indicating that ABC transporters transported more ions and phosphates in WL, ensuring nutrition supply.

Volicitin is a fatty acid-amino acid conjugate obtained after the decomposition of α-linolenic acid. The content of volicitin in SF was 12.54 times that in WL, indicating that fatty acid was decomposed more in SF, while methyl jasmonate, one of the final products of α-linolenic acid metabolism, was relatively low in SF, indicating that fatty acids might conjugatively accumulate during metabolism. 6-Hydroxypseudooxynicotine, a compound of nicotine, in SF was 1.71 times that in WL and played a significant role in nicotinate and nicotinamide metabolism.

## Differentially expressed genes in White Leghorn and Silky Fowl hosts

There was a clear difference in the cecum gene expression profile between WL and SF, as revealed by the PCA plot

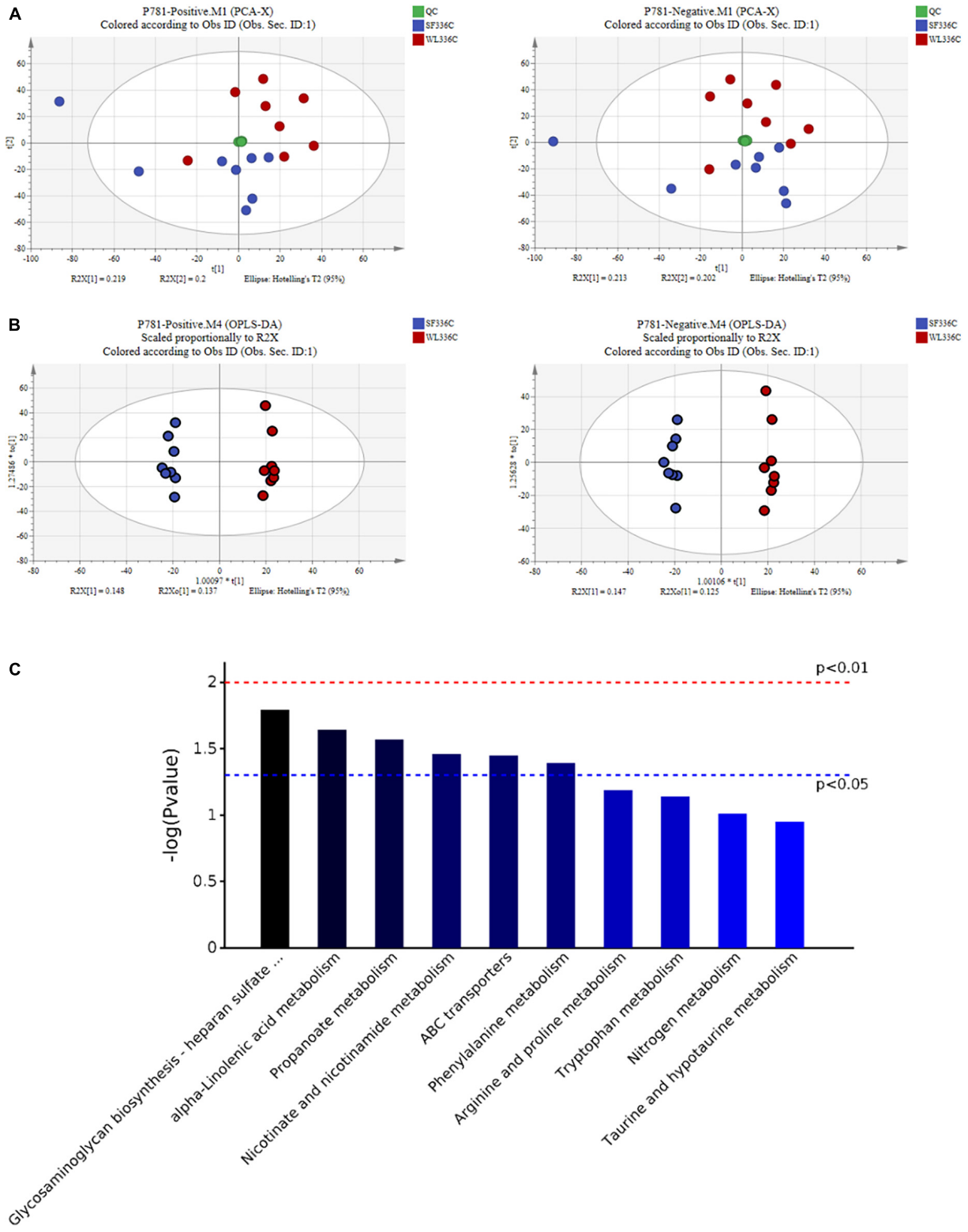


FIGURE 3
The pattern discriminant and composition description of cecal metabolites in WL and SF. **(A)** PCA pattern recognition in positive ion (ESI +) and negative ion (ESI-) mode. **(B)** Discrimination of OPLS-DA mode in positive ion (ESI +) and negative ion (ESI-) mode. **(C)** The Bar chart of differential metabolic pathways.

TABLE 1 Differential metabolites that mapped to KEGG pathways.

| Metabolites name | Metabolites ID | Log2FoldChange | *P*-value (SF/WL) | VIP | KEGG pathway |
|---|---|---|---|---|---|
| Acetate | POSM0714 | −0.6406 | 0.04554 | 1.3226 | Glycosaminoglycan biosynthesis—heparan sulfate/heparin<br>Propanoate metabolism |
| Methyl jasmonate | NEGM0467 | −0.4540 | 0.04338 | 1.3943 | Alpha-Linolenic acid metabolism |
| Volicitin | NEGM0675<br>POSM0009 | 3.6490 | 0.00149<br>0.00145 | 1.8561<br>1.8450 | Alpha-Linolenic acid metabolism |
| Propanoate | NEGM0440<br>POSM0593 | −0.9790 | 0.00453<br>0.00437 | 1.6511<br>1.6651 | Propanoate metabolism<br>Nicotinate and nicotinamide metabolism |
| 6-Hydroxypseudooxynicotine | NEGM0277 | 0.7713 | 0.03439 | 1.4373 | Nicotinate and nicotinamide metabolism |
| Deoxyuridine | NEGM0253<br>POSM0421 | −1.0769 | 0.04252<br>0.04696 | 1.4041<br>1.3637 | ABC transporters |
| L-Proline | POSM0267 | −0.8134 | 0.035234 | 1.3343 | ABC transporters |
| Spermidine | NEGM0007 | −0.6982 | 0.01387 | 1.5735 | ABC transporters |
| Phenylpropanoate | NEGM0692<br>POSM0580 | −1.3395 | 0.03302<br>0.03331 | 1.3664<br>1.3591 | Phenylalanine metabolism |
| D-Cathinone | NEGM0408 | 0.8979 | 0.03081 | 1.4791 | Phenylalanine metabolism |

(Figure 4A) and volcano plot (Figure 4B). A total of 397 DGEs were identified in the cecum tissue between the groups, including 232 upregulated and 165 downregulated genes in the SF group relative to those in the WL group (Supplementary Table 2).

In SF, GO clustering analysis showed that the upregulated DEGs were related to melanin biosynthetic process, melanin metabolic process, and pigment biosynthetic process (Figure 4C). In WL, the upregulated genes were related to RNA-DNA hybrid ribonuclease, endoribonuclease, endonuclease, and peptidase activity (Figure 4D).

The upregulated genes in the SF group were found to be involved in tyrosine metabolism, sphingolipid metabolism, fatty acid biosynthesis, fatty acid degradation, melanogenesis, and PPAR signaling pathway (Figure 4E); whereas those in the WL were involved in histidine metabolism, glycosphingolipid biosynthesis-lacto, and neolacto series, primary bile acid biosynthesis, mannose type O-glycan biosynthesis, starch and sucrose metabolism, fructose and mannose metabolism, and N-glycan biosynthesis (Figure 4F).

## Redundancy analysis and biological relationships integration

RDA was used to determine the relationship between cecal microbiota and metabolites. It showed that *Bacteroides* in WL was positively correlated with acetate ($P < 0.05$), propanoate ($P < 0.05$), and spermidine ($P < 0.05$) (Figure 5A and Supplementary Table 3); while in SF, *Parabacteroides,* and *Veillonellaceae* were positively correlated with volicitin ($P < 0.05$), 6-hydroxypseudooxynicotine, and D-cathinone. These results indicated that *Bacteroidetes* in the WL cecum affected metabolite production of glycosaminoglycan biosynthesis, propanoate metabolism, and ABC transporter pathways, promoting energy generation and ion transport. Bacteria in the SF cecum increased the concentrations of metabolites from α-linolenic acid metabolism, phenylalanine metabolism, and nicotinate and nicotinamide metabolism, which resulted in fatty acid accumulation.

By exploring the correlation between metabolites and DEGs using RDA (Figure 5B and Supplementary Table 4), we found that genes related to melanin production (*MLANA*, *TYRP1*, *PMEL*, *DCT*, and *OCA2*) in SF were positively correlated with *CRP*, which was positively related to 6-hydroxypseudooxynicotine ($P < 0.05$) and D-cathinone ($P < 0.05$). The upregulation of melanin production genes in SF was in accordance with the upregulation of *CRP*. Volicitin ($P < 0.05$) was positively correlated with *ACSBG1*, and increased concentration of the fatty acid metabolite volicitin resulting in a significant upregulation of *ACSBG1* in the PPAR signaling pathway, which improved the level of fatty acid metabolism and immune response.

In WL, *PFKFB4*, *MGAT5B*, *EDA2R*, and *PDSS2* were positively correlated with acetate ($P < 0.05$); *SMAD1*, *NUDT1*, *AKR1D1*, and *SGCZ* were positively correlated with phenylpropanoate, deoxyuridine, L-proline, methyl jasmonate, propanoate ($P < 0.05$), and spermidine ($P < 0.05$). The increase in acetate concentration in WL cecum may cause the upregulation of glucose metabolism-related genes *PFKFB4* and *MGAT5B*, and the improvement in energy metabolism and ion transport could promote the upregulation of egg production-related genes *AKR1D1* and *EDA2R*.

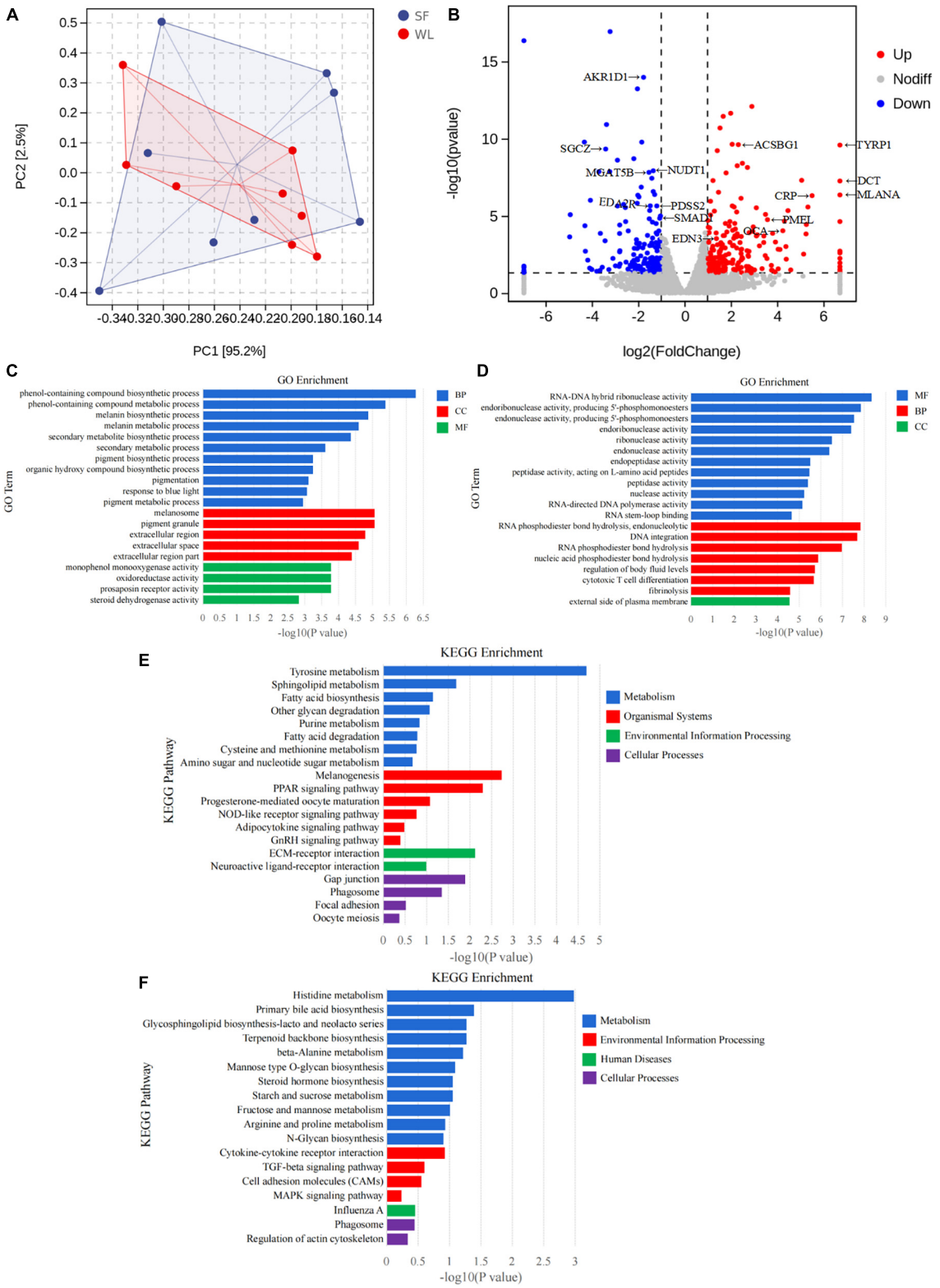


FIGURE 4
Differential characterization of cecum gene expression in hosts. **(A)** PCA map of cecal intestinal wall transcriptome. **(B)** Differential expression volcano plot in the cecal transcriptome of SF and WL. **(C)** GO classification of the up-regulated expressed genes of SF. **(D)** GO classification of the up-regulated expressed genes of WL. **(E)** KEGG classification of the up-regulated expressed genes of SF. **(F)** KEGG classification of the up-regulated expressed genes of WL.

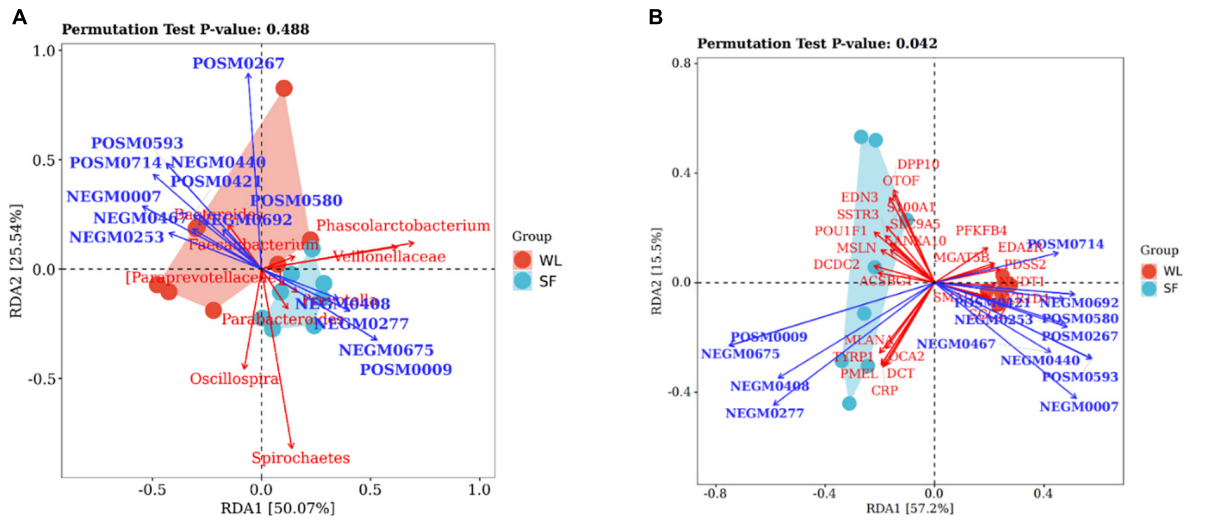


FIGURE 5
Redundancy analyses between microbes, metabolites and DEGs. **(A)** RDA between significantly different microbe and metabolite. **(B)** Relationship between metabolite and DEGs by RDA. Brackets are used to emphasize that this is the official Greengenes database recommended taxonomic information, there may be corrections or improvements to this taxonomic information.

The sources of 271 metabolites with significant differences were obtained using MetOrigin analysis (**Figures 6A,B**), of which 17 were microbial metabolites, 25 were co-metabolites of microbe and hosts, and 229 were other metabolites (53 drug-related, 42 food-related, and 134 unknown). Simultaneously, the metabolites with significant upregulation difference in WL and SF were also traced, the proportion of drug-related metabolites was larger in SF (29.13%) than in WL (13.69%) (**Supplementary Figure 2**). The obtained metabolites were compared with the KEGG database, and the metabolic pathways of bacterial metabolism and co-metabolism were determined (**Supplementary Figure 3**). Tryptophan metabolism, arginine and proline metabolism, nicotinate, and nicotinamide metabolism, tyrosine metabolism, and pyruvate metabolism were significantly different co-metabolism pathways. At the genus level, the Spearman correlation analysis between bacteria and metabolites is shown in **Supplementary Figure 4**, in which *Bacteroides* was significantly positively correlated with propanoic acid, while *Parabacteroides* was significantly positively correlated with volicitin.

Using metabolite traceability to determine bacterial flora participating in metabolic reactions, *Bacteroides* participated in pyruvate metabolism, propanoate metabolism, arginine and proline metabolism, and glycolysis/gluconeogenesis pathways, which significantly positively promoted the metabolites acetic acid, propanoic acid, L-proline, and spermidine, while *Parabacteroides* was significantly negatively correlated with the metabolites acetic acid and propanoic acid in pyruvate metabolism, propanoate metabolism, and glycolysis/gluconeogenesis pathways (**Figure 6C** and **Supplementary Figure 5**). Finally, more specific information on the metabolic changes of genus-level bacteria and metabolites was displayed through microbial-metabolite association network (**Figure 6D**). The co-metabolism network of the seven metabolic pathways showed that eight metabolites were associated with five differential bacteria ($p < 0.01$).

## Discussion

The cecum is thought to be the main site of fermentation. Cecal microbes play a crucial role in decomposing complex polysaccharides, such as uric acid, starch, and cellulose (Stanley et al., 2014). As chickens age, their gut microbial populations became more complex (Wielen et al., 2002). The α-diversity results showed that SF had a higher microbial community richness and unclassified bacteria than did WL. The complex microbiome indicated that SF had better adaptability and immunity to the environment. We found that the dominant phyla in WL and SF cecum were Bacteroidetes and Firmicutes. Consistent with our research, Pandit et al. (2018) found the same cecal microbiota in chickens, which typically accounted for more than 80% of the total number of microorganisms detected. Both Firmicutes and Bacteroidetes participate in fermentation, provide nutrition for the host (Gillilland et al., 2012), and are related to the metabolism of short-chain fatty acids (SCFAS), which not only inhibit the growth of pathogenic bacteria but also provide energy for the host (Józefiak et al., 2004). Studies have reported that a higher Firmicutes/Bacteroides ratio is associated with human obesity (Mariat et al., 2009), while the reverse is related to weight loss (Ley et al., 2005, 2006). Firmicutes promoted the synthesis of butyric as well

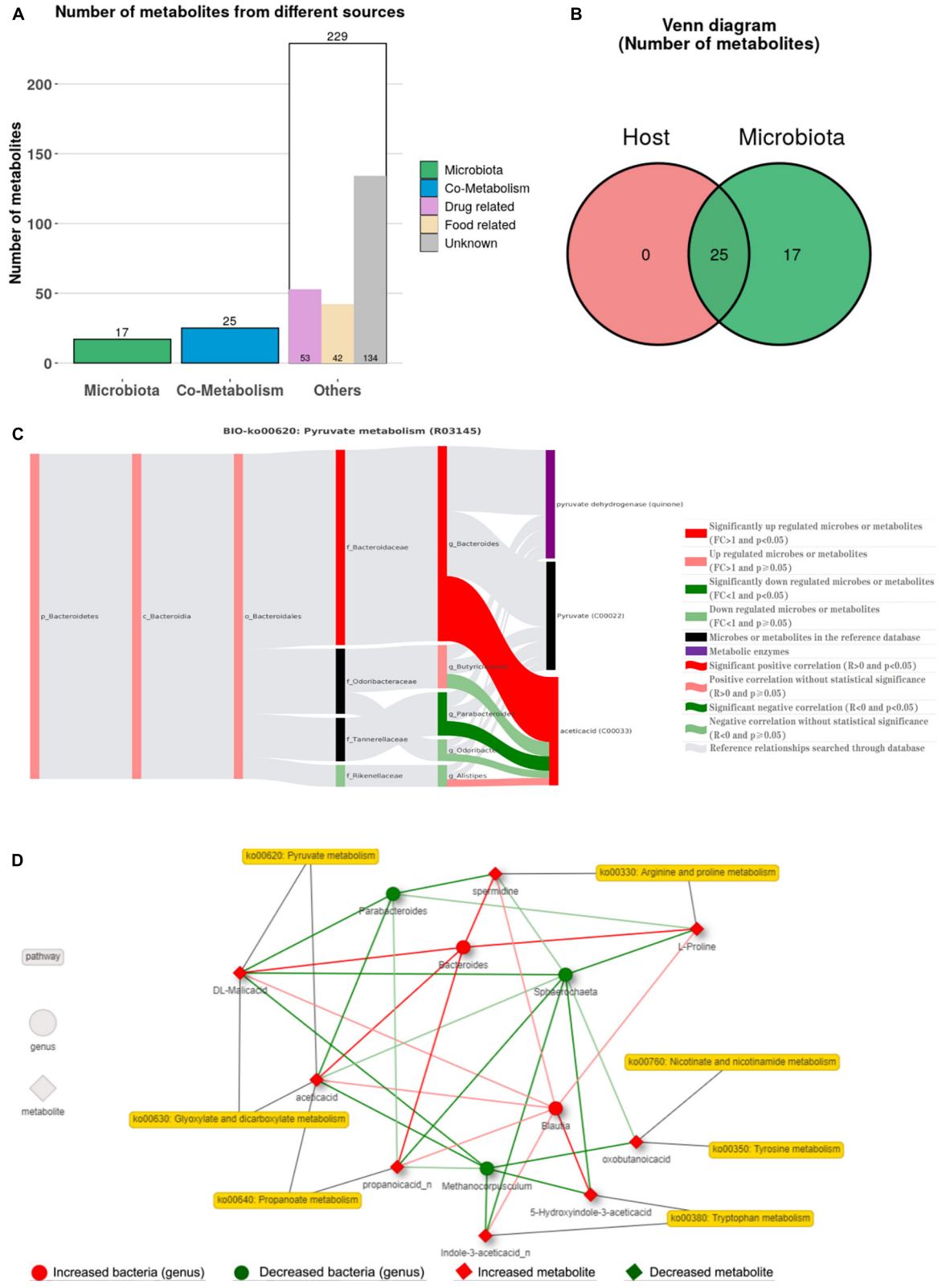


FIGURE 6
Correlation analyses between microbes and metabolites. **(A)** Venn diagram of the number of metabolic pathways in microbial community. **(B)** Venn diagram of the number of metabolites in bacterial communities. **(C)** The BIO-Sankey Network for R03145 metabolic reaction in pyruvate metabolism. **(D)** Network summary of co-metabolism pathways between metabolites and microbes.

as propionic acid and energy harvesting by improving lipid metabolism (Turnbaugh et al., 2008), while Bacteroidetes is usually associated with the degradation of polysaccharides (Degnan et al., 1997) and propionic acid synthesis (Polansky et al., 2016; Saxena et al., 2016) and promote energy metabolism by increasing carbohydrate metabolism (Kim J. K. et al., 2015). This was consistent with our study showing that the cecum of WL contained more Bacteroides and produced more propionic acid, which affected gluconeogenesis in the liver and glucose supply to the body. In chickens, Spirochetes can be potentially pathogenic, resulting in delayed and reduced egg production, slower growth, or diarrhea (Dwars et al., 2007). The disease can also be passed to offspring, making chicks weak, slow-growing, and having impaired gastrointestinal function (Smit et al., 2007). This was consistent with our study in that the content of Spirochetes in the cecum of SF was higher, which might be one of the reasons for their decline in egg production.

*Porphyromonadaceae*, *Veillonellaceae*, and *[Paraprevotellaceae]* were the dominant bacteria in SF. The *Veillonellaceae* family can utilize lactic acid and/or succinic acid to produce propionic acid, butyric acid, and/or valeric acid. *Veillonellaceae* was reported to produce high levels of SCFAS (acetate and propionate) (Lecomte et al., 2015). Cheng et al. (2018) found that the increased abundance of *Prevotellaceae* and *Veillonellaceae* caused glucose intolerance and insulin resistance in mice and that *Veillonellaceae* was related to serum insulin concentration. *Paraprevotellaceae* is associated with fatty acid synthesis and has positive health effects (Round and Mazmanian, 2009). Moreover, the abundance of *Paraprevotellaceae* and *Veillonellaceae* is involved in several functions and different pathways, including metabolic, protective, structural, and histological functions (Gallè et al., 2020). *Bacteroides* can produce carbohydrate metabolism-related enzymes, vitamins, glycans, and cofactor enzymes to promote food digestion (Karlsson et al., 2011). *Bacteroides* more prominently colonized chicken cecum's with better growth performance, which indicated a closer relationship with glycan metabolism, while in chickens with worse growth performance, it was closely related to lipid metabolism (Cui et al., 2021). *Faecalibacterium* plays an important role in host physiology and health. Some studies have reported that *F. prausnitzii* has an anti-inflammatory activity and can secrete active anti-inflammatory substances, regulate its host immune response, and alleviate intestinal inflammation (Lopez-Siles et al., 2011; Heinken et al., 2014). The main metabolic end products of *Parabacteroides* are acetic acid and succinic acid, which are beneficial to the body. Wu et al. (2018) showed that the rate of increase of the body weight of high-fat diet mice was slower after oral administration of living *P. glosteinii* than that of the control group, and the amount of visceral fat, insulin resistance index, pro-inflammatory cytokines, serum endotoxin level, and intestinal permeability decreased. Therefore, *Parabacteroides* can enhance intestinal integrity, reduce inflammation, and potentially treat obesity (Wang et al., 2019). Based on the MetaCyc, KEGG, and COG databases, we found that metabolic pathways, such as glycan biosynthesis and metabolism, carbohydrate decomposition and metabolism, and glycan degradation, were highly enriched in WL, indicating that the bacterial community in WL was highly efficient in degrading carbohydrates and might produce more hydrolysates and energy. Compared to WL, SF were highly enriched in metabolic pathways, such as the TCA cycle, fatty acid and lipid metabolism, lipid transport and metabolism, and xenobiotics biodegradation, indicating that microorganisms in SF have an impact on lipid metabolism and immune metabolism.

The intestinal flora is an important metabolic "organ" in animals and can affect the overall metabolism of the host. There is a process of "co-metabolism" between the host and the flora. If the structure of intestinal flora changes, the physiological metabolism of the host would change correspondingly (Lee and Hase, 2014). The acetic acid and propionic acid contents were high in WL. The microorganisms in WL can fully digest the active ingredients in feed and degrade them to pyruvate, ultimately producing more volatile fatty acid. Acetic acid is a SCFA with the highest concentration in the body and is the center of the carbohydrate pathway. Acetic acid is an important source of host energy, providing approximately 10% of the total daily energy of the body (Cronin et al., 2021). Propionic acid is the main precursor of glucose synthesis in animals, which is conducive to the supply and transformation of energy and provides energy to the body. After being absorbed into the blood, propionic acid is catabolized and metabolized in the liver and participates in the process of reversing pyruvate into glucose, while possibly inhibiting the synthesis of fat (den Besten et al., 2013). Pyruvate enrichment increased the mutual conversion of fat, carbohydrate, and protein, thereby providing the body with more energy, which provided sufficient energy for egg laying. Acetic acid and propionic acid can also play antibacterial roles by promoting the release of host antimicrobial peptides. In addition, the content of deoxyuridine, L-proline, and spermidine in WL was increased, and the ABC transporter pathway enriched in these metabolites played an important role as a multifunctional transmembrane protein in cell osmotic pressure regulation (Jaskulak et al., 2020). SF contained high levels of 6-hydroxypseudooxynicotine, which is involved in the metabolism of nicotinic acid, a B vitamin derived from the synthesis of gut microbes and directly supplied to feed, and was essential for animal growth and development. Nicotinate and nicotinamide participated in lipid metabolism (Kamanna and Kashyap, 2008) and reduced abdominal fat (Jiang et al., 2014), which may increase lipid degradation and metabolism in chickens.

Melanin biosynthesis and metabolism in SF are important pathways related to immunity. Melanin had antioxidant (Liu et al., 2011), anti-virus (Manning et al., 2003), and gastrointestinal health- modulation effects

(El-Obeid et al., 2016). In addition, melanin can also interact with the immune system in a variety of ways, such as by improving the efficacy of antibiotics (Fukuda and Sasaki, 1990), inhibiting inflammation (Nosanchuk and Casadevall, 2003), and enhancing various immune parameters (Pugh et al., 2005). *CRP* was highly expressed in SF, which could play an important role in the natural immunity of the body by enhancing phagocytosis and eliminating necrotic tissue cells (Sproston and Ashworth, 2018). The PPAR metabolism pathway is involved in lipogenesis (Cristancho and Lazar, 2011), fatty acid metabolism (Grygiel-Gorniak, 2014), and immune response (Christofides et al., 2021), and maintains metabolic homeostasis. *ACSBG1* was highly expressed in SF and participates in the PPAR metabolic pathway (Gharib-Naseri et al., 2021), which affects fatty acid metabolism and degradation in animals. *AKR1D1* was highly expressed in WL, promoting bile acid metabolism (Chaudhry et al., 2013) and follicular growth (Zhang et al., 2016), and *EDA2R*, a significantly upregulated gene in WL, was confirmed to be related to egg production (Chen et al., 2021), which may promote egg production in WL. The expression level of *PFKFB4* increased in WL, participating in glucose metabolism and providing energy to the body (Shen et al., 2021), which might be conducive to egg production. *MGAT5B* participates in the metabolic pathway of N-Glycan biosynthesis, and N-glycation modification plays an important role in protein folding, transportation, and other processes (Liu et al., 2014).

The cecal microbes in chicken are closely related to the feeding, health status, and metabolism of their host. However, few studies have explored the relationship among microbial taxa, metabolites, and gene transcription levels. Therefore, how cecal microorganisms interact with their metabolites and gene transcription levels at a deeper taxonomic level remains unclear. In this study, the relative abundances of microorganisms and host cecum genes were analyzed for their correlations with cecal metabolites. We found that the metabolites acetate, propanoate, L-proline, and spermidine were positively correlated with *Bacteroides* in WL. *Bacteroides* played a major role in glycosaminoglycan biosynthesis, propanoate metabolism, and the ABC transporter pathway. Adamberg et al. (2014) showed that acetic acid and propionic acid are fermentation products of *Bacteroides*. Acetate and propionate, the metabolites of *Bacteroides*, had positive effects on glycolysis and propionate metabolism. Genes related to acetic acid and propionic acid were significantly upregulated in the cecum transcriptome. The increase in acetic acid and propionic acid contents enhanced carbohydrate metabolism and provided more energy for the body, which may support an increase in egg production. SF and WL had different dominant bacteria, and the unique bacteria in SF impacted the metabolite acetic acid, inhibiting the production of volatile fatty acids, and had no positive effect on egg production. The metabolites volicitin, 6-hydroxypseudooxynicotine, and D-cathinone were positively correlated with *Parabacteroides* and *Veillonellaceae* in SF. There was a significant positive correlation between *Parabacteroides* and volicitin, which is a metabolite of the α-linolenic acid metabolism pathway, increasing lipid deposition, which occurred in the submucosa of SF cecum. RNA-seq results also showed that melanin production, lipid metabolism, and immune-related genes were significantly upregulated, regulating lipid metabolism, participating in the immune response, and maintaining homeostasis.

## Conclusion

In this study, multi-omics was used to comprehensively analyze differences in cecal metabolism between SF and WL under the same environmental conditions. The high enrichment of *Bacteroidetes* in WL supplied more amino acids and energy to the body and provided the basis for the sustained high level of egg production. *Veillonellaceae* and *Parabacteroides* were enriched in SF, which regulated lipid metabolism and participated in the immune response. This study provides insights into strategies for altering the cecal microbiota to achieve higher egg production and better immunity through feeding management or genetic selection.

## 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

This animal study was reviewed and approved by the Laboratory Animal Welfare Experiment License from China Agricultural University was obtained (permit number: SKLAB-2012-04-07). All experiments were approved by the Committee on the Animal Experimental Ethical Inspection of China Agricultural University (issue number: AW32802202-1-1).

## Author contributions

XD and XY conceived the project and designed the experiments. XY, YT, YM, and JL collected the samples for 16S rDNA gene amplicon sequencing, untargeted metabolomics and RNA-seq, and determined phenotypic index. XY and DH planned the data analyses and oil red O staining. XY wrote the manuscript. DH and XD revised the manuscript. XD supervised

the work and edited manuscripts. 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/fmicb.2022.984654/full#supplementary-material

SUPPLEMENTARY FIGURE 1
Response sequence test analysis and volcano plot of differential metabolite in positive ion and negative ion mode.

SUPPLEMENTARY FIGURE 2
Bar plot and Venn diagram of the number of metabolites in WL and SF separately in different categories.

SUPPLEMENTARY FIGURE 3
The metabolic pathways of bacterial metabolism and cometabolism pathways with the host.

SUPPLEMENTARY FIGURE 4
The Spearman correlation analysis between bacteria at genus level and metabolites.

SUPPLEMENTARY FIGURE 5
The BIO-Sankey network for several metabolic reactions in significantly different metabolism pathways.

## References

Adamberg, S., Tomson, K., Vija, H., Puurand, M., Kabanova, N., Visnapuu, T., et al. (2014). Degradation of Fructans and Production of Propionic Acid by *Bacteroides* thetaiotaomicron are Enhanced by the Shortage of Amino Acids. *Front. Nutr.* 1:21. doi: 10.3389/fnut.2014.00021

Allonby, J. I. H., and Wilson, P. B. (eds) (2018). *British Poultry Standards*, 7th Edn. Hoboken: Wiley.

Binek, M., Cisek, A. A., Rzewuska, M., Chrobak-Chmiel, D., Stefanska, I., and Kizerwetter-Swida, M. (2017). Chicken intestinal microbiome: Development and function. *Med. Weter* 73, 618–625. doi: 10.21521/mw.5790

Bjerrum, L., Engberg, R. M., Leser, T. D., Jensen, B. B., Finster, K., and Pedersen, K. (2006). Microbial community composition of the ileum and cecum of broiler chickens as revealed by molecular and culture-based techniques. *Poult. Sci.* 85, 1151–1164. doi: 10.1093/ps/85.7.1151

Bolyen, E., Rideout, J. R., Dillon, M. R., Bokulich, N. A., Abnet, C. C., Al-Ghalith, G. A., et al. (2019). Author Correction: Reproducible, interactive, scalable and extensible microbiome data science using QIIME 2. *Nat. Biotechnol.* 37:1091. doi: 10.1038/s41587-019-0252-6

Callahan, B. J., Mcmurdie, P. J., Rosen, M. J., Han, A. W., Johnson, A. J. A., and Holmes, S. P. (2016). DADA2: High-resolution sample inference from Illumina amplicon data. *Nat. Methods.* 13, 581–583. doi: 10.1038/nmeth.3869

Caporaso, J. G. (2019). Reproducible, interactive, scalable and extensible microbiome data science using QIIME 2. *Nat. Biotechnol.* 37, 852–857. doi: 10.1038/s41587-019-0209-9

Chaudhry, A. S., Thirumaran, R. K., Yasuda, K., Yang, X., Fan, Y., Strom, S. C., et al. (2013). Genetic Variation in Aldo-Keto Reductase 1D1 (AKR1D1) Affects the Expression and Activity of Multiple Cytochrome P450s. *Drug Metab. Dispos.* 41, 1538–1547. doi: 10.1124/dmd.113.051672

Chen, X., Sun, X., Chimbaka, I. M., Qin, N., Xu, X., Liswaniso, S., et al. (2021). Transcriptome Analysis of Ovarian Follicles Reveals Potential Pivotal Genes Associated With Increased and Decreased Rates of Chicken Egg Production. *Front. Genet.* 12:622751. doi: 10.3389/fgene.2021.622751

Chen, X., Zhao, W., Liu, Y., Demelash, N., Sun, Z., Zhang, X., et al. (2019). Caecal microbiome and metabolites associated with different growth performances of broilers. *Indian J. Anim. Res.* 55, 109–114. doi: 10.18805/ijar.B-1062

Cheng, J., Xue, F., Zhang, M., Cheng, C., Qiao, L., Ma, J., et al. (2018). TRIM31 Deficiency Is Associated with Impaired Glucose Metabolism and Disrupted Gut Microbiota in Mice. *Front. Physiol.* 9:24. doi: 10.3389/fphys.2018.00024

Christofides, A., Konstantinidou, E., Jani, C., and Boussiotis, V. A. (2021). The role of peroxisome proliferator-activated receptors (PPAR) in immune responses. *Metabolism* 114:154338. doi: 10.1016/j.metabol.2020.154338

Collins, S. L., and Patterson, A. D. (2020). The gut microbiome: An orchestrator of xenobiotic metabolism. *Acta Pharm. Sin. B* 10, 19–32. doi: 10.1016/j.apsb.2019.12.001

Cristancho, A. G., and Lazar, M. A. (2011). Forming functional fat: A growing understanding of adipocyte differentiation. *Nat. Rev. Mol. Cell Biol.* 12, 722–734.

Cronin, P., Joyce, S. A., O'Toole, P. W., and O'Connor, E. M. (2021). Dietary Fibre Modulates the Gut Microbiota. *Nutrients* 13:1655. doi: 10.3390/nu13051655

Cui, L., Zhang, X., Cheng, R., Ansari, A. R., Elokil, A. A., Hu, Y., et al. (2021). Sex differences in growth performance are related to cecal microbiota in chicken. *Microb. Pathog.* 150:104710. doi: 10.1016/j.micpath.2020.104710

Degnan, B. A., Macfarlane, S., Quigley, M. E., and Macfarlane, G. T. (1997). Starch Utilization by *Bacteroides* ovatus Isolated from the Human Large Intestine. *Curr. Microbiol.* 34, 290–296. doi: 10.1007/s002849900184

den Besten, G., Lange, K., Havinga, R., van Dijk, T. H., Gerding, A., van Eunen, K., et al. (2013). Gut-derived short-chain fatty acids are vividly assimilated into host carbohydrates and lipids. *Am. J. Physiol. Gastrointest. Liver Physiol.* 305:G900–G910. doi: 10.1152/ajpgi.00265.2013

Diaz Carrasco, J. M., Casanova, N. A., and Fernández Miyakawa, M. E. (2019). Microbiota, Gut Health and Chicken Productivity: What Is the Connection? *Microorganisms* 7:374. doi: 10.3390/microorganisms7100374

Dibner, J. J., Knight, C. D., Kitchell, M. L., Atwell, C. A., Downs, A. C., and Ivey, F. J. (1998). Early Feeding and Development of the Immune System in Neonatal Poultry. *J. Appl. Poult. Res.* 7, 425–436. doi: 10.1093/japr/7.4.425

Ding, J., Zhao, L., Wang, L., Zhao, W., Zhai, Z., Leng, L., et al. (2016). Divergent selection-induced obesity alters the composition and functional pathways of chicken gut microbiota. *Genet. Sel. Evol.* 48:93. doi: 10.1186/s12711-016-0270-5

Dwars, R. M., Davelaar, F. G., and Smit, H. F. (2007). Infection of broiler parent hens with avian intestinal spirochaetes: Effects on egg production and chick quality. *Avian Pathol.* 22, 693–701. doi: 10.1080/03079459308418957

El-Obeid, A., Eltahir, K. E. H., Hamid, E., and Haseeb, A. M. (2016). Anti-ulcerogenic effects of Nigella sativa L. melanin. *World J. Pharm. Res.* 5, 1579–1593.

Fukuda, M., and Sasaki, K. (1990). Changes in the Antibacterial Activity of Melanin-Bound Drugs. *Ophthalmic Res.* 22, 123–127. doi: 10.1159/000267011

Gallè, F., Valeriani, F., Cattaruzza, M. S., Gianfranceschi, G., Liguori, R., Antinozzi, M., et al. (2020). Mediterranean Diet, Physical Activity and Gut Microbiome Composition: A Cross-Sectional Study among Healthy Young Italian Adults. *Nutrients* 12:2164. doi: 10.3390/nu12072164

Gharib-Naseri, K., de Las Heras-Saldana, S., Kheravii, S., Qin, L., Wang, J., and Wu, S. (2021). Necrotic enteritis challenge regulates peroxisome proliferator-1 activated receptors signaling and β-oxidation pathways in broiler chickens. *Anim. Nutr.* 7, 239–251. doi: 10.1016/j.aninu.2020.08.003

Gillilland, M. G., Erb-Downward, J. R., Bassis, C. M., Shen, M. C., Toews, G. B., Young, V. B., et al. (2012). Ecological Succession of Bacterial Communities during Conventionalization of Germ-Free Mice. *Appl. Environ. Microb.* 78, 2359–2366. doi: 10.1128/AEM.05239-11

Grygiel-Gorniak, B. (2014). Peroxisome proliferator-activated receptors and their ligands: Nutritional and clinical implications–a review. *Nutr. J.* 13:17. doi: 10.1186/1475-2891-13-17

Han, D., Tai, Y., Hua, G., Yang, X., Chen, J., Li, J., et al. (2021). Melanocytes in black-boned chicken have immune contribution under infectious bursal disease virus infection. *Poult. Sci.* 100:101498. doi: 10.1016/j.psj.2021.101498

Heinken, A., Khan, M. T., Paglia, G., Rodionov, D. A., Harmsen, H. J. M., and Thiele, I. (2014). Functional Metabolic Map of Faecalibacterium prausnitzii, a Beneficial Human Gut Microbe. *J. Bacteriol.* 196, 3289–3302. doi: 10.1128/JB.01780-14

Hill, J. H., and Round, J. L. (2021). SnapShot: Microbiota effects on host physiology. *Cell* 184, 2796–2796. doi: 10.1016/j.cell.2021.04.026

Jaskulak, M., Grobelak, A., and Vandenbulcke, F. (2020). Effects of sewage sludge supplementation on heavy metal accumulation and the expression of ABC transporters in Sinapis alba L. during assisted phytoremediation of contaminated sites. *Ecotox. Environ. Saf.* 197:110606. doi: 10.1016/j.ecoenv.2020.110606

Jha, R., Das, R., Oak, S., and Mishra, P. (2020). Probiotics (Direct-Fed Microbials) in Poultry Nutrition and Their Effects on Nutrient Utilization, Growth and Laying Performance, and Gut Health: A Systematic Review. *Animals* 10:1863. doi: 10.3390/ani10101863

Jiang, R. R., Zhao, G. P., Zhao, J. P., Chen, J. L., Zheng, M. Q., Liu, R. R., et al. (2014). Influence of dietary nicotinic acid supplementation on lipid metabolism and related gene expression in two distinct broiler breeds of female chickens. *J. Anim. Physiol. Anim. Nutr.* 98, 822–829. doi: 10.1111/jpn.12138

Józefiak, D., Rutkowski, A., and Martin, S. A. (2004). Carbohydrate fermentation in the avian ceca: A review. *Anim. Feed Sci. Tech.* 113, 1–15. doi: 10.1016/j.anifeedsci.2003.09.007

Kamanna, V. S., and Kashyap, M. L. (2008). Mechanism of Action of Niacin. *Am. J. Cardiol.* 101:S20–S26. doi: 10.1016/j.amjcard.2008.02.029

Karlsson, F. H., Ussery, D. W., Nielsen, J., and Nookaew, I. (2011). A Closer Look at *Bacteroides*: Phylogenetic Relationship and Genomic Implications of a Life in the Human Gut. *Microb. Ecol.* 61, 473–485. doi: 10.1007/s00248-010-9796-1

Katoh, K., Misawa, K., Kuma, K., and Miyata, T. (2002). MAFFT: A novel method for rapid multiple sequence alignment based on fast Fourier transform. *Nucl. Acids Res.* 30, 3059–3066. doi: 10.1093/nar/gkf436

Kim, J. E., Lillehoj, H. S., Hong, Y. H., Kim, G. B., Lee, S. H., Lillehoj, E. P., et al. (2015). Dietary Capsicum and Curcuma longa oleoresins increase intestinal microbiome and necrotic enteritis in three commercial broiler breeds. *Res. Vet. Sci.* 102, 150–158. doi: 10.1016/j.rvsc.2015.07.022

Kim, J. K., Shin, S., Moon, J. S., Li, L., Cho, S. K., Kim, T., et al. (2015). Isolation of dextran-hydrolyzing intestinal bacteria and characterization of their dextranolytic activities. *Biopolymers* 103, 321–327. doi: 10.1002/bip.22615

Kundu, A., Singh, D. P., and Mohapatra, S. C. (2016). Immunocompetence based scoring index for evaluating disease resistance status in indigenous and exotic breeds of chicken. *Indian J. Anim. Sci.* 86, 861–864.

Lecomte, V., Kaakoush, N. O., Maloney, C. A., Raipuria, M., Huinao, K. D., Mitchell, H. M., et al. (2015). Changes in gut microbiota in rats fed a high fat diet correlate with obesity-associated metabolic parameters. *PLoS One* 10:e0126931. doi: 10.1371/journal.pone.0126931

Lee, W. J., and Hase, K. (2014). Gut microbiota-generated metabolites in animal health and disease. *Nat. Chem. Biol.* 10, 416–424. doi: 10.1038/nchembio.1535

Ley, R. E., Backhed, F., Turnbaugh, P., Lozupone, C. A., Knight, R. D., and Gordon, J. I. (2005). Obesity alters gut microbial ecology. *Proc. Natl. Acad. Sci. U.S.A.* 102, 11070–11075. doi: 10.1073/pnas.0504978102

Ley, R. E., Turnbaugh, P. J., Klein, S., and Gordon, J. I. (2006). Human gut microbes associated with obesity. *Nature* 444, 1022–1023. doi: 10.1038/4441022a

Liu, J. H., Tian, Y. G., Wang, Y., Nie, S. P., Xie, M. Y., Zhu, S., et al. (2011). Characterization and *in vitro* antioxidation of papain hydrolysate from black-bone silky fowl (Gallus gallus domesticus Brisson) muscle and its fractions. *Food Res. Int.* 44, 133–138.

Liu, T., Zhang, S., Chen, J., Jiang, K., Zhang, Q., Guo, K., et al. (2014). The Transcriptional Profiling of Glycogenes Associated with Hepatocellular Carcinoma Metastasis. *PLoS One* 9:e107941. doi: 10.1371/journal.pone.0107941

Lopez-Siles, M., Khan, T. M., Duncan, S. H., Aldeguer Mante, X., Harmsen, H. J. M., Garcia-Gil, L. J., et al. (2011). Gut Environmental Factors May Shape the Persistence of Faecalibacterium Prausnitzii in the Healthy and Diseased Large Intestine. *Gastroenterology* 140:S665. doi: 10.1016/S0016-5085(11)62761-3

Manning, J. T., Bundred, P. E., and Henzi, P. (2003). Melanin and HIV in sub-Saharan Africa. *J. Theor. Biol.* 223, 131–133. doi: 10.1016/s0022-5193(03)00070-5

Mariat, D., Firmesse, O., Levenez, F., Guimarães, V. D., Sokol, H., Doré, J., et al. (2009). The Firmicutes/Bacteroidetes ratio of the human microbiota changes with age. *BMC Microbiol.* 9:123. doi: 10.1186/1471-2180-9-123

Martin, M. (2011). Cutadapt removes adapter sequences from high-throughput sequencing reads. *Embnet J.* 17, 10–12. doi: 10.1089/cmb.2017.0096

Maruvada, P., Leone, V., Kaplan, L. M., and Chang, E. B. (2017). The Human Microbiome and Obesity: Moving beyond Associations. *Cell Host Microbe* 22, 589–599. doi: 10.1016/j.chom.2017.10.005

Mikulski, D., Jankowski, J., Mikulska, M., and Demey, V. (2020). Effects of dietary probiotic (Pediococcus acidilactici) supplementation on productive performance, egg quality, and body composition in laying hens fed diets varying in energy density. *Poult. Sci.* 99, 2275–2285. doi: 10.1016/j.psj.2019.11.046

Nakphaichit, M., Thanomwongwattana, S., Phraephaisarn, C., Sakamoto, N., Keawsompong, S., Nakayama, J., et al. (2011). The effect of including Lactobacillus reuteri KUB-AC5 during post-hatch feeding on the growth and ileum microbiota of broiler chickens. *Poult. Sci.* 90, 2753–2765. doi: 10.3382/ps.2011-01637

Nosanchuk, J. D., and Casadevall, A. (2003). The contribution of melanin to microbial pathogenesis. *Cell. Microbiol.* 5, 203–223. doi: 10.1046/j.1462-5814.2003.00268.x

Pandit, R. J., Hinsu, A. T., Patel, N. V., Koringa, P. G., Jakhesara, S. J., Thakkar, J. R., et al. (2018). Microbial diversity and community composition of caecal microbiota in commercial and indigenous Indian chickens determined using 16s rDNA amplicon sequencing. *Microbiome* 6:115. doi: 10.1186/s40168-018-0501-9

Polansky, O., Sekelova, Z., Faldynova, M., Sebkova, A., Sisak, F., and Rychlik, I. (2016). Important Metabolic Pathways and Biological Processes Expressed by Chicken Cecal Microbiota. *Appl. Environ. Microb.* 82, 1569–1576. doi: 10.1128/AEM.03473-15

Postler, T. S., and Ghosh, S. (2017). Understanding the Holobiont: How Microbial Metabolites Affect Human Health and Shape the Immune System. *Cell Metab.* 26, 110–130. doi: 10.1016/j.cmet.2017.05.008

Price, M. N., Dehal, P. S., and Arkin, A. P. (2010). FastTree 2–approximately maximum-likelihood trees for large alignments. *PLoS One* 5:e9490. doi: 10.1371/journal.pone.0009490

Pugh, N. D., Balachandran, P., Lata, H., Dayan, F. E., Joshi, V., Bedir, E., et al. (2005). Melanin: Dietary mucosal immune modulator from Echinacea and other botanical supplements. *Int. Immunopharmacol.* 5, 637–647. doi: 10.1016/j.intimp.2004.12.011

Round, J. L., and Mazmanian, S. K. (2009). Erratum: The gut microbiota shapes intestinal immune responses during health and disease. *Nat. Rev. Immunol.* 9, 600–600. doi: 10.1038/nri2614

Rubio, L. A., Peinado, M. J., Ruiz, R., Suárez-Pereira, E., Ortiz Mellet, C., and García Fernández, J. M. (2015). Correlations between changes in intestinal microbiota composition and performance parameters in broiler chickens. *J. Anim. Physiol. Anim. Nutr.* 99, 418–423. doi: 10.1111/jpn.12256

Saxena, S., Saxena, V. K., Tomar, S., Sapcota, D., and Gonmei, G. (2016). Characterisation of caecum and crop microbiota of Indian indigenous chicken

targeting multiple hypervariable regions within 16S rRNA gene. *Brit. Poult. Sci.* 57, 381–389. doi: 10.1080/00071668.2016.1161728

Schokker, D., Veninga, G., Vastenhouw, S. A., Bossers, A., de Bree, F. M., Kaal-Lansbergen, L. M. T. E., et al. (2015). Early life microbial colonization of the gut and intestinal development differ between genetically divergent broiler lines. *BMC Genom.* 16:418. doi: 10.1186/s12864-015-1646-6

Shen, C., Ding, L., Mo, H., Liu, R., Xu, Q., and Tu, K. (2021). Long noncoding RNA FIRRE contributes to the proliferation and glycolysis of hepatocellular carcinoma cells by enhancing PFKFB4 expression. *J. Cancer* 12, 4099–4108. doi: 10.7150/jca.58097

Smit, H. F., Dwars, R. M., Davelaar, F. G., and Wijtten, G. A. W. (2007). Observations on the influence of intestinal spirochaetosis in broiler breeders on the performance of their progeny and on egg production. *Avian Pathol.* 27, 133–141. doi: 10.1080/03079459808419314

Sproston, N. R., and Ashworth, J. J. (2018). Role of C-Reactive Protein at Sites of Inflammation and Infection. *Front. Immunol.* 9:754. doi: 10.3389/fimmu.2018.00754

Stanley, D., Hughes, R. J., and Moore, R. J. (2014). Microbiota of the chicken gastrointestinal tract: Influence on health, productivity and disease. *Appl. Microbiol. Biot.* 98, 4301–4310. doi: 10.1007/s00253-014-5646-2

Turnbaugh, P. J., Bäckhed, F., Fulton, L., and Gordon, J. I. (2008). Diet-Induced Obesity Is Linked to Marked but Reversible Alterations in the Mouse Distal Gut Microbiome. *Cell Host Microbe* 3, 213–223. doi: 10.1016/j.chom.2008.02.015

Waite, D. W., and Taylor, M. W. (2014). Characterizing the avian gut microbiota: Membership, driving influences, and potential function. *Front. Microbiol.* 5:223. doi: 10.3389/fmicb.2014.00223

Wang, K., Liao, M., Zhou, N., Bao, L., Ma, K., Zheng, Z., et al. (2019). Para*bacteroides* distasonis Alleviates Obesity and Metabolic Dysfunctions via Production of Succinate and Secondary Bile Acids. *Cell Rep.* 26, 222–235.e5.

Wielen, P. W. J. J., Keuzenkamp, D. A., Lipman, L. J. A., Knapen, F., and Biesterveld, S. (2002). Spatial and Temporal Variation of the Intestinal Bacterial Community in Commercially Raised Broiler Chickens During Growth. *Microb. Ecol.* 44, 286–293. doi: 10.1007/s00248-002-2015-y

Wu, T. R., Lin, C. S., Chang, C. J., Lin, T. L., and Lai, H. C. (2018). Gut commensal Para*bacteroides* goldsteinii plays a predominant role in the anti-obesity effects of polysaccharides isolated from Hirsutella sinensis. *Gut* 68, 248–262. doi: 10.1136/gutjnl-2017-315458

Yan, W., Sun, C., Yuan, J., and Yang, N. (2017). Gut metagenomic analysis reveals prominent roles of Lactobacillus and cecal microbiota in chicken feed efficiency. *Sci. Rep.* 7:45308. doi: 10.1038/srep45308

Yang, L., Liu, S., Ding, J., Dai, R., He, C., Xu, K., et al. (2017). Gut Microbiota Co-microevolution with Selection for Host Humoral Immunity. *Front. Microbiol.* 8:1243. doi: 10.3389/fmicb.2017.01243

Yang, X., Brisbin, J., Yu, H., Wang, Q., Yin, F., Zhang, Y., et al. (2014). Selected Lactic Acid-Producing Bacterial Isolates with the Capacity to Reduce *Salmonella* Translocation and Virulence Gene Expression in Chickens. *PLoS One* 9:e93022. doi: 10.1371/journal.pone.0093022

Yu, G., Xu, C., Zhang, D., Ju, F., and Ni, Y. (2022). MetOrigin: Discriminating the origins of microbial metabolites for integrative analysis of the gut microbiome and metabolome. *iMeta* 1:e10. doi: 10.1002/imt2.10

Zhang, W., Li, X., Liu, H., and Wang, J. (2016). Molecular Cloning,Bioinformatics of the Goose AKR1D1 Gene cDNA Coding Domain Sequence,and Its Differential Expression Profiles in Ovarian Follicles of Different Stages. *J. Sichuan Agric. Univ.* 34, 365–373.

Zheng, D., Liwinski, T., and Elinav, E. (2020). Interaction between microbiota and immunity in health and disease. *Cell Res.* 30, 492–506. doi: 10.1038/s41422-020-0332-7

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# Metagenomic analysis provides bases on individualized shift of colon microbiome affected by delaying colostrum feeding in neonatal calves

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This study investigated the effect of colostrum feeding time on the colon digesta microbiome of 2-day-old dairy calves using whole-genome-based metagenome sequencing, aiming to understand the dynamic changes of the colon microbiome when the colostrum feeding is delayed. In total, 24 male Holstein calves were grouped to different pasteurized colostrum feeding time treatments randomly: TRT0h (45min after birth, $n$=7); TRT6h (6h after birth, $n$=8); and TRT12h (12h after birth, $n$=9). Bacteria, archaea, eukaryotes, and viruses were identified in the colon microbiome, with bacteria (99.20%) being the most predominant domain. *Streptococcus*, *Clostridium*, *Lactobacillus*, *Ruminococcus*, and *Enterococcus* were the top five abundant bacteria genera. For colon microbiome functions, 114 Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways were identified, with nutrients metabolism-related functions "carbohydrate metabolism," "amino acid metabolism," "metabolism of cofactors and vitamins," "metabolism of terpenoids and polyketides," and "metabolism of other amino acids" being the top five secondary level of KEGG hierarchy functions. When colon microbiomes were compared, they were not affected by delaying first colostrum feeding at both taxonomic and functional levels. However, distinct clusters of colon microbiome profiles were shown based on PERMANOVA analysis despite of different colostrum feeding treatment, suggesting the individualized responses. Moreover, the relative abundance of microbial taxa, microbial functions, and differentially expressed genes was compared between the two distinct clusters, and different relationships were observed among host differentially expressed genes, differential levels of microbial taxa, and microbial functions between the two clusters. Our results suggest that the host may play an important role in shaping the colon microbiome of neonatal dairy calves in response to the

early life feeding management. Whether the observed colon microbiome shifts affect gut health and function in the long term requires further research.

KEYWORDS

delayed colostrum, neonatal calf, colon microbiome, metagenome, individual variance

# Introduction

Calves are born without passive immunity because the maternal placenta separates the blood supply between the dam and fetus during pregnancy (Godden, 2008). Therefore, feeding immunoglobulin-rich colostrum, which is the first milking after calving, is of great importance to neonatal calves. Colostrum contains numerous nutrients, bioactive factors, and immunoglobulins, which play a fundamental role in ensuring the survival and passive immunity of newborn calves (Lopez and Heinrichs, 2022). The apparent efficiency of absorption (AEA) of immunoglobulins decreased from 30.5% to 15.8% when calves were fed colostrum within 1 h compared to those fed 12 to 18 h after birth (Osaka et al., 2014). More recently, Fischer et al. (2018) reported that delaying colostrum feeding from 1 to 12 h results in a decrease in AEA from 51.8% to 35.1%, further corroborating that calves should be fed colostrum immediately after birth.

Newborn calves have an immature GIT at birth, which is quickly colonized by maternal and environmental microbiota in the intestinal tract (DiGiulio, 2012; Aagaard et al., 2014; Weese and Jelinski, 2017; O'hara et al., 2020). Colostrum also contains vast variety of microbe that could affect early intestinal microbial colonization of neonatal calves (Yeoman et al., 2018). However, the pasteurization process is commonly used which could eliminate most of the bacteria in the colostrum. It has been suggested that delaying pasteurized colostrum feeding also influences plasma parameters (Hammon et al., 2000), ileum and colon mucosa-associated bacterial groups (Fischer et al., 2018; Ma et al., 2019), as well as microbial profiles and functions in the ileal digesta of neonatal calves (Song et al., 2021). To date, previous studies have mainly focused on the effect of colostrum feeding time on the transfer of passive immunity, as well as its impact on small intestinal microbial and host functional development of neonatal calves. Few attempts have been made to understand how colostrum feeding time influences the large intestinal digesta-associated microbial colonization and function.

For neonatal calves, colon is the major site of microbial colonization before ruminal development (Song et al., 2018), additionally, colon is related to host energy metabolism, health, immune system modulation, and physiological development (He et al., 2018; Song et al., 2018; Ma et al., 2019; Guo et al., 2021). Recent studies have determined the dynamic changes of the colon bacterial community of Holstein and crossbred calves in early life. Dias et al. (2018) found the relative abundance of *Oscillospira* and *Ruminococcus* genera in the colon remained high and unchanged during the preweaning period of crossbred calves. While Song et al. (2018) reported that digested-associated *Lactobacillus* and *Bacteroides* were the most predominant bacteria in the colon of Holstein dairy claves. Additionally, the effects of pre-weaning feeding management on programming colon microbial colonization have also been reported. It was suggested that the supplementation of low concentrations of antibiotics (Yousif et al., 2018), butyrate-fortified milk replacer (O'hara et al., 2018), as well as direct-fed microbials in the milk replacer, influence the colonization of digesta-associated microbiota in the colon (Fomenky et al., 2018; Samarasinghe et al., 2021). However, these studies only focused on bacterial compositional changes, and the functional shift of the colon microbiome in response to colostrum feeding management in neonatal calves has not been studied. Therefore, the objectives of this study were to characterize colon microbial composition and functional changes under different colostrum feeding time using metagenomic analysis, and to evaluate whether the colonic microbiome shift could be affected by individual differences of neonatal calves.

# Materials and methods

## Calves and colostrum feeding

The animal trail was conducted at the research farm (Dairy Research and Technology Centre) of the University of Alberta (Edmonton, Canada), following the procedures provided by The Livestock Care Committee of the University of Alberta (AUP00001595). In total, 24 Holstein bull calves were selected for the study, with birth body weights (BW) ranging from 35 to 50 kg. All the calves were removed from the dam immediately after birth to avoid contamination. Calves were then dried, ear tagged, treated with navel disinfectant, and housed in individual hutches with straw bedding.

All calves were randomly grouped into one of three treatments: colostrum feeding at 45 min (TRT0h, $n = 7$), 6 h (TRT6h, $n = 8$), or 12 h (TRT12h, $n = 9$) after birth. The pasteurized colostrum (62 g of IgG per L; Saskatoon Colostrum Company Ltd.; Saskatoon, SK, Canada) was fed to the calves at a rate of 7.5% BW at their respective time point. Then, all the calves received milk replacer meals (protein: 26%, fat: 18%; Excel Pro-Gro Calf Milk

Replacer, Grober Nutrition, Cambridge, ON, Canada) at 2.5% BW every 6 h of life starting at 12 h after colostrum feeding.

## Colon digesta collection

The sample collection process was the same as described by Fischer et al. (2018). Briefly, all calves were euthanized at 51 h of age followed by colon digesta sample collection. The colon sample segment was defined as 30 cm distal to the ileocecal junction (Malmuthuge et al., 2019). The digesta contents were squeezed out into a 50-ml Falcon tube, and immediately frozen in liquid nitrogen and then stored at −80°C.

## DNA isolation

Genomic DNA was extracted from colon digesta according to the modified DNA extraction method (Yu and Morrison, 2004). Briefly, the frozen falcon tubes with colon digesta (~0.5 g) were thawed at 4°C the day before DNA extraction process. The digesta were resuspended in 1 ml of cell lysis buffer and were subjected to the BioSpec Mini-BeadBeater 8 (BioSpec, Bartlesville, OK), then cells were mechanically disrupted at 4,800 rpm for 3 min. After bead beating, lysed cells were incubated (70°C, 15 min) and the supernatant was collected for Genomic DNA precipitation. After precipitation, the DNA was purified using QIAamp Fast DNA Stool Mini Kit (QIAGEN Inc. CA, United States). DNA quantity and quality were measured using a NanoDrop 1,000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, United States).

## Metagenomic sequencing and analysis of colon microbiome

After DNA extraction, the libraries of each sample for shot-gun whole-genome-based metagenome sequencing were constructed using the TruSeq DNA PCR-free library preparation kit (Illumina, CA, United States) according to the manufacturer's instructions. Then, the quantity of each library was assessed using a Qubit 2.0 Fluorometer (Thermo Fisher Scientific, MA, United States), and sequenced at McGill University and Génome Québec Innovation Centre (Montreal, QC, Canada) using Illumina HiSeq 4000 (2 × 100 bp paired-end reads).

The detailed procedures of colon metagenome data analysis were described by Song et al. (2021). Quality control was performed to remove low-quality bases (quality scores < 30) and short reads (<75 bp) using fastq-mcf (Aronesty, 2013). Then, the eligible reads were mapped to the bovine genome (UMD 3.1) using TopHat 2 (threads:6, version 2.0.9; Kim et al., 2013) to remove host DNA contamination. To evaluate colon microbiome functional profiles, the filtered DNA sequences from each sample was assembled to contigs using MetaVelvet (Namiki et al., 2012) with a kmer size of 51. The identical contigs were binned from the pooled contigs of all the samples, and unique contigs were constructed. Then, Prodigal program was applied for gene prediction on the purpose of identifying protein-coding sequences (Hyatt et al., 2010). Unique predicted genes (≥100 bp) were preserved for the following analysis. The contigs were annotated against the KEGG database (Kanehisa et al., 2012) with UBLAST program (using the following parameters: E-value ≤ 1e − 5, bit score ≥ 60, sequence identity ≥ 30%), and then the qualified reads of each sample were aligned to annotated contigs for functional annotations. The relative abundance of the KEGG pathways of each colon sample was evaluated through the HUMAnN2 program (Abubucker et al., 2012). Meanwhile, the CPM of each pathway was calculated with the following formula: CPM = (number of reads mapped to a gene) ÷ (total number of reads mapped to all annotated genes) × $10^6$. The taxonomic analysis of colon digesta microbiome was performed using Metagenomic Rapid Annotations using Subsystems Technology (MG-RAST) version 3.3.9. The microbial taxa were assigned to phylum, family, and genus levels referencing to Refseq database, following e-value ≤ 1e − 5, identity ≥ 60%, and alignment ≥ 50 bp (Meyer et al., 2008).

## Short chain fatty acids measurement in the colon of newborn calves

Detailed procedures were the same as described previously (Guan et al., 2008). In this study, colon digesta samples (~ 0.1 g) were dissolved in 25% phosphoric acid solutions in a 5 ml tube, at a ratio of 4:1 according to volume. Acetate, propionate, butyrate, isobutyrate, isovalerate, and valerate concentrations were measured using gas chromatography (GC) with Short chain fatty acids (SCFA) concentrations presented as μmol/g fresh weight of digesta sample.

## Colonic transcriptome profiles analysis between calves classified into different groups based on metagenomes

Colon tissue transcriptome profiles were retrieved from our previous publication (He et al., 2018) and the profiles were compared between calves that were grouped based on their metagenomes (Group A and Group B). The levels of expression of genes between groups A and B in the colon tissue were compared. Log2 fold change of each gene was calculated using the equation: log2 fold change = log2(average cpm of Group A/average cpm of Group B). The threshold for differential expressed genes (DE genes) was defined using the following cut-off, with $p < 0.05$, log2 fold change ≤ −1 and ≥1, with positive values indicating upregulated in group A, and negative values indicating downregulated genes in group A. In addition, the unique genes and commonly expressed genes in groups A and B were plotted

with a Venn Diagram. The DE genes and unique genes in Groups A and B were subjected to Annotation, Visualization, and integrated Discovery (DAVID) for up_keywords, gene ontology (Go) terms annotation and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways analysis, with enrichment score > 1, and $p$-value < 0.05 defined as significant.

## Statistical analyses

Microbial taxa, SCFAs concentration and molar proportion, the phenotypic parameters retrieved from our previous studies, including the concentration of plasma glucagon-like peptide (GLP-1), glucagon-like peptide (GLP-2), insulin (Inabu et al., 2018), and serum IgG (Fischer et al., 2018), the copy number of total bacteria, the abundance of *Clostridium* cluster XIVa, *Faecalibacterium prausnitzii*, and *E. coli*, as well as the transcriptomic profiles of colon tissues (He et al., 2018) were analyzed using R version 4.1.3 and SPSS 26.0 packages (IBM Corp., Armonk, NY). Microbial taxa with the relative abundance > 0.05% and present in more than half of the total animals within each treatment (TRT0h, TRT6h, or TRT12h) were considered as detected microbial taxa. Additionally, the metabolic pathways with CPM > 5 in at least 50% of the animals in each treatment were defined as detected microbial functions. The detected microbial taxa and functions were further analyzed in the downstream analysis. To identify the differences of bacterial and archaeal composition, as well as SCFAs concentration and molar proportion among three treatments, the non-parametric Kruskal–Wallis test was applied, and Dunn's test was used to test the difference between any two treatment groups. The $p$-value was adjusted with Benjamini–Hochberg method for false discovery rate (FDR; Yoav and Yosef, 1995), with $p < 0.05$, and $0.05 \leq p < 0.10$ declared as statistical significance and tendency, respectively. Additionally, the abundance of colon microbiome functions was compared using linear discriminant analysis (LDA) effect size (LEfse; Segata et al., 2011), and features with LDA score > 2 and $p < 0.05$ were considered to be significantly different (Mottawea et al., 2016). Bacterial and archaeal general communities among different treatments were analyzed using Bray–Curtis dissimilarity matrices-based principal coordinate analysis (PCoA). Microbial metabolic functions were analyzed with principal component analysis (PCA), and PERMANOVA analysis was conducted to test the statistical difference among treatments. The original phenotypic data from the same animal trial in previous publications were obtained for deep comparison between calves with two type of colon metagenomes (groups A and B) using Welch's $T$-test. Spearman's rank correlation was performed between the varied microbial taxa, functions, and phenotypic parameters (butyrate concentration and DE genes) of groups A and B, with significance declared at $|\rho| > 0.8$ and $p$-value < 0.01.

# Results

## Colon digesta metagenomes of neonatal calves

In total, 744,939,525 reads were generated, and 378,452,001 reads were retained after quality control, with 15,768,833 ± 1,976,165 reads (mean ± SEM) per calf for colon microbiome. After subjecting the predicted genes to the KEGG database for functional annotation, 692,370 genes were successfully annotated. Then, the metagenome of each calf was mapped to the reference comprised of the above-annotated genes to define colon microbiome function. In total, 19.18% ± 5.64% (mean ± SEM) metagenomic sequences of each calf were mapped (Supplementary Dataset 1).

## Microbial functions in the colon of neonatal calves

Among all the generated metabolic pathways using HUMAnN2, 164 KEGG pathways were annotated from the colon metagenomes of neonatal calves. However, 25 pathways were removed as exogenous pathways. In total, 114 core pathways were identified in all the 24 colon samples with CPM > 5 (Supplementary Dataset 1), which belonged to four first-level KEGG functions, including "Cellular Processes" (2.47% ± 0.08%), "Environmental Information Processing" (3.63% ± 0.07%), "Genetic Information Processing" (12.23% ± 0.13%) and "Metabolism" (76.41% ± 0.18%). At the secondary level of KEGG hierarchy, 21 KEGG functions were identified, with "Carbohydrate metabolism" (15.43% ± 0.08%), "Amino acid metabolism" (12.13% ± 0.13%), "Metabolism of cofactors and vitamins" (10.39% ± 0.14%), "Metabolism of terpenoids and polyketides" (8.68% ± 0.13%), and "Metabolism of other amino acids" (7% ± 0.09%) being the top five functions (Figure 1). Meanwhile, the top five KEGG pathways were "ko01051: Biosynthesis of ansamycins" (3.94% ± 0.16%), "ko01055: Biosynthesis of vancomycin group antibiotics" (1.94% ± 0.09%), "ko00471: D-Glutamine and D-glutamate metabolism" (1.82% ± 0.03%), "ko00473: D-Alanine metabolism" (1.79% ± 0.04%), and "ko00290: Valine, leucine and isoleucine biosynthesis" (1.78% ± 0.04%; Supplementary Dataset 1).

## Taxonomic and functional composition of neonatal calves' colon microbiome

In general, four domains including bacteria, archaea, eukaryota, and viruses comprised the microbiome of 2-day-old calves. Regardless of the effect of colostrum feeding time treatment, bacteria was the predominant domain, with a relative abundance of 99.20% ± 0.09%, and archaea, eukaryotes, and viruses followed with relative abundances of 0.13 ± 0.01%, 0.09% ± 0.01%,




FIGURE 1
Profiles of neonatal calves' colon microbiome. Microbial metabolic pathways based on their First and Second Level functions in the KEGG hierarchy.

0.50% ± 0.09%, respectively (Supplementary Dataset 1). Bacterial and archaeal taxa with average relative abundance > 0.05%, and presented in more than 50% of the animals in at least one treatment, were defined as detected microbiota. For bacteria, colon microbiota consisted of nine bacterial phyla, including Firmicutes, Proteobacteria, Actinobacteria, Bacteroidetes, Fusobacteria, Spirochaetes, Cyanobacteria, Chloroflexi, and Thermotogae. Meanwhile, 35 bacterial families were detected, with *Enterobacteriaceae* (20.38% ± 3.17%), *Streptococcaceae* (16.96% ± 2.20%), *Lactobacillaceae* (14.79% ± 2.48%), *Clostridiaceae* (12.73% ± 1.15%), and *Ruminococcaceae* (11.47% ± 1.36%) being the top five most predominant bacterial families (Supplementary Dataset 1). Additionally, 441 bacterial genera were detected, with *Streptococcus* (16.08% ± 10.35%), *Clostridium* (12.54% ± 5.54%), *Lactobacillus* (12.37% ± 12.07%), *Ruminococcus* (9.38% ± 5.39%), and *Enterococcus* (6.71% ± 5.39%) being the top five bacterial genera (Figure 2).

Based on previous publications (Zhou et al., 2014; Nkamga et al., 2017; Song et al., 2021), the "non-gut archaeal taxa" were removed before microbial composition analysis. Taxonomic profiling revealed that sequences belonged to three archaeal phyla, 23 archaeal genera, and 20 archaeal species. The top five archaeal genera were *Methanobrevibacter* (15.89% ± 0.76%), *Methanosarcina* (11.83% ± 0.24%), *Methanocorpusculum* (10.59% ± 0.46%), *Methanococcus* (10.35% ± 0.65%), and *Methanosphaera* (4.51% ± 0.15%). Meanwhile, the top five archaeal species were *Methanocorpusculum labreanum* (11.34% ± 0.51%), *Methanobrevibacter smithii* (10.32% ± 0.63%), *Methanobrevibacter ruminantium* (6.63% ± 0.23%), *Methanosarcina acetivorans* (6.19% ± 0.16%), and *Methanosphaera stadtmanae* (4.81% ± 0.15%). It was found that 58% of sequences belonged to less abundant, unclassified, and non-gut Eukaryota phyla. In addition, viruses were unclassified at the phylum level.

## Effect of delayed colostrum feeding on colon microbiome composition

Colon bacterial community was compared using the Bray–Curtis dissimilarity matrices-based principal-coordinate analysis

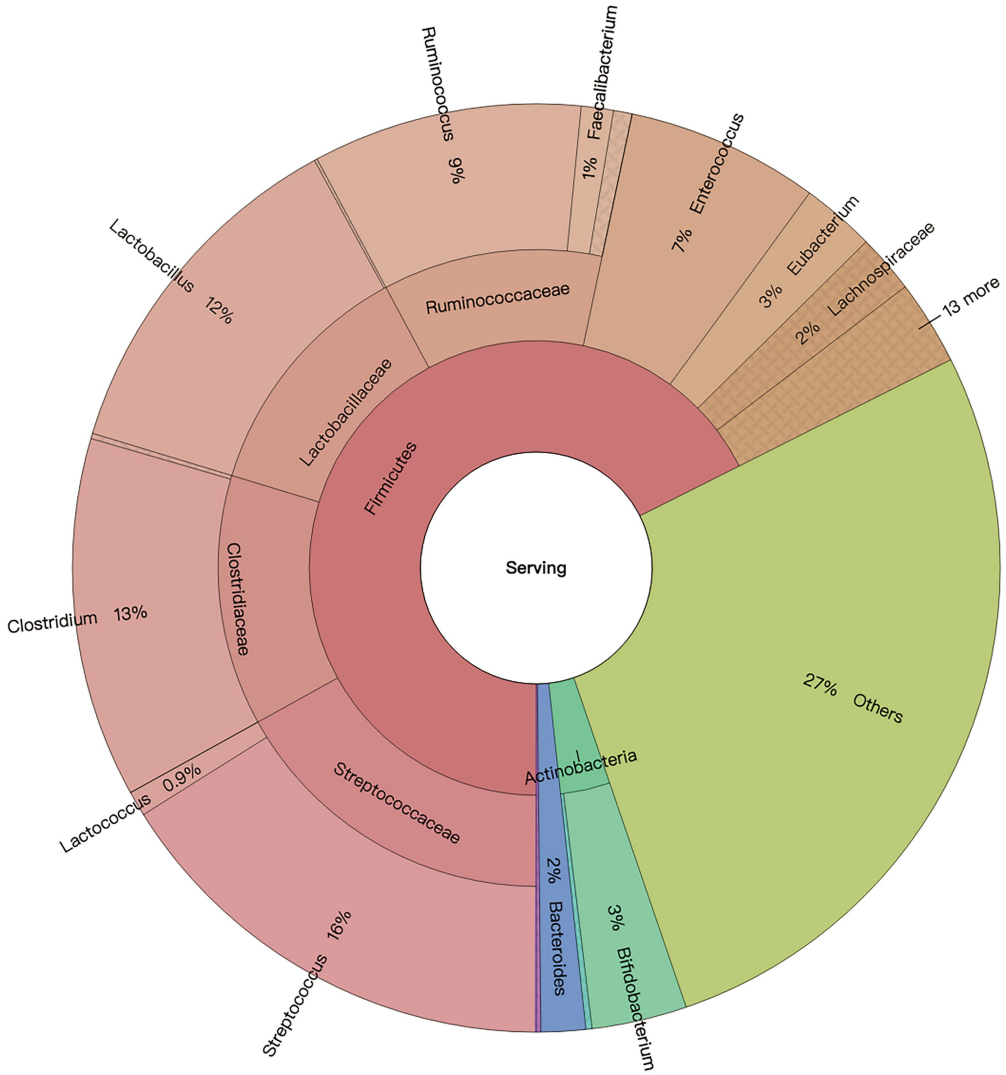


FIGURE 2
Colon bacterial composition of neonatal calves.

TABLE 1 Effect of delayed colostrum feeding on colon bacterial diversity (mean±SEM).

| Diversity | Treatment | | | *P*-value |
|---|---|---|---|---|
| Matrix | TRT0h | TRT6h | TRT12h | |
| Shannon | 2.39 ± 0.14[b] | 2.80 ± 0.10[a] | 2.76 ± 0.07[a] | 0.02 |
| Simpson | 0.80 ± 0.02[b] | 0.87 ± 0.01[a] | 0.87 ± 0.01[a] | 0.01 |

$P < 0.05$ means there is significant difference among groups; $P < 0.1$ means there is a tendency to be significant among groups.
[a,b]Different superscripts within a row represent significance.

(PCoA), which revealed no clear separation among treatments (PERMANOVA, $p = 0.65$; Supplementary Figure 1A). Meanwhile, Shannon and Simpson indices were compared among treatments, and they were significantly higher in the calves from TRT6h and TRT12h groups compared to those in TRT0h group (Table 1). When the bacterial genera were compared at genus level, the relative abundance of four bacterial genera including *Parascardovia* ($P < 0.01$), *Scardovia* ($P < 0.01$), *Arthrobacter* ($p = 0.01$), and *Micrococcus* ($p = 0.02$) were found to be significantly higher in calves from TRT0h treatment, compared to calves in TRT6h and TRT12h treatment groups. In addition, the relative abundance of 14 bacterial groups was significantly higher in the colon digesta of the calves from TRT6h and TRT12h, compared to those calves in TRT0h group (Table 2). Additionally, Principal-coordinate analysis using Bray–Curtis dissimilarity matrices was also performed on the archaeal community, and no clear separated clusters were detected among different treatment groups (PERMANOVA, $p = 0.80$; Supplementary Figure 1B). Furthermore, the overall KEGG pathways in the colon digesta of calves were compared among three treatments using PCA analysis (Figure 3), and no clear separations among treatments (PERMANOVA, $p = 0.11$) were observed. Meanwhile, none of the KEGG pathways differed significantly among the three treatment groups.

TABLE 2 Comparison of the relative abundance of bacterial genera among calves fed colostrum immediately after birth (TRT0h, *n*=7), at 6h after birth (TRT6h, *n*=8) and at 12h after birth (TRT12h, *n*=9).

| | Phylum | Family | Genus | Treatments | | | SEM | *P*-value* |
|---|---|---|---|---|---|---|---|---|
| | | | | TRT0h | TRT6h | TRT12h | | |
| Bacteria | Actinobacteria | *Bifidobacteriaceae* | *Parascardovia* | 0.012[a] | 0.003[b] | 0.002[b] | 0.002 | 0.00 |
| | | | *Scardovia* | 0.009[a] | 0.003[b] | 0.002[b] | 0.001 | 0.00 |
| | | *Micrococcaceae* | *Arthrobacter* | 0.026[a] | 0.017[b] | 0.013[b] | 0.002 | 0.01 |
| | | | *Micrococcus* | 0.003[a] | 0.002[b] | 0.002[b] | 0.001 | 0.02 |
| | | | *Rothia* | 0.031[a] | 0.020[ab] | 0.013[b] | 0.004 | 0.02 |
| | Firmicutes | *Leuconostocaceae* | *Weissella* | 0.009[a] | 0.006[ab] | 0.004[b] | 0.001 | 0.02 |
| | Proteobacteria | *Aeromonadaceae* | *Tolumonas* | 0.004[b] | 0.007[a] | 0.008[a] | 0.001 | 0.01 |
| | | *Ferrimonadaceae* | *Ferrimonas* | 0.001[b] | 0.002[a] | 0.002[a] | 0.001 | 0.01 |
| | | *Vibrionaceae* | *Photobacterium* | 0.011[b] | 0.018[a] | 0.018[a] | 0.001 | 0.01 |
| | | *Vibrionaceae* | *Aliivibrio* | 0.007[b] | 0.010[a] | 0.010[a] | 0.001 | 0.02 |
| | | *Halomonadaceae* | *Chromohalobacter* | 0.003[b] | 0.005[a] | 0.005[a] | 0.001 | 0.02 |
| | | *Pseudomonadaceae* | *Pseudomonas* | 0.034[b] | 0.055[a] | 0.055[a] | 0.004 | 0.02 |
| | | *Vibrionaceae* | *Vibrio* | 0.033[b] | 0.051[a] | 0.047[a] | 0.003 | 0.02 |
| | | *Alcanivoracaceae* | *Kangiella* | 0.001[b] | 0.002[a] | 0.002[a] | 0.001 | 0.03 |
| | | *Enterobacteriaceae* | *Xenorhabdus* | 0.004[b] | 0.005[ab] | 0.007[a] | 0.001 | 0.03 |
| | | *Francisellaceae* | *Francisella* | 0.006[b] | 0.009[a] | 0.008[a] | 0.001 | 0.03 |
| | | *Rhodobacteraceae* | *Sulfitobacter* | 0.001[b] | 0.002[a] | 0.001[b] | 0.001 | 0.03 |
| | | *Shewanellaceae* | *Shewanella* | 0.034[b] | 0.054[a] | 0.051[a] | 0.004 | 0.03 |
| | | *Aeromonadaceae* | *Aeromonas* | 0.011[b] | 0.017[a] | 0.018[a] | 0.002 | 0.04 |
| | | *Desulfobulbaceae* | *Desulfotalea* | 0.013[b] | 0.027[a] | 0.019[ab] | 0.002 | 0.04 |
| | | *Oxalobacteraceae* | *Herminiimonas* | 0.001[b] | 0.002[a] | 0.002[a] | 0.001 | 0.04 |
| | Tenericutes | *Acholeplasmataceae* | *Candidatus Phytoplasma* | 0.002[b] | 0.003[a] | 0.003[a] | 0.001 | 0.03 |
| Archaea | Crenarchaeota | *Sulfolobaceae* | *Sulfolobus* | 1.573[a] | 0.884[b] | 0.753[b] | 0.151 | 0.04 |
| | Euryarchaeota | *Methanocellaceae* | *Methanocella* | 0.840[b] | 0.959[ab] | 1.149[a] | 0.047 | 0.01 |

*$P$-values among the three colostrum treatment groups were obtained using non-parametric Kruskal–Wallis test statistical method. The significant difference between any two treatment groups was tested using Dunn's test. The $P$-value was adjusted with Benjamini–Hochberg method for false discovery rate (FDR; Yoav and Yosef, 1995), with statistical significances declared at $P < 0.05$.

[a,b]Different superscripts within a row represent significance.

## Effect of colostrum feeding time on SCFAs profiles

SCFAs concentrations were measured using GC, and acetate, propionate, and butyrate were detected. Generally, acetate had the highest concentration in the colon digesta. Butyrate concentration and its molar proportion were significantly higher in the colon digesta of calves in TRT0h compared to calves in TRT6h and TRT12h ($p = 0.01$). On the contrary, calves in TRT0h had lower acetate molar proportion compared to calves in TRT6h and TRT12h (Table 3).

## Individualized colon microbiome of 2-day-old calves

Although the PCA did not show clear separation based on KEGG pathways among different colostrum feeding time treatments, it showed two major clusters regardless of treatments (PERMANOVA, $p = 0.001$; Figure 3). Cluster one consisted of 13 calves (TRT0h: $n = 6$, TRT6h: $n = 2$, TRT12h: $n = 5$), while cluster two included 11 calves (TRT0h: $n = 1$, TRT6h: $n = 6$, TRT12h: $n = 4$). Additionally, calves from the same treatment (TRT12h) were selected from clusters 1 and 2, defined as Group A ($n = 5$) and B ($n = 4$). Although these calves in Groups A and B were from the same colostrum feeding time treatment, we observed noticeable variations in the relative abundance of KEGG functions (Figure 4) through conducting the LEfSe analysis, and 12 differentially expressed KEGG pathways were identified (LDA score > 2 and $p < 0.05$) between Groups A and B. Specifically, seven KEGG pathways were more abundant in Group A, including "ko00520: Amino sugar and nucleotide sugar metabolism," "ko00051: Fructose and mannose metabolism," "ko00010: Glycolysis/Gluconeogenesis," "ko00230: Purine metabolism," "ko00906: Carotenoid biosynthesis," "ko00620: Pyruvate metabolism," and "ko04210: Apoptosis" (Figure 4). On the contrary, five KEGG pathways had higher relative abundance in Group B, including "ko00190: Oxidative phosphorylation,"

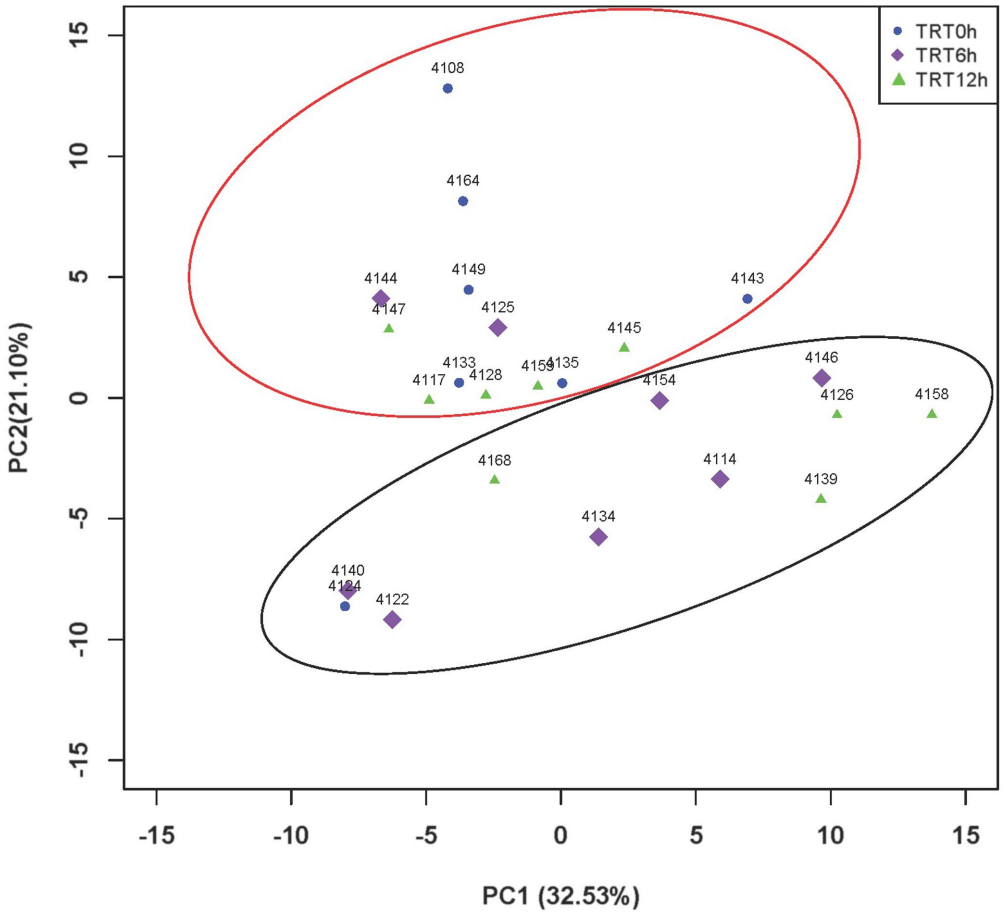


FIGURE 3
PCA plot of KEGG pathways among different treatments. Principal component analysis (PCA) plots of colon microbial metabolic Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways among three colostrum feeding time treatments in neonatal dairy calves. TRT0h, TRT6h, and TRT12h indicate calves were fed colostrum at 45min, 6h, and 12h after birth, respectively. PC1, principal component 1; PC2, principal component 2.

**TABLE 3** Short chain fatty acid (SCFA) concentrations (umol/g) and molar proportions among calves fed colostrum immediately after birth (TRT0h, *n*=7), at 6h after birth (TRT6h, *n*=8) and at 12h after birth (TRT12h, *n*=9).

| SCFAs | Treatments | | | SEM | *P*-value |
|---|---|---|---|---|---|
| | TRT0h | TRT6h | TRT12h | | |
| Acetate[A] | 11.75 | 13.31 | 9.26 | 1.25 | 0.46 |
| Propionate[A] | 1.98 | 2.51 | 2.19 | 0.37 | 0.88 |
| Butyrate[A] | 3.62[a] | 0.90[b] | 0.91[b] | 0.40 | 0.01 |
| Total SCFA[A] | 17.36 | 16.71 | 12.36 | 1.65 | 0.41 |
| Acetate[B] | 0.68[b] | 0.80[a] | 0.76[a] | 0.02 | 0.01 |
| Propionate[B] | 0.11 | 0.14 | 0.15 | 0.02 | 0.61 |
| Butyrate[B] | 0.21[a] | 0.06[b] | 0.10[b] | 0.02 | 0.01 |

[A]Indicates differences in SCFA concentration.
[B]Indicates differences in the molar proportion of SCFA. Significance between treatment groups was declared at $p < 0.05$ and tendencies were declared at $p < 0.10$.
[a,b]Different superscripts within a row represent significance.

"ko00860: Porphyrin and chlorophyll metabolism," "ko00630: Glyoxylate and dicarboxylate metabolism," "ko00633: Nitrotoluene degradation," and "ko03070: Bacterial secretion system" (Figure 4).

Additionally, the taxonomic profiles were compared between Groups A and B. *Pediococcus* ($p = 0.03$), *Carnobacterium* ($p = 0.02$), *Oenococcus* ($p = 0.02$), *Staphylococcus* ($p = 0.02$), and *Thermoplasma* ($p = 0.03$) were more abundant in group A, while *Aeromonas* ($p = 0.03$), *Alcanivorax* ($p = 0.03$), *Edwardsiella* ($p = 0.03$), *Enterobacter* ($p = 0.03$), *Erwinia* ($p = 0.03$), *Escherichia* ($p = 0.03$), *Providencia* ($p = 0.03$), *Serratia* ($p = 0.03$), *Shigella* ($p = 0.03$), *Xenorhabdus* ($p = 0.03$), *Histophilus* ($p = 0.03$), *Photobacterium* ($p = 0.03$), *Candidatus Blochmannia* ($p = 0.02$), *Idiomarina* ($p = 0.02$), *Aggregatibacter* ($p = 0.02$), *Haemophilus* ($p = 0.02$), *Pseudomonas* ($p = 0.02$), *Shewanella* ($p = 0.02$), *Aliivibrio* ($p = 0.02$), and *Vibrio* ($p = 0.02$) had higher relative abundances in group B (Table 2).

## Colonic transcriptome profiles varied between calves with different microbiomes

In total, 62 DE genes were detected between groups A and B. Among them, 46 DE genes were upregulated in group A, while

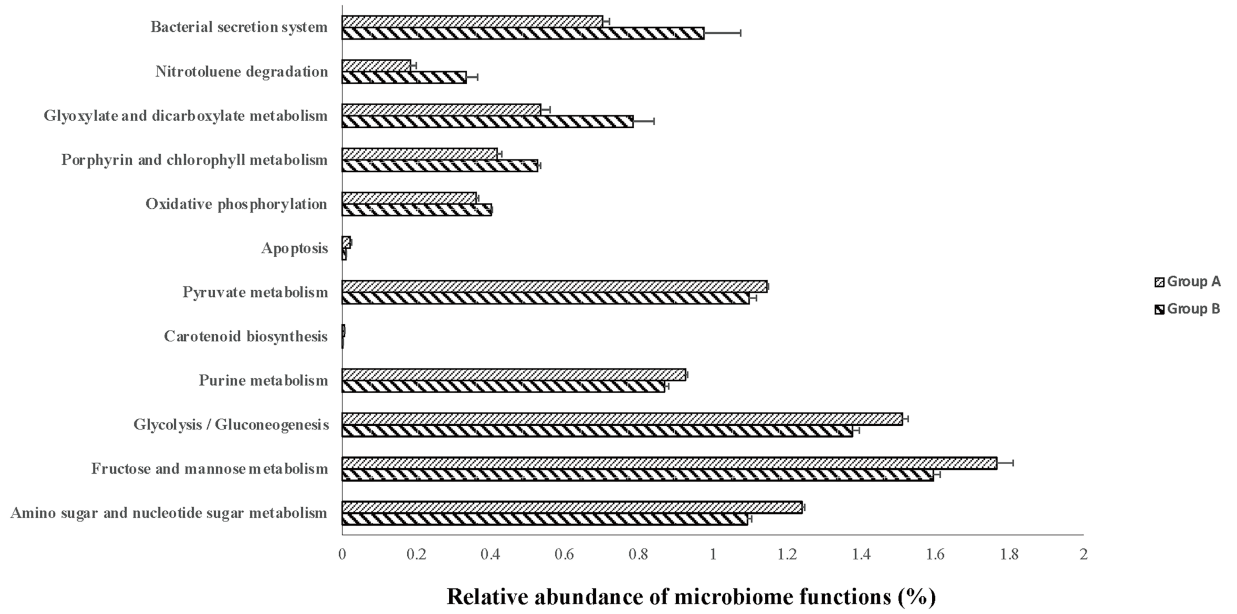


FIGURE 4
The relative abundance of varied second level Kyoto Encyclopedia of Genes and Genomes (KEGG) functions between calves with two type of colon metagenomes. Group A include five animals from the TRT12h treatment based on the principal component analysis plot (Figure 3) clearly separated from the other four animals in TRT12h treatment (group B).

16 genes were downregulated in group A (Supplementary Dataset 1). Further Venn Diagram analysis revealed that 13,992 genes were commonly expressed in the colon of groups A and B, and 189, 702 genes were uniquely expressed in groups A and B, respectively (Supplementary Figure 2). Meanwhile, the phenotypic parameters including the concentration of serum IgG, plasma GLP-1, GLP-2, insulin, acetate, propionate, and butyrate, as well as colon weight and length were compared between calves in groups A and B. However, only butyrate concentration was significantly different between groups A and B (A:1.47 ± 0.06 μmol/g; B:0.35 ± 0.04 μmol/g; $p < 0.01$).

## Correlation networks for identified differentially expressed genes, microbial taxa, and microbial functions between calves with different microbiomes

The expression of DE genes between groups A and B was significantly correlated with the abundance of colon microbial taxa and functions. In total, the expression of 25 DE genes showed at least one correlation with 36 bacteria, and 19 DE genes were significantly correlated with 12 microbial functions (Figure 5). Generally, the expression of ankyrin repeat, SAM, and basic leucine zipper domain containing 1 (ASZ1), Cyclin J like (CCNJL), cytochrome P450, family 2, subfamily S, polypeptide 1 (CYP2S1), Histamine receptor H2 (HRH2), NTPase KAP family P-loop domain containing 1 (NKPD1), Ras homolog family member D (RHOD), Ring finger protein, transmembrane 2 (RNFT2), Solute carrier family 7 member 14 (SLC7A14), and Transmembrane protein 169 (TMEM169) was significantly correlated with no less than five bacterial taxa in the colon (Figure 5A). More specifically, the expression of *CCNJL* was negatively correlated with *Staphylococcus*, *Oenococcus*, *Pediococcus*, and *Oceanobacillus* ($\rho < -0.8$; $p < 0.01$), while it was positively correlated with *Pseudomonas*, *Shewanella*, and *Vibrio* ($\rho$ values > 0.8; $p < 0.01$). The expression of *HRH2* was positively correlated with *Erwinia*, *Xenorhabdus*, *Pseudomonas*, *Aggregatibacter*, *Vibrio*, *Tolumonas*, and *Shewanella* ($\rho$ values > 0.8; $p < 0.01$). The expression of *RHOD* was negatively correlated with *Candidatus Blochmannia, Pseudomonas, Escherichia, Shigella,* and *Haemophilus* ($\rho$ values< −0.8; $p < 0.01$). The expression of *TMEM169* was negatively correlated with *Candidatus Blochmannia, Pseudomonas, Pasteurella, Aggregatibacter,* and *Haemophilus* ($\rho$ values< −0.8; $p < 0.01$).

Meanwhile, the expression of *CBLN4*, *CCNJL*, *HRH2*, and *RHOD* was closely correlated with no less than five colon microbial functions. The expression of *CCNJL* was negatively correlated with the relative abundances of amino sugar and nucleotide sugar metabolism, fructose and mannose metabolism, and Glycolysis/Gluconeogenesis ($\rho$ values< −0.8; $p < 0.01$), and was positively correlated with oxidative phosphorylation and porphyrin and chlorophyll metabolism ($\rho$ values > 0.8; $p < 0.01$). The expression of *HRH2* was negatively correlated with the relative abundances of amino sugar and nucleotide sugar metabolism, apoptosis, carotenoid biosynthesis, fructose and mannose metabolism, purine metabolism, and pyruvate metabolism ($\rho$ values< −0.8; $p < 0.01$), and was positively correlated with the

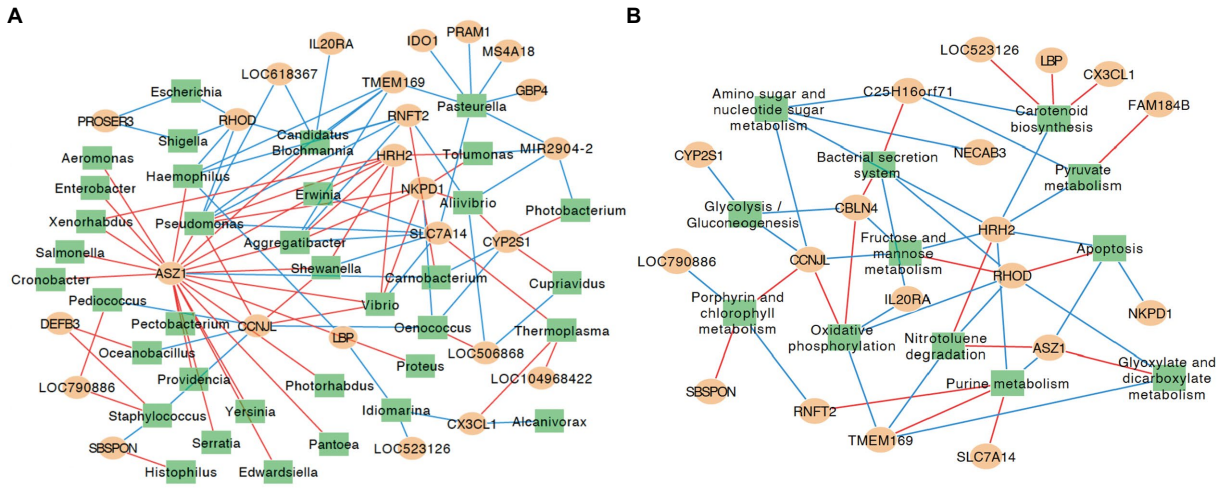


FIGURE 5
Correlation networks for identified differentially expressed genes, microbial taxa and microbial functions at KEGG pathway hierarchical level 3. **(A)** Correlation networks for identified differentially expressed genes and microbial taxa. **(B)** Correlation networks for identified differentially expressed genes and microbial functions at KEGG pathway hierarchical level 3. The yellow circles represent genes differentially expressed between groups A and B, and green rectangle represent the microbial taxa that were significantly different between groups A and B. The red lines indicate positive correlation, and blue lines indicate negative correlation; only correlations with rho values ≥ 0.8 and $p \leq 0.01$ are shown. Data for genes/microbial taxa and functions that did not show a significant correlation are not shown.

relative abundance of nitrotoluene degradation ($\rho = 0.82$, $p < 0.01$). The expression of *RHOD* was negatively correlated with the relative abundances of bacterial secretion system, glyoxylate and dicarboxylate metabolism, nitrotoluene degradation, and oxidative phosphorylation ($\rho$ values$< -0.8$; $p < 0.01$), and was positively correlated with the relative abundance of apoptosis and fructose and mannose metabolism ($\rho$ values $> 0.8$; $p < 0.01$; Figure 5B).

## Discussion

Similar to our previous finding of microbial composition in the ileum of 2-day-old calves (Song et al., 2021), bacteria, archaea, eukaryotes, and viruses constituted colon microbial microbiome, with bacteria being the most predominant. Compared with calves at 7-days old (Song et al., 2018), Firmicutes and Proteobacteria also dominated in the colon digesta at phylum level. However, a lower relative abundance of Bacteroidetes (20.81% ± 0.89% at D7, 1.71% ± 0.72% at D2) was detected at 2 days old. Meanwhile, the most abundant bacterial family (*Lactobacillaceae* with 22.36% ± 0.90% at D7; *Enterobacteriaceae* with 20.38% ± 3.17% at D2) and genera (*Lactobacillus* with 22.36% ± 1.37% at D7; *Streptococcus* with 16.08% ± 10.35% at D2) also differed between the two studies (Song et al., 2018). Such discrepancy may be caused by maternal factors, sequencing methods (amplicon sequencing vs. metagenomic sequencing), and colostrum feeding management strategies.

Although large intestinal microbiomes were mainly colonized by bacteria, the presence of archaea was revealed through metagenomic sequencing analysis. Similar to the predominant archaeal phyla in the human intestine at the first day of birth (Nkamga et al., 2017), Euryarchaeota (93.73%), Crenarchaeota (5.42%), and Thaumarchaeota (0.58%) were the most dominant archaea phyla in the colon of 2-day-old neonatal calves. This suggests that archaea may start to colonize the large intestine during the first 1–2 days after birth similar as the observation on their colonization in the rumen (Malmuthuge et al., 2019; Meale et al., 2021). Additionally, in agreement with our previous publication that *Methanobrevibacter*, *Methanosarcina*, and *Methanocorpusculum* were the three major archaeal genera in the small intestine using metagenomic sequencing analysis (Song et al., 2021), these three genera were also the predominant genera in the colon. Meanwhile, the archaeal species that related to methane synthesis accounted for 57.96% of all archaeal species, which may be closely associated with their functions in depleting $H_2$ and carbohydrates degradation in the gut (Guindo et al., 2020). It was speculated that utilization of $H_2$ for methane production could improve polysaccharide fermentation efficiency (Stams, 1994), which is beneficial for nutrient digestion in the large intestine, since the pre-weaning rumen has an undeveloped rumen. However, the functions of other archaeal species were not well studied and future studies are needed to explore the role that archaea may play in the colon of neonatal calves.

In addition, this study has provided the fundamental knowledge of colon microbial functions of 2-day-old dairy calves. The top five KEGG pathways at second level were all associated with nutrient metabolism. Moreover, "carbohydrate metabolism" (15.43% ± 0.08%) and "amino acid metabolism" (12.13% ± 0.13%) were the top two gene annotated KEGG pathways, which is similar to our previous findings of hindgut microbial functions of neonatal calves through functional prediction using 16S rRNA genes (Song et al., 2018). These results suggest that colon colonizers may play important roles in nutrient metabolism and energy harvest when the rumen is not

well developed during the early neonatal period. Meanwhile, the top three metagenomic functions at level two of the KEGG pathways in the colon were similar to those in the ileum (Song et al., 2021), suggesting similar microbial functions regardless of gut sections of neonatal calves. Furthermore, two major third-level KEGG functions, including "biosynthesis of ansamycins" (3.94% ± 0.16%) and "biosynthesis of vancomycin group antibiotics" (1.94% ± 0.09%), were linked to antibiotics production, indicating that the early colon microbiome may inhibit the growth of other colonizers through producing antibiotics in scenarios of nutrient competition.

Notably, microbial diversity and richness were higher for calves that received colostrum at a 6 h and 12 h delay compared with calves fed colostrum immediately after birth, suggesting a complex microbiome establishment of the animals within these treatment groups. The observed changes of the colon digesta microbiome were driven by colostrum feeding time confounded with the total amount of nutrients that calves received over the 2-d study period. Overall, colostrum feeding time did not affect microbial functions, which may have been caused by high individual variation of animals among groups. Nevertheless, the relative abundances of some microbial taxa were influenced by different colostrum feeding time. The observed higher relative abundance of *Micrococcus* for TRT0h calves may be related to the higher concentration of butyrate in the colon digesta, since *Micrococcus* spp., such as *Micrococcus aerogenes,* could utilize colostrum glutamic acid for butyrate production (Horler et al., 1966). However, further studies are required to measure specific butyric acid-producing *Micrococcus* species using qPCR to confirm our speculation. Meanwhile, we speculate that increased relative abundance of *Aeromonas* genera in the 12-h delayed feeding group may lead to greater incidences of intestinal inflammation due to its classification as an opportunistic pathogen and its association with enteric lesions in cattle (Al-Mashat and Taylor, 1983). However, isolating specific pathogenic *Aeromonas* species is warranted to confirm such speculation. Of note, higher butyrate concentrations were observed in calves from the TRT0h group, which may promote intestinal development in these calves due to this microbial fermentation product's ability to increase intestinal tissue mass and thickness (Tappenden et al., 2003), as well as enhance epithelial tight junctions, provide energy to epithelial cells (Lopetuso et al., 2013), and regulate immune system development (Arpaia et al., 2013). Therefore, we suggest that calves in TRT0h group may have the potential for a healthier gut. However, proof of this supposition needs to be further obtained by measuring intestinal permeability and histological examination.

Similar to previous studies reporting individualized small intestinal microbiome of neonatal calves (Malmuthuge et al., 2019; Song et al., 2021), the differences in colon microbiome were detected between individual animal groups (Groups A and B), even in animals within the same colostrum feeding treatment. Generally, the microbiota composition and functions of individual calf could be attributed to environmental, maternal, and host factors (Song et al., 2021). In detail, environmental factors include the farm environment (O'hara et al., 2020), the processing of colostrum and milk (fresh vs. pasteurized; Song et al., 2019), and feed additives in the milk (Badman et al., 2019; Wang et al., 2022). Maternal factors consist of the parity of the dam, maternal amniotic fluid (DiGiulio, 2012), placenta (Aagaard et al., 2014), as well as maternal colostrum (Yeoman et al., 2018). As such, several of these variables, including the bedding, disinfection strategy of the hutches, colostrum, milk replacer, and the dam parity in each colostrum feeding time treatment, were controlled in this study. Specifically, pasteurized colostrum was utilized for the calves in each treatment. Pasteurization would leave the nutritional and immunological factors intact but kill the vast diversity of microorganisms present in colostrum (Yeoman et al., 2018; Chen et al., 2021). Hence, all the calves were practically in the absence of maternally sourced and especially colostrum-sourced bacteria. Since environmental and management were controlled to be consistent for all calves, we suggest that host factors may be the main driver for the individualization of colon microbiota of neonatal calves. Host genetics have been reported to shape gut microbiomes of mice (Benson et al., 2010) and human infants (Goodrich et al., 2016). However, the extent to which the host may contribute to the individualized early colon microbiome colonization of neonatal calves is still unknown.

In this study, the difference of phenotypic traits, including DE genes, uniquely expressed genes in the colon tissue, and butyrate concentration in the colon digesta were detected between animals with two types of colon metagenomes, suggesting individual differences. Consistent with the phenotypic parameters, the variation in microbial taxa and functions were also detected between calves with two types of colon metagenomes. The microbial functions associated with nutrients metabolism, including amino sugar and nucleotide sugar metabolism, fructose and mannose metabolism, glycolysis/gluconeogenesis, purine metabolism, carotenoid biosynthesis, and pyruvate metabolism had higher levels of abundance in neonatal calves within group A than group B, highlighting that microbial nutrient metabolism functions are greatly affected by individual difference.

To further validate the extent by which individual host differences could affect the colon digesta microbiome, we explored how host gene expression may play a role. It is worth noting that significant correlations were identified between the expression levels of DE genes and the abundances of several microbial genus, such as Ras homolog family member D (*RHOD*) and the relative abundance of *Escherichia*. The enzyme rhodanese encoded by *RHOD* proved to be responsible for detoxifying hydrogen sulfide ($H_2S$) in the colon (Picton et al., 2002), which was suggested to inhibit butyrate metabolism and cellular energy synthesis (Moore et al., 1997). Hence, the removal of $H_2S$ is beneficial for butyrate metabolism. Meanwhile, butyrate was reported to suppress *E. coli* colonization through increasing *Lactobacillus acidophilus* and *Bifidobacterium longum* adherence (Jung et al., 2022). Therefore, the expression of the *RHOD* may play a negative role in *E. coli* colonization, which is in agreement with our finding of a negative relationship between *RHOD* expression and Escherichia abundance. Similarly, multiple correlations were observed between the expression of host DE genes and the abundance of colon microbial pathways, which highlights the close interactions between the host and colon microbiome. Also, the identified correlations between the abundance of microbial nutrient utilization-related functions, such as pyruvate metabolism, fructose and mannose

metabolism, amino sugar and nucleotide sugar metabolism, glycolysis/gluconeogenesis, and the expression of host DE genes implies host intestinal cell gene expression level may play important roles in regulating colon microbial metabolism. Meanwhile, other microbial functions, including bacterial secretion system and apoptosis were also affected by the different expression level of host genes. However, future studies through a combination of gene knockout technology and calf feeding trials are needed to elucidate the roles of the host on the intestinal microbiome.

## Conclusion

In conclusion, this study characterized the colon microbiome of 2-day-old dairy calves and how it altered when colostrum feeding time differed. The top five second-level KEGG pathways were all related to nutrients metabolism, with "carbohydrate metabolism" and "amino acid metabolism" being the top two gene annotated KEGG pathways, which suggested that colon colonizers may play important roles in nutrient metabolism and energy utilization during the early neonatal period. In general, delaying colostrum feeding did not influence the overall colon microbiome of 2-day-old dairy calves at both the taxonomic and functional levels, and the short duration of the animal trial could be one of the reasons. Long-term effects of delaying first colostrum feeding to colon microbiome are needed in the further study. Individualized colon microbiome profiles were observed regardless of colostrum feeding time treatment. This suggests that host factors may play an important role in shaping early life colon microbiome of the dairy calves. The correlation between identified DE genes, microbial taxa and microbial functions between calves with varied colon microbiomes further proved the effect of host on colon microbiome. However, further studies are encouraged to explore the extent by which the host may have an effect on the initial colonization of the colon microbiome by gene knockout technology of the DE genes.

## Data availability statement

The data presented in the study are deposited in the MG-RAST repository, accession number: https://www.mg-rast.org/linkin.cgi?project=mgp82806.

## Ethics statement

The animal study was reviewed and approved by The Livestock Care Committee of the University of Alberta (AUP00001595).

## Author contributions

YS participated in animal sampling and metagenomic library construction. YS and SW contributed to data analysis and writing and revision of the manuscript. FL helped with data processing. AF-T helped with colon digesta sample collection. ZH provided the original data of colon metatranscriptomic data. MS participated in the experiment design and paper revise. LG participated in the study design and 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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## Supplementary material

The Supplementary material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2022.1035331/full#supplementary-material

# References


Aagaard, K., Ma, J., Antony, K. M., Ganu, R., Petrosino, J., and Versalovic, J. (2014). The placenta harbors a unique microbiome. *Sci. Transl. Med.* 6:237ra65. doi: 10.1126/scitranslmed.3008599

Abubucker, S., Segata, N., Goll, J., Schubert, A. M., Izard, J., Cantarel, B. L., et al. (2012). Metabolic reconstruction for metagenomic data and its application to the human microbiome. *PLoS Comput. Biol.* 8:e1002358. doi: 10.1371/journal.pcbi.1002358

Al-Mashat, R. R., and Taylor, D. J. (1983). Bacteria in enteric lesions of cattle. *Vet. Rec.* 112, 5–10. doi: 10.1136/vr.112.1.5

Aronesty, E. (2013). Comparison of sequencing utility programs. *Open Bioinform. J.* 7, 1–8.

Arpaia, N., Campbell, C., Fan, X., Dikiy, S., Van Der Veeken, J., Deroos, P., et al. (2013). Metabolites produced by commensal bacteria promote peripheral regulatory T-cell generation. *Nature* 504, 451–455. doi: 10.1038/nature12726

Badman, J., Daly, K., Kelly, J., Moran, A. W., Cameron, J., Watson, I., et al. (2019). The effect of Milk replacer composition on the intestinal microbiota of pre-ruminant dairy calves. *Front. Vet. Sci.* 6:371. doi: 10.3389/fvets.2019.00371

Benson, A. K., Kelly, S. A., Legge, R., Ma, F., Low, S. J., Kim, J., et al. (2010). Individuality in gut microbiota composition is a complex polygenic trait shaped by multiple environmental and host genetic factors. *Proc. Natl. Acad. Sci. U. S. A.* 107, 18933–18938. doi: 10.1073/pnas.1007028107

Chen, B., Tang, G., Guo, W., Lei, J., Yao, J., and Xu, X. (2021). Detection of the Core bacteria in colostrum and their association with the rectal microbiota and with Milk composition in two dairy cow farms. *Animals* 11:3363. doi: 10.3390/ani11123363

Dias, J., Marcondes, M. I., Motta De Souza, S., Cardoso Da Mata, E. S. B., Fontes Noronha, M., Tassinari Resende, R., et al. (2018). Bacterial community dynamics across the gastrointestinal tracts of dairy calves during Preweaning development. *Appl. Environ. Microbiol.* 84, e02675–17. doi: 10.1128/AEM.02675-17

DiGiulio, D. B. (2012). Diversity of microbes in amniotic fluid. *Semin. Semin Fetal Neonatal Med* 17, 2–11. doi: 10.1016/j.siny.2011.10.001

Fischer, A. J., Song, Y., He, Z., Haines, D. M., Guan, L. L., and Steele, M. A. (2018). Effect of delaying colostrum feeding on passive transfer and intestinal bacterial colonization in neonatal male Holstein calves. *J. Dairy Sci.* 101, 3099–3109. doi: 10.3168/jds.2017-13397

Fomenky, B. E., Do, D. N., Talbot, G., Chiquette, J., Bissonnette, N., Chouinard, Y. P., et al. (2018). Direct-fed microbial supplementation influences the bacteria community composition of the gastrointestinal tract of pre- and post-weaned calves. *Sci. Rep.* 8:14147. doi: 10.1038/s41598-018-32375-5

Godden, S. (2008). Colostrum management for dairy calves. *Vet. Clin. North Am. Food Anim. Pract.* 24, 19–39. doi: 10.1016/j.cvfa.2007.10.005

Goodrich, J. K., Davenport, E. R., Beaumont, M., Jackson, M. A., Knight, R., Ober, C., et al. (2016). Genetic determinants of the gut microbiome in UK twins. *Cell Host Microbe* 19, 731–743. doi: 10.1016/j.chom.2016.04.017

Guan, L. L., Nkrumah, J. D., Basarab, J. A., and Moore, S. S. (2008). Linkage of microbial ecology to phenotype: correlation of rumen microbial ecology to cattle's feed efficiency. *FEMS Microbiol. Lett.* 288, 85–91. doi: 10.1111/j.1574-6968.2008.01343.x

Guindo, C. O., Drancourt, M., and Grine, G. (2020). Digestive tract methanodrome: physiological roles of human microbiota-associated methanogens. *Microb. Pathog.* 149:104425. doi: 10.1016/j.micpath.2020.104425

Guo, W., Van Niekerk, J. K., Zhou, M., Steele, M. A., and Guan, L. L. (2021). Longitudinal assessment revealed the shifts in rumen and colon mucosal-attached microbiota of dairy calves during weaning transition. *J. Dairy Sci.* 104, 5948–5963. doi: 10.3168/jds.2020-19252

Hammon, H. M., Zanker, I. A., and Blum, J. W. (2000). Delayed colostrum feeding affects IGF-I and insulin plasma concentrations in neonatal calves. *J. Dairy Sci.* 83, 85–92. doi: 10.3168/jds.S0022-0302(00)74859-4

He, Z., Fischer, A., Song, Y., Steele, M., and Guan, L. L. (2018). Genome wide transcriptome analysis provides bases on colonic mucosal immune system development affected by colostrum feeding strategies in neonatal calves. *BMC Genomics* 19:635. doi: 10.1186/s12864-018-5017-y

Horler, D. F., Westlake, D. W., and Mcconnell, W. B. (1966). Conversion of glutamic acid to volatile acids by Micrococcus aerogenes. *Can. J. Microbiol.* 12, 47–53. doi: 10.1139/m66-008

Hyatt, D., Chen, G. L., Locascio, P. F., Land, M. L., Larimer, F. W., and Hauser, L. J. (2010). Prodigal: prokaryotic gene recognition and translation initiation site identification. *BMC Bioinformatics* 11:119. doi: 10.1186/1471-2105-11-119

Inabu, Y., Fischer, A., Song, Y., Guan, L. L., Oba, M., Steele, M. A., et al. (2018). Short communication: the effect of delayed colostrum feeding on plasma concentrations of glucagon-like peptide 1 and 2 in newborn calves. *J. Dairy Sci.* 101, 6627–6631. doi: 10.3168/jds.2018-14412

Jung, T. H., Han, K. S., Park, J. H., and Hwang, H. J. (2022). Butyrate modulates mucin secretion and bacterial adherence in LoVo cells via MAPK signaling. *PLoS One* 17:e0269872. doi: 10.1371/journal.pone.0269872

Kanehisa, M., Goto, S., Sato, Y., Furumichi, M., and Tanabe, M. (2012). KEGG for integration and interpretation of large-scale molecular data sets. *Nucleic Acids Res.* 40, D109–D114. doi: 10.1093/nar/gkr988

Kim, D., Pertea, G., Trapnell, C., Pimentel, H., Kelley, R., and Salzberg, S. L. (2013). TopHat2: accurate alignment of transcriptomes in the presence of insertions, deletions and gene fusions. *Genome Biol.* 14:R36. doi: 10.1186/gb-2013-14-4-r36

Lopetuso, L. R., Scaldaferri, F., Petito, V., and Gasbarrini, A. (2013). Commensal clostridia: leading players in the maintenance of gut homeostasis. *Gut Pathog* 5:23. doi: 10.1186/1757-4749-5-23

Lopez, A. J., and Heinrichs, A. J. (2022). Invited review: the importance of colostrum in the newborn dairy calf. *J. Dairy Sci.* 105, 2733–2749. doi: 10.3168/jds.2020-20114

Ma, T., O'hara, E., Song, Y., Fischer, A. J., He, Z., Steele, M. A., et al. (2019). Altered mucosa-associated microbiota in the ileum and colon of neonatal calves in response to delayed first colostrum feeding. *J. Dairy Sci.* 102, 7073–7086. doi: 10.3168/jds.2018-16130

Malmuthuge, N., Liang, G., GriebeL, P. J., and Guan, L. L. (2019). Taxonomic and functional compositions of the small intestinal microbiome in neonatal calves provide a framework for understanding early life gut health. *Appl. Environ. Microbiol.* 85, e02534-18. doi: 10.1128/AEM.02534-18

Meale, S. J., Popova, M., Saro, C., Martin, C., Bernard, A., Lagree, M., et al. (2021). Early life dietary intervention in dairy calves results in a long-term reduction in methane emissions. *Sci. Rep.* 11:3003. doi: 10.1038/s41598-021-82084-9

Meyer, F., Paarmann, D., D'souza, M., Olson, R., Glass, E. M., Kubal, M., et al. (2008). The metagenomics RAST server - a public resource for the automatic phylogenetic and functional analysis of metagenomes. *BMC Bioinformatics* 9:386. doi: 10.1186/1471-2105-9-386

Moore, J. W., Babidge, W., Millard, S., and Roediger, W. E. (1997). Effect of sulphide on short chain acyl-CoA metabolism in rat colonocytes. *Gut* 41, 77–81. doi: 10.1136/gut.41.1.77

Mottawea, W., Chiang, C. K., Mühlbauer, M., Starr, A. E., Butcher, J., Abujamel, T., et al. (2016). Altered intestinal microbiota-host mitochondria crosstalk in new onset Crohn's disease. *Nat. Commun.* 7:13419. doi: 10.1038/ncomms13419

Namiki, T., Hachiya, T., Tanaka, H., and Sakakibara, Y. (2012). MetaVelvet: an extension of velvet assembler to de novo metagenome assembly from short sequence reads. *Nucleic Acids Res.* 40:e155. doi: 10.1093/nar/gks678

Nkamga, V. D., Henrissat, B., and Drancourt, M. (2017). Archaea: essential inhabitants of the human digestive microbiota. *Hum. Microb. J.* 3, 1–8. doi: 10.1016/j.humic.2016.11.005

O'hara, E., Kelly, A., Mccabe, M. S., Kenny, D. A., Guan, L. L., and Waters, S. M. (2018). Effect of a butyrate-fortified milk replacer on gastrointestinal microbiota and products of fermentation in artificially reared dairy calves at weaning. *Sci. Rep.* 8:14901. doi: 10.1038/s41598-018-33122-6

O'hara, E., Kenny, D. A., Mcgovern, E., Byrne, C. J., Mccabe, M. S., Guan, L. L., et al. (2020). Investigating temporal microbial dynamics in the rumen of beef calves raised on two farms during early life. *FEMS Microbiol. Ecol.* 96:fiz203. doi: 10.1093/femsec/fiz203

Osaka, I., Matsui, Y., and Terada, F. (2014). Effect of the mass of immunoglobulin (Ig)G intake and age at first colostrum feeding on serum IgG concentration in Holstein calves. *J. Dairy Sci.* 97, 6608–6612. doi: 10.3168/jds.2013-7571

Picton, R., Eggo, M. C., Merrill, G. A., Langman, M. J., and Singh, S. (2002). Mucosal protection against sulphide: importance of the enzyme rhodanese. *Gut* 50, 201–205. doi: 10.1136/gut.50.2.201

Samarasinghe, M. B., Sehested, J., Weisbjerg, M. R., Van Der Heide, M. E., Nørgaard, J. V., Vestergaard, M., et al. (2021). Feeding milk supplemented with Ulva sp., Ascophyllum nodosum, or Saccharina latissima to preweaning dairy calves: effects on growth, gut microbiota, gut histomorphology, and short-chain fatty acids in digesta. *J. Dairy Sci.* 104, 12117–12126. doi: 10.3168/jds.2021-20680

Segata, N., Izard, J., Waldron, L., Gevers, D., Miropolsky, L., Garrett, W. S., et al. (2011). Metagenomic biomarker discovery and explanation. *Genome Biol.* 12:R60. doi: 10.1186/gb-2011-12-6-r60

Song, Y., Li, F., Fischer-Tlustos, A. J., Neves, A. L. A., He, Z., Steele, M. A., et al. (2021). Metagenomic analysis revealed the individualized shift in ileal microbiome of neonatal calves in response to delaying the first colostrum feeding. *J. Dairy Sci.* 104, 8783–8797. doi: 10.3168/jds.2020-20068

Song, Y., Malmuthuge, N., Li, F., and Guan, L. L. (2019). Colostrum feeding shapes the hindgut microbiota of dairy calves during the first 12 h of life. *FEMS Microbiol. Ecol.* 95. doi: 10.1093/femsec/fiy203

Song, Y., Malmuthuge, N., Steele, M. A., and Guan, L. L. (2018). Shift of hindgut microbiota and microbial short chain fatty acids profiles in dairy calves from birth to pre-weaning. *FEMS Microbiol. Ecol.* 94. doi: 10.1093/femsec/fix179

Stams, A. J. (1994). Metabolic interactions between anaerobic bacteria in methanogenic environments. *Antonie Van Leeuwenhoek* 66, 271–294. doi: 10.1007/BF00871644

Tappenden, K. A., Albin, D. M., Bartholome, A. L., and Mangian, H. F. (2003). Glucagon-like peptide-2 and short-chain fatty acids: a new twist to an old story. *J. Nutr.* 133, 3717–3720. doi: 10.1093/jn/133.11.3717

Wang, Y., An, M., Zhang, Z., Zhang, W., Kulyar, M. F., IqbaL, M., et al. (2022). Effects of Milk replacer-based lactobacillus on growth and gut development of Yaks' calves: A gut microbiome and metabolic study. *Microbiol. Spectr.* 10:e0115522. doi: 10.1128/spectrum.01155-22

Weese, J. S., and Jelinski, M. (2017). Assessment of the fecal microbiota in beef calves. *J. Vet. Intern. Med.* 31, 176–185. doi: 10.1111/jvim.14611

Yeoman, C. J., Ishaq, S. L., Bichi, E., Olivo, S. K., Lowe, J., and Aldridge, B. M. (2018). Biogeographical differences in the influence of maternal microbial sources on the early successional development of the bovine neonatal gastrointestinal tract. *Sci. Rep.* 8:3197. doi: 10.1038/s41598-018-21440-8

Yoav, B., and Yosef, H. (1995). Controlling the false discovery rate: a practical and powerful approach to multiple testing. *J. R. Stat. Soc. Ser. B.* 57, 289–300.

Yousif, M. H., Li, J. H., Li, Z. Q., Maswayi Alugongo, G., Ji, S. K., Li, Y. X., et al. (2018). Low concentration of antibiotics modulates gut microbiota at different levels in pre-weaning dairy calves. *Microorganisms* 6:118. doi: 10.3390/microorganisms6040118

Yu, Z., and Morrison, M. (2004). Improved extraction of PCR-quality community DNA from digesta and fecal samples. *Biotechniques* 36, 808–812. doi: 10.2144/04365ST04

Zhou, M., Chen, Y., Griebel, P. J., and Guan Le, L. (2014). Methanogen prevalence throughout the gastrointestinal tract of pre-weaned dairy calves. *Gut Microbes* 5, 628–638. doi: 10.4161/19490976.2014.969649

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# Chlorogenic acid improves growth performance of weaned rabbits *via* modulating the intestinal epithelium functions and intestinal microbiota

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This study was conducted to investigate the impacts of chlorogenic acid (CGA) on growth performance, intestinal permeability, intestinal digestion and absorption-related enzyme activities, immune responses, antioxidant capacity and cecum microbial composition in weaned rabbits. One hundred and sixty weaned rabbits were allotted to four treatment groups and fed with a basal diet or a basal diet supplemented with 400, 800, or 1,600mg/kg CGA, respectively. After a 35-d trial, rabbits on the 800mg/kg CGA-supplemented group had higher ($p$<0.05) ADG and lower ($p$<0.05) F/G than those in control (CON) group. According to the result of growth performance, eight rabbits per group were randomly selected from the CON group and 800mg/kg CGA group to collect serum, intestinal tissue samples and cecum chyme samples. Results showed that compared with the CON group, supplementation with 800mg/kg CGA decreased ($p$<0.05) levels of D-lactate, diamine oxidase, IL-1β, IL-6, and malondialdehyde (MDA), and increased IL-10 concentration in the serum; increased ($p$<0.05) jejunal ratio of villus height to crypt depth, enhanced ($p$<0.05) activities of maltase and sucrase, increased ($p$<0.05) concentrations of IL-10, T-AOC, MHCII and transforming growth factor-α, and decreased ($p$<0.05) levels of TNF-α and MDA in the jejunum of weaned rabbits. In addition, results of high-throughput sequencing showed that CGA supplementation elevated ($p$<0.05) microbial diversity and richness, and increased ($p$<0.05) the abundances of butyrate-producing bacteria (including genera *V9D2013_group*, *Monoglobus*, *Papillibacter*, *UCG-005*, and *Ruminococcus*). These results indicated that dietary supplementation with 800mg/kg CGA could improve the growth performance of weaned rabbits by enhancing intestinal structural integrity, improving the intestinal epithelium functions, and modulating the composition and diversity of gut microbiota.

KEYWORDS

**rabbit, chlorogenic acid, growth performance, small intestine, microbiota**

# Introduction

Weaning is an inevitable and stressful process for mammals, as it may affect the morphological structure, barrier function, and intestinal microbiota of the gastrointestinal tract with altered food status, resulting in reduced feed intake and growth retardation, and consequent economic losses (Gharib et al., 2018). Moreover, accumulated evidences showed that weaning stress could cause mammalian intestinal barrier dysfunction by inducing physiological inflammation and oxidative stress within a short time after weaning (Smith et al., 2010; Hu et al., 2013; Gharib et al., 2018). It is reported that digestive diseases account for 70% of all rabbit diseases (Carabaño et al., 2008) and up to 60% mortality were cause by epizootic rabbit enteropathy, especially for weaned rabbits (Fann and O'Rourke, 2001; Carabaño et al., 2008). As we all know, intestine is not only the main site for nutrient absorption but also provides a primary barrier against pathogenic microorganisms (Martens et al., 2018). Therefore, it is a priority to explore effectively and safely strategies to prevent gastrointestinal mucosal damage in weaned rabbits.

Plant polyphenols have been reported to be able to ameliorate the damage of animals induced by stress (Relja et al., 2012; Yan et al., 2020). Chlorogenic acid (CGA) is one kind of natural polyphenols that formed by esterification of caffeic acid and quinic acid and widely found in coffee, fruits, and vegetables (Bouayed et al., 2007). It is safe with minimal side effects (Metwally et al., 2020) and possesses various biological properties, including antioxidant, anti-inflammatory, and antimicrobial activities, all of which contributed to maintain intestinal health (Chen et al., 2018b, 2021b). Previous study showed that CGA could restore intestinal epithelial tight-junction integrity and ameliorate intestinal inflammation in lipopolysaccharide (LPS)-challenged Caco-2 cells (Yu et al., 2021) and enhance the growth performance of weaned rats (Ruan et al., 2014). Moreover, an early study in weaned pigs showed that dietary CGA supplementation could improve the growth performance by enhancing the intestinal digestion and absorption function (Chen et al., 2018a). However, until now, there is little literature available evaluating the growth-promoting effect of CGA on weaned rabbits whose intestine is more vulnerable and sensitive to weaning stress. In addition, as CGA is partially consumed by symbiotic microorganisms in the large intestine to generate short-chain fatty acids (Lu et al., 2020), it has the potential to be employed as prebiotics to repair damaged intestinal barrier (Chen et al., 2019).

Therefore, in view of the foregoing, it is plausible to hypothesize that CGA has the potential in enhancing the growth performance of weaned rabbits by maintaining the intestinal barrier structure and function. This study was carried out to verify the above hypothesis by evaluating the effects of CGA on intestinal permeability, intestinal digestion and absorption-related enzyme activities, immune responses, antioxidant capacity and cecum microbial composition in weaned rabbits.

# Materials and methods

## Experimental animals, diet and management

A total of one hundred and sixty weaned rabbits, with an initial average body weight (BW) of 1.05 ± 0.01 kg, were randomly allotted to four dietary treatments ($n = 40$) in a randomized complete block design. The treatments consist of a basal diet (control, CON) and a basal diet supplemented with 400, 800, or 1,600 mg/kg CGA (provided by Chengdu Hengfeng Tiancheng Technology Co., Ltd., Chengdu, China). The experiment lasted for 35 days. The basal diet was formulated based on the recommendation of Mateos et al. (2010), and the feed was made into 4-mm pellets by using steam. Ingredients and compositions of the basal diet are shown in Table 1. All rabbits were housed in cages (60 cm × 40 cm × 40 cm) individually and were given *ad libitum* access to fresh water and feed. Temperature and lighting were maintained according to commercial conditions (20 to 23°C, 12 light/12 dark).

## Growth performance

Feed consumption of each rabbit were measured daily throughout the trial. The BWs of rabbits were individually measured after 12 h fasting on the morning of days 1, 15 and 36 of the feeding trial. The value of average daily BW gain (ADG),

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

| Ingredients | Content (%) | Nutrient levels[2] | Content (%) |
|---|---|---|---|
| Corn | 13.30 | Dry matter | 84.49 |
| Bean pulp | 13.00 | Crude protein | 16.38 |
| Wheat bran | 19.00 | Crude fat | 3.10 |
| Corn germ meal | 19.00 | Crude fiber | 15.44 |
| Alfalfa meal | 12.00 | Acid detergent fiber | 20.60 |
| Soya bean stem meal | 19.00 | Neutral detergent fiber | 37.85 |
| Soya oil | 0.70 | Ash | 5.36 |
| Premix[1] | 4.00 | Calcium | 1.13 |
| Total | 100.00 | Phosphorus | 0.49 |

[1]The following premix ingredients were added to each kg of diet: vitamin A, 8,000 IU; vitamin D3, 1,000 IU; vitamin E, 50 mg; vitamin K3, 2.3 mg; thiamine, 1.75 mg; riboflavin, 6.9 mg; niacin, 28.45 mg; pantothenic acid, 6.7 mg; biotin, 2.75 mg; folic acid, 0.6 mg; vitamin B12, 2.2 mg; choline, 420 mg; lysine, 1.5 g; methionine, 1.5 g; copper, 50 mg; iron, 100 mg; manganese, 30 mg; magnesium, 150 mg; iodine, 0.1 mg.
[2]Values were calculated.

average daily feed intake (ADFI), and the feed-to-gain ratio (F/G) were then calculated.

## Sampling collection and preparation

According to the result of growth performance, 800 mg/kg feed is the optimum dose of CGA for growth promotion. Therefore, to further explain the growth-promoting effect of CGA, eight rabbits with the average BW of CON and 800 mg/kg CGA treatment groups were selected for sample collection. Blood samples were collected from the ear edge vein, and serum samples were then harvested after centrifugation at 3,000×*g* for 10 min at 4°C and stored at-20°C for further analysis. The same sixteen rabbits were then sacrificed by cervical dislocation, and the abdomen was immediately opened to remove the entire small intestine. Two segments (about 2-cm) of the mid-jejunum were quickly isolated, and washed gently by cold saline solution. One segment was fixed in 4% paraformaldehyde for morphology measurements; another segment was divided into different packages, and stored at-80°C for further analysis after being rapidly frozen in liquid nitrogen. Furthermore, the chyme samples of cecum were quickly collected as described in Li et al. (2019), and stored at-80°C for microbiological analysis.

## Intestinal morphology measurement

The jejunum morphology was conducted as previously described by Li J. et al. (2022). After fixation for 24 h, jejunal segments were removed from the 4% paraformaldehyde solution, dehydrated with normal saline, and embedded in paraffin wax. Tissue sections were cut into ~5 μm slices using a microtome (Leica RM2235), followed by being fixed on slides, and stained with hematoxylin and eosin. Jejunum mucosal morphology including villus height and crypt depth was determined with ImageJ analysis software (Version 1.47, Bethesda, MD, United States). A minimum of 10 well-orientated villi and their adjoined crypts from each sample were chosen for statistical analysis.

## Serum cytokines, antioxidant parameters, diamine oxidase (DAO) and D-lactic acid levels

The serum levels of serum interleukin 1 beta (IL-1β), tumor necrosis factor-alpha (TNF-α), interleukin 10 (IL-10), interleukin 6 (IL-6) and D-lactic acid were measured spectrophotometrically by the corresponding rabbit enzyme-linked immunosorbent assay (ELISA) kit (Jiangsu Meimian Industrial Co., Ltd., Jiangsu, China) according to the previous study (Li J. et al., 2022). The concentrations of malondialdehyde (MDA), total antioxidant capacity (T-AOC), glutathione peroxidase (GSH-Px), catalase (CAT) and diamine oxidase (DAO) in serum were measured by corresponding assay kits (Nanjing Jiancheng Institute of Bioengineering, Nanjing, China) according to the manufacturer's instructions.

## Jejunum mucosa parameters measurements

The mucosa samples of jejunum were homogenized with ice-cold saline solution in a weight (g): volume (ml) ratio of 1: 9. The homogenates were centrifuged at 2,500 × g for 10 min at 4°C, and the jejunum mucosal supernatant was collected for the further detection. The concentration of intestinal mucosa cytokines (IL-1β, TNF-α, IL-10 and IL-6), transforming growth factor-α (TGF-α), trefoil factor family (TFF) and major histocompatibility complex II (MHCII) were measured by the corresponding rabbit ELISA kit (Jiangsu Meimian Industrial Co., Ltd., Jiangsu, China) according to the previous study (Li J. et al., 2022). The concentration of disaccharide enzyme (lactase, maltase and sucrase) and antioxidant indexes (MDA, T-AOC, GSH-Px and CAT) were measured by the corresponding detection kits purchased from Nanjing Jiancheng Institute of Bioengineering (Nanjing, China) according to the manufacturer's instructions.

## 16S rRNA sequencing and analysis

Microbial composition and diversity were analyzed as described in Li et al. (2020). Briefly, microbial DNA was extracted from frozen fecal samples using an E.Z.N.A.™ Stool DNA kit (Omega Bio-Tek, Norcross, GA, United States) according to the manufacturer's protocols. After the final DNA concentration and purity determining, DNA quality was checked by 1% agarose gel electrophoresis. DNA was diluted to 1 ng/μL using sterile water, and the V4 hypervariable regions of the bacterial 16S rRNA gene were amplified with the primers 515F (5′-GTGCCAGCMGCCGCGGTAA-3′) and 806R (5′-GGACTACHVGGGTWTCTAAT-3′) on the Illumina HiSeq PE2500 platform by Novogene (Beijing, China). The filtered, non-chimeric high-quality sequences (tags) were clustered into the same operational taxonomic units (OTUs) by 97% sequence similarity using UPARSE software. Then the OTUs were classified to different taxonomic levels using SILVA database based on Mothur algorithm to annotate taxonomic information. Alpha diversity and beta diversity were analyzed after the operational taxonomic unit abundance information were normalized. Shannon, Simpson, Chao 1, and ACE indexes were chosen to ascertain differences in alpha diversity between the different groups (Chen L. et al., 2018; Gallego et al., 2021), and Bray-Curtis distances were calculated for comparison of taxonomic data in beta diversity, and visualized using Principal Coordinate Analysis (PCoA; Lozupone and Knight, 2005). The statistical differences in alpha and beta diversity of bacterial communities between the two groups were examined using the Wilcoxon rank-sum test. The analysis of

similarity (ANOSIM) test method was used to access the significant difference among the microbial communities.

## Statistical analysis

All data were assessed for normal distribution using the Shapiro–Wilk's statistic ($W > 0.05$). Data on growth performance were analyzed using one-way analysis of variance (ANOVA) procedure of SAS 9.4 (Institute Inc., Cary, NC), followed by Tukey's multiple-range test to compare the statistical differences among groups. The linear and quadratic responses to the dietary CGA levels were detected by using the REG procedure in SAS. The results of the experiments were expressed as mean and SEM. Statistical significance was presented at $p < 0.05$ and a tendency toward difference was considered as $p < 0.10$. For other indexes, data were analyzed by *T*-test using the statistical program SAS 9.4. Spearman's rank test was used to evaluate the correlations between the abundances of differential bacteria and the levels of inflammatory cytokines and antioxidant indices. Multiple testing was corrected by using the Benjamini-Hochberg false discovery rate. The data were plotted in the figures as mean and standard error. Significant differences are displayed in the figures by $^{*}0.01 < p < 0.05$, and $^{**}p < 0.01$, while $0.05 < p < 0.10$ was considered as a trend to significance.

# Results

## Growth performance

As shown in Table 2, higher ($p < 0.05$) BW on day 35 of the trial was observed in rabbits fed the 800 mg/kg CGA-supplemented diet compared with those fed the CON and 1,600 mg/kg CGA-supplemented diets and demonstrated quadratic ($p < 0.05$) effects in response to increasing CGA levels. However, dietary CGA supplementation had no significant effect ($p > 0.05$) on the growth performance of weaned rabbits on days 1–14 of the trial. From days 15 to 35, the rabbits fed CGA-supplemented diets tended ($p < 0.10$) to have an increased ADFI compared with those fed the CON diet; the ADG in 400 and 800 mg/kg CGA-supplemented groups was higher ($p < 0.05$) than that in CON group; The ADG in 800 mg/kg CGA-supplemented group was higher ($p < 0.05$) than that in 1600 mg/kg CGA-supplemented group and showed a quadratic ($p < 0.05$) effect in response to increasing CGA levels; but no significant difference ($p > 0.05$) was observed in F/G among the groups. For the whole trial period, the ADG of rabbits in 800 mg/kg CGA-supplemented group were higher ($p < 0.05$) than that in CON and 1,600 mg/kg CGA-supplemented groups; lower ($p < 0.05$) F/G was observed in rabbits fed the 800 mg/kg CGA-supplemented diet compared with those fed the CON and 1,600 mg/kg CGA-supplemented diets; the ADG and F/G demonstrated quadratic ($p < 0.05$) effects in response to increasing CGA levels; in addition, dietary supplemented with CGA tended ($p < 0.10$) to affect the ADFI of rabbits.

## Intestinal morphology

The intestinal morphology of rabbits is showed in Figure 1. Rabbits in CGA group had higher ($p < 0.05$) villus height to crypt depth ratio than rabbits in CON group. However, no significant

TABLE 2 Effects of CGA on growth performance of weaned rabbits.

| | Dietary CGA levels (mg/kg) | | | | | *p*-Values | | |
|---|---|---|---|---|---|---|---|---|
| **Items** | **0** | **400** | **800** | **1,600** | **SEM** | **Treatment** | **Linear** | **Quadratic** |
| Initial BW (kg) | 1.05 | 1.04 | 1.05 | 1.05 | 0.012 | 0.996 | 0.998 | 0.985 |
| d 14 BW (kg) | 1.58 | 1.58 | 1.64 | 1.55 | 0.018 | 0.308 | 0.694 | 0.121 |
| d 35 BW (kg) | 2.47$^{b}$ | 2.59$^{ab}$ | 2.66$^{a}$ | 2.46$^{b}$ | 0.027 | 0.018 | 0.734 | 0.002 |
| d 1–14 | | | | | | | | |
| ADFI (g/d) | 124.59 | 125.83 | 128.59 | 121.42 | 1.635 | 0.486 | 0.495 | 0.189 |
| ADG (g/d) | 38.39 | 38.37 | 44.03 | 36.23 | 1.201 | 0.115 | 0.670 | 0.057 |
| F/G | 3.47 | 3.68 | 3.11 | 3.78 | 0.103 | 0.101 | 0.494 | 0.148 |
| d 15–35 | | | | | | | | |
| ADFI (g/d) | 155.38 | 166.91 | 166.54 | 156.25 | 2.154 | 0.090 | 0.813 | 0.014 |
| ADG (g/d) | 42.43$^{c}$ | 47.99$^{ab}$ | 48.34$^{a}$ | 43.18$^{bc}$ | 0.886 | 0.023 | 0.911 | 0.002 |
| F/G | 3.78 | 3.52 | 3.55 | 3.76 | 0.058 | 0.243 | 0.862 | 0.050 |
| d 1–35 | | | | | | | | |
| ADFI (g/d) | 143.07 | 150.48 | 151.36 | 142.32 | 1.553 | 0.067 | 0.628 | 0.009 |
| ADG (g/d) | 40.81$^{b}$ | 44.14$^{ab}$ | 46.61$^{a}$ | 40.40$^{b}$ | 0.775 | 0.010 | 0.733 | 0.001 |
| F/G | 3.61$^{a}$ | 3.45$^{ab}$ | 3.31$^{b}$ | 3.63$^{a}$ | 0.046 | 0.048 | 0.843 | 0.008 |

Results are presented as mean and SEM. $^{ab}$Means in the same row with different letter differ ($P < 0.05$). $n = 40$. BW, bodyweight; ADFI, average daily feed intake; ADG, average daily gain; F/G, feed efficiency (the ratio of ADFI and ADG).

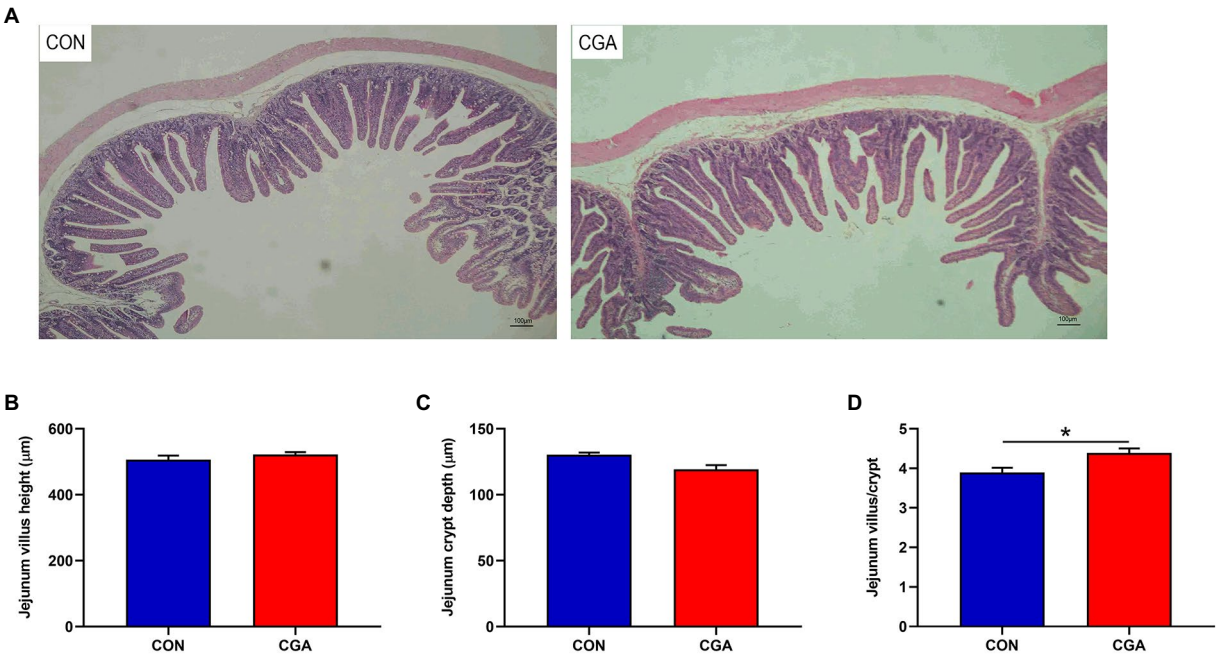


FIGURE 1
Effects of CGA on intestinal morphology in the jejunum of weaned rabbits. **(A)** Hematoxylin and eosin photomicrographs obtained at 100×magnification; **(B)** Villus height; **(C)** Crypt depth; **(D)** Villus height/crypt depth ratio. CON, rabbits fed a basal diet; CGA, rabbits fed the basal diet supplemented with 800mg/kg chlorogenic acid. Results are presented as mean and SEM ($n$=8). Significant difference was recorded by $0.01<p<0.05$*.

effect of dietary CGA supplementation was detected on villus height and crypt depth.

## Intestinal barrier and function

As shown in Figure 2, the levels of DAO (Figure 2A) and D-lactase (Figure 2B) in the serum were decreased ($p<0.05$) by CGA supplementation. Compared with the CON group, CGA supplementation increased ($p<0.05$) the activities of maltase (Figure 2D) and sucrase (Figure 2E) in the jejunum mucosa of rabbits. However, there was no significant difference in lactase (Figure 2C) activity between the two groups. Besides, higher ($p<0.05$) TGF-α (Figure 2H) concentration in the jejunum mucosa was also observed in rabbits fed the CGA-supplemented diet, meanwhile rabbits fed CGA diet tended to ($0.05<p<0.10$) have an increased jejunum MHCII (Figure 2F) concentration. But no significant difference was observed in jejunal concentration of TFF (Figure 2G) in rabbits between the two groups.

## Systemic and intestinal inflammatory cytokines

The effects of CGA on serum and jejunum inflammatory cytokines are presented in Figure 3. Compared with the CON group, the serum IL-1β (Figure 3A) and IL-6 (Figure 3D) levels were decreased ($p<0.05$), whereas the serum IL-10 (Figure 3C) level was increased ($p<0.05$) in CGA group. Meanwhile, rabbits fed the CGA diet had lower ($p<0.05$) TNF-α (Figure 3F) level and higher ($p<0.05$) IL-10 (Figure 3G) level in the jejunum were observed in rabbits on CGA group. However, no significant differences in serum TNF-α (Figure 3B) concentration and jejunal IL-1β (Figure 3E) and IL-6 (Figure 3H) levels were detected between the two treatment groups.

## Systemic and intestinal antioxidant capacity

As showed in Figure 4, dietary CGA supplementation decreased ($p<0.05$) the activity of CAT (Figure 4D) and the content of MDA (Figure 4A) but had no significant effects on the activities of T-AOC (Figure 4B) and GSH-Px (Figure 4C) in the serum of rabbits. In jejunum, the content of T-AOC (Figure 4F) was increased ($p<0.05$) and the MDA (Figure 4E) content was decreased ($p<0.05$) by CGA supplementation. There were no significant differences in the activities of GSH-Px (Figure 4G) and CAT (Figure 4H) between the two treatment groups.

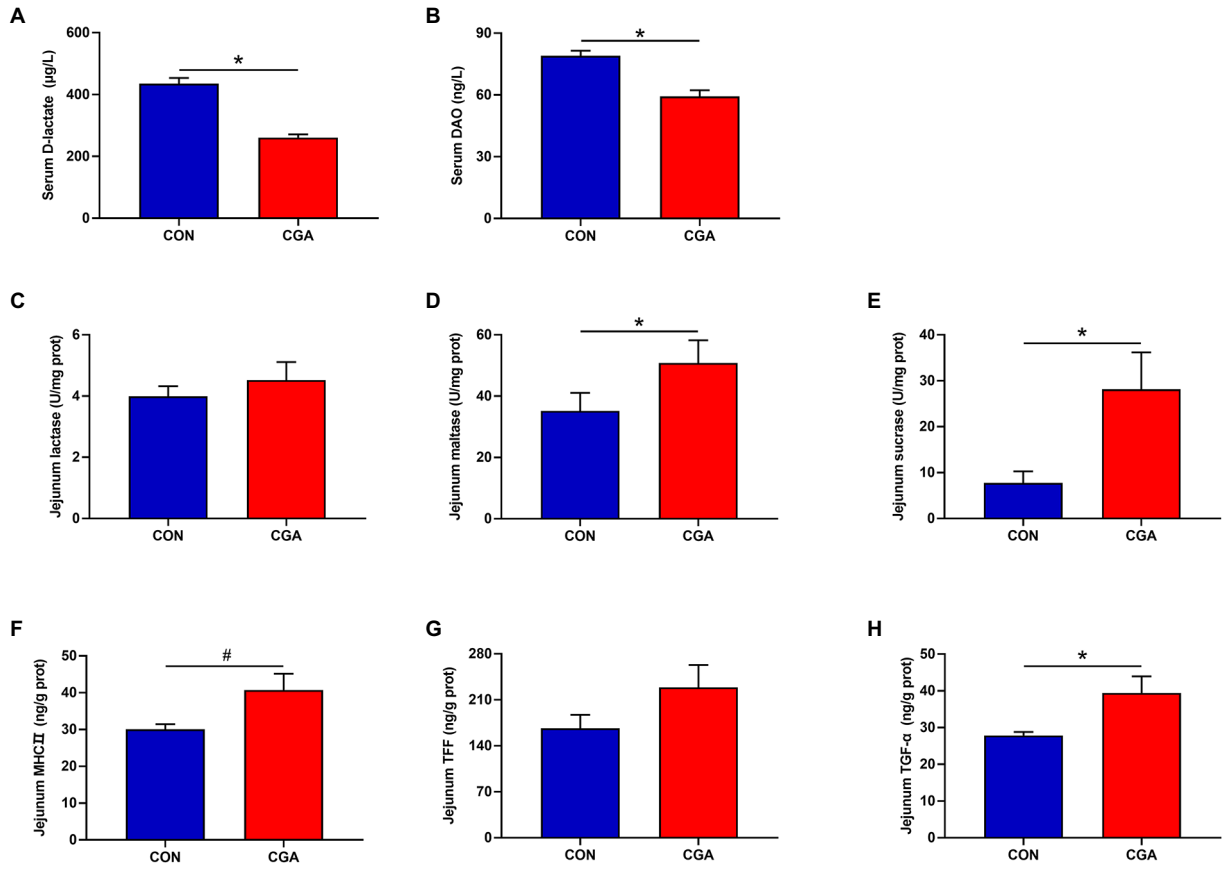


FIGURE 2
Effects of CGA on the maturity and integrity of small intestine in weaned rabbits. **(A)** serum D-lactate; **(B)** serum diamine oxidase (DAO); **(C)** jejunal lactase; **(D)** jejunal maltase; **(E)** jejunal sucrase; **(F)** jejunal major histocompatibility complex II (MHCII); **(G)** jejunal trefoil factor family (TFF); **(H)** jejunal transforming growth factor-α (TGF-α). CON, rabbits fed a basal diet; CGA, rabbits fed the basal diet supplemented with 800mg/kg chlorogenic acid. Results are presented as mean and SEM ($n$=8). Significant difference was recorded by $0.01<p<0.05$*. #Means a tendency to difference ($0.05<p<0.10$).

## Analysis of bacterial community and diversity

As shown in Supplementary Figure 1, a total of 1,140,800 total tags, 1,067,486 taxon tags, 74 unclassified tags, and 73,240 unique tags were obtained from 16 cecal digesta samples. After taxonomic information was annotated, a total of 23,085 OTUs were obtained, with an average of 1,272 ± 92 in CON group and 1,614 ± 118 in CGA group. The bacteria community diversity and richness are shown in Figure 5. The two groups shared 2,217 common OTUs, and rabbits in CGA group showed an increased number of unique OTUs (Figure 5A). Compared with the CON group, CGA group showed significantly higher ($p<0.05$) observed species (Figure 5B), Shannon index (Figure 5C), and Chao 1 index (Figure 5E), and tended to ($0.05<p<0.10$) increase Simpson index (Figure 5D) and ACE index (Figure 5F) of cecal microbiota in weaned rabbits.

The beta diversity of microbial community is shown in Figure 6. The results of heat-map (Figure 6A) and PCoA plot (Figure 6B) drawn based on the bray_curtis distances displayed that the PFA samples tended to cluster separately from the CON samples, and ANOSIM (Figure 6C) showed the two groups tended to have different bacterial community structures ($R=0.081$, $p=0.059$).

## Relative abundance of cecal microbiota

The top 10 phyla in relative abundance of cecal microbiota are shown in shown in Figure 7 and Supplementary Table S1. The most predominant phyla in cecal samples of rabbits were Firmicutes and Bacteroidetes. However, no significant differences were observed in the relative abundances of top ten phyla between the two groups.

The relative abundances at genus level in rabbit cecal microbiota (top 30 genera) are shown in Figure 8 and Supplementary Table S2. The identified most plentiful genera in cecal samples were *Akkermansia*, *NK4A214_group*, and *Christensenellaceae_R-7_group*. Of the top 35 genera, the relative abundances of *V9D2013_group*, *Monoglobus*, *Papillibacter*, and

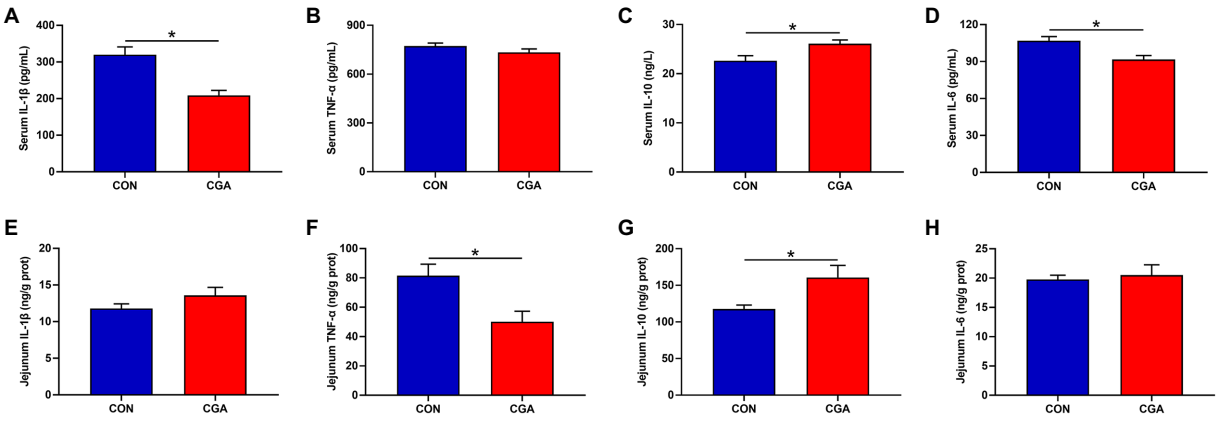


FIGURE 3
Effects of CGA on the levels of critical inflammation-related molecules in the serum **(A–D)** and jejunum **(E–H)** of weaned rabbits. IL-1β, interleukin 1 beta; TNF-α, tumor necrosis factor-alpha; IL-10, interleukin 10; IL-6, interleukin 6; CON, rabbits fed a basal diet; CGA, rabbits fed the basal diet supplemented with 800mg/kg chlorogenic acid. Results are presented as mean and SEM ($n$=8). Significant difference was recorded by $0.01<p<0.05$*.

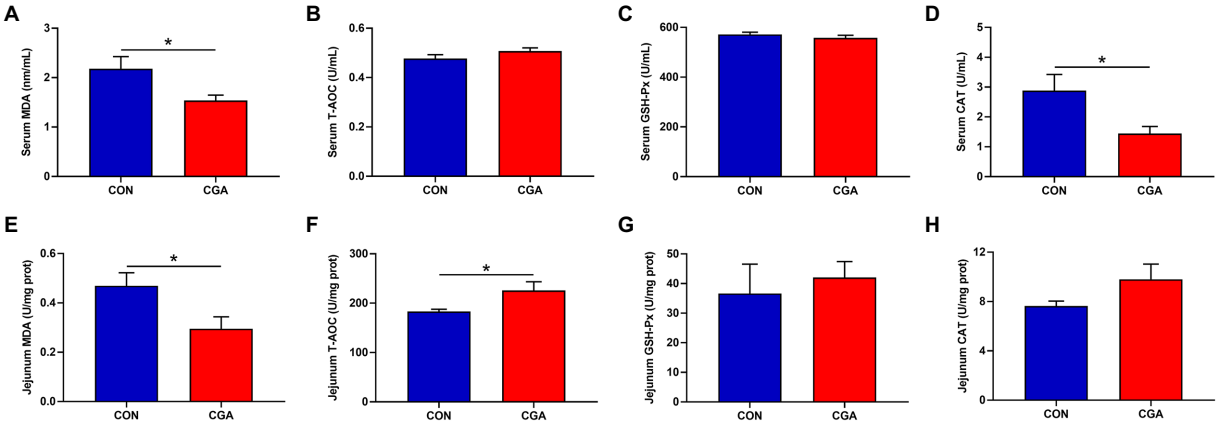


FIGURE 4
Effects of CGA on the levels of antioxidant indicators in the serum **(A–D)** and jejunum **(E–H)** of weaned rabbits. MDA, malondialdehyde; T-AOC, total antioxidant capacity; GSH-Px, glutathione peroxidase; CAT, catalase; CON, rabbits fed a basal diet; CGA, rabbits fed the basal diet supplemented with 800mg/kg chlorogenic acid. Results are presented as mean and SEM ($n$=8). Significant difference was recorded by $0.01<p<0.05$*.

*UCG-005* were higher ($p<0.05$) in CGA group than in CON group. Besides, dietary CGA supplementation tended to increase the relative abundance of *Ruminococcus* compared with the CON group ($p=0.095$).

## Correlation analysis between the abundances of differential bacteria and the levels of inflammatory cytokines and antioxidant indices

As shown in Figure 9, the abundances of *V9D2013* and *Papillibacter* showed negative correlation with the serum MDA concentration ($p<0.05$). The abundance of *Monoglobus* had significant positive correlation with the CAT activity ($p<0.05$). The abundance of *UCG-005* showed positive correlations with jejunal IL-10 and T-AOC levels ($p<0.05$), and negative correlations with jejunal TNF-α concentration ($p<0.05$). In addition, the abundance of *Ruminococcus* had significant positive correlation with jejunal T-AOC concentration ($p<0.05$).

# Discussion

Numerous studies have verified the beneficial effects of CGA on the health of human beings and animals (Naveed et al., 2018;

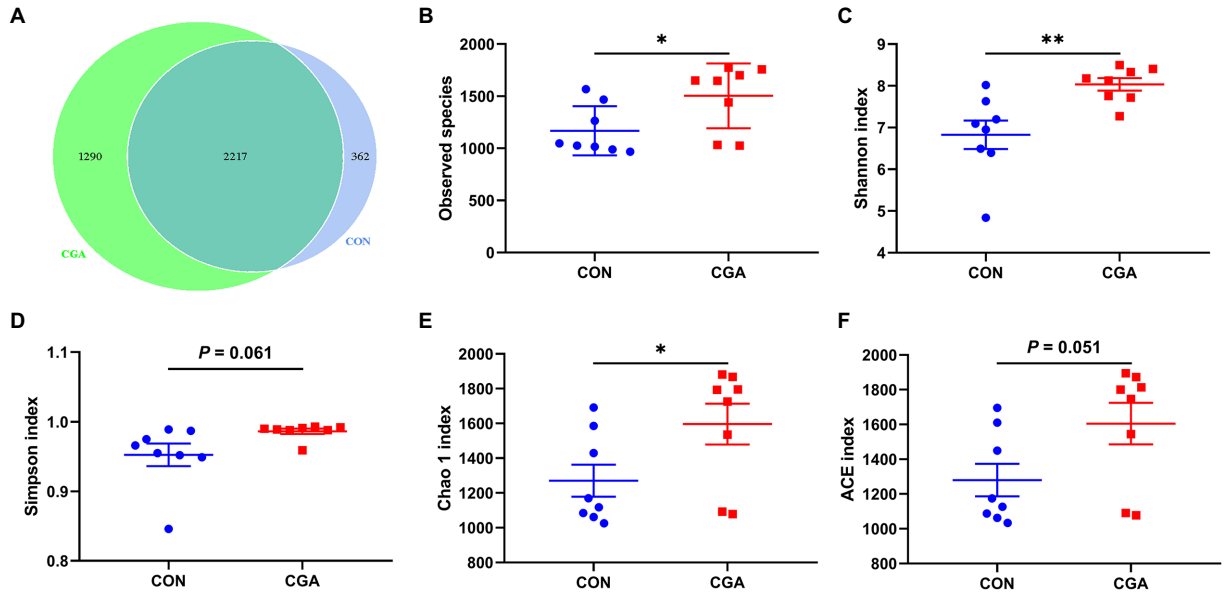


FIGURE 5
Differences on bacteria community diversity and richness between the two groups. **(A)** A Venn diagram generated to depict shared and nique sequences between the two groups; **(B)** Observed species; **(C)** Shannon index; **(D)** Simpson index; **(E)** Chao 1 index; **(F)** ACE index. CON, rabbits fed a basal diet; CGA, rabbits fed the basal diet supplemented with 800mg/kg chlorogenic acid. Results are presented as mean and SEM ($n$=8). Significant difference was recorded by 0.01<$p$<0.05*, $p$<0.01**.

Lu et al., 2020). The present study showed that dietary supplemented with 800 mg/kg CGA increased the ADG and F/G in rabbits though had no significant effect on feed intake, which was in accordance with previous studies in weaned pigs and rats (Ruan et al., 2014; Chen et al., 2018a). It suggested that dietary supplementation with 800 mg/kg CGA could increase the bioavailability of dietary nutrients and CGA can also play a good growth promoter for weaned rabbits. These interesting results provided a foundation for further study and the application of CGA in rabbits.

The small intestine is the leading site for nutrient digestion and absorption. A previous study has indicated that the complete intestinal structure is the basis for its normal function (Duan et al., 2022). Maintenance of normal small intestine morphology and structural integrity are imperative for improving nutrient absorption and preventing bacterial translocation from the intestinal tract. However, weaning stress often induces dramatic changes in intestinal morphology, such as villous shedding, villus atrophy, and crypt hyperplasia (Bivolarski and Vachkova, 2014), which adversely influences the digestion and absorption of nutrients and consequently causes growth retardation (Cera et al., 1988). The villi are critical components of the digestive tract, and determine the absorption capacity of the small intestine (Heo et al., 2013). Moreover, the ratio of villus height to crypt depth is an important parameter in evaluating the absorptive capacity of the small intestine (Xu et al., 2017). The findings of Ruan et al. (2014) showed that CGA significantly increased the villus height and the ratio of villus height to crypt depth, and decreased the crypt depth in the jejunum of LPS-challenged weaned rodents. Similarly, in present study, we found that the small intestinal mucosal morphology was significantly elevated by increasing the ratio of villus height to crypt depth in the jejunum, suggesting that dietary CGA supplementation could maintain favorable mucosa structure and enlarge the absorptive area of luminal villous, which benefited to promote the intestinal health of weaned rabbits.

Changes of the intestinal morphology is usually correlated to decreased digestion and absorption function, mainly characterized by digestive enzyme alteration (Kirmiz et al., 2018). Disaccharide enzymes, including maltase, lactase and sucrose, are the main enzymes in brush border of the intestinal epithelium, which play important roles in promoting carbohydrate breakdown, providing continuous energy reserves, and then promoting the growth performance (Fleige et al., 2007). In this study, 800 mg/kg CGA supplementation significantly improved the activities of maltase and sucrase in the jejunum of weaned rabbits, implying that dietary supplementation with 800 mg/kg CGA helped to improve digestion function of small intestine in weaned rabbits. Similarly, Chen et al. (2018a) also found that dietary supplemented 1,000 mg/kg CGA enhanced jejunal activities of lactase and maltase in weaned piglets. Moreover, previous studies have indicated that phenolic compounds (including CGA) possess potential roles in regulating nutrient (such as protein and lipid) metabolism in liver tissues (Lafay et al., 2006; Bagdas et al., 2020). These results in current study, together with similar results, might be a possible explanation for the positive effects of CGA on the growth performance of CGA-supplemented weaned rabbits.

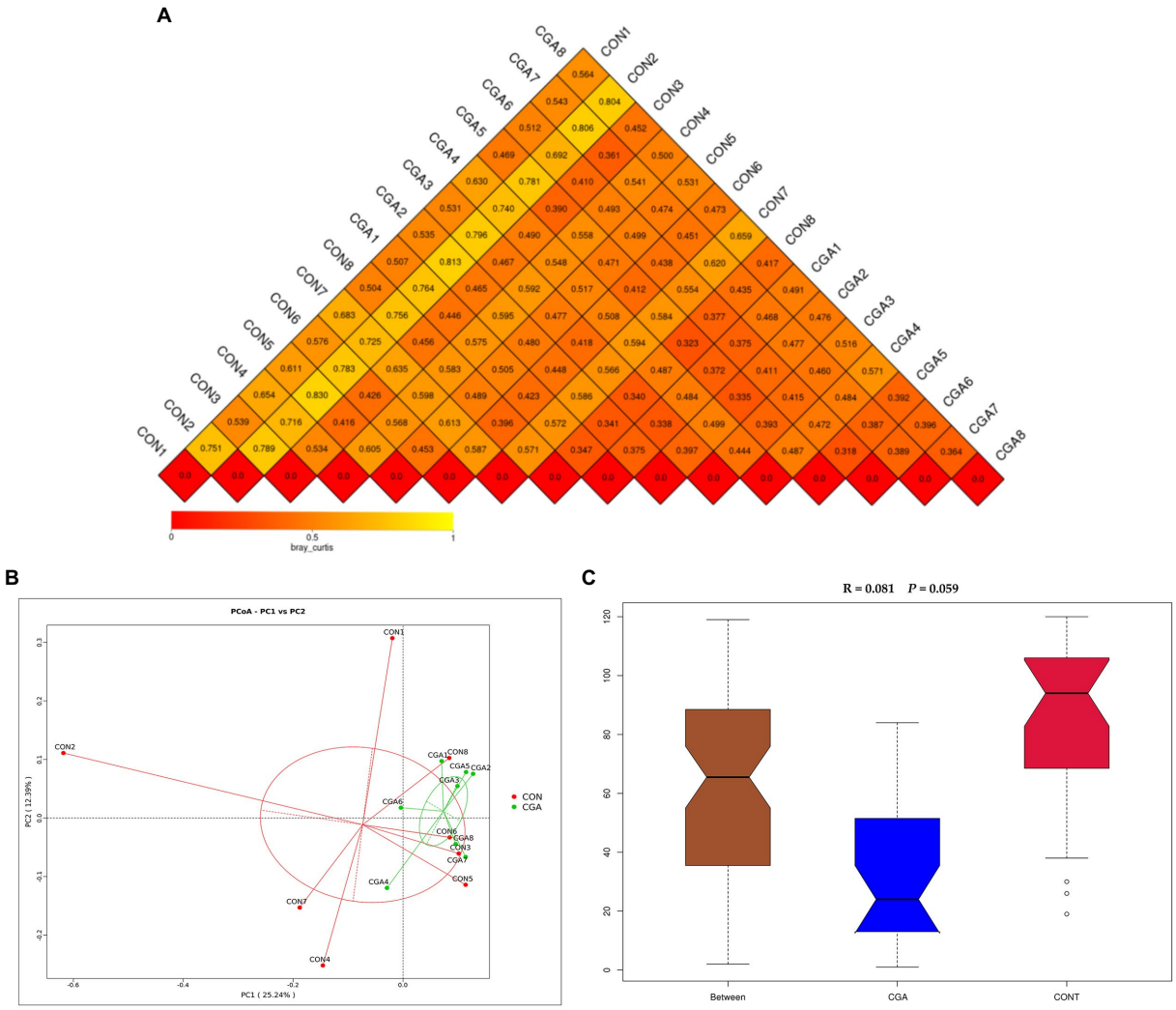


FIGURE 6
Beta diversity of microbial community analysis. **(A)** Heat-map of beta diversity for each two samples in the two groups by weighted Unifrac distance. **(B)** The principal coordinate analysis (PCoA) profile of weighted Unifrac distance. **(C)** Analysis of ANOSIM. *R* value is scaled to lie between −1 and+1. Generally, 0<*R*<1 and *p*<0.05 represents that there were significant differences between the groups. CON, rabbits fed a basal diet; CGA, rabbits fed the basal diet supplemented with 800mg/kg chlorogenic acid. *n*=8.

The integrity of intestinal barrier is closely related to the maintenance of intestinal function. However, previous studies in mammal indicated that weaning stress could induce seriously impairment of small intestinal barrier function within a short time after weaning (Smith et al., 2010; Bivolarski and Vachkova, 2014). It is well known that intestinal permeability can be commonly assessed using some blood indices. Diamine oxidase, an intracellular enzyme, is primarily expressed in the small intestine, but rare in the serum under normal circumstances (Zhao et al., 2014). Once the intestinal epithelial barrier is injured, the serum DAO levels will increase (Nieto et al., 2000). Moreover, D-lactic acid, a metabolite of intestinal bacteria, is primarily found in the small intestine, but it will be released into the blood circulation when intestinal injury occurs (Li J. et al., 2021). Therefore, increased serum DAO and D-lactic acid levels can be considered as circulating markers for reflecting an injury degree of intestinal mucosal barrier. In the present study, serum D-lactic acid concentration and DAO activity were found to be decreased in weaned rabbits fed the CGA diet, indicating that CGA had beneficial effects on attenuating intestinal permeability of weaned rabbits. In addition, another meaningful discovery in the present study was that dietary CGA supplementation could also improve the concentration of TGF-α in the jejunum of weaned rabbits. TGF-α is another crucial molecule in maintaining the integrity of intestinal epithelial cells (Chen et al., 2019). This result further suggested the improved effect of CGA on maintaining the integrity of intestinal permeability in weaned rabbits.

Intestinal inflammatory is one of the activators causing intestinal barrier dysfunction (Wang et al., 2020). Luissint et al. (2016) reported that the inflammatory microenvironment could

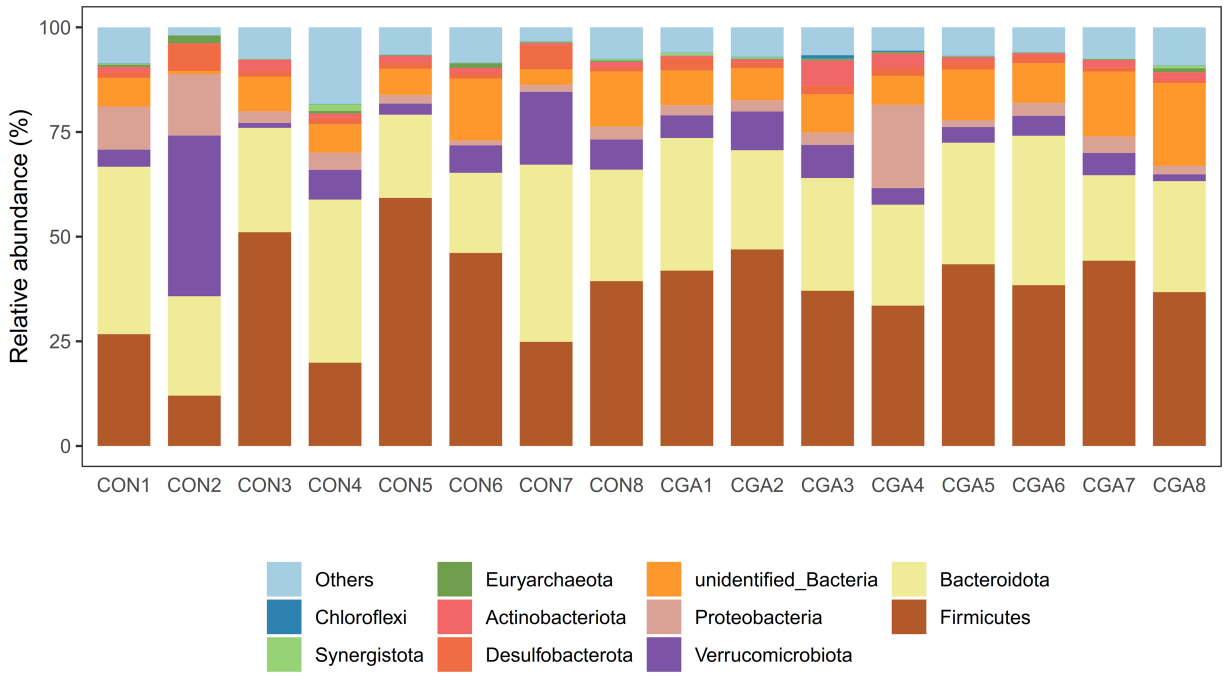


FIGURE 7
Bar graph shows the phylum level composition of bacteria. Color-coded bar plot shows the relative abundance of bacterial phylum across the different samples. CON 1, 2, 3, 4, 5, 6, 7, 8 are cecal digesta samples from rabbits fed with a basal diet; CGA 1, 2, 3, 4, 5, 6, 7, 8 are cecal digesta samples from rabbits fed with a basal diet supplemented with 800mg/kg chlorogenic acid.

destroy the structure and function of epithelial cell-to-cell junctions, impairing the epithelial barrier and mucosal homeostasis. The inflammatory biomarkers, including TNF-α, IL-6 and IL-1β, were reported to be elevated in weaned animals (Hu et al., 2013; Ma et al., 2021). In the present study, we found that CGA decreased levels of IL-6 and IL-1β in serum and TNF-α in jejunum mucosa, and increased levels of IL-10 in serum and jejunum mucosa. Similar results were also found in previous research in weaned pigs (Chen et al., 2018b). Increased TNF-α concentration usually results in the dysfunction of intestinal mucosal barrier by amplifying local and systemic inflammation (Wang et al., 2019). Yu et al. (2021) pointed out that TNF-α and IL-6 could induce inflammatory activation and tight junction conformation change, and consequently increase the permeability of Caco-2 cell monolayer. Besides, IL-10, as one of the key anti-inflammatory cytokines, can inhibit immune responses to antigens and suppress the expression of pro-inflammatory factors such as TNF-α, IL-6, and IL-1β (Xu et al., 2020). A previous study in IL-10-deficient mice showed that IL-10-deficiency was a primary initiating event in chronic intestinal inflammation (Simon et al., 2016). Therefore, the results in current study indicated that CGA could alleviate the systemic and intestinal inflammatory responses of weaned rabbits by maintaining the cytokines at normal levels, further protecting the integrity of the intestinal barrier. Moreover, MHCII is one of the key molecules in the adaptive immune system, and plays an important role in presenting peptide antigens to T cell scrutiny (Reynisson et al., 2020). In the present study, CGA supplementation significantly increased the concentration of MHCII in the jejunum mucosa, further suggesting that CGA could improve the intestinal anti-inflammatory ability.

In addition, intestinal oxidative stress is closely related to the intestinal barrier dysfunction (Wang et al., 2020). Under normal physiological conditions, the oxidation and antioxidant defense systems are in dynamic equilibrium (Metwally et al., 2020). A previous study indicated that weaning could induce oxidative stress by disrupting the physiologic equilibrium of oxidants and antioxidants (Zhu et al., 2012). Therefore, antioxidant ability of intestinal tissues is important for animal growth. A previous study reported that MDA is an important index reflecting the degree of membrane lipid peroxidation (Cheng et al., 2020). In addition, CAT, one of the most important endogenous antioxidant enzymes, plays a key role in alleviating oxidative stress by converting hydrogen peroxide to $H_2O$ (David et al., 2008). In the present study, dietary CGA supplementation reduced the MDA contents, while decreased the activity of CAT in serum, indicating that CGA relieved the oxidative stress of weaned rabbits. In jejunal mucosa, the MDA contents was also reduced, but the content of T-AOC was dramatically increased when rabbits were fed CGA-supplemented diet. The value of T-AOC represents the ability of antioxidants to scavenge free radicals, which reflects the total antioxidant capacity of the body's defense system and is closely related to the health of body (Tian et al., 2021). Han et al. (2010) pointed that CGA could inhibit cellular oxidative stress as

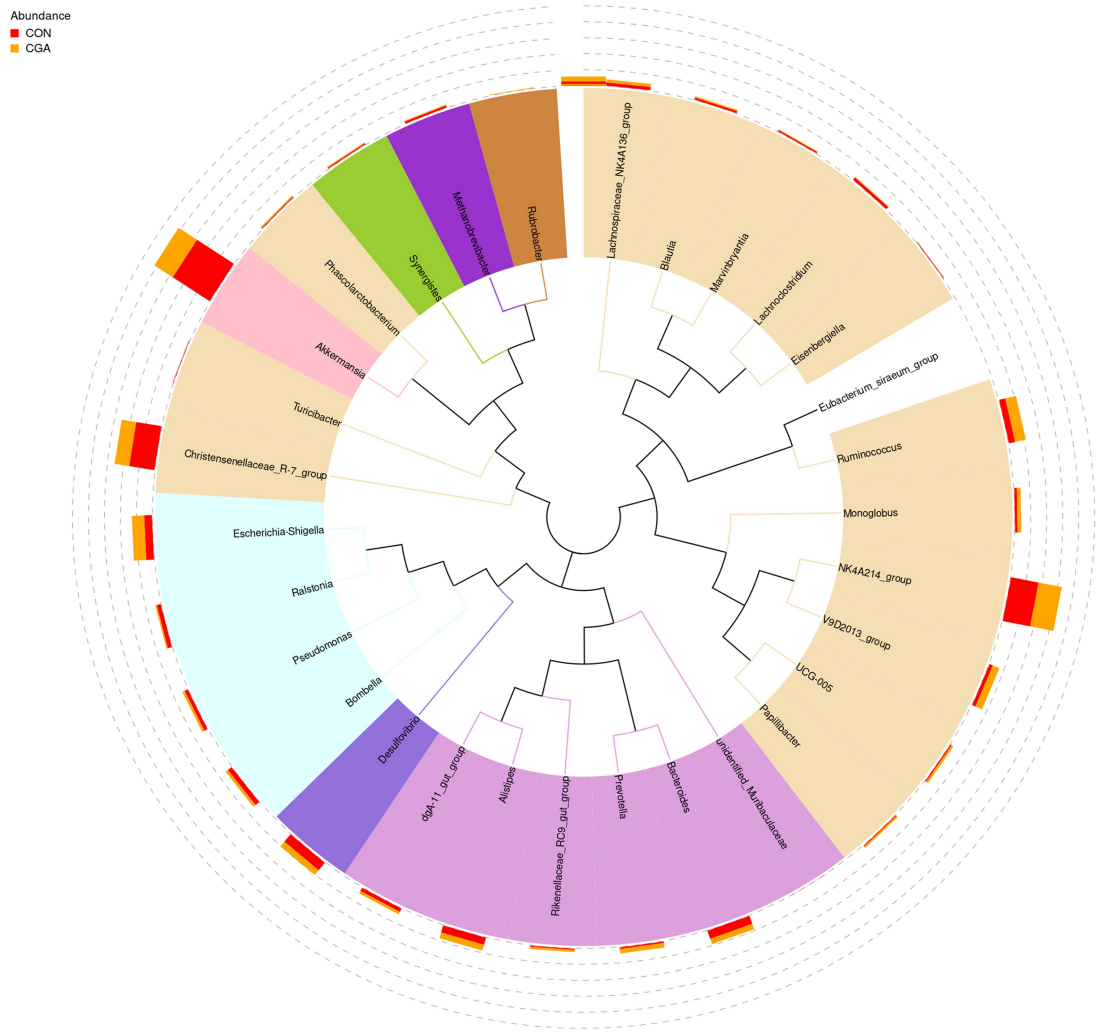


FIGURE 8
Changes of the relative abundance at genus levels. The phylogenetic tree constructed based on the sequence of the top 35 genera. The branches with different colors in the inner circle represent their corresponding phylum, and the stacked column chart in the outer circle indicates the relative abundance of each genus in different treatments. CON, rabbits fed a basal diet; CGA, rabbits fed the basal diet supplemented with 800mg/kg chlorogenic acid.

a chemical protective antioxidant by enhancing the cellular antioxidant system's ability. Therefore, according to those results, we speculated that CGA exhibited an alleviation effect on weaning stress-induced oxidative stress in weaned rabbits partly by improving the antioxidant capacity of the small intestine. But further studies are needed to obtain evidence of the antioxidant effect of CGA.

Based on previous results from Gonthier et al. (2003), dietary CGA is only absorbed at a rate of approximately 33% in the small intestine, and consequently, most dietary CGA is able to reach the large intestine. Gut health often refers to interaction between the intestinal wall barrier, the microbiota, and physiological and immunological components, which enable different animals to cope with internal and external stressors (Placha et al., 2022). The intestinal mucosa, serving as cellular barrier, is the primary location of interaction with external materials and bacteria (Perez-Lopez et al., 2016). An integrated and mature intestinal mucosa plays a vital role in suppressing the colonization and proliferation of pathogenic bacteria (Chen et al., 2021a). In the present study, we found that CGA supplementation increased alpha diversity indexes including observed species, Shannon index, Simpson index, Chao 1 index, and ACE index of cecal microbiota. Previous study in weaned piglet also demonstrated that dietary CGA supplementation increased Shannon index, Simpson index, Chao 1 index, and ACE index of cecal microbiota (Chen et al., 2019). Generally, the observed species was used to calculate unique OTUs, Shannon and Simpson indexes were used to measure community diversity, and Chao 1 and ACE indexes were used to estimate community richness (Li et al., 2020). The results in this study indicated that CGA supplementation could increase bacteria community diversity and richness in cecum of rabbits. The gut microbiota

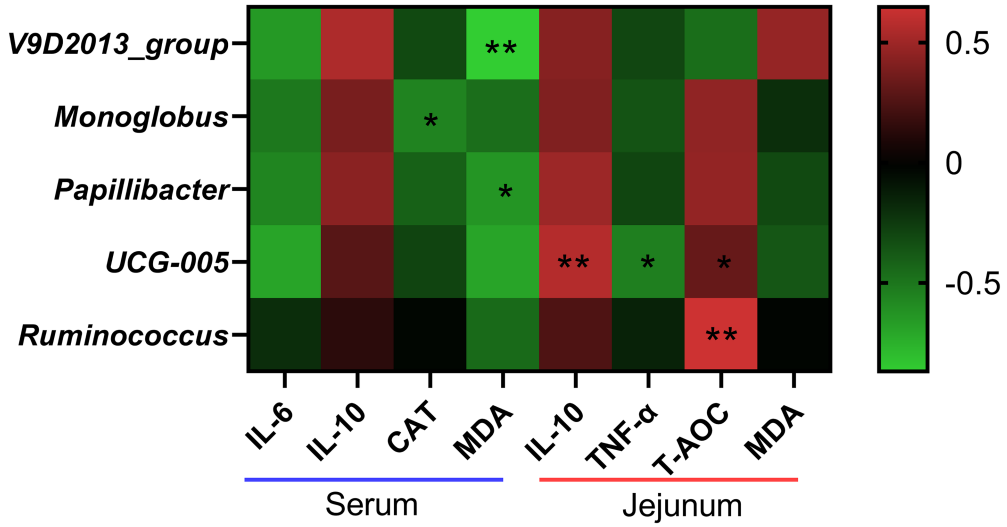


FIGURE 9
Correlation analysis between the abundances of differential bacteria at genus level and the levels of inflammatory cytokines and antioxidant indices. IL-6, interleukin 6; IL-10, interleukin 10; CAT, catalase; MDA, malondialdehyde; TNF-α, tumor necrosis factor-alpha; T-AOC, total antioxidant capacity. *the correlation is significant at a level of 0.05. **the correlation is significant at a level of 0.01.

diversity is a reliable indicator of host health. Inflammatory response and oxidative stress were often accompanied by a decreased diversity of gut microbiota which was reproducibly observed in the individuals with gut microbiota dysbiosis (Vasquez et al., 2019). Moreover, we also found that rabbits in CGA group had higher abundances of *V9D2013_group*, *Monoglobus*, *Papillibacter*, *UCG-005*, and *Ruminococcus* than those in CON group. *V9D2013_group*, *Monoglobus*, *Papillibacter*, *UCG-005*, and *Ruminococcus* were butyrate-producing bacteria (Hao et al., 2022; Yan et al., 2022). Chen et al. (2019) has proven that CGA diet intake increases cecal concentrations of short-chain fatty acids (including acetate, propionate, and butyrate) in weaned piglets. Short-chain fatty acids, especially butyrate, produced by gut microbiota were reported to suppress inflammatory response and oxidative stress (Hu et al., 2013). Besides, *Monoglobus* was enriched in health individuals, and its abundance was positively correlated with CD4+ T cell counts (Li R. et al., 2022; Wang et al., 2022). CD4(+) T cells play crucial roles in adaptive immune response against pathogens (Yang et al., 2020). *Papillibacter* was considered as a kind of beneficial bacteria that enriched in healthy gut (Yasoob et al., 2021). Lower *Papillibacter* abundance was observed in the damaged cecum induced by heat stress in rabbits (Yasoob et al., 2021). *Ruminococcus* was one of the keystone bacteria of the rabbit cecal microbiota, and could degrade and convert complex polysaccharides into a variety of nutrients for the hosts (Bäuerl et al., 2014; La Reau and Suen, 2018). Therefore, CGA supplementation increased the abundance of beneficial bacteria in the cecum of rabbits. A previous study showed that the cecal microbiota of rabbit could influence the host growth and feed efficiency (Velasco-Galilea et al., 2021). Increased beneficial bacteria is conducive to shaping mucosal immunity, inhibiting the inflammatory response and oxidative stress, and maintaining mucosa integrity of the gut (Perez-Lopez et al., 2016; Li W. et al., 2021). Consistently, in the present study, the changes of bacterial abundances also showed significant correlations with the parameters related inflammatory response and oxidative stress in serum and small intestine of weaned rabbits. Above all, increased bacteria community diversity and richness and beneficial bacteria abundance might be another reason for the positive effect of CGA supplementation on intestinal epithelial barrier functions.

## Conclusion

In conclusion, dietary supplemented with 800 mg/kg CGA exerts growth-promoting effects by enhancing intestinal structural integrity, improving intestinal barrier and absorption function, as well as maintaining intestinal microbial homeostasis, which give insight into the protective effects of CGA on the growth retardant of weaned rabbits. These findings allow for a broader understanding of the growth-promoting effects of CGA on the weaned 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 at: https://www.ncbi.nlm.nih.gov/, PRJNA848144.

## Ethics statement

The animal study was reviewed and approved by the Care and Use committee of Shandong Agricultural University (protocol code SDAUA-2021-050).

## Author contributions

JC, LL, and FL: conceptualization. JC, ZS, and RJ: data curation and project administration. JC and ZS: formal analysis. FL: funding acquisition. RJ and HZ: investigation. YL and HZ: methodology. ZS, RJ, and YL: resources. JC and ZS: software. LL and FL: supervision. JC and ZS: visualization. JC: writing—original draft. LL and FL: writing—review and editing. All authors contributed to the article and approved the submitted version.

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## Conflict of interest

YL and HZ were employed by Qingdao Kangda Food Co., Ltd.

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

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## Supplementary material

The Supplementary material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2022.1027101/full#supplementary-material

## References

Bagdas, D., Gul, Z., Meade, J. A., Cam, B., Cinkilic, N., and Gurun, M. S. (2020). Pharmacologic overview of chlorogenic acid and its metabolites in chronic pain and inflammation. *Curr. Neuropharmacol.* 18, 216–228. doi: 10.2174/1570159X17666191021111809

Bäuerl, C., Collado, M. C., Zuniga, M., Blas, E., and Perez, M. G. (2014). Changes in cecal microbiota and mucosal gene expression revealed new aspects of epizootic rabbit enteropathy. *PLoS One* 9:e105707. doi: 10.1371/journal.pone.0105707

Bivolarski, B., and Vachkova, E. (2014). Morphological and functional events associated to weaning in rabbits. *J. Anim. Physiol. Anim Nutr.* 98, 9–18. doi: 10.1111/jpn.12058

Bouayed, J., Rammal, H., Dicko, A., Younos, C., and Soulimani, R. (2007). Chlorogenic acid, a polyphenol from *Prunus domestica* (Mirabelle), with coupled anxiolytic and antioxidant effects. *J. Neurol. Sci.* 262, 77–84. doi: 10.1016/j.jns.2007.06.028

Carabaño, R., Badiola, I., Chamorro, S., García, J., García-Ruiz, A., García-Rebollar, P., et al. (2008). New trends in rabbit feeding: influence of nutrition on intestinal health. A review. *Span J. Agric. Res.* 6, 15–25. doi: 10.5424/sjar/200806S1-5346

Cera, K., Mahan, D., Cross, R., Reinhart, G., and Whitmoyer, R. (1988). Effect of age, weaning and postweaning diet on small intestinal growth and jejunal morphology in young swine. *J. Anim. Sci.* 66, 574–584. doi: 10.2527/jas1988.662574x

Chen, J., Li, F., Yang, W., Jiang, S., and Li, Y. (2021a). Supplementation with exogenous catalase from *Penicillium notatum* in the diet ameliorates lipopolysaccharide-induced intestinal oxidative damage through affecting intestinal antioxidant capacity and microbiota in weaned pigs. *Microbiol. Spectr.* 9:e0065421. doi: 10.1128/Spectrum.00654-21

Chen, J., Li, Y., Yu, B., Chen, D., Mao, X., Zheng, P., et al. (2018a). Dietary chlorogenic acid improves growth performance of weaned pigs through maintaining antioxidant capacity and intestinal digestion and absorption function. *J. Anim. Sci.* 96, 1108–1118. doi: 10.1093/jas/skx078

Chen, L., Li, S., Zheng, J., Li, W., Jiang, X., Zhao, X., et al. (2018). Effects of dietary clostridium butyricum supplementation on growth performance, intestinal development, and immune response of weaned piglets challenged with lipopolysaccharide. *J. Anim. Sci. Biotechnol.* 9, 62–14. doi: 10.1186/s40104-018-0275-8

Chen, J., Luo, Y., Li, Y., Chen, D., Yu, B., and He, J. (2021b). Chlorogenic acid attenuates oxidative stress-induced intestinal epithelium injury by co-regulating the PI3K/Akt and IκBα/NF-κB signaling. *Antioxidants* 10:1915. doi: 10.3390/antiox10121915

Chen, J., Yu, B., Chen, D., Huang, Z., Mao, X., Zheng, P., et al. (2018b). Chlorogenic acid improves intestinal barrier functions by suppressing mucosa inflammation and improving antioxidant capacity in weaned pigs. *J. Nutr. Biochem.* 59, 84–92. doi: 10.1016/j.jnutbio.2018.06.005

Chen, J., Yu, B., Chen, D., Zheng, P., Luo, Y., Huang, Z., et al. (2019). Changes of porcine gut microbiota in response to dietary chlorogenic acid supplementation. *Appl. Microbiol. Biot.* 103, 8157–8168. doi: 10.1007/s00253-019-10025-8

Cheng, D., Wang, G., Wang, X., Tang, J., Yu, Q., Zhang, X., et al. (2020). Neuroprotection of chlorogenic acid against Al-induced apoptosis in PC12 cells via modulation of Al metabolism and Akt/GSK-3β pathway. *J. Funct. Foods* 70:103984. doi: 10.1016/j.jff.2020.103984

David, M., Munaswamy, V., Halappa, R., and Marigoudar, S. R. (2008). Impact of sodium cyanide on catalase activity in the freshwater exotic carp, *Cyprinus carpio* (Linnaeus). *Pestic. Biochem. Phys.* 92, 15–18. doi: 10.1016/j.pestbp.2008.03.013

Duan, Q., Chen, D., Yu, B., Huang, Z., Luo, Y., Zheng, P., et al. (2022). Effect of sialyllactose on growth performance and intestinal epithelium functions in weaned pigs challenged by enterotoxigenic Escherichia Coli. *J. Anim. Sci. Biotechnol.* 13:30. doi: 10.1186/s40104-022-00673-8

Fann, M. K., and O'Rourke, D. (2001). Normal bacterial flora of the rabbit gastrointestinal tract: a clinical approach. *Semin. Avian Exot. Pet Med.* 10, 45–47. doi: 10.1016/S1055-937X(01)80022-0

Fleige, S., Preißinger, W., Meyer, H., and Pfaffl, M. (2007). Effect of lactulose on growth performance and intestinal morphology of pre-ruminant calves using a milk replacer containing *Enterococcus faecium*. *Animal* 1, 367–373. doi: 10.1017/S1751731107661850

Gallego, S., Esbrí, J. M., Campos, J. A., Peco, J. D., Martin-Laurent, F., and Higueras, P. (2021). Microbial diversity and activity assessment in a 100-year-old lead mine. *J. Hazard. Mater.* 410:124618. doi: 10.1016/j.jhazmat.2020.124618

Gharib, H. S., Abdel-Fattah, A. F., Mohammed, H. A., and Abdel-Fattah, D. M. (2018). Weaning induces changes in behavior and stress indicators in young New Zealand rabbits. *J. Adv. Vet. Anim. Res.* 5, 166–172. doi: 10.5455/javar.2018.e262

Gonthier, M. P., Verny, M. A., Besson, C., Rémésy, C., and Scalbert, A. (2003). Chlorogenic acid bioavailability largely depends on its metabolism by the gut microflora in rats. *J. Nutr.* 133, 1853–1859. doi: 10.1093/jn/133.6.1853

Han, J., Miyamae, Y., Shigemori, H., and Isoda, H. (2010). Neuroprotective effect of 3, 5-di-O-caffeoylquinic acid on SH-SY5Y cells and senescence-accelerated-prone mice 8 through the up-regulation of phosphoglycerate kinase-1. *Neuroscience* 169, 1039–1045. doi: 10.1016/j.neuroscience.2010.05.049

Hao, Y., Ji, Z., Shen, Z., Xue, Y., Zhang, B., Yu, D., et al. (2022). Increase dietary fiber intake ameliorates Cecal morphology and drives Cecal species-specific of short-chain fatty acids in white Pekin ducks. *Front. Microbiol.* 13:853797. doi: 10.3389/fmicb.2022.853797

Heo, J. M., Opapeju, F., Pluske, J., Kim, J., Hampson, D., and Nyachoti, C. M. (2013). Gastrointestinal health and function in weaned pigs: a review of feeding strategies to control post-weaning diarrhoea without using in-feed antimicrobial compounds. *J. Anim. Physiol. Anim. Nutr.* 97, 207–237. doi: 10.1111/j.1439-0396.2012.01284.x

Hu, C. H., Xiao, K., Luan, Z. S., and Song, J. (2013). Early weaning increases intestinal permeability, alters expression of cytokine and tight junction proteins, and activates mitogen-activated protein kinases in pigs. *J. Anim. Sci.* 91, 1094–1101. doi: 10.2527/jas.2012-5796

Kirmiz, N., Robinson, R. C., Shah, I. M., Barile, D., and Mills, D. A. (2018). Milk glycans and their interaction with the infant-gut microbiota. *Annu. Rev. Food Sci Technol.* 9, 429–450. doi: 10.1146/annurev-food-030216-030207

La Reau, A. J., and Suen, G. (2018). The Ruminococci: key symbionts of the gut ecosystem. *J. Microbiol.* 56, 199–208. doi: 10.1007/s12275-018-8024-4

Lafay, S., Gil-Izquierdo, A., Manach, C., Morand, C., Besson, C., and Scalbert, A. (2006). Chlorogenic acid is absorbed in its intact form in the stomach of rats. *J. Nutr.* 136, 1192–1197. doi: 10.1093/jn/136.5.1192

Li, J., Liu, Y., Niu, J., Jing, C., Jiao, N., Huang, L., et al. (2022). Supplementation with paraformic acid in the diet improved intestinal development through modulating intestinal inflammation and microbiota in broiler chickens. *Front. Microbiol.* 13:975056. doi: 10.3389/fmicb.2022.975056

Li, Y., Liu, H., Zhang, L., Yang, Y., Lin, Y., Zhuo, Y., et al. (2019). Maternal dietary fiber composition during gestation induces changes in offspring antioxidative capacity, inflammatory response, and gut microbiota in a sow model. *Int. J. Mol. Sci.* 21:31. doi: 10.3390/ijms21010031

Li, J., Ren, Y., Gao, C., Zhang, K., Zheng, F., and Kang, J. (2021). Evaluation of fecal calprotectin, D-lactic acid and bedside gastrointestinal ultrasound image data for the prediction of acute gastrointestinal injury in sepsis patients. *Front. Med. Technol.* 3:733940. doi: 10.3389/fmedt.2021.733940

Li, W., Xu, B., Wang, L., Sun, Q., Deng, W., Wei, F., et al. (2021). Effects of clostridium butyricum on growth performance, gut microbiota and intestinal barrier function of broilers. *Front. Microbiol.* 12:777456. doi: 10.3389/fmicb.2021.777456

Li, R., Yi, X., Yang, J., Zhu, Z., Wang, Y., Liu, X., et al. (2022). Gut microbiome signatures in the progression of hepatitis B virus-induced liver disease. *Front. Microbiol.* 13:916061. doi: 10.3389/fmicb.2022.916061

Li, Y., Zhao, X., Zhang, L., Zhan, X., Liu, Z., Zhuo, Y., et al. (2020). Effects of a diet supplemented with exogenous catalase from *Penicillium notatum* on intestinal development and microbiota in weaned piglets. *Microorganisms* 8:391. doi: 10.3390/microorganisms8030391

Lozupone, C., and Knight, R. (2005). UniFrac: a new phylogenetic method for comparing microbial communities. *Appl. Environ. Microb.* 71, 8228–8235. doi: 10.1128/AEM.71.12.8228-8235.2005

Lu, H., Tian, Z., Cui, Y., Liu, Z., and Ma, X. (2020). Chlorogenic acid: a comprehensive review of the dietary sources, processing effects, bioavailability, beneficial properties, mechanisms of action, and future directions. *Compr. Rev. Food Sci. Food Sci.* 19, 3130–3158. doi: 10.1111/1541-4337.12620

Luissint, A. C., Parkos, C. A., and Nusrat, A. (2016). Inflammation and the intestinal barrier: leukocyte–epithelial cell interactions, cell junction remodeling, and mucosal repair. *Gastroenterology* 151, 616–632. doi: 10.1053/j.gastro.2016.07.008

Ma, X., Zhang, Y., Xu, T., Qian, M., Yang, Z., Zhan, X., et al. (2021). Early-life intervention using exogenous fecal microbiota alleviates gut injury and reduce inflammation caused by weaning stress in piglets. *Front. Microbiol.* 12:671683. doi: 10.3389/fmicb.2021.671683

Martens, E. C., Neumann, M., and Desai, M. S. (2018). Interactions of commensal and pathogenic microorganisms with the intestinal mucosal barrier. *Nat. Rev. Microbiol.* 16, 457–470. doi: 10.1038/s41579-018-0036-x

Mateos, G. G., Rebollar, P. G., and De Blas, C. (2010). Minerals, vitamins and additives. *Nutrit. Rabbit* 2, 119–150. doi: 10.1079/9781845936693.0222

Metwally, D. M., Alajmi, R. A., El-Khadragy, M. F., Yehia, H. M., WA, A. L.-M., Akabawy, A. M., et al. (2020). Chlorogenic acid confers robust neuroprotection against arsenite toxicity in mice by reversing oxidative stress, inflammation, and apoptosis. *J. Funct. Foods* 75:104202. doi: 10.1016/j.jff.2020.104202

Naveed, M., Hejazi, V., Abbas, M., Kamboh, A. A., Khan, G. J., Shumzaid, M., et al. (2018). Chlorogenic acid (CGA): a pharmacological review and call for further research. *Biomed. Pharmacother.* 97, 67–74. doi: 10.1016/j.biopha.2017.10.064

Nieto, N., Torres, M., Fernandez, M., Giron, M., Rios, A., Suarez, M., et al. (2000). Experimental ulcerative colitis impairs antioxidant defense system in rat intestine. *Digest Dis. Sci.* 45, 1820–1827. doi: 10.1023/a:1005565708038

Perez-Lopez, A., Behnsen, J., Nuccio, S.-P., and Raffatellu, M. (2016). Mucosal immunity to pathogenic intestinal bacteria. *Nat. Rev. Immunol.* 16, 135–148. doi: 10.1038/nri.2015.17

Placha, I., Bacova, K., Zitterl-Eglseer, K., Laukova, A., Chrastinova, L., Madarova, M., et al. (2022). Thymol in fattening rabbit diet, its bioavailability and effects on intestinal morphology, microbiota from caecal content and immunity. *J. Anim. Physiol. Nutr.* 106, 368–377. doi: 10.1111/jpn.13595

Relja, B., Töttel, E., Breig, L., Henrich, D., Schneider, H., Marzi, I., et al. (2012). Plant polyphenols attenuate hepatic injury after hemorrhage/resuscitation by inhibition of apoptosis, oxidative stress, and inflammation via NF-kappaB in rats. *Eur. J. Nutr.* 51, 311–321. doi: 10.1007/s00394-011-0216-1

Reynisson, B., Barra, C., Kaabinejadian, S., Hildebrand, W. H., Peters, B., and Nielsen, M. (2020). Improved prediction of MHC II antigen presentation through integration and motif deconvolution of mass spectrometry MHC eluted ligand data. *J. Proteome Res.* 19, 2304–2315. doi: 10.1021/acs.jproteome.9b00874

Ruan, Z., Liu, S., Zhou, Y., Mi, S., Liu, G., Wu, X., et al. (2014). Chlorogenic acid decreases intestinal permeability and increases expression of intestinal tight junction proteins in weaned rats challenged with LPS. *PLoS One* 9:e97815. doi: 10.1371/journal.pone.0097815

Simon, J. M., Davis, J. P., Lee, S. E., Schaner, M. R., Gipson, G. R., Weiser, M., et al. (2016). Alterations to chromatin in intestinal macrophages link IL-10 deficiency to inappropriate inflammatory responses. *Eur. J. Immunol.* 46, 1912–1925. doi: 10.1002/eji.201546237

Smith, F., Clark, J. E., Overman, B. L., Tozel, C. C., Huang, J. H., Rivier, J. E., et al. (2010). Early weaning stress impairs development of mucosal barrier function in the porcine intestine. *Am. J. Physiol. Gastr. Liver Physiol.* 298, G352–G363. doi: 10.1152/ajpgi.00081.2009

Tian, W., Wu, B., Sun, L., and Zhuang, Y. (2021). Protective effect against d-gal-induced aging mice and components of polypeptides and polyphenols in defatted walnut kernel during simulated gastrointestinal digestion. *J. Food Sci.* 86, 2736–2752. doi: 10.1111/1750-3841.15744

Vasquez, E. C., Pereira, T., Campos-Toimil, M., Baldo, M. P., and Peotta, V. A. (2019). Gut microbiota, diet, and chronic diseases: the role played by oxidative stress. *Oxidative Med. Cell. Longev.* 2019, 1–3. doi: 10.1155/2019/7092032

Velasco-Galilea, M., Piles, M., Ramayo-Caldas, Y., and Sánchez, J. P. (2021). The value of gut microbiota to predict feed efficiency and growth of rabbits under different feeding regimes. *Sci. Rep.* 11, 19495–19418. doi: 10.1038/s41598-021-99028-y

Wang, Y., Chen, Y., Zhang, X., Lu, Y., and Chen, H. (2020). New insights in intestinal oxidative stress damage and the health intervention effects of nutrients: a review. *J. Funct. Foods* 75:104248. doi: 10.1016/j.jff.2020.104248

Wang, Q., Zhang, S., Chang, M., Qiao, J., Wang, C., Li, X., et al. (2022). Characteristics of the gut microbiome and its relationship with peripheral CD4+ T cell subpopulations and cytokines in rheumatoid arthritis. *Front. Microbiol.* 13:799602. doi: 10.3389/fmicb.2022.799602

Wang, J., Zhang, C., Guo, C., and Li, X. (2019). Chitosan ameliorates DSS-induced ulcerative colitis mice by enhancing intestinal barrier function and improving microflora. *Int. J. Mol. Sci.* 20:5751. doi: 10.3390/ijms20225751

Xu, Z., Chen, W., Deng, Q., Huang, Q., Wang, X., Yang, C., et al. (2020). Flaxseed oligosaccharides alleviate DSS-induced colitis through modulation of gut microbiota and repair of the intestinal barrier in mice. *Food Funct.* 11, 8077–8088. doi: 10.1039/d0fo01105c

Xu, Q., Jitkaew, S., Choksi, S., Kadigamuwa, C., Qu, J., Choe, M., et al. (2017). The cytoplasmic nuclear receptor RARγ controls RIP1 initiated cell death when cIAP activity is inhibited. *Nat. Commun.* 8:425. doi: 10.1038/s41467-017-00496-6

Yan, X., Zhang, H., Lin, A., and Su, Y. (2022). Antagonization of ghrelin suppresses muscle protein deposition by altering gut microbiota and serum amino acid composition in a pig model. *Biology* 11:840. doi: 10.3390/biology11060840

Yan, Y., Zhou, X., Guo, K., Zhou, F., and Yang, H. (2020). Chlorogenic acid protects against indomethacin-induced inflammation and mucosa damage by decreasing bacteroides-derived LPS. *Front. Immunol.* 11:1125. doi: 10.3389/fimmu.2020.01125

Yang, W., Chen, X., and Hu, H. (2020). CD4+ T-cell differentiation in vitro. *T-Cell Recept. Signal.* 2111, 91–99. doi: 10.1007/978-1-0716-0266-9_8

Yasoob, T. B., Yu, D., Khalid, A. R., Zhang, Z., Zhu, X., Saad, H. M., et al. (2021). Oral administration of *Moringa oleifera* leaf powder relieves oxidative stress,

modulates mucosal immune response and cecal microbiota after exposure to heat stress in New Zealand white rabbits. *J. Anim. Sci. Biotechnol.* 12:66. doi: 10.1186/s40104-021-00586-y

Yu, L., Mao, L., Wu, C., Ye, W., and Wang, X. (2021). Chlorogenic acid improves intestinal barrier function by downregulating CD14 to inhibit the NF-κB signaling pathway. *J. Funct. Foods* 85:104640. doi: 10.1016/j.jff.2021.104640

Zhao, L., Luo, L., Jia, W., Xiao, J., Huang, G., Tian, G., et al. (2014). Serum diamine oxidase as a hemorrhagic shock biomarker in a rabbit model. *PLoS One* 9:e102285. doi: 10.1371/journal.pone.0102285

Zhu, L., Zhao, K., Chen, X., and Xu, J. (2012). Impact of weaning and an antioxidant blend on intestinal barrier function and antioxidant status in pigs. *J. Anim. Sci.* 90, 2581–2589. doi: 10.2527/jas.2012-4444

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# Long-term effects of early-life rumen microbiota modulation on dairy cow production performance and methane emissions

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Rumen microbiota modulation during the pre-weaning period has been suggested as means to affect animal performance later in life. In this follow-up study, we examined the post-weaning rumen microbiota development differences in monozygotic twin-heifers that were inoculated (T-group) or not inoculated (C-group) ($n$=4 each) with fresh adult rumen liquid during their pre-weaning period. We also assessed the treatment effect on production parameters and methane emissions of cows during their 1st lactation period. The rumen microbiota was determined by the 16S rRNA gene, 18S rRNA gene, and ITS1 amplicon sequencing. Animal weight gain and rumen fermentation parameters were monitored from 2 to 12months of age. The weight gain was not affected by treatment, but butyrate proportion was higher in T-group in month 3 ($p$=0.04). Apart from archaea ($p$=0.084), the richness of bacteria ($p$<0.0001) and ciliate protozoa increased until month 7 ($p$=0.004) and anaerobic fungi until month 11 ($p$=0.005). The microbiota structure, measured as Bray–Curtis distances, continued to develop until months 3, 6, 7, and 10, in archaea, ciliate protozoa, bacteria, and anaerobic fungi, respectively (for all: $p$=0.001). Treatment or age × treatment interaction had a significant ($p$<0.05) effect on 18 bacterial, 2 archaeal, and 6 ciliate protozoan taxonomic groups, with differences occurring mostly before month 4 in bacteria, and month 3 in archaea and ciliate protozoa. Treatment stimulated earlier maturation of prokaryote community in T-group before month 4 and earlier maturation of ciliate protozoa at month 2 (Random Forest: 0.75month for bacteria and 1.5month for protozoa). No treatment effect on the maturity of anaerobic fungi was observed. The milk production and quality, feed efficiency, and methane emissions were monitored during cow's 1st lactation. The T-group had lower variation in energy-corrected milk yield ($p$<0.001), tended to differ in pattern of residual energy intake over time ($p$=0.069), and had numerically lower somatic cell count throughout their 1st lactation period ($p$=0.081), but no differences between the groups in methane emissions (g/d, g/kg DMI, or g/kg milk) were observed. Our results demonstrated that the orally administered microbial inoculant induced transient changes in early rumen microbiome maturation. In addition, the treatment may influence the later production

performance, although the mechanisms that mediate these effects need to be further explored.

KEYWORDS

rumen function, microbiome modulation, microbiome establishment, heifer, dairy cow

# Introduction

Global demand for food is increasing, but reduction of greenhouse gas emissions from the agricultural sectors is required to mitigate climate change (FAO, 2019). One way the livestock sector can contribute to the reduction of greenhouse gas emissions is by increasing animal's efficiency in production. Ruminants are adapted to utilize plant material that is indigestible to many monogastric animals, including humans, due to symbiotic interaction with the microbiota inhabiting the rumen. The efficiency of digestion is linked to both animal genetics (Brito et al., 2020) and characteristics of microbial ecosystem in the gut (Kruger Ben Shabat et al., 2016). The function of microbiota can be improved by, e.g., optimizing the feeding or adding feed supplements (Beauchemin et al., 2020; Erickson and Kalscheur, 2020), by modulating the microbial communities with antimicrobials or pro-and prebiotics (Cameron and McAllister, 2016; Bąkowski and Kiczorowska, 2021). However, the fully established gut microbiota is resistant to changes, and often reverts to pre-manipulation state shortly after the end of the treatment (Weimer, 2015; Weimer et al., 2017). On the contrary, modulation of rumen microbiota while it is still developing in young ruminants may present a window of opportunity to affect animal performance or health later in life (Yáñez-Ruiz et al., 2015; Malmuthuge and Guan, 2017; Huws et al., 2018; Meale et al., 2021).

Many early-life rumen modulation studies have reported short-to medium-term effects of the treatments on the microbiota development and/or phenotypic characteristics although only a few studies have followed animals long after the treatment ended. Natural versus artificial rearing management practices were found to improve the microbial establishment in young ruminants (Abecia et al., 2014a, 2017; Belanche et al., 2019a,b). Belanche et al. (2019b) showed that natural nurturing of lambs with ewes accelerated the rumen microbiota development and eased the transition to solid diet. These lambs also retained higher fungal diversity that was suggested to contribute to better feed digestibility and growth. The pre-weaning diet influence on the rumen microbiota establishment was demonstrated by Dill-McFarland et al. (2019). They showed that feeding starter, silage, or mixed feed to calves during the pre-weaning period induced differences in bacterial, archaeal, and anaerobic fungal communities up to 2 years of age, but no later differences in milk production efficiency or overall efficiency of the animals were observed. On the contrary, Cristobal-Carballo et al. (2021) found no long-lasting effect of pre-weaning diet on microbial community structure later in life. The direct inoculation of live or processed microbial fluids as means of rumen modulation demonstrated promotion of microbial community establishment during and after weaning, with positive effect on weaning health and growth (e.g., Cersosimo et al., 2019; Belanche et al., 2020; Palma-Hidalgo et al., 2021; Park et al., 2021; Rosa et al., 2021). Park et al. (2021) demonstrated that the inoculation induced differences in community composition persisted at least 3 weeks after the end of the treatment. Studies that aimed to reduce methane emissions through antimicrobial and other feed supplements have been successful in showing medium (Abecia et al., 2013, 2014b; de Barbieri et al., 2015a,b; Lyons et al., 2017) to long-term (Meale et al., 2021) treatment effects. The observations by Meale et al. (2021) that calves treated with 3-NOP early in life had long-term reduced methane emissions suggest that rumen microbiome pre-programming is possible.

For our study, we recruited monozygotic twin calves to eliminate possible differences in response to the treatment caused by the host genetics. During the pre-weaning period, we repeatedly treated half of them with fresh rumen liquid inoculation from a feed efficient adult cow. We demonstrated that the inoculation enhanced the maturation of bacterial, archaeal, and ciliate protozoan communities, but not of rumen anaerobic fungi. Moreover, the treatment had a positive impact on animal growth, and it tended to increase the feed intake during the pre-weaning period (Huuki et al., 2022). The aim of this follow-up study was to examine the impact of early life rumen modulation on the rumen microbiota development post-weaning until animals reached 12 months of age. The second aim was to assess the possible long-term effects on animal production performance and methane emissions during their first lactation. We hypothesized that the treatment would continue to influence the microbial community development after the treatment ended and would lead to improved production efficiency later in life.

# Materials and methods

## Animals and experimental design

Four pairs of identical twin female calves produced by embryo splitting were separated into individual pens (147 cm × 172 cm) after

birth and were randomly assigned to either a treatment (T-group) or a control (C-group) group as described by Huuki et al. (2022). Starting from the 2nd week, the T-group calves received an oral dose of fresh rumen liquid, obtained from a fistulated adult cow, ranked previously as feed efficient animal (Mäntysaari et al., 2012). During weeks 2 and 3, the oral dose was 5 ml, given 3 times a week. During weeks 4–8, the dose was increased to 10 ml. The inoculations were stopped after weaning. The microbial composition of the inoculum and the effect of inoculum on calves' microbiota establishment during the pre-weaning period have been reported by Huuki et al. (2022). The calves were weaned at the age of 8 weeks and the animals from both the T- and C-groups were placed into a group pen and housed together first at the Natural Resources Institute Finland (Luke) research barn, and later in Viking Genetics breeding facility (Hollola, Finland) until their first calving. Right after weaning the calves were offered silage and concentrate (Pikkumullin herkku, Raisio agro, Finland) supplement *ad libitum*. After moving to breeding facility, the weight gain of the heifers was controlled to avoid fattening. The goal for growth was to reach 280 kg by 10 ± 0.5 months of age. The weight gain was maintained at maximum of 750 g/d until 18 months old, and maximum of 550 g/d after 18 months of age by offering 2 different forage mixes with different metabolizable energy levels (~9 or ~ 10 MJ/kg DM). The feed was offered once a day. Heifers remained in good general health throughout the experiment. Weight was estimated monthly by measuring hearth-girth with a weight tape measurer (H. Hauptner und Richard Herberholz GmbH & Co. LP, Germany). The monthly weight gain was calculated by subtracting the previous month's weight from the current weight. Two months before calving the heifers returned to Luke's research barn. The cows were fed grass silage and concentrate mix, provided through the feeding stations. After calving the proportion of concentrate in the diet was adjusted based on the stage of lactation and the digestibility of the grass silage. When the concentration of digestible organic matter in dry matter (DM) of silage was between 680 and 700 g/kg DM, the concentrates were offered so that the proportion of concentrate in the diet DM became 52% during lactation days 1–150, 47% during days 151–250 and 37% thereafter. The amount of concentrate decreased or increased 2%-units for each 10 g /kg DM increase or decrease in digestibility of grass silage. Adjustment of cow's individual concentrate offering was based on measured daily silage DM intake of the cow. On average the proportion of concentrate in the diet of twin cows was 49.2%. The composition of feeds is presented in Supplementary Table S1. During the first lactation month, two cows from the C-group and one from the T-group were treated with Penovet vet (Boehringer Ingelheim Animal Health Nordics A/S, Denmark) and Carepen vet (aniMedica GmbH, Germany) because of clinical signs of mastitis.

## Rumen sample collection

Rumen samples from heifers were collected monthly starting from month 2 until animals reached 12 months of age. During the 1st lactation, rumen samples were collected from each cow once when they were 147 ± 11 (Average ± SD) days in milk. Rumen fluid from heifers was collected between 1,000 and 1,100 h before feeding *via* the esophageal PVC tube (10/16 or 12/18 mm inner/outer diameter) and from cows using Ruminator (Profs Products, Wittybreut, Germany). Sample preparation for VFA analysis was performed as described by Ahvenjärvi and Huhtanen (2018). In brief, the rumen liquid was filtered through two layers of cheesecloth, and a 5-mL aliquot was mixed with 0.5 ml of saturated mercuric (II) chloride solution and 2 ml of 1 M sodium hydroxide solution and stored at −20°C for later VFA analysis with gas chromatography (Huhtanen et al., 1998). For microbial analysis, 0.5 ml rumen liquid aliquots were snap-frozen in dry ice and stored at −80°C until DNA extraction.

## DNA extraction, sequencing

The total DNA was extracted from 0.5 ml of rumen liquid with a method combining bead-beating and column extraction as described by Rius et al. (2012). Universal primers 515F and 806R (Caporaso et al., 2011) targeting the V4 region of the 16S rRNA gene were used for bacterial and archaeal amplicon sequencing. Primers 316F and 539R targeting the 18S rRNA gene area were used for ciliate protozoa (Sylvester et al., 2004), and primers Neo 18SF and Neo5.8SR targeting the ITS1 region (Edwards et al., 2008) were used for amplicon sequencing of anaerobic fungi. The sequencing libraries were prepared as described by Huuki et al. (2022) and sequenced in Finnish Functional Genomics Centre (Turku, Finland) on Illumina MiSeq platform by using 2 × 250 bp chemistry for bacteria and archaea, and 2 × 300 bp for ciliate protozoa and anaerobic fungi.

## Sequence data processing

The demultiplexing of sequences, adapter removal, and sorting sequences by barcode were performed by the sequencing center. Bacterial and archaeal sequencing data were processed using QIIME 2 (Bolyen et al., 2019). Briefly, quality control, filtering of chimeric reads, and clustering of bacterial sequences into amplicon sequence variants (ASV) were performed using DADA2 (Callahan et al., 2016). The ASVs with less than 2 reads in total were removed. The bacterial ASV taxonomy was assigned using the SILVA 138 database (Quast et al., 2013) and the archaeal taxonomy was assigned using the RIM-DB database (Seedorf et al., 2014). The ciliate protozoa and anaerobic fungi sequence data were processed using QIIME v. 1.9.1 (Caporaso et al., 2010) as described by Huuki et al. (2022). In short, the reads were joined using SeqPrep and filtered for quality (>Q20) and length (protozoa: 220–300 bp; anaerobic fungi: 350–410 bp). Open reference OTU clustering (97% similarity) was performed with UCLUST, and chimeric reads were removed with usearch61 (Edgar, 2010). The OTUs with less than two reads were removed.

The taxonomy was assigned with BLAST using ciliate protozoa reference database (Kittelmann et al., 2015), and anaerobic fungi ITS reference database v. 3.5, released on 9th of December 2019 (Koetschan et al., 2014). The sequence reads are available in the NCBI Sequence Read Archive under BioProject PRJNA713003 with corresponding BioSample Accessions SAMN28869958 - SAMN28870037.

## Quantitation of microbial communities

The quantities of bacteria, archaea, ciliate protozoa, and anaerobic fungi were estimated with qPCR by quantifying rRNA gene copy numbers of each taxonomic group in 1 ng of extracted DNA. The quantitation of archaea, ciliate protozoa, and anaerobic fungi was performed as described by Huuki et al. (2022). The bacteria were quantified by amplifying the 16S rRNA gene area (279 bp) using primers UniF (3′ - GTG STG CAY GGY TGT CGT CA - 5′) and UniR (3′ - ACG TCR TCC MCA CCT TCC TC - 5′; Maeda et al., 2003). Amplification reaction (10 µl) contained 1 x Power SYBR Green PCR master mix (Applied Biosystems by Thermo Fischer Scientific, Life Technologies Ltd., United Kingdom), 0.2 µM of each primer, and 10 ng of DNA. The amplification was done in a Viia7 thermocycler (Applied Biosystems), with denaturation at 95°C for 10 min, 40 cycles of denaturation at 95°C for 15 s, annealing at 60°C for 30 s, and extension at 72°C for 30 s. A melt curve analysis was performed after amplification with denaturation at 95°C for 15 s, annealing at 60°C for 1 min, and denaturation at 95°C for 15 s, with a ramp increment of 0.4°C. A more detailed description of methods is provided in Supplementary material 1.

## First lactation production data

Twin cows calved between August 2019 and January 2020, when cows were 23–29 months old. The body weight (BW, kg) of the cows was recorded daily after each milking using an automatic scale. Daily milk yield (kg/day) and dry matter intake (kg/day) were monitored throughout the lactation period. Morning and evening milk samples for analyses of fat, protein, and lactose were taken once on the lactation weeks 2 and 3 and every fourth week thereafter. Milk analyses were provided by Valio Ltd. milk laboratory in Seinäjoki using MilkoScan FT+ spectrometer (Foss Electric, Hillerød, DK). Daily average milk fat, protein, and lactose content were calculated based on the morning and evening milk yields. Energy-corrected milk yield (ECM) was calculated as proposed by Sjaunja et al. (1990). Metabolizable energy intake (MEI, MJ/d) and average residual energy intake (REI, ME MJ/d) were calculated for each lactation month, except when cows were on summer pasture. REI was calculated by fitting a multiple linear regression model with ECM, metabolic BW ($BW^{0.75}$), and piecewise regressions of BW gain or BW loss on the MEI. Thereafter, the daily residuals from the prediction equations were defined to be REI (Mäntysaari et al., 2012). Cows with low REI values are defined as efficient.

## Methane emissions

The daily methane ($CH_4$) production was measured for the first 5 months after calving by photoacoustic IR spectroscopy (PAS) with F10 multigas analyzer (Gasera Ltd., Turku, Finland) as previously described by Negussie et al. (2017). Briefly, the gas analyzer was attached to two feeding stations and was recording the $CH_4$ and $CO_2$ gas contents of cow's breath while the animal was visiting the feeding station. The average daily $CH_4$ production was calculated for each lactation month from measurements of daily visits from both stations. Two T-group cows and one C-group cow were missing the data from lactation month 1, and one T-group cow from month 2. For one T-group cow, only data from lactation months 1 and 2 were available.

## Statistical analyses

To explore treatment and age effect on the changes in microbial community structure, between-sample diversity was evaluated as Bray-Curtis dissimilarities following Hellinger transformation and visualized using principal coordinate analysis (PCoA) with *Phyloseq* (McMurdie and Holmes, 2013) R package. The significance for the effect of age, treatment, and treatment × age interaction was estimated using distance-based permutational multivariate analysis (Adonis) as implemented in *vegan* R package (Oksanen et al., 2019) and the pairwise comparisons between age × treatment with Permutational multivariate analysis of variance with False discovery rate adjustment with *RVAideMemoire* R package (Herve, 2020). The effect of host genetics on microbial community composition was estimated from the Bray-Curtis distance matrix. Significant differences between months were estimated by comparing the average distances between twins to distances between not related heifers with Kruskal–Wallis test, and pairwise comparison with Wilcoxon signed-rank test.

The taxonomic differences between the groups were evaluated at genus level for bacteria and at species level for the rest. The significance of age and treatment × age interaction was evaluated using ANOVA. Apart from the archaea dataset, the taxa with less than 0.1% relative abundance in the dataset were filtered out. All data were log(1 + x) transformed for normalization before the analysis.

Microbial community alpha diversity in T- and C-groups was estimated using Shannon and Simpson indices, and the richness as number of observed OTUs/ASVs as implemented in *Phyloseq*. The data were evenly subsampled to 9,600 reads for bacteria, 54,394 reads for ciliate protozoa, 25,369 reads for anaerobic fungi, and 115 reads for archaea. The statistical analysis was performed with Linear Mixed Model GLIMMIX in SAS 9.4 (SAS Institute

Inc., Cary, NC, United States). Treatment, age, and the treatment × age interaction were treated as fixed effects. Pair was treated as random while age (month) as a repeated effect. Based on fit statistics, the compound symmetry covariance structure was chosen for alpha diversity measures. The difference in variance between groups was considered in bacteria and archaea alpha diversity models. The Simpson index was $(x)^{20}$ transformed for anaerobic fungi and log(x) transformed for archaea, to achieve the normal distribution of residuals, and least square means were later reverse transformed. The effects were estimated using Residual Maximum Likelihood (REML) method and were declared significant at $p \leq 0.05$. The alpha diversity estimates during the lactation period were compared between the groups using Wilcoxon signed-rank test.

Microbial quantities, measured as gene amplicon copy numbers, were tested using Linear Mixed Model as described above. Based on the Model Fit Statistics, the covariance structure was set to compound symmetry for archaea and ciliate protozoa, and to spatial power for bacteria and anaerobic fungi to improve the model fit.

The rumen VFA proportions (mmol/1,000 mol) until 12 months of age, production performance data (BW, weight gain, dry matter intake (DMI), REI, ECM, methane emissions and milk fat, protein, and lactose proportions) were analyzed using Mixed procedure in SAS v 9.4 with fixed and random effects as above. Pair and interaction of pair and age were treated as random effects with production performance data. Spatial power covariance structure was selected for VFA proportions and autoregressive covariance structure for production data. Milk somatic cell counts (SCC) were log(x) transformed to meet model assumptions and analyzed with the program GLIMMIX using Linear Mixed Model approach. The unequal variance of residuals between groups was considered. The total VFA concentration and individual VFA proportions from the first lactation period were compared between the groups using Wilcoxon signed-rank test.

Random Forests regression was used to regress rarefied ASVs at 9,700 sequences per sample against their chronological age using default parameters as implemented in R package *randomForest* with ntree – 10,000 (Liaw and Wiener, 2002). The statistically significant OTUs were identified by Boruta algorithm in R package *Boruta* (Kursa and Rudnicki, 2010). Analysis was done with default confidence level ($p = 0.01$) but increasing the '*maxRuns*' to 1,000. Of the tentatively significant OTUs, those with higher median Z-score than the best shadow OTU were included as implemented in '*TentativeRoughFix*'-function. The C-group heifers were used in training the model to estimate the microbial maturity of the animals. A thin plate regression spline (Wood, 2003) was fit between microbiota age and chronological age of the animal separately for C-and T-groups as implemented in R-package *mgcv* (Wood, 2017). To analyze the contribution of specific OTUs on the model prediction, the approximate Shapley values were calculated with *fastshap* (Greenwell, 2021).

# Results

After quality filtering and removal of rare sequences, there were a total of 1,796,922 bacterial, 29,461 archaeal, 7,296,045 ciliate protozoan, and 6,156,516 anaerobic fungal sequences from the pre-parturition period, with an average number of 22,462 ± 6,067, 368 ± 152, 92,355 ± 13,382, and 81,007 ± 17,188 reads per sample, respectively. The data of bacteria, archaea, ciliate protozoa, and anaerobic fungi from lactation period contained a total of 258,120, 5,199, 447,438, 515,997 reads with an average of 32,265 ± 10,020, 650 ± 204, 55,930 ± 38,364, 64,500 ± 24,997 reads per sample, respectively.

## Treatment had minor effect on the archaea community

Treatment or interaction of age and treatment had no effect on archaeal 16S rRNA gene copy numbers (Figure 1A), ASV richness (Figure 1B), alpha diversity indexes (Supplementary Figure S1), or community distances (Figure 1C). At a taxonomical level, treatment affected the relative abundance of two species (Figure 1D; Supplementary Table S2). C-group had a higher overall abundance of *Methanosphaera stadtmanae* (treatment: $p = 0.018$) and a higher abundance of *Methanomassiliicoccaceae* group 10 sp. in month 2 (interaction: $p = 0.04$). After month 3, differences between the groups were no longer observed.

## Archaea community develops with age

The rumen archaeal quantity, estimated as 16S rRNA gene copy numbers, tended to decrease until month 9, and then increased again (Age: $p = 0.084$; Figure 1A). The archaeal ASV richness numerically decreased after month 2 and increased again after month 6, reaching the highest values in the C-group in month 7 and in the T-group in month 8 (Figure 1B). Similarly, Shannon and Simpson diversity indexes numerically decreased between months 2–6 and increased after month 6 reaching the highest values in month 7 in the C-group and month 8 in the T-group (Supplementary Figure S1). Age explained 20% of the variance in Bray–Curtis distances (treatment: $R^2 = 0.012$, $p = 0.400$; Age: $R^2 = 0.2$, $p = 0.001$; Interaction: $R^2 = 0.105$, $p = 0.403$; Figure 1C). The community at month 2 was distant from the later months, but between months 3 and 10 the variation in the community composition was high and no clear separation into age groups was observed. Among the 18 archaeal species detected, *Methanosphaera* sp. ISO3-F5, *Methanobrevibacter gottschalkii* clade, and *Mbb. ruminantium* clade (71–93%) were the most abundant in both groups (Supplementary Table S2; Figure 1D). *Methanosphaera* sp. ISO3-F5 and *Methanomicrobium mobile* had their highest abundances in month 2 in both groups, but the abundance reduced by month 3. *Mbb. gottschalkii* became

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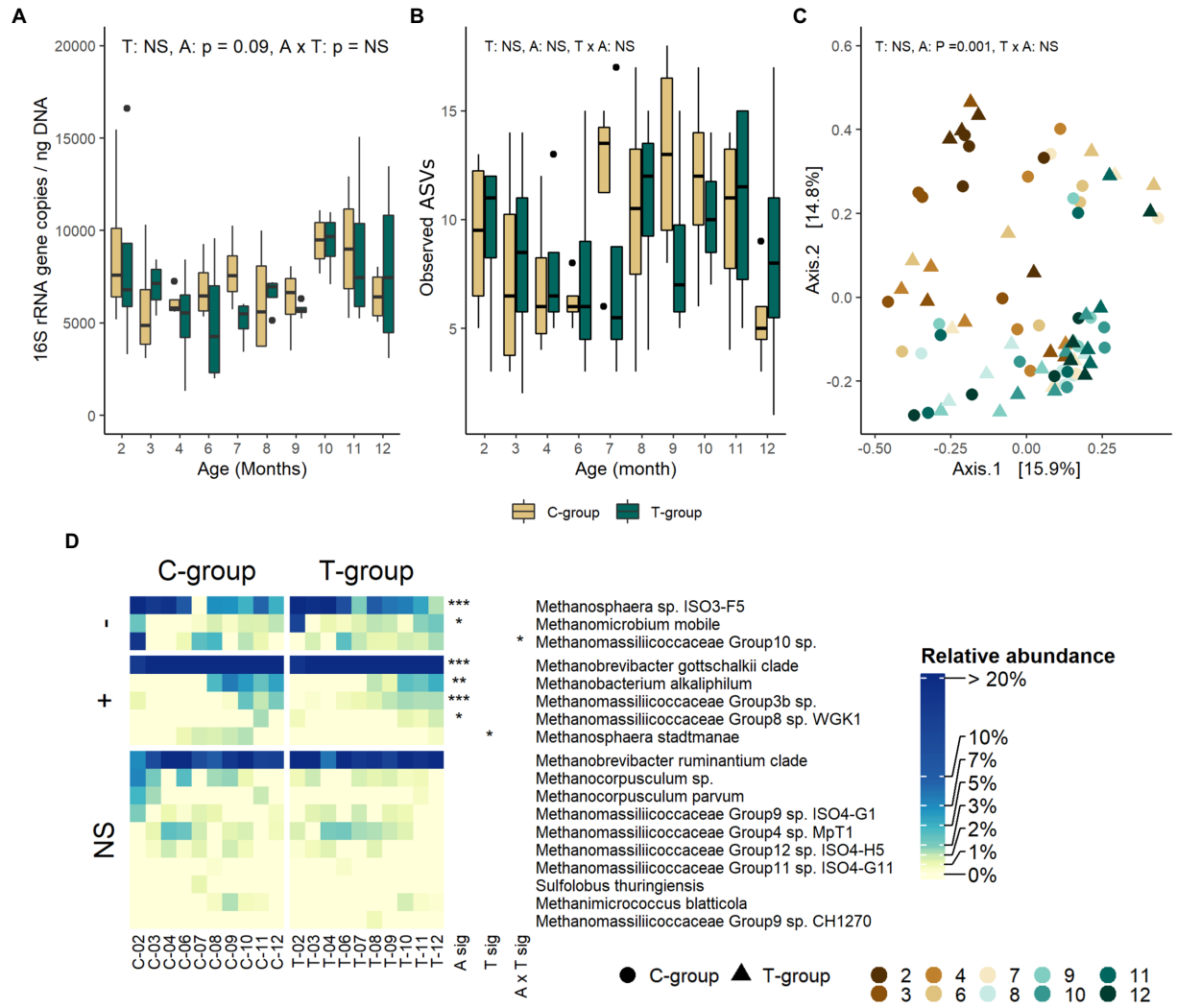


FIGURE 1
The rumen archaeal community in treatment (T-group) and control (C-group) heifers at 2–12months of age. **(A)** The rumen archaea 16S rRNA gene copy numbers, **(B)** the number of observed ASVs, **(C)** principal coordinate analysis visualization of Bray-Curtis community distances, and **(D)** the relative abundances of rumen archaea species. Only taxa with statistical significances are shown. The taxa are grouped by increasing (+) or decreasing (–) trend with time, or no significant effects (NS). Statistical significance of Treatment (T-sig), Age (A-sig), and TreatmentxAge interaction (TxA sig): $p<0.05$: *, $p<0.01$: **, $p<0.001$: ***.

the predominant species from month 3 onward, while *Methanobacterium alkaliphilum*, *Mmc*. Group 3b sp. and *Mmc*. Group8 sp. WGK1 did not become abundant before heifers reached 8 months of age.

## Treatment effect on bacterial community gradually diminished

Treatment or interaction of age and treatment had no significant effect on the rumen bacteria 16S rRNA gene copy numbers measured with RT-qPCR (Figure 2A), the ASV richness (Figure 2B), or alpha diversity metrics (Supplementary Figure S1). Treatment induced minor differences in bacterial community composition, but the effect of treatment only explained 1.4% of the variance (Adonis; treatment: $R^2$: 0.014, $p = 0.04$) (Figure 2C). Overall, the T-group had higher abundances of *Prevotella* (treatment: $p = 0.033$), *Prevotellaceae* Ga6A1 group spp. ($p = 0.008$), *Prevotellaceae* UCG-001 spp. ($p = 0.005$), (*Eubacterium*) *nodatum* group ($p = 0.023$), and *Lachnospiraceae* UCG-009 spp., while *Butyrivibrio* ($p = 0.019$) and *Saccharofermentans* ($p = 0.011$) were less abundant as compared to C-group (Figure 2D; Supplementary Table S4). Most of the differences between the groups were detected during months 2 and 3, after which the communities became more alike (Figure 2D; Supplementary Table S4). In month 2, the T-group had higher abundances of *Prevotellaceae* spp. ($p < 0.001$), *Paraprevotella*

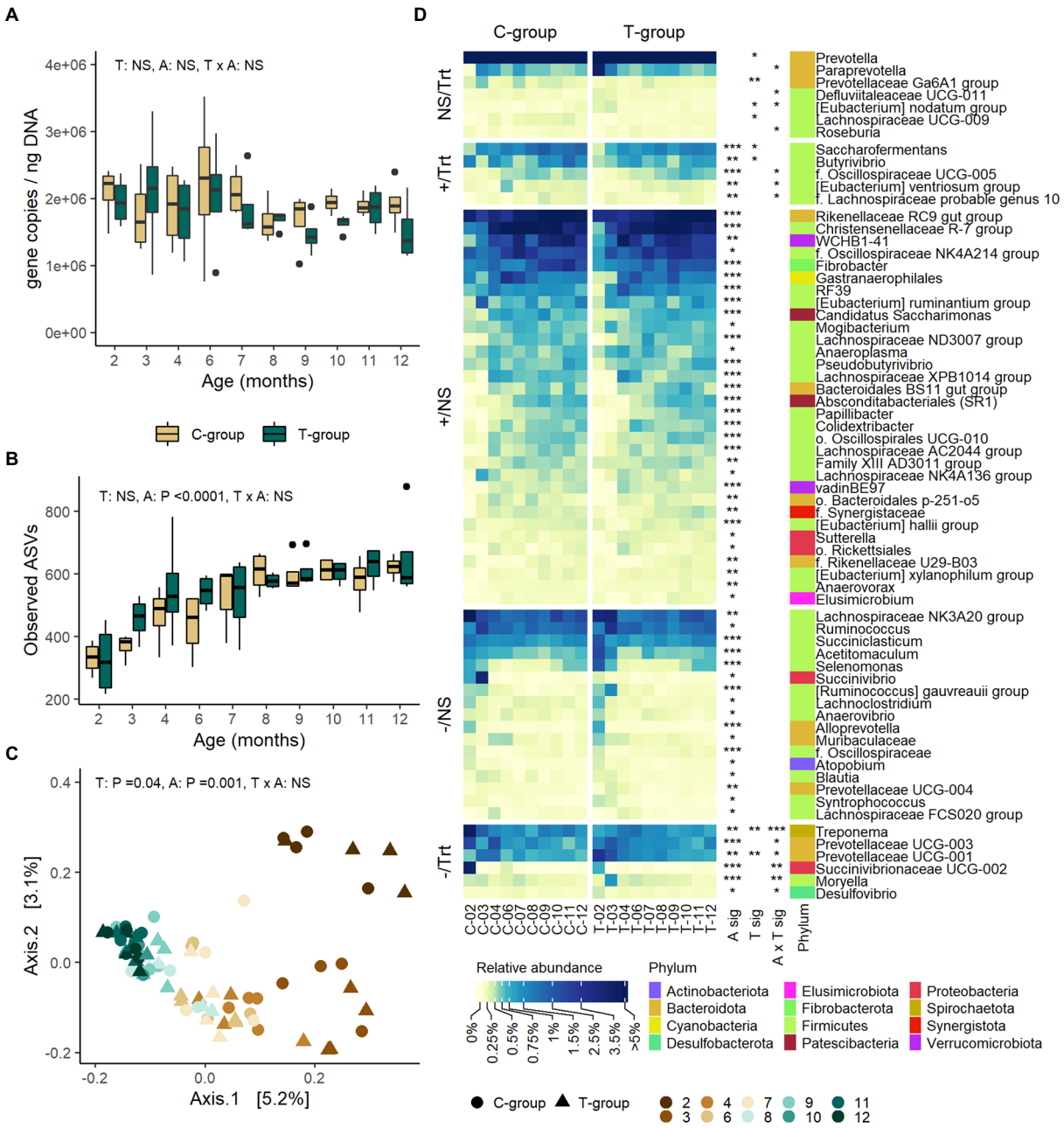


FIGURE 2
The rumen bacterial community in treatment (T-group) and control (C-group) heifers at 2–12months of age. **(A)** The rumen bacteria 16S rRNA gene copy numbers, **(B)** the number of observed ASVs, **(C)** principal coordinate analysis visualization of Bray-Curtis community distances, and **(D)** the relative abundances of rumen bacterial genera with abundance >0.1%. Only taxa with statistical significances are shown. The taxa are grouped by increasing (+) or decreasing (–) trend with time, and with significant treatment effects (Trt) or no treatment effects (NS). Statistical significance of Treatment (T-sig), Age (A-sig) and TreatmentxAge interaction (TxA sig): $p<0.05$: *, $p<0.01$: **, $p<0.001$: ***.

(Tukey: $p=0.004$), and *Desulfovibrio* ($p=0.005$), while lower abundances of genus *Treponema* (Tukey $p<0.0001$) and an unidentified genus affiliated with *Succinivibrionaceae* ($p<0.001$), as compared to C-group. After month 2, only minor differences wereobserved in the taxonomic composition between the groups, which were mainly related to genera within Firmicutes phylum.

## Bacteria community develops with age

The rumen bacterial 16S rRNA gene copy numbers remained at a similar level from month 2 to month 12 (Age: $p=0.727$; Figure 2A). ASV richness increased until month 7 in both groups ($p<0.0001$) and then stabilized (Figure 2B). Shannon diversity increased until month 7 (Age: $p<0.0001$), while

Simpson's evenness index was low in month 2, but no longer increased after month 3 (Age: $p < 0.0001$; Supplementary Figure S1). As indicated by Adonis analysis of Bray–Curtis dissimilarities, age explained 16.5% of the variation in the bacterial community structure (treatment: $R^2 = 0.014$, $p = 0.04$; age: $R^2 = 0.165$; $p = 0.001$; interaction: $R^2 = 0.106$, $p = 0.04$; Figure 2C). The first axis separated the communities by age in months 3–6, but not from month 7 onward. The second axis separated the month 2 from the rest. The age differences between the communities were due to decreasing relative abundances of phyla Bacteroidota, Proteobacteria, and Spirochaetota and increasing abundances of Firmicutes, Fibrobacterota, Patescibacteria, and Verrucomicrobiota (Anova age: $p < 0.05$; Supplementary Table S3). At the genus level, 60 of the 94 observed genera (> 0.1% abundance) were affected by age, of which 23 decreased and 37 increased in abundance over time (Figure 2D; Supplementary Table S4). More than half of the taxa affected by age (36 genera) were affiliated with Firmicutes. The differences in bacterial community structure between months 2 and 3 were related to reducing abundances of *Lachnospiraceae* NK3A20 group, *Succiniclasticum*, *Acetitomaculum*, *Selenomonas*, *Lachnoclostridium*, *Alloprevotella*, *Succinivibrio*, and *Succinivibrionaceae* UCG-002, while *Lachnospiraceae* NK4A136 group, (*Eubacterium*) *ruminantium* group and RF39 became more abundant in month 3. Between months 4 to 9 the changes in taxonomic composition were mainly caused by increasing abundance of WCHB1-41, *Christensenellaceae* R-7 group, *Saccharofermentas*, *Rikenellaceae* RC9 gut group, and *Fibrobacter*. *Rikenellaceae RC9* gut group, *Absconditabacteriales* (SR1) sp., *Papillibacter,* and *Pseudobutyrivibrio* were among the last genera to stabilize in abundance after month 7.

To assess early life modulation effect on the rumen prokaryote community maturation during the post-weaning period, we applied the Random Forest analysis. The Random Forest prediction of rumen microbiome age, based on the 29 bacterial and archaeal ASVs, revealed that the treatment accelerated early post-weaning microbiome succession. In month 4, the maturity of rumen bacteria in the T-group was by 0.75 months ahead of the maturity of C-group (Supplementary Figure S2A). The main gradual maturation-related changes caused by the treatment were assessed by calculating the approximate Shapley values for prediction on month 4. They showed that the low abundance ASVs affiliated with, e.g., *Methanobrevibacter millerae* and some *Prevotella* ASVs, and a high abundance of other *Prevotella* ASVs strongly reduced the predicted age, while a higher abundance of, e.g., *Pseudobutyrivibrio*, other *Prevotella*, *Methanobrevibacter*, and *Clostridiales bacterium* strongly increased the predicted age of T-group (Supplementary Figure S2B). To summarize the patterns on ASVs identified to have an impact on age estimates, several *Christensenellaceae R-7* group ASVs were most abundant between 4–6 months of age, and some *Prevotella* and *Lachnospiraceae* NK3A20 group ASVs increased in abundance with age, while other *Prevotella* and *Succinivibrionaceae* UCG-002 ASVs reduced in abundance with age (Supplementary Figure S2C).

## Treatment effect on ciliate protozoa community diminished

The ciliate protozoa copy numbers (Figure 3A) or the number of observed OTUs (Figure 3B) were not significantly affected by the treatment. However, the effect of treatment was visible in the Bray-Curtis distances, which separated the groups until month 3, but not anymore thereafter (treatment: $R^2 = 0.0229$, $p = 0.016$; interaction: $R^2 = 0.1114$, $p = 0.031$) (Figure 3C). The management of calves in individual pens during the pre-weaning period resulted in differences in the ciliate protozoa community composition between the groups. All but one C-group heifer received ciliate protozoa by month 2, having a community with 6 abundant (>1%) species, but were highly dominated by *Eremoplastron dilobum* and *Entodinium furca monolobum*. On the contrary, the T-group was richer, inhabited by 10 species and dominated by *Isotricha* sp., *Epidinium caudatum,* and *Isotricha prostoma* (Supplementary Table S5; Figure 3D). The Shannon and Simpson indexes were significantly lower in the C-group in month 2 (interaction: $p < 0.05$), but in month 3 when the heifers were moved to a group pen, the differences between the groups disappeared (Supplementary Figure S1).

## Ciliate protozoa community develops with age

The ciliate protozoan 18S rRNA gene copy numbers were low in month 2 but dramatically increased in month 3, and thereafter gradually reduced until month 9 when the quantity stabilized (age: $p = 0.004$; Figure 3A). The number of observed OTUs increased until month 10 after which it stabilized ($p < 0.0001$; Figure 3B). The Shannon and Simpson indexes were significantly lower in month 2 in comparison to the following months (age: $p < 0.0001$; Supplementary Figure S1). Both indexes reached the stable level in month 4 but experienced a drop around month 7, after which the levels gradually increased by month 10. The age explained 32% of the variation observed in the Bray-Curtis distances of ciliate protozoa ($R^2$: 0.321, $p = 0.001$; Figure 3C). The first axis of principal coordinate analysis separated the communities by age, months 2–7 being separated from the communities of following months, while the second axis separated the T-group communities at month 2 from the other communities. The abundance of 12 species was affected by age (Figure 3D; Supplementary Table S5). The relative abundance of six protozoa species reduced with age, *Eremoplastron dilobum*, *Epidinium caudatum,* and *Isotricha* sp. being among the most abundant of these. The abundance of *Isotricha* sp. reduced in both groups by month 4, while *Eremoplastron dilobum* reduced in both groups by month 8, and *Epidinium caudatum* by month 9. The abundance of six species increased toward adulthood, of which *Isotricha prostoma* became the dominant species after month 8, *Entodinium longinucleatum* increased in abundance between months 3 and 11, while

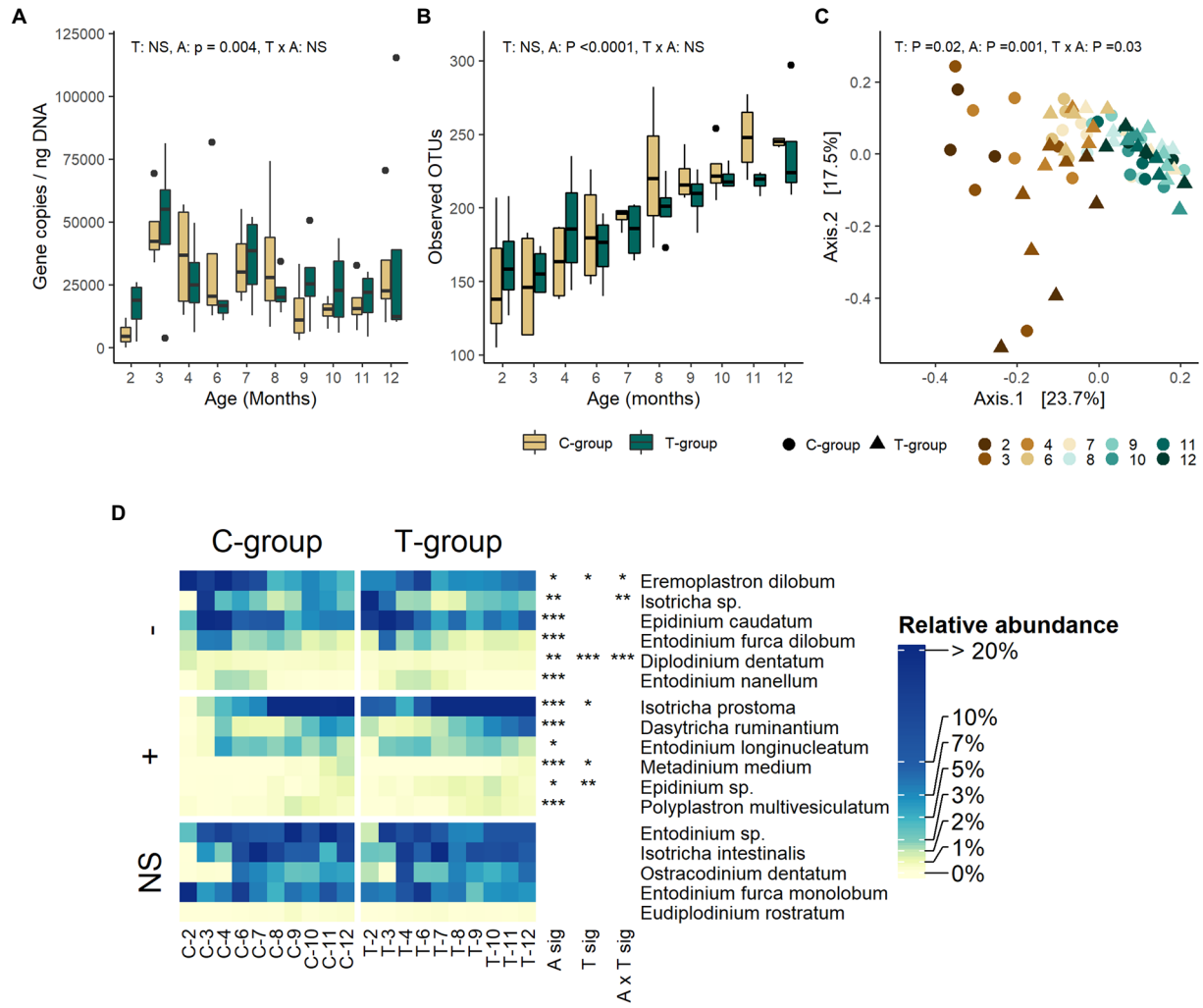


FIGURE 3
The rumen ciliate protozoa community in treatment (T-group) and control (C-group) heifers at 2–12months of age. **(A)** The rumen protozoa 18S rRNA gene copy numbers, **(B)** the number of observed OTUs, **(C)** Principal coordinate analysis visualization of Bray-Curtis community distances, and **(D)** the relative abundances of protozoa species. Only taxa with statistical significances are shown. The taxa are grouped by increasing (+) or decreasing (–) trend with time, and no significant effects (NS). Statistical significance of Treatment (T-sig), Age (A-sig), and Treatment×Age interaction (T×A sig): $p<0.05$: *, $p<0.01$: **, $p<0.001$: ***.

*Dasytricha ruminantium* and other rarer species became more abundant only after month 9.

The Random Forest model of ciliate protozoa community predicted a significantly faster community maturation of in the T-group in month 2, being 1.5 months ahead of the maturity of C-group. Thereafter, the predicted ages of two groups became similar (Supplementary Figure S3A). When looking at the approximate Shapley values for the T-group in month 2, the low abundance of OTUs affiliated with *Ostracodinium dentatum*, *Polyplastron multivesiculatum*, *Isotricha prostoma*, *Entodinium longinucleatum*, and *Isotricha intestinalis* was among the taxa that reduced the age prediction, while the low abundance of OTUs affiliated with *Eremoplastron dilobum* increased the predicted age (Supplementary Figure S3B). Looking at all the 31-maturation associated ciliate protozoa OTUs (Supplementary Figure S3C) taxa such as *Metadinium medium*, *Polyplastron multivesiculatum*, *Epidinium* spp., and *Isotricha* spp. were getting more abundant at later ages, while *Eremoplastron dilobum*, *Entodinium* sp., and *E. nagellum* were more abundant when the animals were young.

## The anaerobic fungi community was unaffected by treatment

The treatment had no effect on the anaerobic fungi ITS1 gene copy numbers (treatment: $p=0.385$; Figure 4A), OTU richness ($p=0.626$; Figure 4B), alpha diversity (Supplementary Figure S1), or beta diversity ($R^2=0.012$, $p=0.19$; Figure 4C). There were no statistically significant differences in relative abundance of any of the anaerobic fungi species between the groups (Figure 4D)

## The anaerobic fungi community established late

Evidenced by the low fungal ITS1 gene copy numbers, the communities were still established in both groups between months 2–3 (age: $p = 0.005$; Figure 4A). Anaerobic fungi were detected in month 2 in only one sample in the T-group, and three samples in the C-group. Between months 4–10, the copy numbers remained at a stable level, but again increased after month 11. The OTU richness increased significantly with age, reaching its peak in month 11, but temporarily declined between months 7–8 ($p < 0.0001$; Figure 4B). The similar stepwise increase was observed in Shannon index continuing up to 10 months (age: $p < 0.0001$; Supplementary Figure S1). Age explained 35% of variation between fungal community distances ($R^2 = 0.355$, $p = 0.001$; Figure 4C). The PCoA visualization of Bray-Curtis distances separated the communities by age, where the communities in month 2–3, month 4–7, and month 9–12 were more similar to each other. The species-level taxonomic composition showed that in months 2 and 3, *Piromyces* 2 and *Piromyces* 7 dominated the community in both groups (Figure 4D; Supplementary Table S6). The anaerobic fungal richness started increasing in month 3, and the gradual development of fungal community structure continued until month 10, after which it stabilized (all pairwise: $p < 0.05$). From month 3 onward, *Piromyces* 2 and *Piromyces* 7 reduced in abundance in both groups and were replaced by *Neocallimastix* 1 as the dominant species accompanied by *Caecomyces* 1 and SK3 in month 4. Both community diversity and evenness further increased with increasing abundances of, e.g., *Cyllamyces* 2, *Piromyces* 3 and *BlackRhino* in month 6, KF1 and *Orpinomyces* 1b in month 7, and *Orpinomyces* 5, *Orpinomyces* 1a, and AL8 in month 8. By month 11, the evenness and diversity of the fungal community had reached its peak, as 11 species were observed with a relative abundance of more than 1.5%.

Random Forest model, based on the 45 age-related OTUs, indicated similar maturities in both groups (Supplementary Figure S4A). Similar to C-group, the Shapley values of the T-group's model prediction from 2 to 12 months of age showed that higher abundances of *Neocallimastix* 1, *Orpinomyces 1a*, *Caecomyces* 1, *BlackRhino,* and *Orpinomyces* 1b were associated with a more mature community, whereas *Piromyces* 7, *Tahromyces munnarensis*, and *Caecomyces* 1 were associated with young community (Supplementary Figures S4B,C).

## Rumen function and growth of heifers

There were no significant differences between the groups in the weight of the heifers (treatment: $p = 0.627$, interaction: $p = 0.992$; Supplementary Table S7). The T-group gained on average 267 ± 9 kg and C-group 272 ± 9 kg during the 1st year period.

The total VFA concentration decreased significantly with age (age: $p < 0.019$), and acetate proportion increased, while the proportions of all other individual VFAs reduced or had monthly fluctuation (Supplementary Figure S5). Butyrate (interaction: $p = 0.04$) was significantly affected by the treatment. The butyrate proportion was at its highest in month 3, with numerically higher proportion in the T-group.

## Host genetic effect on the microbial community similarity

To assess if the host genetics influences the rumen microbial community composition, we compared Bray-Curtis distances within twin-pairs against non-sibling animals (Figure 5). We hypothesized that inoculation will be the major factor influencing rumen microbiota differences between the animals right after the weaning. If host genetics has an impact, the community distances between the twins will be smaller as compared to the distances between non-related animals during rumen maturation. Bray-Curtis distances reduced over time and were smaller between the twins than between the non-related animals at the end of the 12-month period for bacteria (Figure 5A), ciliate protozoa (Figure 5C) and anaerobic fungi (Figure 5D). A similar trend was not observed for archaea (Figure 5B).

## Rumen microbiome and rumen function during the first lactation

There were no significant differences between the groups in the total quantity of rumen bacteria, archaea, ciliate protozoa, or anaerobic fungi (Supplementary Table S8), or ASV/OTU alpha diversity (Supplementary Table S8) during the first lactation period. The Bray-Curtis distances of bacteria and fungi did not significantly differ between the groups (Adonis $p > 0.05$; Supplementary Figure S6), while differences in archaea and ciliate protozoa community structure between the groups were significant (Adonis $p < 0.05$). From the total of 83 bacterial genera observed (Supplementary Table S9), the T-group had higher relative abundances of unidentified genera affiliated with *Lachnospiraceae* ($p = 0.025$), *Lachnospiraceae NK3A20* group ($p = 0.03$), *Lachnospiraceae XPB1014* group ($p = 0.066$) and (*Eubacterium*) *xylanophilum* group ($p = 0.067$) as well as lower abundance of unidentified genera affiliated with *Prevotellaceae* ($p = 0.058$). *Methanobrevibacter gottschalkii*, *Mbb. ruminantium*, *Methanosphaera ISO3-F5,* and 6 less abundant (<5% relative abundance) archaeal species were observed within the dataset, but no significant differences between the groups were detected (Supplementary Table S10). In both groups, ciliate protozoa were represented by 16 species with *Entodinium* spp. and *Entodinium furca monolobum* being predominant, although two C-group heifers had high abundance of *Epidinium caudatum* (Supplementary Table S11). Only *Diplodinium dentatum* was more abundant in the C-group ($p = 0.037$), while *Entodinium*

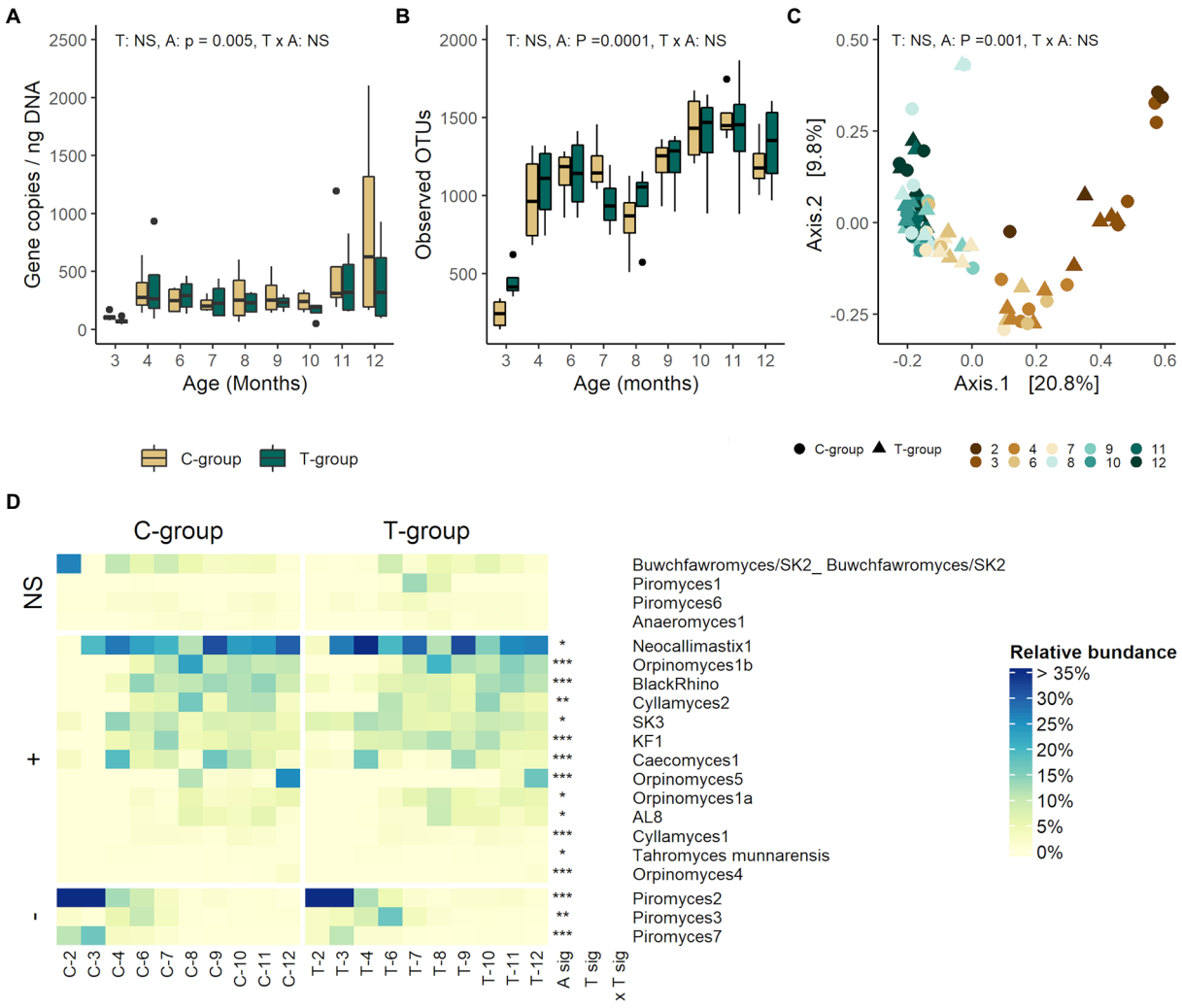


FIGURE 4
The rumen anaerobic fungi community in treatment (T-group) and control (C-group) heifers at 2–12months of age. **(A)** The rumen anaerobic fungi ITS1 area copy numbers, **(B)** the number of observed OTUs, **(C)** principal coordinate analysis visualization of Bray-Curtis community distances, and **(D)** the relative abundances of protozoa species. Only taxa with statistical significances are shown. The taxa are grouped by increasing (+) or decreasing (–) trend with time, and with no significant effects (NS). Statistical significance of Treatment (T-sig), Age (A-sig), and TreatmentxAge interaction (TxA sig): $p<0.05$: *, $p<0.01$: **, $p<0.001$: ***.

*longinucleatum* was significantly more abundant in the T-group ($p=0.043$). The fungal community was represented by 10 genera in both groups (Supplementary Table S12). *Neocallimastix* and *Buwchfawromyces/SK2* were predominant fungi, but there was a high between-sample variation.

No significant differences between the groups were observed in total VFA concentration or in any other individual VFA proportions (Supplementary Table S13).

## Early life modulation effect on cows' production performance

During the first lactation, no significant differences were observed in the DMI between the groups (Figure 6A). The C-group ate on average 18.6 kg DM/d corresponding to 204 ± 10 ME MJ/d, while the T-group ate on average 18.0 kg DM/d corresponding to 197 ± 10 ME MJ/d. The DMI increased until month 4 in the C-group and month 5 in the T-group after which it started to reduce (month: $p<0.0001$).

There was no significant difference in the average BW between the groups, although the body weight of T-group was numerically lower (523 ± 26 kg) in comparison to C-group (563 ± 26 kg) at the beginning of lactation. The BW increased from 543 ± 18 kg in the first lactation month to 628 ± 18 kg in the end of the lactation period (Month: $p<0.0001$) (Supplementary Table S14). Due to high between-animal variation, there were no significant differences between the groups or lactation months in the weekly weight gain of the cows (Figure 6B). The cows from T-group gained on average 2.6 ± 1.6 kg/week and from the

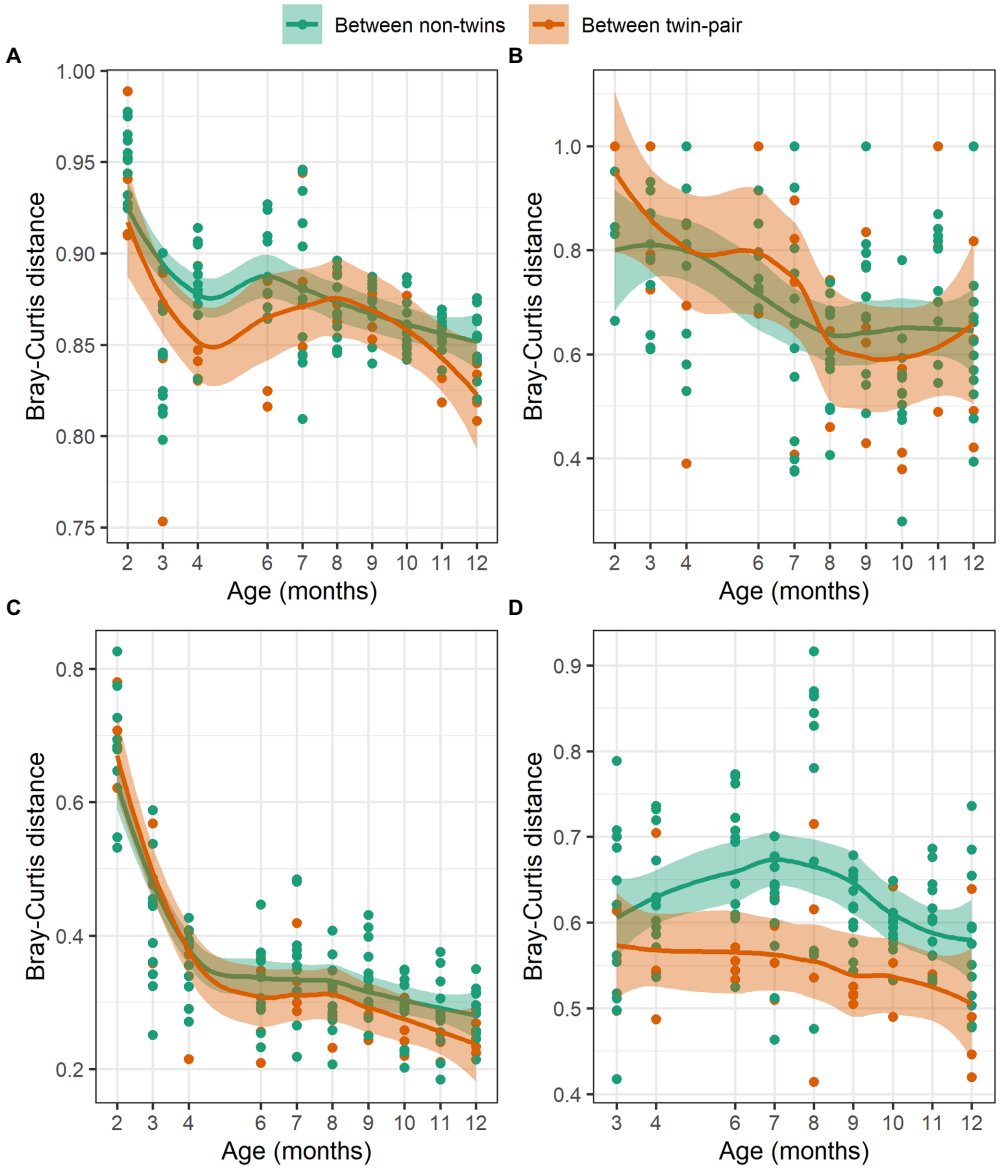


FIGURE 5
The Hellinger transformed Bray-Curtis distances of **(A)** bacteria, **(B)** archaea, **(C)** ciliate protozoa, and **(D)** anaerobic fungi communities between twin pairs (red) and between non-related heifers (green) at 2–12months of age.

C-group 1.6 ± 1.6 kg/week during the 1st lactation. However, the T-group maintained a more even weight gain through the whole lactation as compared with the C-group, while C-group tended to lose weight during the second lactation month.

The average daily ECM yield was 24.8 ± 1.6 kg for C-and 23.3 ± 1.6 kg for the T-group and varied with month (treatment: $p = 0.504$, Month: $p < 0.0001$, interaction: $p = 0.0009$; Figure 6C). The peak in ECM yield in the C-group was observed in month 4 (28.8 ± 1.8 kg), but from 5 months onward the ECM rapidly diminished. In the T-group the ECM reached the highest yield (25.4 ± 1.8 kg) at 3 months after calving and remained more stable throughout the remaining lactation period. There were no significant differences in milk lactose, fat, or protein percent between the groups (Supplementary Table S15). The overall protein production (C-group:0.88 ± 0.05 kg; T-group: 0.83 ± 0.05 kg, $p = 0.498$), lactose production (C-group: 1.01 ± 0.06 kg; T-group: 0.96 ± 0.06 kg, $p = 0.595$), and fat production (C-group:1.03 ± 0.08 kg; T-group: 0.95 ± 0.08 kg, $p = 0.494$) did not differ between groups (Supplementary Table S15). However, numerically higher milk yield in the C-group during the early lactation resulted into a numerically higher lactose (treatment × month: $p = 0.002$), protein (treatment × month: $p = 0.001$), and fat production (treatment × month: $p = 0.001$) during the first 5 months, in comparison to later months, while the production in T-group increased after first month, and remained at the same level thereafter. Treatment tended to affect the milk

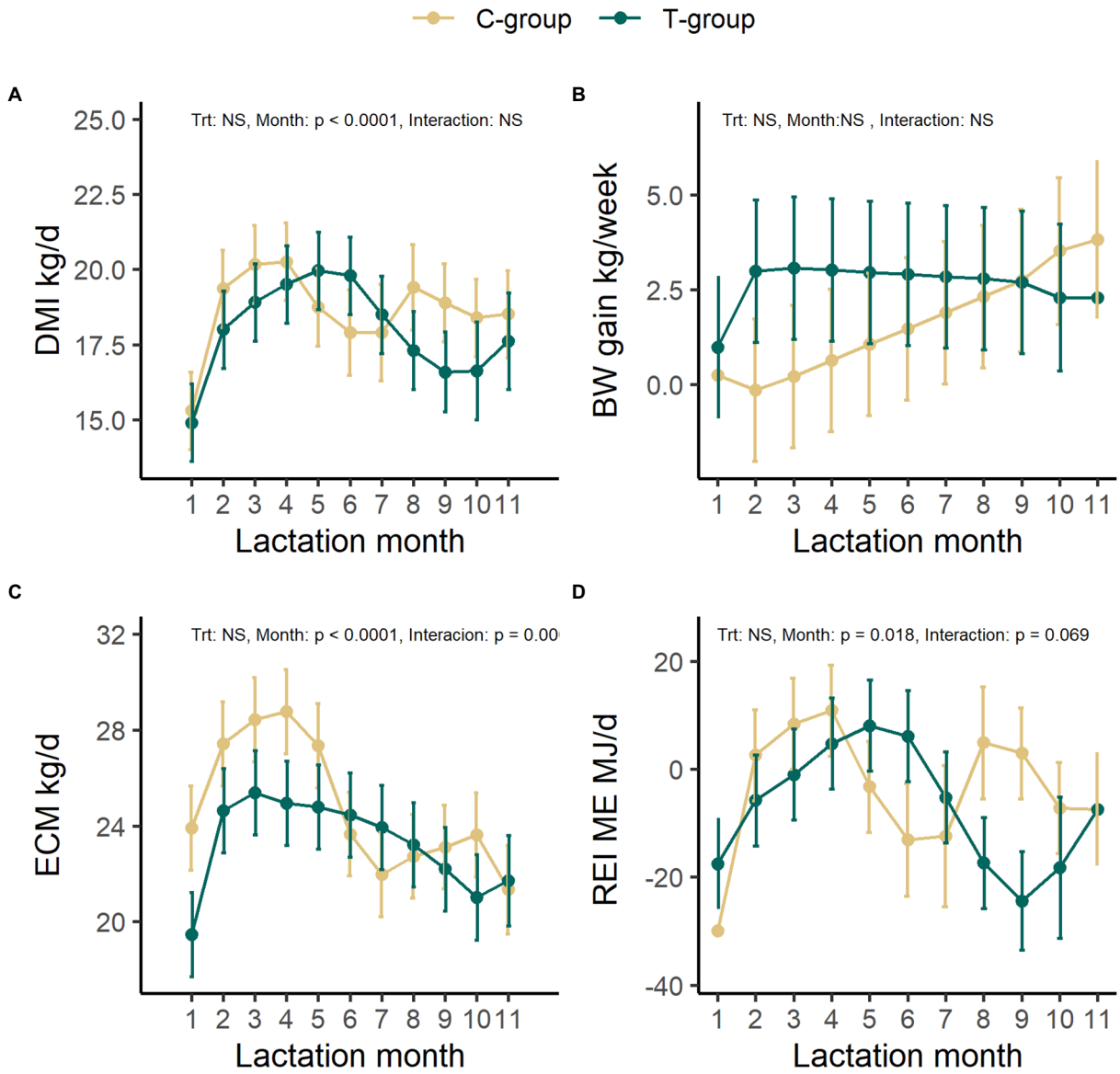


FIGURE 6
The average feed dry matter intake (DMI, kg/day) **(A)**, average weekly body weight gain **(B)**, energy-corrected milk yield (ECM, kg/day) **(C)**, and residual energy intake (REI) **(D)** in treatment (T-group) and control (C-group) cows during the 1st lactation period. Data is presented in least square means±standard error.

somatic cell count (SCC; treatment: $p = 0.081$; Supplementary Figure S6). Throughout the first lactation period the C-group had a numerically higher SCC (36 ± 1.1 × 1,000/ml) in comparison to the T-group (14 ± 1.1 × 1,000/ml).

The REI was affected by the stage of lactation in both groups ($p = 0.018$) but tended to have a different pattern of cycles of low and high REI between the group (interaction: $p = 0.069$; Figure 6D). Numerically, the mean REI was negative in the beginning and at the end of lactation in both C-and T-groups. The C-group had numerically lowest REI (−30 ± 8 ME MJ/d) in 1st lactation month, while the T-group in 9th month (−24 ± ME MJ/d). The shifts from negative to positive REI were delayed in the T-group, occurring *ca.* 2 months later in comparison to C-group. The T-group showed a longer period of negative REI in the early lactation, and lower and longer negative REI in the late lactation in comparison to C-group, while the C-group returned to negative REI earlier in mid-lactation and experienced a second positive REI peak in the late lactation.

The average methane production did not differ between the groups (C-group: 335 ± 14 g/d; T-group: 336 ± 16 g/d, LS mean ± SE, $p = 0.951$), but increased from 286.5 ± 19.9 g/d during the first lactation month to 373.3 ± 23.8 g/d in fifth lactation month ($p = 0.02$; Supplementary Table S14). After the first lactation month, the methane production in the C-group increased and remained stable, while the methane output in the T-group was more variable. There were no significant differences

between the groups in the average methane yield (C-group: 18.1 ± 0.6, T-group: 17.4 ± 0.8 g/kg DMI; $p = 0.378$) or in the methane intensity (C-group: 13.7 ± 0.4, T-group: 15.0 ± 0.6 g/kg milk; $p = 0.123$). Both the methane yield and the methane production intensity maintained a similar level during 5 months measurement period (Month: $p > 0.05$; Supplementary Table S14).

# Discussion

In this study, we investigated the effect of rumen microbiota modulation during the pre-weaning period on the rumen microbiota maturation post-weaning and further elucidated its influence on the production performance of cows during their first lactation period. The present study builds upon our previous findings that inoculation with fresh rumen liquid can enhance the bacterial and archaeal community maturation before calves are weaned (Huuki et al., 2022). Here, we show that the effect of inoculation persisted post-weaning but gradually diminished, making the C-and T-groups similar in rumen microbiota structure before they reached 12 months of age. We also demonstrated that the inoculum treatment may affect the production performance during the 1st lactation period in monozygotic twins, but a larger number of animals would be needed to reach significance.

## The transfer of microbes from inoculum donor into calves' rumen before weaning

In our previous study (Huuki et al., 2022; Supplementary Tables S12–S14) we investigated the transfer of microbes between the donor cow and the recipient calves. We did this by comparing the number of shared OTUs between the donor and the core microbial community within the T-and C-groups at each sampling week during the pre-weaning period. The inoculum increased the OTU richness of the prokaryote and eukaryote core community in T-group starting from week 2. The number of OTUs shared with the donor increased with age in both groups, so that by weaning both groups shared all their prokaryote core OTUs with the donor. In a similar way, all anaerobic fungal and ciliate protozoan core OTUs detected within T-and C-groups at weaning were shared with the donor (Huuki et al., 2022). In the present study we no longer followed the donor community transfer, because, by weaning, all the core community microbes were shared with the donor. Instead, we focused on the further microbiota development with age, and on the community differences between the groups.

## Microbial community development until 1 year of age

### Bacteria

The rumen modulation during the pre-weaning period influenced the bacterial community maturation for at least 2 months after the treatment. Based on the age-determinant taxa, the T-group calves were predicted to be older in comparison to the C-group until around month 4, after which the difference in predicted age was no longer apparent. The more mature community in the T-group was associated with increased abundance of *Prevotella*, *Christensenellaceae R-7* group, and *Lachnospiraceae* NK3A20 group as well as higher abundance of *Pseudobutyrivibrio*, and *Clostridiales* bacterium. Interestingly, ASVs affiliated with these taxa were significant contributors to maturation also in grazing yaks (Guo et al., 2020). Our observations are in line with the results of de Barbieri et al. (2015a), where inoculation of lambs with rumen liquid from ewes fed either coconut oil or rumen protected fat induced changes in the bacterial community lasting up to 3 months after the treatment. Similarly, different diets induced significant differences in the bacteria community composition in pre-weaned calves, but later these differences greatly diminished or disappeared (Dill-McFarland et al., 2019). The microbiome composition in the rumen is controlled by deterministic effects of both diets, in terms of available substrates for the function of microbes, and age of the animal, in terms of anatomical and physiological constraints, but also stochasticity that especially may affect the microbial communities in early life (Furman et al., 2020). It is possible that the plasticity of the rumen microbial community allowed the microbes obtained through the pre-weaning inoculation to be still active in microbial interactions, but with shared diet and environment, the communities gradually became more alike.

We saw major changes occurring in the bacterial community composition right after weaning (2–3 months) and continuing until 7 months of age, after which only minor changes were observed. The taxonomical changes around weaning were related to the decreasing abundance of Proteobacteria, such as *Succinivibrio* or *Succinivibrionaceae* spp., and several Bacteroidota genera, such as *Alloprevotella* and *Prevotellaceae* spp., but also shifts in the taxonomic composition of Firmicutes. Bacteroidota are known to be abundant in the pre-weaned calves (Jami et al., 2013; Rey et al., 2014), but major shifts within Bacteroidota occur by weaning due to increasing dominance of *Prevotellaceae* family that is linked to increasing diet fiber content (Rey et al., 2014; Furman et al., 2020). Similarly, the Proteobacteria are more abundant in young calves (Jami et al., 2013), and *Succinivibrionaceae* has been observed to decrease around weaning, which was speculated to be associated with increasing fiber content in the diet and increasing abundance of *Methanobacteriaceae* family archaea (Furman et al., 2020). On the other hand, our results showed, that the bacterial community continued to develop until month 7, mainly due to shifts in abundances of several Firmicutes genera. Changes in some rare taxa were observed even up to 10 months of age. Firmicutes contain many important cellulolytic taxa, that have a role in fiber degradation (Deusch et al., 2017; Terry et al., 2019). The observed shifts in the Firmicutes taxonomical composition with age could be an adjustment to the changing diet or could be an indication of changes in the rumen microbial interactions, as an increase in

abundance of, e.g., *Lachnospiraceae* family has been previously found without changes in the diet (Furman et al., 2020). Our results on the timeline of the rumen maturation agree with some of the few studies that have followed the natural progress of rumen bacteria community development until adulthood, which showed that rumen core community establishes by 5 months of age, but few and less persistent changes related to other taxa continued to occur until adulthood (Furman et al., 2020).

## Archaea

The archaea community post-weaning was not affected by treatment, and experienced small taxonomical changes until month 6. These changes were related to *Methanosphaera* spp. being more abundant in younger heifers but being replaced by *Methanobrevibacter gottchanlkii* as the dominant species after month 3. *Methanobacterium alkaliphilum*, *Methanomassilii coccaceae* Group3b sp., and *Mmc*. Group8 sp. were late arrivals and increased in abundance after month 8. The increasing taxonomical diversity of archaea is possibly linked to the rumen metabolite diversity, which increases with age due to the more complex microbial functional interactions. Our observations are in line with Dias et al. (2017) who demonstrated that *Methanosphaera* was dominating in the pre-weaning calves fed milk and concentrate diet, while *Mbb. gottschalkii* dominated in calves receiving milk. While *Mbb. gottschalkii* is a hydrogenotrophic methanogen (Miller and Lin, 2002), *Methanosphaera* uses methanol for methanogenesis (Miller and Wolin, 1985). Methanol is produced in pectin degradation, with *Prevotella* spp. being regarded as the main producer of methanol in the rumen (Kelly et al., 2019). *Methanomassiliicoccaceae* spp. can also utilize methanol as an electron acceptor, but unlike *Methanosphaera*, they are also able to utilize methylamines (Poulsen et al., 2013; Söllinger et al., 2018), and have advantage in competition for substrates with *Methanosphaera*. *Methanobacterium alkaliphilum,* on the other hand, is known to tolerate high pH (Worakit, 1986). The greater abundance of *Methanobacterium alkaliphilum* during the last months could be related to potential changes in the rumen pH caused by changes in feed/silage quality, but unfortunately, rumen pH was not measured to confirm this hypothesis.

## Ciliate protozoa

The initial housing conditions for transmission of ciliate protozoa differed between the two groups, resulting in *Eremoplastron dilobum* dominating the C-group, whereas *Isotricha* sp. and *Epidinium caudatum* being predominant in the T-group by the end of the pre-weaning period (Huuki et al., 2022). While the T-group received ciliate protozoa with rumen liquid inoculum, and in all the T-group heifers protozoa were observed already in month 1 (Huuki et al., 2022), majority of the C-group calves remained defaunated and acquired ciliate protozoa only by month 3, when they were moved to a group pen. Therefore, the T-group community had more time to establish, and the treatment continued to enhance the maturation of ciliate protozoa community until animals reached 4 months of age. However, from month 3 onward, the communities of both groups started to become more alike, and the C-group quickly caught-up in maturation. The ciliate protozoa are transmitted through a contact with conspecifics and contaminated environment (Bird et al., 2010). The translocation of heifers into a different barn, and the housing of calves from both groups inside the same group pen may have caused exchange of different ciliate protozoan taxa, and shifts in the relative abundance of species, that resulted in similar community composition between the groups at the age of 7–8 months, dominated by *Isotricha prostoma*. While, the ciliate protozoa have been intensively studied in terms of methane mitigation and protein metabolism, recent research on the long-term community changes is sparse. Despite ciliate protozoa being robust in their group persistence (Eadie, 1962a,b), it looks that C-group, with different taxa abundant at month 2, was still flexible for changing ciliate community composition.

## Anaerobic fungi

In our previous study, we demonstrated that the adult animal rumen liquid inoculum, provided to the calves during the pre-weaning period, did not stimulate earlier anaerobic fungi establishment and rather had the opposite effect (Huuki et al., 2022). By following rumen microbial community development post-weaning, we demonstrated that the quantity of rumen fungi remained low until month 4, and that the development of the anaerobic fungi community structure and the increase in richness was gradual and continued at least until month 10, after which it stabilized.

In our study, the pre-weaning treatment did not induce fungal community composition differences between the groups in any of the community diversity measures post-weaning. Dill-McFarland et al. (2019) found that the pre-weaning diet affected the fungal community later in life in cows, and Belanche et al. (2019a) showed that natural rearing with lambs enhanced the fungal community establishment and had long-term impact on fungal richness when lambs were grazing. Together these observations suggest that the diet determines the fungal community composition, and the establishment of anaerobic fungi in the rumen cannot be aided by the pre-weaning inoculation with rumen fluid from an adult cow. It is possible that in the case of anaerobic fungi, the best time window for modulation is only after the weaning, when the rumen environment can maintain the community and the establishment properly begins.

The anaerobic fungi taxonomical composition continued to develop at least until month 10. During milk feeding period, *Caecomyces* and SK3 were the most abundant taxa (Huuki et al., 2022). The diet before weaning is rich in fast fermentable carbohydrates, and high levels of fermentation products could have an inhibiting effect on anaerobic fungi (Joblin and Naylor, 1993). As the solid feed intake increased toward the weaning, *Caecomyces* and SK3 were gradually replaced by *Buwchfawromyces*/SK2, and *Piromyces 2* and 7 became dominant around month 3. These *Piromyces* species have not yet been cultured and their functional properties are not yet characterized. However, some

*Piromyces* strains are known to possess very high hydrolytic activities in comparison to other anaerobic fungi (Tripathi et al., 2007), which could give them advantage in competition for increasing substrates during the early stages of rumen development. From month 4 onward, the *Piromyces* spp. were replaced by *Neocallimastix* 1, which retained the dominant status until the end of the experiment.

Secondly, the taxonomic richness and evenness of the fungal community increased with age. While at weaning the *Piromyces* 2 abundance reached 62%, and only five other taxa were detected at a relative abundance >1%, the fungal taxonomic richness and evenness greatly increased between months 4 and 10. This period in rumen anaerobic fungi development was described by fluctuation among various fungal species, including *Orpinomyces* spp. It is likely, that the increasing solid feed intake, the more stable rumen environment, and more developed rumen microbial ecosystem supported the establishment of increasing number of fungal species, but also induced competition between the species, which was seen in increased evenness estimate. Competition between *Orpinomyces* and *Neocallimastix* has been previously proposed by (Kittelmann et al., 2012) as they seem to have structurally similar cellulase and xylanase enzymes (Li et al., 1997). The taxonomic composition of rumen anaerobic fungi has been found to vary with the diet in adult animals, e.g., the *Neocallimastix* being more dominant in high concentrate-to-forage ratio diet (Tapio et al., 2017).

## Rumen volatile fatty acids until 1 year of age

The age-dependent patterns of rumen VFA likely reflected the changes in the feeding regime and quality of the feed. The proportion of acetate had a slight increase, while the propionate and butyrate proportions reduced with age, most significant changes occurring after weaning at month 3. A high concentrate diet has been shown to increase the production of butyrate and propionate due to breakdown of carbohydrates into sugars by amylolytic bacteria. The hexoses are further broken down to pyruvate, which is used to produce butyrate, and propionate through intermediate metabolites such as lactate and succinate (Zhang et al., 2020). We saw a reduction in the relative abundance of several sugar utilizing, succinate, and/or lactate-producing taxa, e.g., *Selenomonas* (Silley and Armstrong, 1985), *Succinivibrio* (Bryant and Small, 1956), *Alloprevotella* (Downes et al., 2013), *Blautia* (Liu et al., 2021), and, e.g., in propionate producing taxa such as *Succiniclasticum* (Van Gylswyk, 1995). Therefore, it is tempting to speculate that the changes in diet around weaning may have contributed to the reduction of fermentation intermediates such as lactate and succinate and led to the decrease of propionate and butyrate proportions. This is further supported by previous studies, which have reported higher concentrations of fermentation intermediates in calves, as well as a decrease in propionate and increase in acetate proportions in response to changing from milk diet toward fiber and starch diet (Dill-McFarland et al., 2019), or decreasing acetate to propionate ratio and butyrate concentration (Anderson et al., 1987). Dill-McFarland et al. (2019) suggested that the decrease in propionate is related to increased maturation of gastrointestinal tract, as propionate is the main substrate of gluconeogenesis in ruminants.

Despite some differences in microbial community composition between the T-and C-group, we observed only minor differences in the VFA proportions between the groups, which was likely due to functional plasticity within the rumen (Taxis et al., 2015). In comparison to C-group, the T-group tended to have higher proportion of butyrate around month 3. Many of the taxa, identified by Random Forest to be related to more mature community in T-group around the same time, are known to include butyrate-producing species, e.g., *Christensenellaceae* (Morotomi et al., 2012) and *Pseudobutyrivibrio* (Van Gylswyk et al., 1996). The overall higher abundance of butyrate-producing taxa may have contributed to the numerically higher butyrate proportion in T-group. Moreover, the ciliate protozoa quantity peaked in month 3 in both groups, being slightly higher in T-group in comparison to the C-group. The ciliate protozoa have been shown to contribute to the VFA production to a varying degree, depending on the protozoa community structure (Solomon et al., 2022). Also, it has been shown that the defaunation of ruminants from ciliate protozoa may have a negative effect on the butyrate production (Newbold et al., 2015). We therefore speculate, that the ciliate protozoa contributed to the butyrate concentration peak around month 3 and can partially explain the numerically higher butyrate proportion in T-group.

## Host genetics effect on rumen microbiome

The host genetics is known to affect the ruminant microbiota (Li F. et al., 2019; Wallace et al., 2019). An increasing body of evidence suggests that the transfer of bacteria and archaea from the mother to fetus starts already in the uterus (Guzman et al., 2020; Husso et al., 2021) and microbiome between the twins in humans (Turnbaugh et al., 2009; Goodrich et al., 2014) and bovine (Mayer et al., 2012) is more similar than between unrelated individuals. To minimize the variation caused by the host genetics in the present study, we used monozygotic twin calves, produced by embryo splitting. Due to inoculation treatment during the pre-weaning period, we observed differences in the rumen microbial community composition between the C-and T-groups (Huuki et al., 2022). However, we hypothesized that if the hosts' genetics affects its own microbiome development, the impact will be visible after rumen modulation is over and animals are left to

develop until adulthood. In this study, we observed that the treatment effect gradually diminished and the microbial communities in all individuals became more alike with age. However, apart from the archaea, the Bray-Curtis distances in all other domains became smaller with age between the twin pairs in comparison to distances between the non-siblings. The impact of host genetics on ciliate protozoa and fungi has not been extensively studied, and currently existing data suggest that the rumen eukaryome is not considered to be highly hereditary (Gonzalez-Recio et al., 2018; Wallace et al., 2019; Martínez-Álvaro et al., 2022). Yet, we found that the communities tended to be more similar within the twin-pairs in comparison to non-siblings. It is tempting to speculate that this greater similarity could be a result of host genetic impact on own pro-and eukaryome development, that became apparent when the effect of inoculation diminished. However, a study with larger number of animals is needed to confirm our preliminary findings.

## First lactation period animal performance

We observed some differences in production parameters between the T-and C-groups during their 1st lactation period. During the first four lactation months, the C-group maintained numerically higher milk production in comparison to the T-group. The differences between the groups in early lactation could be due to numerically lower body weight in the T-group after parturition caused by slightly younger parturition age due to earlier insemination success. The lower body reserves at the start of the lactation period have been shown to direct the energy use toward growth and reduce the milk yield, because cows with higher BW are able to mobilize more energy from the body reserves, especially during early lactation (Mäntysaari et al., 1999; Mäntysaari and Mäntysaari, 2010). Between 2nd and 4th lactation month the C-group also started to compensate for the earlier body mass loss. In comparison, the T-group did not lose BW, although the BW gain temporarily slowed down during the first lactation month. The C-group had its highest efficiency (lowest REI) during 1st month of lactation, while the T-group reached highest efficiency between 8th and 10th month of lactation, when the ECM and DMI gradually reduced, and more energy was used toward growth. These observations may suggest that the T-group partitioned more of the energy into growth than into milk production, while keeping a steady milk yield, whereas the C-group used more of the energy into milk production especially during the early lactation but had to invest more energy into growth in the late lactation. Previously, high, and low merit producers have been found to vary in partitioning of metabolizable energy into different energy sinks, and that there is genetic variation within these traits (Mehtiö et al., 2018).

There were no differences in milk composition between the groups, but differences were detected in SCC, with C-group having on average higher SCC in comparison to T-group throughout the whole lactation. Two of the C-group cows and one T-group cow were diagnosed with mastitis during their early lactation. The SCC of all these cows reduced to a healthy level (<150,000 cell/ml) after treatment. However, one C-group cow with mastitis and another C-group cow without clinical signs of mastitis retained SCC on average higher than 50,000 cells/ml throughout the experiment. A link between the rumen microbiome and clinical mastitis has been demonstrated in cows (Zhong et al., 2018, 2020) and in sheep (Boggio et al., 2021). Zhong et al. (2018) showed that the high SCC associated cows had a higher rumen bacterial diversity, changes in the bacterial taxonomical composition, a lower milk yield, and a lower milk lactose (%), but a higher milk fat and protein (%). No similar observations were made in our study, as the C-group had numerically higher milk yield in comparison to T-group, and the milk composition did not differ between the groups. The C-group showed signs of lower REI during the first lactation month in comparison to the T-group, which could have affected the higher SCC. It has been previously shown that the low REI often leads to greater negative energy balance, which may increase the risk of diseases (Becker et al., 2021).

Because the microbial community composition and rumen fermentation characteristics were similar between the groups, and the genetic background of individuals was identical, the production performance of the animals would be expected to be similar. Yet, subtle differences in production performance were observed. In general, the different seasons and the age of the cow can cause variation in the production performance experiments, which is normally resolved with a higher number of study subjects. Due to the limited number of animals in the present experiment, and the differences in calving times, we cannot rule out the possibility that these factors may have influenced the results. However, in the current experiment the genetic background, housing and dietary conditions were controlled, apart from pasture season. Therefore, the differences between the groups could be speculated to be affected by the rumen modulation treatment, especially during the first 5 lactation months, when none of the cows were on pasture and the diet was strictly controlled. We cannot rule out the possibility, that the rumen microbiome modulation during the pre-weaning period induced permanent changes in, e.g., calf's metabolism (Li W. et al., 2019), immune development (Raabis et al., 2019), or intestinal development and/or function, that could indirectly impact the performance. Further studies are also needed to assess influence of the lower gut microbiome on the differences in the phenotypic characteristics, as some indications of changes caused by rumen liquid inoculum on colon microbiota exist (Yu et al., 2020) and in lambs' differences in lower intestine microbiota have been associated with feed efficiency phenotype (Perea et al., 2017).

## Conclusion

Our results showed that the orally administered microbial inoculant enhanced the maturation of T-group bacteria and ciliate protozoa until month 4, and in both groups, the community development reached the adult animal stage between 8 and 9 months after birth. The pre-weaning treatment did not enhance the anaerobic fungi establishment, as the anaerobic fungi were the last domain that started efficient rumen colonization only post-weaning, and their gradual development to adult stage continued until month 10. The age-related changes in the archaea community were not as apparent as in bacteria, and the community tended to stabilize at 6 months of age. Some differences in production parameters were observed between the T-and C-groups during the 1st lactation period. The T-group tended to have a more uniform energy-corrected milk yield and residual energy intake during the different phases of lactation, and had lower somatic cell count, as compared to the C-group animals. No differences in methane production were detected. However, further studies with a larger number of animals are needed to elucidate the mechanisms through which the early-life microbiome modulation influences the production performance in ruminants.

## 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 PRJNA713003 with corresponding BioSample Accessions SAMN28869958 - SAMN28870037.

## Ethics statement

The animal study was reviewed and approved by National Ethics Committee (ESAVI/5687/04.10.07/2017, Hämeenlinna, Finland).

## Author contributions

IT and JV: conceptualization and funding acquisition. HH, IT, PM, EN, and SA: methodology and data acquisition. HH and MT: data analysis. HH: writing original draft. IT, AV, MT, PM, SA, JV, and EN: 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.

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## Supplementary material

The Supplementary material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2022.983823/full#supplementary-material

# References


Abecia, L., Jimenez, E., Martinez-Fernandez, G., Martín-García, A. I., Ramos-Morales, E., Pinloche, E., et al. (2017). Natural and artificial feeding management before weaning promote different rumen microbial colonization but not differences in gene expression levels at the rumen epithelium of newborn goats. *PLoS One* 12:e0182235. doi: 10.1371/journal.pone.0182235

Abecia, L., Martín-García, A. I., Martinez, G., Newbold, C. J., and Yáñez-Ruiz, D. R. (2013). Nutritional intervention in early life to manipulate rumen microbial colonization and methane output by kid goats postweaning. *J. Anim. Sci.* 91, 4832–4840. doi: 10.2527/jas.2012-6142

Abecia, L., Ramos-Morales, E., Martínez-Fernandez, G., Arco, A., Martín-García, A. I., Newbold, C. J., et al. (2014a). Feeding management in early life influences microbial colonisation and fermentation in the rumen of newborn goat kids. *Anim. Prod. Sci.* 54, 1449–1454. doi: 10.1071/AN14337

Abecia, L., Waddams, K. E., Martínez-Fernandez, G., Martín-García, A. I., Ramos-Morales, E., Newbold, C. J., et al. (2014b). An antimethanogenic nutritional intervention in early life of ruminants modifies ruminal colonization by archaea. *Archaea* 2014:841463. doi: 10.1155/2014/841463

Ahvenjärvi, S., and Huhtanen, P. (2018). Effects of intraruminal urea-nitrogen infusion on feed intake, nitrogen utilization, and milk yield in dairy cows. *J. Dairy Sci.* 101, 9004–9015. doi: 10.3168/jds.2018-14617

Anderson, K. L., Nagaraja, T. G., Morrill, J. L., Avery, T. B., Galitzer, S. J., and Boyer, J. E. (1987). Ruminal microbial development in conventionally or early-weaned calves. *J. Anim. Sci.* 64, 1215–1226. doi: 10.2527/jas1987.6441215x

Bąkowski, M., and Kiczorowska, B. (2021). Probiotic microorganisms and herbs in ruminant nutrition as natural modulators of health and production efficiency – A review. *Ann. Anim. Sci.* 21, 3–28. doi: 10.2478/aoas-2020-0081

Beauchemin, K. A., Ungerfeld, E. M., Eckard, R. J., and Wang, M. (2020). Review: fifty years of research on rumen methanogenesis: lessons learned and future challenges for mitigation. *Animal* 14, S2–S16. doi: 10.1017/S1751731119003100

Becker, V. A. E., Stamer, E., Spiekers, H., and Thaller, G. (2021). Residual energy intake, energy balance, and liability to diseases: genetic parameters and relationships in German Holstein dairy cows. *J. Dairy Sci.* 104, 10970–10978. doi: 10.3168/jds.2021-20382

Belanche, A., Cooke, J., Jones, E., Worgan, H. J., and Newbold, C. J. (2019a). Short- and long-term effects of conventional and artificial rearing strategies on the health and performance of growing lambs. *Animal* 13, 740–749. doi: 10.1017/S1751731118002100

Belanche, A., Palma-Hidalgo, J. M., Nejjam, I., Jiménez, E., Martín-García, A. I., and Yáñez-Ruiz, D. R. (2020). Inoculation with rumen fluid in early life as a strategy to optimize the weaning process in intensive dairy goat systems. *J. Dairy Sci.* 103, 5047–5060. doi: 10.3168/jds.2019-18002

Belanche, A., Yáñez-Ruiz, D. R., Detheridge, A. P., Griffith, G. W., Kingston-Smith, A. H., and Newbold, C. J. (2019b). Maternal versus artificial rearing shapes the rumen microbiome having minor long-term physiological implications. *Environ. Microbiol.* 21, 4360–4377. doi: 10.1111/1462-2920.14801

Bird, S. H., Hegarty, R. S., and Woodgate, R. (2010). Modes of transmission of rumen protozoa between mature sheep. *Anim. Prod. Sci.* 50, 414–417. doi: 10.1071/AN09216

Boggio, G. M., Meynadier, A., Daunis-I-Estadella, P., and Marie-Etancelin, C. (2021). Compositional analysis of ruminal bacteria from ewes selected for somatic cell score and milk persistency. *PLoS One* 16, e0254874–e0254816. doi: 10.1371/journal.pone.0254874

Bolyen, E., Rideout, J. R., Dillon, M. R., Bokulich, N. A., Abnet, C. C., Al-Ghalith, G. A., et al. (2019). Reproducible, interactive, scalable and extensible microbiome data science using QIIME 2. *Nat. Biotechnol.* 37, 852–857. doi: 10.1038/s41587-019-0209-9

Brito, L. F., Oliveira, H. R., Houlahan, K., Fonseca, P. A. S., Lam, S., Butty, A. M., et al. (2020). Genetic mechanisms underlying feed utilization and implementation of genomic selection for improved feed efficiency in dairy cattle. *Can. J. Anim. Sci.* 100, 587–604. doi: 10.1139/cjas-2019-0193

Bryant, M. P., and Small, N. (1956). Characteristics of two new genera of anaerobic curved rods isolated from the rumen of cattle. *J. Bacteriol.* 72, 22–26. doi: 10.1128/jb.72.1.22-26.1956

Callahan, B. J., McMurdie, P. J., Rosen, M. J., Han, A. W., Johnson, A. J. A., and Holmes, S. P. (2016). DADA2: high-resolution sample inference from Illumina amplicon data. *Nat. Methods* 13, 581–583. doi: 10.1038/nmeth.3869

Cameron, A., and McAllister, T. A. (2016). Antimicrobial usage and resistance in beef production. *J. Animal Sci. Biotechnol.* 7:68. doi: 10.1186/s40104-016-0127-3

Caporaso, J. G., Kuczynski, J., Stombaugh, J., Bittinger, K., Bushman, F. D., Costello, E. K., et al. (2010). QIIME allows analysis of high-throughput community sequencing data. *Nat. Methods* 7, 335–336. doi: 10.1038/nmeth.f.303

Caporaso, J. G., Lauber, C. L., Walters, W. A., Berg-lyons, D., Lozupone, C. A., Turnbaugh, P. J., et al. (2011). Global patterns of 16S rRNA diversity at a depth of millions of sequences per sample. *PNAS* 108, 4516–4522. doi: 10.1073/pnas.1000080107

Cersosimo, L. M., Radloff, W., and Zanton, G. I. (2019). Microbial inoculum composition and pre-weaned dairy calf age Alter the developing rumen microbial environment. *Front. Microbiol.* 10:1651. doi: 10.3389/fmicb.2019.01651

Cristobal-Carballo, O., McCoard, S. A., Cookson, A. L., Laven, R. A., Ganesh, S., Lewis, S. J., et al. (2021). Effect of divergent feeding regimes during early life on the rumen microbiota in calves. *Front. Microbiol.* 12:711040. doi: 10.3389/fmicb.2021.711040

de Barbieri, I., Hegarty, R. S., Silveira, C., Gulino, L. M., Oddy, V. H., Gilbert, R. A., et al. (2015a). Programming rumen bacterial communities in newborn merino lambs. *Small Rumin. Res.* 129, 48–59. doi: 10.1016/j.smallrumres.2015.05.015

de Barbieri, I., Hegarty, R. S., Silveira, C., and Oddy, V. H. (2015b). Positive consequences of maternal diet and post-natal rumen inoculation on rumen function and animal performance of merino lambs. *Small Rumin. Res.* 129, 37–47. doi: 10.1016/j.smallrumres.2015.05.017

Deusch, S., Camarinha-Silva, A., Conrad, J., Beifuss, U., Rodehutscord, M., and Seifert, J. (2017). A structural and functional elucidation of the rumen microbiome influenced by various diets and microenvironments. *Front. Microbiol.* 8:1605. doi: 10.3389/fmicb.2017.01605

Dias, J., Marcondes, M. I., Noronha, M. F., Resende, R. T., Machado, F. S., Mantovani, H. C., et al. (2017). Effect of pre-weaning diet on the ruminal archaeal, bacterial, and fungal communities of dairy calves. *Front. Microbiol.* 8:1553. doi: 10.3389/fmicb.2017.01553

Dill-McFarland, K. A., Weimer, P. J., Breaker, J. D., and Suen, G. (2019). Diet influences early microbiota development in dairy calves without Long-term impacts on Milk production. *Appl. Environ. Microbiol.* 85, 1–12. doi: 10.1128/AEM.02141-18

Downes, J., Dewhirst, F. E., Tanner, A. C. R., Wade, W. G., Tanner, A. C. R., and Wade, W. G. (2013). Description of *Alloprevotella rava* gen. nov., sp. nov., isolated from the human oral cavity, and reclassification of *Prevotella* tannerae Moore et al. 1994 as *Alloprevotella tannerae* gen. nov., comb. nov. *Int. J. Syst. Evol. Microbiol.* 63, 1214–1218. doi: 10.1099/ijs.0.041376-0

Eadie, J. (1962a). The development of rumen microbial populations in lambs and calves under various conditions of management. *J. Gen. Microbiol.* 29, 563–578. doi: 10.1099/00221287-29-4-563

Eadie, J. (1962b). Inter-relationships between certain rumen ciliate protozoa. *J. Gen. Microbiol.* 29, 579–588. doi: 10.1099/00221287-29-4-579

Edgar, R. C. (2010). Search and clustering orders of magnitude faster than BLAST. *Bioinformatics* 26, 2460–2461. doi: 10.1093/bioinformatics/btq461

Edwards, J. E., Kingston-Smith, A. H., Jimenez, H. R., Huws, S. A., SkAt, K. P., Griffith, G. W., et al. (2008). Dynamics of initial colonization of nonconserved perennial ryegrass by anaerobic fungi in the bovine rumen. *FEMS Microbiol. Ecol.* 66, 537–545. doi: 10.1111/j.1574-6941.2008.00563.x

Erickson, P. S., and Kalscheur, K. F. (2020). "Nutrition and feeding of dairy cattle" in *Animal Agriculture: Sustainability, Challenges and Innovations*. eds. F. W. Bazer, G. C. Lamb and G. B. T. Wu (London, United Kingdom: Academic Press), 157–180.

FAO (2019). Five Practical Actions Towards Low-Carbon Livestock. Available at: http://www.fao.org/3/ca7089en/CA7089EN.pdf (Accessed October 18, 2022).

Furman, O., Shenhav, L., Sasson, G., Kokou, F., Honig, H., Jacoby, S., et al. (2020). Stochasticity constrained by deterministic effects of diet and age drive rumen microbiome assembly dynamics. *Nat. Commun.* 11:1904. doi: 10.1038/s41467-020-15652-8

Gonzalez-Recio, O., Zubiria, I., García-Rodríguez, A., Hurtado, A., and Atxaerandio, R. (2018). Short communication: signs of host genetic regulation in the microbiome composition in 2 dairy breeds: Holstein and Brown Swiss. *J. Dairy Sci.* 101, 2285–2292. doi: 10.3168/jds.2017-13179

Goodrich, J. K., Waters, J. L., Poole, A. C., Sutter, J. L., Koren, O., Blekhman, R., et al. (2014). Human genetics shape the gut microbiome. *Cells* 159, 789–799. doi: 10.1016/j.cell.2014.09.053

Greenwell, B. (2021). Fastshap: Fast Approximate Shapley Values. R package version 0.0.7. R package version 0.0.7., 10. Available at: https://cran.r-project.org/package=fastshap (Accessed October 18, 2022).

Guo, W., Zhou, M., Ma, T., Bi, S., Wang, W., Zhang, Y., et al. (2020). Survey of rumen microbiota of domestic grazing yak during different growth stages revealed novel maturation patterns of four key microbial groups and their dynamic interactions. *Animal Microbiome* 2:23. doi: 10.1186/s42523-020-00042-8

Guzman, C. E., Wood, J. L., Egidi, E., White-Monsant, A. C., Semenec, L., Grommen, S. V. H., et al. (2020). A pioneer calf foetus microbiome. *Sci. Rep.* 10, 17712–17713. doi: 10.1038/s41598-020-74677-7

Herve, M. (2020). Package "RVAideMemoire". Version 0.9-75. Available at: https://cran.r-project.org/web/packages/RVAideMemoire/RVAideMemoire.pdf (Accessed October 18, 2022).

Huhtanen, P. J., Blauwiekel, R., and Saastamoinen, I. (1998). Effects of intraruminal infusions of propionate and butyrate with two different protein supplements on milk production and blood metabolites in dairy cows receiving grass silage-based diet. *J. Sci. Food Agric.* 77, 213–222. doi: 10.1002/(SICI)1097-0010(199806)77:2<213::AID-JSFA28>3.0.CO;2-6

Husso, A., Lietaer, L., Pessa-Morikawa, T., Grönthal, T., Govaere, J., van Soom, A., et al. (2021). The composition of the microbiota in the full-term fetal gut and amniotic fluid: A bovine cesarean section study. *Front. Microbiol.* 12:626421. doi: 10.3389/fmicb.2021.626421

Huuki, H., Ahvenjärvi, S., Lidauer, P., Popova, M., Vilkki, J., Vanhatalo, A., et al. (2022). Fresh rumen liquid inoculant enhances the rumen microbial community establishment in pre-weaned dairy calves. *Front. Microbiol.* 12:758395. doi: 10.3389/fmicb.2021.758395

Huws, S. A., Creevey, C. J., Oyama, L. B., Mizrahi, I., Denman, S. E., Popova, M., et al. (2018). Addressing global ruminant agricultural challenges through understanding the rumen microbiome: past, present, and future. *Front. Microbiol.* 9:2161. doi: 10.3389/fmicb.2018.02161

Jami, E., Israel, A., Kotser, A., and Mizrahi, I. (2013). Exploring the bovine rumen bacterial community from birth to adulthood. *ISME J.* 7, 1069–1079. doi: 10.1038/ismej.2013.2

Joblin, K. N., and Naylor, G. E. (1993). Inhibition of the rumen anaerobic fungus Neocallimastix frontalis by fermentation products. *Lett. Appl. Microbiol.* 16, 254–256. doi: 10.1111/j.1472-765X.1993.tb01412.x

Kelly, W. J., Leahy, S. C., Kamke, J., Soni, P., Koike, S., Mackie, R., et al. (2019). Occurrence and expression of genes encoding methyl-compound production in rumen bacteria. *Animal Microbiome* 1, 15–13. doi: 10.1186/s42523-019-0016-0

Kittelmann, S., Devente, S. R., Kirk, M. R., Seedorf, H., Dehority, B. A., and Janssen, P. H. (2015). Phylogeny of intestinal ciliates, including Charonina ventriculi, and comparison of microscopy and 18S rRNA gene pyrosequencing for rumen ciliate community structure analysis. *Appl. Environ. Microbiol.* 81, 2433–2444. doi: 10.1128/AEM.03697-14

Kittelmann, S., Naylor, G. E., Koolaard, J. P., and Janssen, P. H. (2012). A proposed taxonomy of anaerobic fungi (class neocallimastigomycetes) suitable for large-scale sequence-based community structure analysis. *PLoS One* 7:36866. doi: 10.1371/journal.pone.0036866

Koetschan, C., Kittelmann, S., Lu, J., Al-Halbouni, D., Jarvis, G. N., Müller, T., et al. (2014). Internal transcribed spacer 1 secondary structure analysis reveals a common core throughout the anaerobic fungi (Neocallimastigomycota). *PLoS One* 9:e91928. doi: 10.1371/journal.pone.0091928

Kruger Ben Shabat, S., Sasson, G., Doron-Faigenboim, A., Durman, T., Yaacoby, S., Berg Miller, M. E., et al. (2016). Specific microbiome-dependent mechanisms underlie the energy harvest efficiency of ruminants. *ISME J.* 10, 2958–2972. doi: 10.1038/ismej.2016.62

Kursa, M. B., and Rudnicki, W. R. (2010). Feature selection with the Boruta package. *J. Stat. Softw.* 36, 1–13. doi: 10.18637/jss.v036.i11

Li, X. L., Chen, H., and Ljungdahl, L. G. (1997). Monocentric and polycentric anaerobic fungi produce structurally related cellulases and xylanases. *Appl. Environ. Microbiol.* 63, 628–635. doi: 10.1128/aem.63.2.628-635.1997

Li, W., Edwards, A., Riehle, C., Cox, M., Raabis, S., Skarlupka, J., et al. (2019). Transcriptomics analysis of host liver and meta-transcriptome analysis of rumen epimural microbial community in young calves treated with artificial dosing of rumen content from adult donor cow. *Sci. Rep.* 9, 790–711. doi: 10.1038/s41598-018-37033-4

Li, F., Li, C., Chen, Y., Liu, J., Zhang, C., Irving, B., et al. (2019). Host genetics influence the rumen microbiota and heritable rumen microbial features associate with feed efficiency in cattle. *Microbiome* 7:92. doi: 10.1186/s40168-019-0699-1

Liaw, A., and Wiener, M. (2002). Classification and regression by randomForest. *R News* 2, 18–22.

Liu, X., Mao, B., Gu, J., Wu, J., Cui, S., Wang, G., et al. (2021). Blautia—a new functional genus with potential probiotic properties? *Gut Microbes* 13, 1–21. doi: 10.1080/19490976.2021.1875796

Lyons, T., Boland, T., Storey, S., and Doyle, E. (2017). Linseed oil supplementation of lambs' diet in early life leads to persistent changes in rumen microbiome structure. *Front. Microbiol.* 8:1656. doi: 10.3389/fmicb.2017.01656

Maeda, H., Fujimoto, C., Haruki, Y., and Maeda, T. (2003). Quantitative real-time PCR using TaqMan and SYBR green for *Actinobacillus actinomycetemcomitans*, *Porphyromonas gingivalis*, *Prevotella intermedia*, tetQ gene and total bacteria. *FEMS Immunol. Med. Microbiol.* 39, 81–86. doi: 10.1016/S0928-8244(03)00224-4

Malmuthuge, N., and Guan, L. L. (2017). Understanding host-microbial interactions in rumen: searching the best opportunity for microbiota manipulation. *J. Animal Sci. Biotechnol.* 8:8. doi: 10.1186/s40104-016-0135-3

Mäntysaari, P., Ingvartsen, K. L., and Toivonen, V. (1999). Feeding intensity of pregnant heifers effect of feeding intensity during gestation on performance and plasma parameters of primiparous Ayrshire cows. *Livest. Prod. Sci.* 62, 29–41. doi: 10.1016/S0301-6226(99)00055-X

Mäntysaari, P., Liinamo, A. E., and Mäntysaari, E. A. (2012). Energy efficiency and its relationship with milk, body, and intake traits and energy status among primiparous Nordic red dairy cattle. *J. Dairy Sci.* 95, 3200–3211. doi: 10.3168/jds.2011-4685

Mäntysaari, P., and Mäntysaari, E. A. (2010). Predicting early lactation energy balance in primiparous red dairy cattle using milk and body traits. *Acta Agric. Scand. A Animal Sci.* 60, 79–87. doi: 10.1080/09064702.2010.496002

Martínez-Álvaro, M., Auffret, M., Duthie, C., Dewhurst, R., Cleveland, M., Watson, M., et al. (2022). Bovine host genome acts on rumen microbiome function linked to methane emissions. *Commun. Biol.* 5:350. doi: 10.1038/s42003-022-03293-0

Mayer, M., Abenthum, A., Matthes, J. M., Kleeberger, D., Ege, M. J., Hölzel, C., et al. (2012). Development and genetic influence of the rectal bacterial flora of newborn calves. *Vet. Microbiol.* 161, 179–185. doi: 10.1016/j.vetmic.2012.07.023

McMurdie, P. J., and Holmes, S. (2013). Phyloseq: an R package for reproducible interactive analysis and graphics of microbiome census data. *PLoS One* 8:e61217. doi: 10.1371/journal.pone.0061217

Meale, S. J., Popova, M., Saro, C., Martin, C., Bernard, A., Lagree, M., et al. (2021). Early life dietary intervention in dairy calves results in a long-term reduction in methane emissions. *Sci. Rep.* 11:3003. doi: 10.1038/s41598-021-82084-9

Mehtiö, T., Negussie, E., Mäntysaari, P., Mäntysaari, E. A., and Lidauer, M. H. (2018). Genetic background in partitioning of metabolizable energy efficiency in dairy cows. *J. Dairy Sci.* 101, 4268–4278. doi: 10.3168/jds.2017-13936

Miller, T. L., and Lin, C. (2002). Description of *Methanobrevibacter gottschalkii* sp. nov., *Methanobrevibacter thaueri* sp. nov., *Methanobrevibacter woesei* sp. nov. and *Methanobrevibacter wolinii* sp. nov. *Int. J. Syst. Evol. Microbiol.* 52, 819–822. doi: 10.1099/ijs.0.02022-0

Miller, T. L., and Wolin, M. J. (1985). *Methanosphaera stadtmaniae* gen. Nov., sp. nov.: a species that forms methane by reducing methanol with hydrogen. *Arch. Microbiol.* 141, 116–122. doi: 10.1007/BF00423270

Morotomi, M., Nagai, F., and Watanabe, Y. (2012). Description of Christensenella minuta gen. Nov., sp. nov., isolated from human faeces, which forms a distinct branch in the order *Clostridiales*, and proposal of *Christensenellaceae* fam. Nov. *Int. J. Syst. Ecol. Microbiol.* 62, 144–149. doi: 10.1099/ijs.0.026989-0

Negussie, E., Lehtinen, J., Mäntysaari, P., Bayat, A. R., Liinamo, A., Mäntysaari, E. A., et al. (2017). Non-invasive individual methane measurement in dairy cows. *Animal* 11, 890–899. doi: 10.1017/S1751731116002718

Newbold, C., de la Fuente, G., Belanche, A., Ramos-Morales, E., and McEwan, N. (2015). The role of ciliate protozoa in the rumen. *Front. Microbiol.* 6:1313. doi: 10.3389/fmicb.2015.01313

Oksanen, J., Blanchet, F. G., Kindt, R., Legendre, P., Minchin, P. R., O'Hara, R.B., et al. (2019). Vegan: Community Ecology Package. R package version 2.5-6. Available at: https://cran.r-project.org/package=vegan (Accessed October 18, 2022).

Palma-Hidalgo, J. M., Jiménez, E., Popova, M., Morgavi, D. P., Martín-García, A. I., Yáñez-Ruiz, D. R., et al. (2021). Inoculation with rumen fluid in early life accelerates the rumen microbial development and favours the weaning process in goats. *Animal Microbiome* 3:11. doi: 10.1186/s42523-021-00073-9

Park, T., Cersosimo, L. M., Li, W., Radloff, W., and Zanton, G. I. (2021). Pre-weaning ruminal administration of differentially-enriched, rumen-derived inocula shaped rumen bacterial communities and co-occurrence networks of post-weaned dairy calves. *Front. Microbiol.* 12:625488. doi: 10.3389/fmicb.2021.625488

Perea, K., Perz, K., Olivo, S. K., Williams, A., Lachman, M., Ishaq, S. L., et al. (2017). Feed efficiency phenotypes in lambs involve changes in ruminal, colonic, and small-intestine-located microbiota. *J. Anim. Sci.* 95, 2585–2592. doi: 10.2527/jas2016.1222

Poulsen, M., Schwab, C., Borg Jensen, B., Engberg, R. M., Spang, A., Canibe, N., et al. (2013). Methylotrophic methanogenic thermoplasmata implicated in reduced methane emissions from bovine rumen. *Nat. Commun.* 4:1428. doi: 10.1038/ncomms2432

Quast, C., Pruesse, E., Yilmaz, P., Gerken, J., Schweer, T., Yarza, P., et al. (2013). The SILVA ribosomal RNA gene database project: improved data processing and web-based tools. *Nucleic Acids Res.* 41, D590–D596. doi: 10.1093/nar/gks1219

Raabis, S., Li, W., and Cersosimo, L. (2019). Effects and immune responses of probiotic treatment in ruminants. *Vet. Immunol. Immunopathol.* 208, 58–66. doi: 10.1016/j.vetimm.2018.12.006

Rey, M., Enjalbert, F., Combes, S., Cauquil, L., Bouchez, O., and Monteils, V. (2014). Establishment of ruminal bacterial community in dairy calves from birth to weaning is sequential. *J. Appl. Microbiol.* 116, 245–257. doi: 10.1111/jam.12405

Rius, A. G., Kittelmann, S., Macdonald, K. A., Waghorn, G. C., Janssen, P. H., and Sikkema, E. (2012). Nitrogen metabolism and rumen microbial enumeration in

lactating cows with divergent residual feed intake fed high-digestibility pasture. *J. Dairy Sci.* 95, 5024–5034. doi: 10.3168/jds.2012-5392

Rosa, F., Michelotti, T. C., St-Pierre, B., Trevisi, E., and Osorio, J. S. (2021). Early life fecal microbiota transplantation in neonatal dairy calves promotes growth performance and alleviates inflammation and oxidative stress during weaning. *Animals* 11:2704. doi: 10.3390/ani11092704

Seedorf, H., Kittelmann, S., Henderson, G., and Janssen, P. H. (2014). RIM-DB: A taxonomic framework for community structure analysis of methanogenic archaea fromthe rumen and other intestinal environments. *PeerJ* 2:e494. doi: 10.7717/peerj.494

Silley, P., and Armstrong, D. G. (1985). Metabolism of the rumen bacterium *Selenomonas* ruminantium grown in continuous culture. *Lett. Appl. Microbiol.* 1, 53–55. doi: 10.1111/j.1472-765X.1985.tb01488.x

Sjaunja, L. O., Baevre, L., Junkkarinen, L., Pedersen, J., and Setälä, J. (1990). *A Nordic Proposal for an Energy Corrected Milk (ECM) Formula. In Proc. 27th Session of the International Committee of Recording and Productivity of Milk Animals, Paris, France, July 2-9, 1990*. EAAP Publication no. 50. European Association for Animal Production, Rome, Italy, 156–157.

Söllinger, A., Tveit, A. T., Poulsen, M., Noel, S. J., Bengtsson, M., Bernhardt, J., et al. (2018). Holistic assessment of rumen microbiome dynamics through quantitative Metatranscriptomics reveals multifunctional redundancy during key steps of anaerobic feed degradation. *mSystems* 3:e00038-18. doi: 10.1128/msystems.00038-18

Solomon, R., Wein, T., Levy, B., Eshed, S., Dror, R., Reiss, V., et al. (2022). Protozoa populations are ecosystem engineers that shape prokaryotic community structure and function of the rumen microbial ecosystem. *ISME J.* 16, 1187–1197. doi: 10.1038/s41396-021-01170-y

Sylvester, J. T., Karnati, S. K. R., Yu, Z., Morrison, M., and Firkins, J. L. (2004). Development of an assay to quantify rumen ciliate Protozoal biomass in cows using real-time PCR. *Nut. Methodol.* 134, 3378–3384. doi: 10.1093/jn/134.12.3378

Tapio, I., Fischer, D., Blasco, L., Tapio, M., Wallace, R. J., Bayat, A. R., et al. (2017). Taxon abundance, diversity, co-occurrence and network analysis of the ruminal microbiota in response to dietary changes in dairy cows. *PLoS One* 12:e0180260. doi: 10.1371/journal.pone.0180260

Taxis, T., Wolff, S., Gregg, S., Minton, N. O., Zhang, C., Dai, J., et al. (2015). The players may change but the game remains: network analyses of ruminal microbiomes suggest taxonomic differences mask functional similarity. *Nucleic Acids Res.* 43, 9600–9612. doi: 10.1093/nar/gkv973

Terry, S. A., Badhan, A., Wang, Y., Chaves, A. V., and Mcallister, T. A. (2019). Fibre digestion by rumen microbiota – a review of recent metagenomic and metatranscriptomic studies. *Can. J. Anim. Sci.* 99, 678–692. doi: 10.1139/cjas-2019-0024

Tripathi, V. K., Sehgal, P. J., Puniya, K. A., and Singh, K. (2007). Hydrolytic activities of anaerobic fungi from wild blue bull (*Boselaphus tragocamelus*). *Vet. Anaerobes Dis.* 13, 36–39. doi: 10.1016/j.anaerobe.2006.11.001

Turnbaugh, P. J., Hamady, M., Yatsunenko, T., Cantarel, B. L., Duncan, A., Ley, R. E., et al. (2009). A core gut microbiome in obese and lean twins. *Nature* 457, 480–484. doi: 10.1038/nature07540

Van Gylswyk, N. O. (1995). Succiniclasticum ruminis gen. Nov., sp. nov., a ruminal bacterium converting succinate to propionate as the sole energy-yielding mechanism. *Int. J. Syst. Bacteriol.* 45, 297–300. doi: 10.1099/00207713-45-2-297

Van Gylswyk, N. O., Hippe, H., and Rainey, F. A. (1996). *Pseudobutyrivibrio ruminis* gen. Nov., sp. nov., a butyrate-producing bacterium from the rumen that closely resembles *Butyrivibrio* fibrisolvens in phenotype. *Int. J. Syst. Evol. Microbiol.* 46, 559–563. doi: 10.1099/00207713-46-2-559

Wallace, J. R., Sasson, G., Garnsworthy, P. C., Tapio, I., Gregson, E., Bani, P., et al. (2019). A heritable subset of the core rumen microbiome dictates dairy cow productivity and emissions. Science. *Advances* 5:eaav8391. doi: 10.1126/sciadv.aav8391

Weimer, P. J. (2015). Redundancy, resilience, and host specificity of the ruminal microbiota: implications for engineering improved ruminal fermentations. *Front. Microbiol.* 6:296. doi: 10.3389/fmicb.2015.00296

Weimer, P. J., Cox, M. S., Vieira de Paula, T., Lin, M., Hall, M. B., and Suen, G. (2017). Transient changes in milk production efficiency and bacterial community composition resulting from near-total exchange of ruminal contents between high- and low-efficiency holstein cows. *J. Dairy Sci.* 100, 7165–7182. doi: 10.3168/jds.2017-12746

Wood, S. N. (2003). Thin plate regression splines. Journal of Royal Statistical Society. *Series B* 65, 95–114. doi: 10.1111/1467-9868.00374

Wood, S. N. (2017). *Generalized additive models: An introduction with R. 2nd Edn.* Boca Raton: Chapman and Hall/CRC.

Worakit, S. (1986). Methanobacterium alcaliphilum. *Int. J. Syst. Bacteriol.* 36, 380–382.

Yáñez-Ruiz, D. R., Abecia, L., and Newbold, C. J. (2015). Manipulating rumen microbiome and fermentation through interventions during early life: a review. *Front. Microbiol.* 6:1133. doi: 10.3389/fmicb.2015.01133

Yu, S., Zhang, G., Liu, Z., Wu, P., Yu, Z., and Wang, J. (2020). Repeated inoculation with fresh rumen fluid before or during weaning modulates the microbiota composition and co-occurrence of the rumen and colon of lambs. *BMC Microbiol.* 20:29. doi: 10.1186/s12866-020-1716-z

Zhang, R., Liu, J., Jiang, L., and Mao, S. (2020). Effect of high-concentrate diets on microbial composition, function and the VFAs formation process in the rumen of dairy cows. *Anim. Feed Sci. Technol.* 269:114619. doi: 10.1016/j.anifeedsci.2020.114619

Zhong, Y., Xue, M., and Liu, J. (2018). Composition of rumen bacterial community in dairy cows with different levels of somatic cell counts. *Front. Microbiol.* 9:3217. doi: 10.3389/fmicb.2018.03217

Zhong, Y., Xue, M. Y., Sun, H. Z., Valencak, T. G., Guan, L. L., and Liu, J. (2020). Rumen and hindgut bacteria are potential indicators for mastitis of mid-lactating Holstein dairy cows. *Microorganisms* 8, 1–13. doi: 10.3390/microorganisms8122042

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# A preliminary study on the possibility of fermented pineapple peel residue partially replacing whole corn silage in feeding Chuanzhong black goats

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This study aims to assess the effects of the partial replacement of whole corn silage (WCS) with fermented pineapple peel residue (FPPR) on growth, serological parameters, muscle quality, rumen microorganisms, and fecal microorganisms. A total of 24 Chuanzhong black goats weighing 10.23 ± 1.42 kg were evaluated in a randomized complete trial design in accordance with the following treatments: (1) 0% FPPR in the diet, (2) 25% FPPR in the diet, and (3) 50% FPPR in the diet. In goats, the partial substitution of FPPR for WCS increased the abundance of probiotics, such as *Blautia*, *Butyrivibrio fibrisolvens*, and *Ruminococcus albus*, and did not exert significant effects on overall serological parameters and muscle quality. In conclusion, the partial substitution of FPPR for WCS in the diet did not impair or affect the productive performance of goats.

KEYWORDS

**agricultural by-products, fermented feeds, Chuanzhong black goats, pineapple peel, rumen microfora, fecal microfora, meat quality**

## Introduction

Roughages are essential for ruminant production because they stimulate the chewing response and enhance saliva output to stabilize the rumen microbial environment (Tafaj et al., 2006). In Chinese rangelands, corn silage is the most common source of roughage for ruminants; however, continuous maize monoculture can negatively affect nutrient extraction from the soil and increase the chance of erosion (Silva et al., 2021). In addition, there is an urgent need to develop new sources of roughage for the progression of ruminant farming in many areas where maize cultivation is unsuitable due to climatic, geographical, or economic conditions.

Pineapple, which is widely distributed in the tropics, is well received by consumers for its unique fragrance and rich nutrition. Moreover, it promotes the body's health (Brito et al., 2020; Padrón-Mederos et al., 2020). In addition to being consumed fresh, pineapple is often used to produce fruit juices, jams, dried fruits, and syrups (Sanya et al., 2020). However, the edible part of pineapple only accounts for 30–45% of its total weight, and the rest parts are often discarded or buried in landfills, causing great environmental pollution and resource waste (Gasmi Benahmed et al., 2021). Pineapple peel residue (PPR) is a by-product of pineapple processing that is rich in nutrients and bioactive substances (Roda and Lambri, 2019). This waste is mainly composed of dietary fibers, flavonoids, minerals, sugars, polyphenols, and vitamins and plays an important role in antibiosis, antioxidation, and gastrointestinal protection (Hu et al., 2019). Therefore, it can be developed as a feed material. Studies on the use of pineapple residue as feed have been reported. These works demonstrated that in animals, the favorable palatability of pineapple residue can improve feed intake (Shivakumar Gowda et al., 2015; Kiggundu and Kabi, 2019; Liu et al., 2021) and that PPR has no adverse effects on health and metabolism (Shivakumar Gowda et al., 2015; Kiggundu and Kabi, 2019).

Rumen microorganisms can transform fibrous materials into energy needed for daily activities of ruminants and execute important roles in regulating host physiological functions, metabolic reactions, and resistance to pathogens (Jami et al., 2013; Wang et al., 2013). Bacteria dominate rumen microorganisms and play a crucial role in the digestion and conversion of feed into short-chain fatty acids and mycoproteins (Brulc et al., 2009). Diet composition is an important factor affecting rumen microorganisms. A previous study found that in lambs, substituting a certain proportion of buckwheat straw for corn straw in the feed reduced the diversity of rumen microflora. Another study (Wang et al., 2021) reported that grapeseed decreased *Firmicutes* and increased *Bacteroidetes* and *Prevotella_1* in the rumen microflora of lambs. Nevertheless, studies reporting the effects of PPR on the rumen microflora of ruminants are limited.

Intestinal microorganisms also perform an important role in host health, and the disorder of microbial composition may lead to inflammation and diseases (Lee et al., 2020). Intestinal flora is affected by numerous factors, such as physiological status, nutrient composition, environmental changes, drug treatments, pathogens, and stress (Kunz et al., 2019). In animal husbandry, numerous works have demonstrated that different feed ingredients cause changes in the composition of fecal microbiota (Sun et al., 2017; Xie et al., 2020). In our previous study, we found that replacing whole corn silage (WCS) with a certain proportion of *Broussonetia papyrifera* silage could affect the composition and function of fecal microbiota in Holstein heifers (Tian et al., 2020). However, related studies on the effects of fermented PPR (FPPR) as a new feed material on the intestinal microflora in ruminants are still limited.

Although FPPR has the potential to replace corn silage as a new source of roughage due to its nutritional richness, there is a lack of the literature assessing its feasibility in ruminant feeding. This study aims to use FPPR as a partial replacement for maize silage as a source of roughage for Chuanzhong black goats and to evaluate growth performance, serological parameters, muscle quality, rumen microbiology, and fecal microbiology for complementing the theoretical basis to determine the feasibility of FPPR as a source of roughage for ruminants.

## Materials and methods

All experimental procedures used in this study were approved by the Committee of Animal Experiments of South China Agricultural University (No. 2021G018).

### Experimental materials

Pineapple peel residue was purchased from a technology company in Leizhou city. After being crushed and pressed, the PPR measurement was approximately 2–3 cm. Then, it was evenly sprayed with a commercial starter (moisture $\leq$ 12%; *Bacillus subtilis* $\geq 2 \times 10^9$ cfu/g; *Peeltococcus acitilactict* $\geq 2 \times 10^9$ cfu/g; and *Candida utilis* $\geq 4 \times 10^9$ cfu/g) solution. Approximately 40 kg of PPR was sealed into a polyethylene fermentation bag, and after 20 days of fermentation, FPPR was obtained.

### Determination of nutrients in diets

The nutrient composition of the feed used in the experiment was determined as follows: dry matter, crude protein, ether extract, and ash were measured based on the method described by the Association of Official Analytical Chemists (2002). Neutral detergent fiber and acid detergent fiber were determined in accordance with a previous study (Van Soest et al., 1991). Calcium and phosphorus were tested *via* the ethylenediaminetetraacetic acid (EDTA) complexometric titration method and vanadium molybdate yellow colorimetric method, respectively.

### Experimental animals

The trial was conducted at a commercial farm in Zhaoqing (Guangdong province, China). It included a preliminary feeding period of 7 days and a formal experimental period of 35 days.

TABLE 1 The ingredients and chemical composition of the diets.

| Item[a] | Diets treatments[b] | | |
|---|---|---|---|
| | CON | B25 | B50 |
| Ingredient (as fed-basis %) | | | |
| WCS | 76.20 | 57.20 | 38.10 |
| FPPR | 0.00 | 19.00 | 38.10 |
| Peanut seedling hay | 19.00 | 19.00 | 19.00 |
| Indian meal | 2.93 | 2.93 | 2.93 |
| Soybean meal | 1.20 | 1.20 | 1.20 |
| Alfalfa hay | 0.47 | 0.47 | 0.47 |
| Calcium hydrophosphate | 0.05 | 0.05 | 0.05 |
| Mountain flour | 0.10 | 0.10 | 0.10 |
| Salt | 0.05 | 0.05 | 0.05 |
| Nutrient content | | | |
| DM (%) | 30.84 | 30.39 | 30.34 |
| CP (%/DM) | 11.68 | 11.99 | 11.45 |
| EE (%/DM) | 0.01 | 0.02 | 0.02 |
| Ash (%/DM) | 0.13 | 0.12 | 0.12 |
| NDF (%/DM) | 58.11 | 52.98 | 54.29 |
| ADF (%/DM) | 36.20 | 33.35 | 33.12 |
| Ca (%/DM) | 0.90 | 0.97 | 0.97 |
| P (%/DM) | 0.44 | 0.42 | 0.42 |
| NEm (mcal/kg) | 1.18 | 1.25 | 1.29 |
| NEg (macl/kg) | 0.62 | 0.69 | 0.72 |

[a]WCS, whole corn silage; FPPR, fermented pineapple peel residue; DM, dry matter; CP, crude protein; EE, ether extract; NDF, neutral detergent fiber; ADF, acid detergent fiber; NEm, net energy for maintenance; NEg, net energy for gain. [b]CON, FPPR replaces 0% of WCS; B25, FPPR replaces 25% of WCS; B50, FPPR replaces 50% of WCS.

A total of 24 healthy 4-month-old female Chuanzhong black goats (10.23 ± 1.42 kg) with similar genetic backgrounds were evenly and randomly assigned into three groups. In all three groups, the substitution amounts of FPPR for WCS in the diet were 0% (CON), 25% (B25), and 50% (B50). The diets were mixed in accordance with the National Research Council (2016), and their nutrient compositions were analyzed according to Xie et al. (2020). The ingredients and nutrient composition of these diets are shown in Table 1. Feeding was performed at 09:00 and 17:00 every day, and the goats were allowed to eat and drink freely during the experiment. During the experimental period, feed intake and average daily gain (ADG) were measured.

## Collection of samples

Serum sample collection was carried out on days 17 and 35 of the formal experimental period. Goats were fasted for 12 h before blood sampling, and blood was collected from the jugular vein. After centrifugation at 4,000 r/min for 15 min, the upper serum was removed and stored at −20°C.

Fecal samples were collected from the rectum on the last day of the experimental period. Approximately 2 g of the sample was stored in a 2-ml frozen pipe at −80°C for the determination of bacterial communities.

A total of 16 goats from CON and B50, which showed a strong effect of FPPR on ADG and feed intake, were selected and mercy killed on the last day of the experimental period. The rumen fluid from each goat was filtered through the four layers of sterile gauze, and 150 ml was collected. Filtered rumen fluid was poured into a 2-ml frozen pipe and 50-ml centrifuge tube. The samples in the frozen pipes were stored at −80°C for the analysis of bacterial community. The pH value of the rumen fluid in the centrifuge tube was determined using a pH meter (FE28-Standard, METTLER-TOLEDO). Then, the rumen fluid was centrifuged at 5,000 r/min for 15 min. The supernatant obtained through centrifugation was removed and stored at −20°C for the determination of fermentation parameters.

The longissimus dorsi muscles of the killed goats were removed and divided into two parts along their sections. One was used for the determination of pH, meat color (brightness, L*; redness, a*; and yellowness, b*), drip loss, shear force, and water-holding capacity (WHC) through the determination methods reported by Abdel-Wareth et al. (2019) and Wassie et al. (2020). Another part was used for the determination of amino acid content in muscles with an L-8900 amino acid analyzer (Hitachi, Japan) in accordance with the method of Fu et al. (2016).

## Determination of serum indicators

Serum biochemical indicators, including albumin, total protein, globulin, uric acid, blood urea (UREA), nonesterified fatty acids (NEFA), creatine kinase, and blood glucose, were determined using a HITACHI Automatic Analyzer 7600 (Hitachi, Ltd., Tokyo, Japan). Interferon-γ, C-reactive protein, β-hydroxybutyric acid, heat shock protein-70, triiodothyronine, and immunoglobulin family were measured *via* the ELISA method. Catalase, glutathione peroxidase, and total antioxidant capacity were measured using commercial colorimetric ABTS kits (Nanjing Jiancheng Institute of Bioengineering, Nanjing).

## Analysis of rumen and fecal bacterial communities

The cetyltrimethyl ammonium bromide method was adopted to extract genomic deoxyribonucleic acid (DNA) from the samples. The genomic DNA sample were tested for purity, concentration, and integrity. The 16S rDNA V1–V9 regions were amplified with a tansStart® FastPfu DNA polymerase kit. The forward and reverse primers were designed as 27F (GAGAGTTTGATCCTGGCTCAG) and 1541R

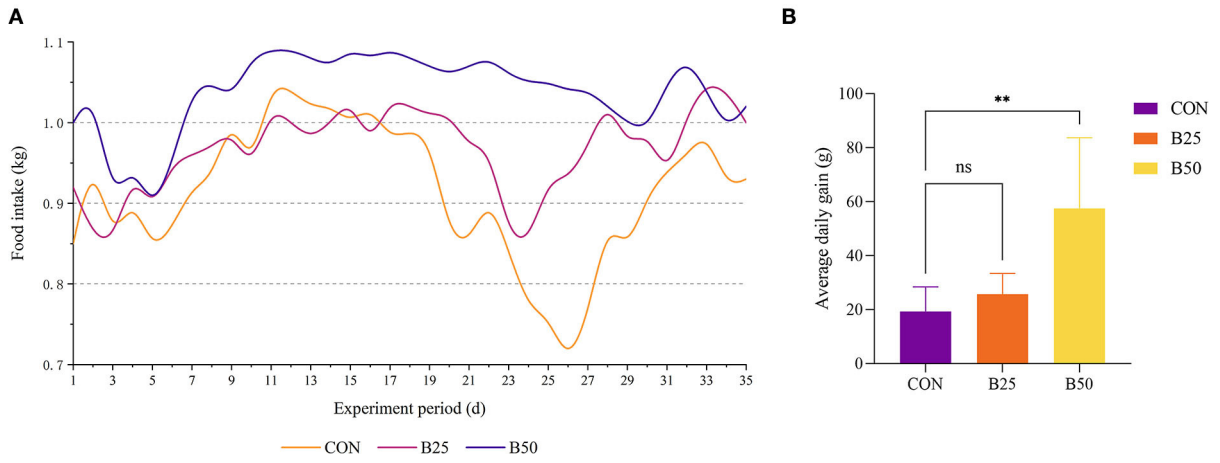


FIGURE 1
Effect of fermented pineapple peel residue (FPPR) on the growth performance of black goats. **(A)** Feed intake and **(B)** average daily gain (ADG). $^{**}p < 0.01$; ns, No Significant.

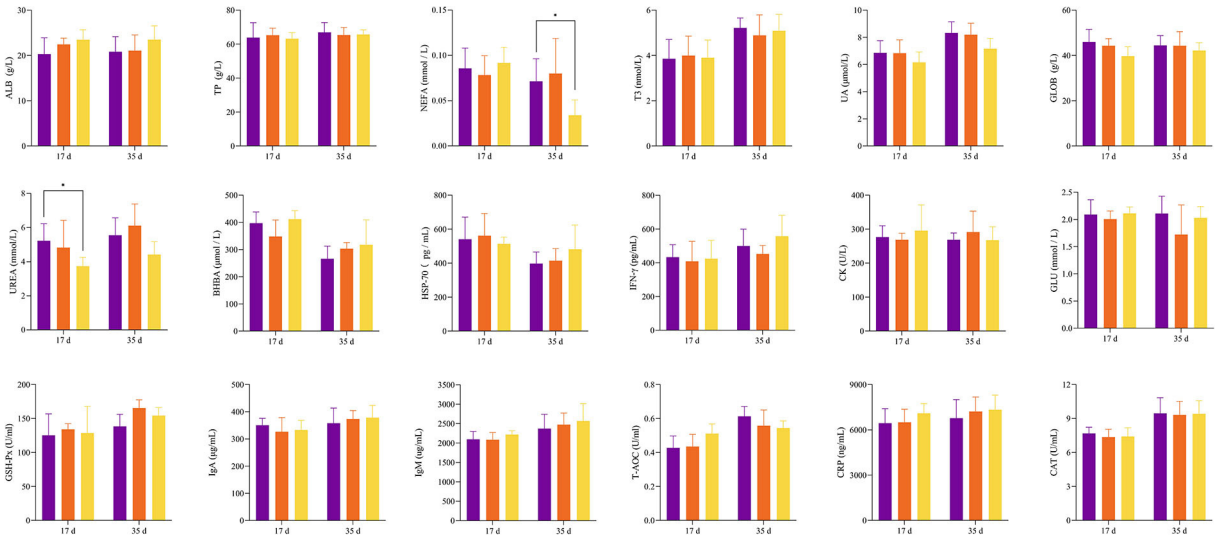


FIGURE 2
Effect of FPPR on the serum indicators of black goats.

(AAGGAGGTGATCCAGCCGCA), respectively (Jin et al., 2018). Reaction systems were established as follows: Trans Fastpfu, 1 μl; 5× buffer, 10 μl; 5× Stimulate, 5 μl; dNTP, 5 μl; forward primer, 1 μl; reverse primer, 1 μl; gDNA 5 μl; and nuclease-free water, 23 μl. The reaction conditions were set as follows: initial denaturation, 95°C for 2 min; denaturation (95°C, 30 s), annealing (60°C, 45 s), and extension (72°C, 90 s) for 35 cycles; and final extension, 72°C for 10 min. After amplification, the quality and integrity of the PCR products were determined by electrophoresis with 2% agarose gel. Then, the products were purified using a QIAquick Gel Extraction kit (QIAGEN, Inc., Valencia, CA, USA). The sequencing library was generated with a SMRTbellTM template preparation kit (Pacific Biosciences, Inc., Menlo Park, CA, USA) (Song et al., 2019). Library quality was evaluated by using a Qubit@ 2.0 fluorimeter and FEMTO Pulse system. Finally, the PacBio Sequel platform (Pacific Biosciences, Inc., Menlo Park, CA, USA) was selected for library sequencing, and the data were subjected to bioinformatics analyses based on the methods described by Yang et al. (2020). α-diversity, including Ace, Chao1, Shannon, and Simpson indexes, was calculated using Qiime software, and β-diversity was analyzed with the "vegan" package in R software (De Filippis et al., 2018). Functional prediction of the microflora was performed using Tax4Fun (Sharma et al., 2020). Linear

TABLE 2 Effect of the partial substitution of fermented pineapple peel residue (FPPR) for WSC on the meat quality of longissimus dorsi in Chuangzhong black goats.

| Items[a] | | Treatments | | *P*-value |
|---|---|---|---|---|
| | | CON | FPPR | |
| pH | 45 min | 6.05 ± 0.10 | 5.62 ± 0.12 | 0.002 |
| | 24 h | 5.80 ± 0.09 | 5.68 ± 0.07 | 0.096 |
| | 48 h | 6.03 ± 0.05 | 5.81 ± 0.06 | 0.002 |
| L* | 45 min | 55.14 ± 2.82 | 54.63 ± 2.66 | 0.796 |
| | 24 h | 53.60 ± 3.78 | 52.00 ± 2.18 | 0.483 |
| | 48 h | 54.25 ± 1.81 | 54.40 ± 1.24 | 0.889 |
| a* | 45 min | 15.45 ± 1.17 | 14.22 ± 0.81 | 0.123 |
| | 24 h | 15.99 ± 2.00 | 14.18 ± 0.99 | 0.143 |
| | 48 h | 16.15 ± 1.14 | 15.01 ± 1.86 | 0.325 |
| b* | 45 min | 8.56 ± 0.85 | 8.10 ± 0.77 | 0.438 |
| | 24 h | 10.05 ± 0.89 | 9.02 ± 0.59 | 0.145 |
| | 48 h | 9.97 ± 0.89 | 9.15 ± 1.70 | 0.858 |
| DL (%) | | 21.12 ± 5.23 | 17.48 ± 4.55 | 0.365 |
| SF (N) | | 51.36 ± 4.51 | 50.02 ± 1.19 | 0.581 |
| WHC (%) | | 94.26 ± 1.42 | 92.63 ± 1.71 | 0.182 |

[a]DL, drip loss; SF, shear force; WHC, water-holding capacity.

TABLE 3 Effect of the partial substitution of FPPR for WSC on the amino acid composition of longissimus dorsi in Chuangzhong black goats (g/100 g protein).

| Items[a] | Treatments | | *P*-value |
|---|---|---|---|
| | CON | FPPR | |
| Alanine[$] | 4.35 ± 0.12 | 4.37 ± 0.19 | 0.844 |
| Arginine[#] | 5.04 ± 0.16 | 4.94 ± 0.17 | 0.468 |
| Aspartic acid[$] | 6.85 ± 0.36 | 6.63 ± 0.25 | 0.372 |
| Cysteine | 0.78 ± 0.16 | 0.64 ± 0.09 | 0.185 |
| Glutamic acid[$] | 11.65 ± 0.66 | 11.04 ± 0.61 | 0.241 |
| Glycine[$] | 3.47 ± 0.11 | 4.24 ± 0.83 | 0.149 |
| Histidine[#] | 1.92 ± 0.40 | 2.40 ± 0.25 | 0.091 |
| Isoleucine[#] | 3.54 ± 0.18 | 3.36 ± 0.19 | 0.235 |
| Leucine[#] | 6.36 ± 0.23 | 5.95 ± 0.25 | 0.061 |
| Lysine[#] | 6.95 ± 0.27 | 6.48 ± 0.29 | 0.064 |
| Methionine[#] | 1.99 ± 0.16 | 1.91 ± 0.10 | 0.465 |
| Phenylalanine[#,$] | 3.20 ± 0.18 | 2.95 ± 0.07 | 0.041 |
| Proline | 3.08 ± 0.08 | 3.34 ± 0.43 | 0.335 |
| Serine | 2.88 ± 0.12 | 2.79 ± 0.11 | 0.322 |
| Threonine[#] | 3.50 ± 0.16 | 3.33 ± 0.14 | 0.170 |
| Tyrosine[$] | 2.70 ± 0.28 | 2.45 ± 0.11 | 0.149 |
| Valine[#] | 3.78 ± 0.17 | 3.61 ± 0.14 | 0.198 |
| EAA | 36.28 ± 1.77 | 34.94 ± 1.38 | 0.297 |
| DAA | 32.23 ± 1.20 | 31.69 ± 0.99 | 0.536 |
| TAA | 72.04 ± 2.90 | 70.44 ± 1.90 | 0.408 |

[a]EAA, essential amino acids, including Arginine, Histidine, Isoleucine, Leucine, Lysine, Methionine, Phenylalanine, Threonine, and Valine, marked with "#" as a superscript in the table; DAA, delicious amino acids, including Alanine, Aspartic acid, Glutamic acid, Glycine, Phenylalanine, and Tyrosine, marked with "$" as a superscript in the table; TAA, total amino acids. Asparagine, Glutamine, and Tryptophane were undetected in this study.

discriminant analysis (LDA) effect size (LEfSe) and functional prediction of the microflora were carried out using an online tool (http://huttenhower.sph.harvard.edu/galaxy/), and $p < 0.05$ and LDA score >2 were selected as cutoffs (Cadena et al., 2019).

## Statistical analysis

Test data were preliminarily sorted and analyzed using Excel software (Microsoft, Redmond, WA, USA) and then analyzed with SAS 9.4 software (SAS Institute, Inc., Cary, NC, USA). The normality of the data was tested using the UNIVARIATE procedure. Outliers were processed based on the Studentized absolute residual values > 2.5.

The MIXED procedure model used for the data from meat quality processing is $Y_{ij} = \mu + B_i + T_j + \varepsilon_i$, where $Y_{ij}$ is the value of the dependent variable of the test goats under different treatments, $\mu$ is the overall mean, $B_i$ are the dietary treatment effects, $T_j$ are the time treatment effects, and $\varepsilon_{ij}$ is the random error.

The generalized linear model (GLM) procedure model used for data other than meat quality processing data is $Y_i = \mu + B_i + \varepsilon_i$, where $Y_i$ is the value of the dependent variable of the test goats under different treatments, $\mu$ is the overall mean, $B_i$ are the dietary treatment effects, and $\varepsilon_i$ is the random error.

Unless otherwise noted, test data are shown in figures and tables as mean and standard deviation (SD), and $p < 0.05$ indicates a significant difference.

## Results

### Performance and serum indicators

Throughout the experiment, the feed intake of the CON group was always lower than that of the B50 group (Figure 1A) and the ADG of B50 was significantly higher than that of CON ($p < 0.05$). However, the B25 group did not significantly differ from the CON group ($p > 0.05$) (Figure 1B). Figure 2 shows that on day 17, the UREA in B50 was significantly lower than that in CON ($p < 0.05$). On day 35, NEFA in B50 was significantly lower than that in CON ($p < 0.05$). The contents of other serum indicators did not differ significantly among all groups.

### Meat quality

The results of meat quality and amino acid composition of longissimus dorsi in goats are shown in Tables 2, 3.

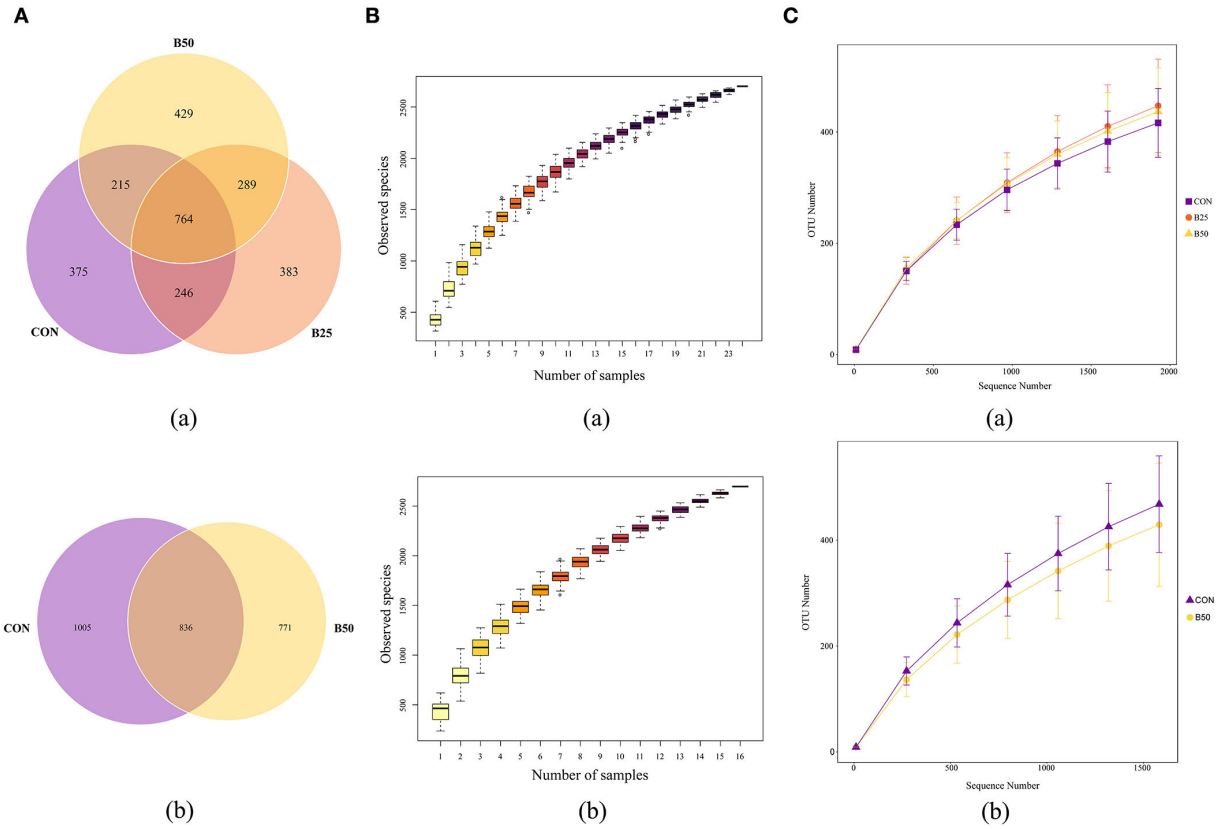


FIGURE 3
Overall microbial species composition **(A)** a Venn diagram of operational taxonomic units (OTUs); **(B)** species accumulation boxplot; and **(C)** rarefaction curve.

The physical properties presented in Table 2 indicated that FPPR decreased the pH value of longissimus dorsi at 45 min and 48 h ($p < 0.05$). The results in Table 3 demonstrated that FPPR reduced the content of phenylalanine ($p < 0.05$), whereas the contents of total amino acids (TAA), essential amino acids (EAA), and delicious amino acids (DAA) did not change significantly ($p > 0.05$).

## The diversity of fecal and rumen fluid microflora

The 24 fecal samples were sequenced using the PacBio platform, and 278,008 raw read sequences were obtained. After removing primers and mosaics, 193,765 optimized sequences were retained. The optimized sequences were 1,432–1,441 bp in length and had an average length of 1,438 bp. The 16 samples of rumen fluid from CON and B50 were also sequenced using the PacBio platform, and 233,489 raw reads and 170,176 clean reads were obtained. The average lengths of these valid sequences were 1,429–1,477 nt. Clean reads from all samples were clustered to study the diversity of the species composition of the samples, and their sequences were grouped into operational taxonomic units (OTUs) with 97% identity. Then, species annotation was performed on the representative sequences of OTUs. A total of 2,701 and 2,612 effective OTUs were obtained from fecal and rumen fluid samples, respectively. A Venn diagram was used to illustrate the composition of unique and shared fecal and rumen fluid species. The Venn diagram of the fecal samples (Figure 3Aa) showed that there were 764 OTUs under all the three treatments; 375, 383, and 429 OTUs were exclusive to CON, B25, and B50, respectively. In the rumen fluid, 836 OTUs were present in both treatments (Figure 3Ab), and 1,005 and 771 OTUs were exclusively present in the CON and B50 treatments, respectively. The observed species curve (Figure 3B) and species accumulation boxplot (Figure 3C) show that with increasing sequence numbers, the curves tended to become level gradually, indicating that sampling was sufficient for data analysis.

The results of the α-diversity of fecal and rumen fluid microflora (Figures 4A,B, a,c) were obtained. In terms of

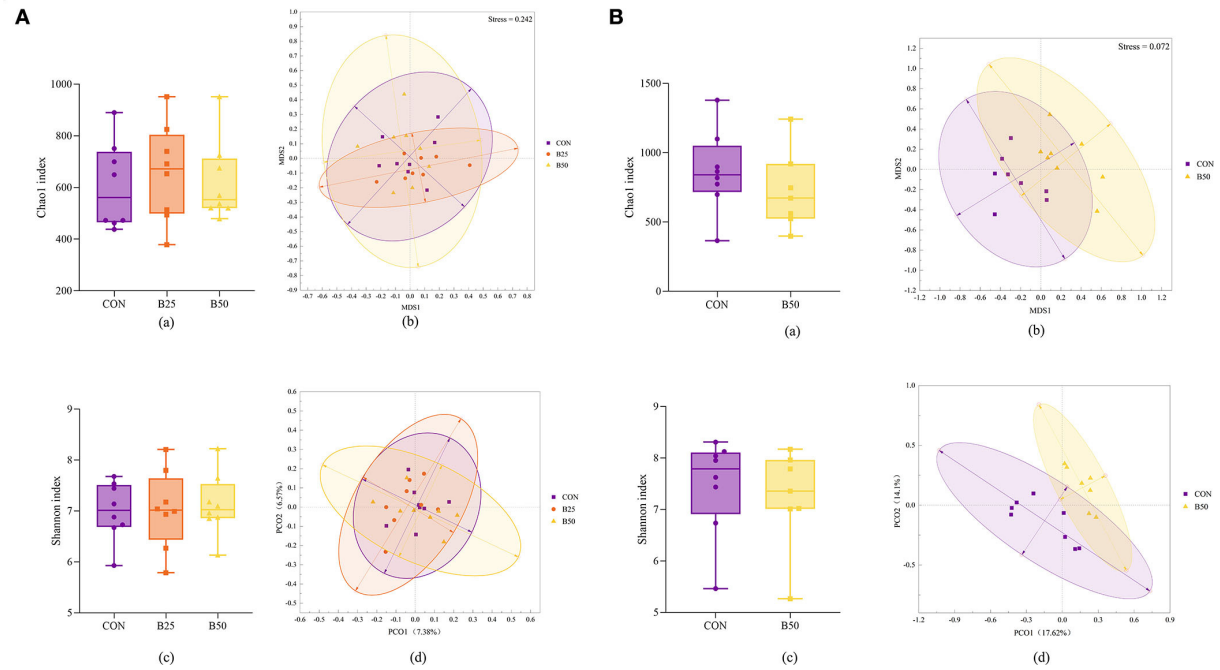


FIGURE 4
α- and β-diversity of fecal and rumen fluid microflora affected by FPPR. **(A)** α- and β-diversity for fecal microflora: (a) Chao1 index; (b) non-metric multidimensional scaling (NMDS) plot; (c) Shannon index; (d) principal co-ordinates analysis (PCoA) plot; **(B)** α- and β-diversity for rumen fluid microflora: (a) Chao1 index; (b) NMDS plot; (c) Shannon index; (d) PCoA plot.

α-diversity, both fecal and rumen fluid microbiota were not affected significantly by FPPR ($p > 0.05$). Separation among the CON, B25, and B50 fecal samples was not noticeable (Figure 4Ab,d). However, the result of the analysis of similarities (ANOSIM) (Table 4) revealed that the *R*-values of CON/B25, CON/B50, and B25/B50 were all greater than 0, indicating that the differences between the groups were significantly greater than those within the groups. Furthermore, in this study, the *p*-values for CON/B50 and B25/B50 were less than 0.05, demonstrating that there were significant differences between the two treatments. The results of principal co-ordinates analysis (PCoA) and nonmetric multidimensional scaling (NMDS) for the β-diversity of rumen fluid (Figure 4Bb,d) revealed a noticeable separation between CON and B50. The ANOSIM results (Table 4) unanimously showed that the *R*-value of CON/B50 was greater than 0 and the *p*-value of CON/B50 was less than 0.05. These results indicated that FPPR significantly affected the β-diversity of rumen fluid microbiota.

## The composition of fecal and rumen fluid microbiota

The effects of FPPR on fecal microflora are shown in Figure 5A. *Firmicutes, Bacteroidetes, Spirochaetes, unidentified bacteria, Tenericutes, Proteobacteria, Melainabacteria, Deferribacteres, Verrucomicrobia, and Lentisphaerae* were the most abundant bacteria at the phylum level. *Lysinibacillus*, *Anaerovibrio*, *Bacteroides*, *Campylobacter*, *Roseburia*, *unidentified Ruminococcaceae*, *Alistipes*, *Phascolarctobacterium*, *Anaerosporobacter*, and *Mucispirillum* were the dominant bacteria at the genus level. Among the dominant phyla, significant differences were found in Firmicutes and Bacteroidetes. In addition, among the dominant genera, *Lysinibacillus* had significantly greater abundance in B25 than in CON and B50. LEfSe analysis is an analytical tool for discovering and interpreting high-dimensional biomarkers in microbial research. It emphasizes statistical significance and biological correlation and can be used to find biomarkers with statistical differences among groups. Two differential OTUs were identified in fecal samples under the three treatments (Figure 5B), including one genus and one class that were each distributed in CON and B50. B25 had no biomarker. The LDA score plot showed that Alphaproteobacteria was upregulated and obviously enriched in CON, whereas *Blautia* was downregulated and obviously enriched in B50.

The composition of rumen fluid in CON and B50 are shown in Figure 6A. At the phylum level, the dominant bacteria were *Firmicutes, Tenericutes, Bacteroidetes, unidentified_bacteria, Synergistetes, Lentisphaerae, Melainabacteria, Proteobacteria, Kiritimatiellaeota, and Planctomycetes*. Compared to CON,

TABLE 4 Analysis of similarities (ANOSIM) of the composition and predicted function in fecal and rumen fluid microflora.

| Item | | Microflora | | Predicted function | |
|---|---|---|---|---|---|
| | | *R*-value | *P*-value | *R*-value | *P*-value |
| Feces | CON-B25 | 0.0993 | 0.055 | 0.0614 | 0.136 |
| | CON-B50 | 0.1588 | 0.047 | 0.0915 | 0.106 |
| | B25-B50 | 0.1828 | 0.012 | −0.0530 | 0.739 |
| Rumen Fluid | CON-B50 | 0.4446 | 0.001 | 0.0357 | 0.265 |

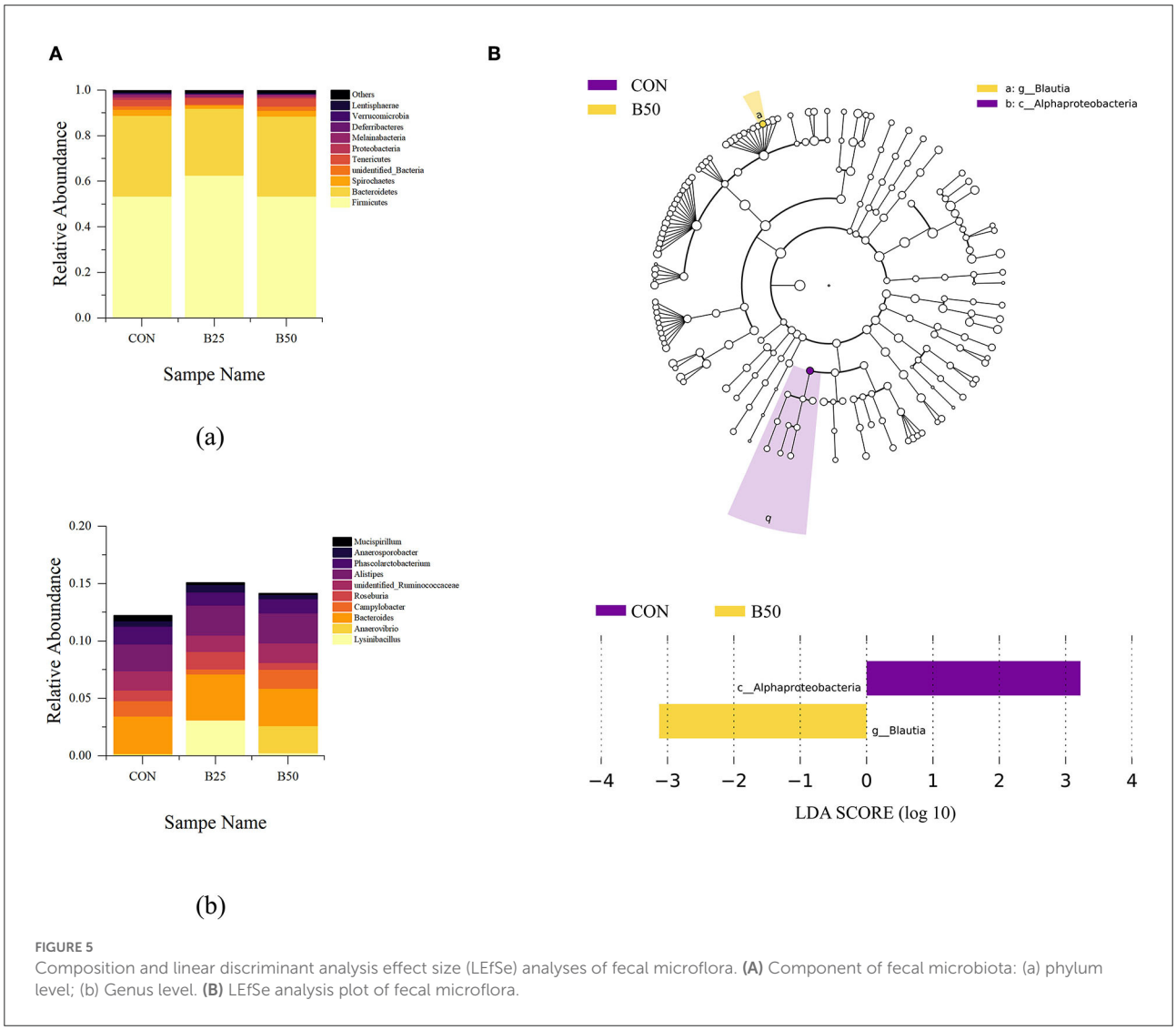


FIGURE 5
Composition and linear discriminant analysis effect size (LEfSe) analyses of fecal microflora. **(A)** Component of fecal microbiota: (a) phylum level; (b) Genus level. **(B)** LEfSe analysis plot of fecal microflora.

the relative abundance of Bacteroidetes had decreased in B50 ($p < 0.05$). At the genus level, the dominant bacteria were *Candidatus_Saccharimonas*, *Quinella*, *Saccharofermentans*, *Fretibacterium*, *unidentified_Ruminococcaceae*, *unidentified_Bacteroidales*, *unidentified_Lachnospiraceae*, *Anaeroplasma*, *Anaerovorax*, and *Succiniclasticum*. The microflora compositions of CON and B50 did not significantly differ. In addition, LEfSe analyses found 26 differential OTUs between CON and B50 (Figure 6B). Among these OTUs, six were upregulated and 30 were downregulated in B50.

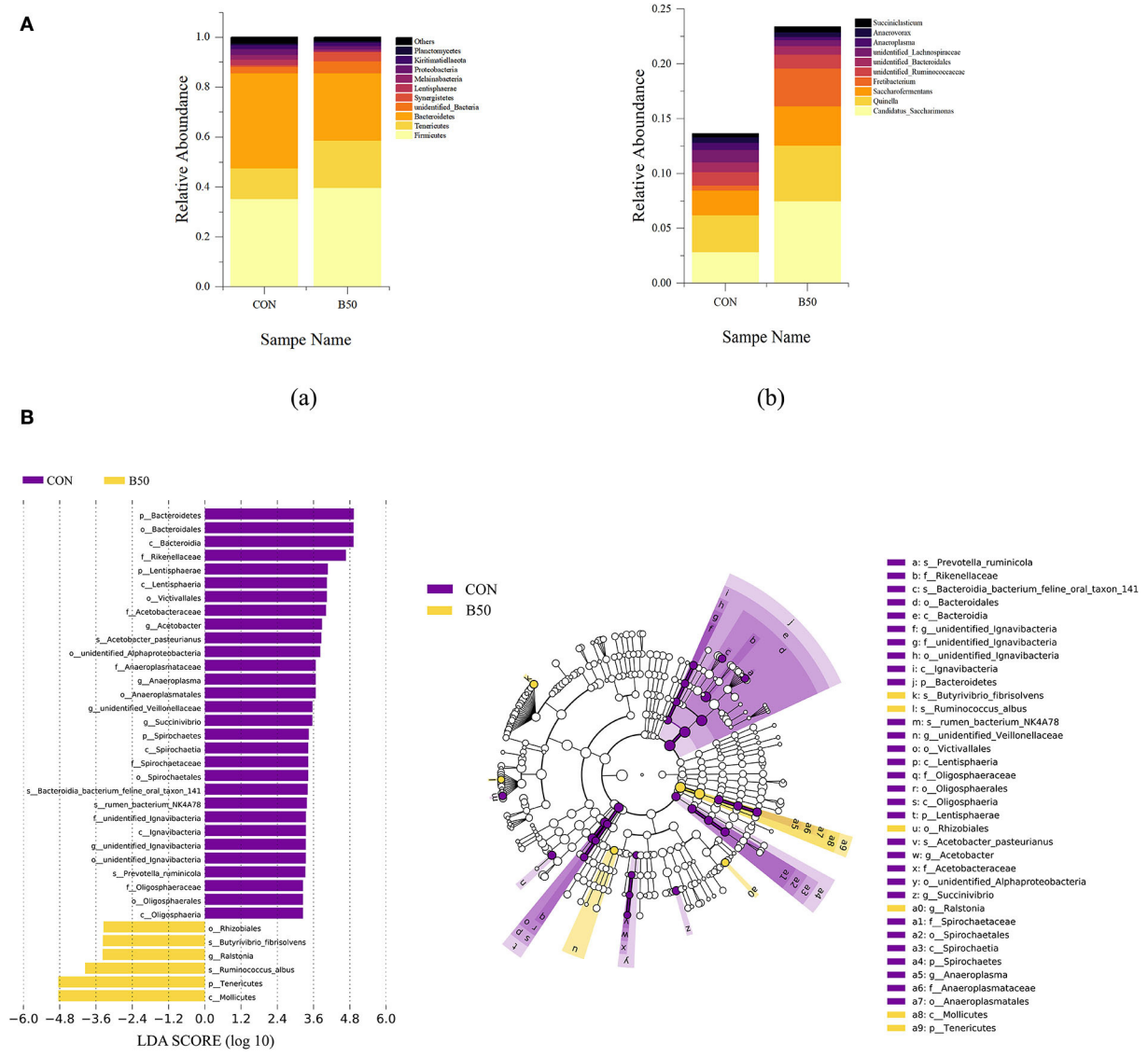


FIGURE 6
Composition and LEfSe analyses of rumen fluid microflora. **(A)** Component of rumen fluid microbiota: (a) phylum level; (b) genus level. **(B)** LEfSe analysis plot of rumen fluid microflora.

## Predicted function of fecal and rumen fluid microflora

In this study, 6,043 Kyoto Encyclopedia of Genes and Genomes (KEGG) orthologs in fecal microflora were predicted by Tax4Fun. In the first level, these KEGG orthologs were related to organismal systems, human diseases, cellular process, environmental information processing, genetic information processing, and metabolism (Figure 7Aa). In the second level, fecal microflora were connected with the metabolism of cofactors and vitamins, signal transduction, glycan biosynthesis and metabolism, carbohydrate metabolism, and replication and repair (Figure 7Ab). Figure 7Ac shows that at the third level, the dominant pathways were transporters, DNA repair and recombination proteins, a two-component system, transfer ribonucleic acid (RNA) biogenesis, and purine metabolism. Figure 7Ad illustrates that the top 5 KEGG orthologs were K03406, K06147, K02014, K02004, and K07497. The β-diversity of the predicted functions in feces was determined after pathway annotation (Figure 7B). The PCoA and NMDS score plots revealed no obvious separations among the three treatments. ANOSIM analyses were also performed and, as shown in Table 4, no significant difference was found among the three treatments ($p > 0.05$). The results indicated that FPPR had no effect on the

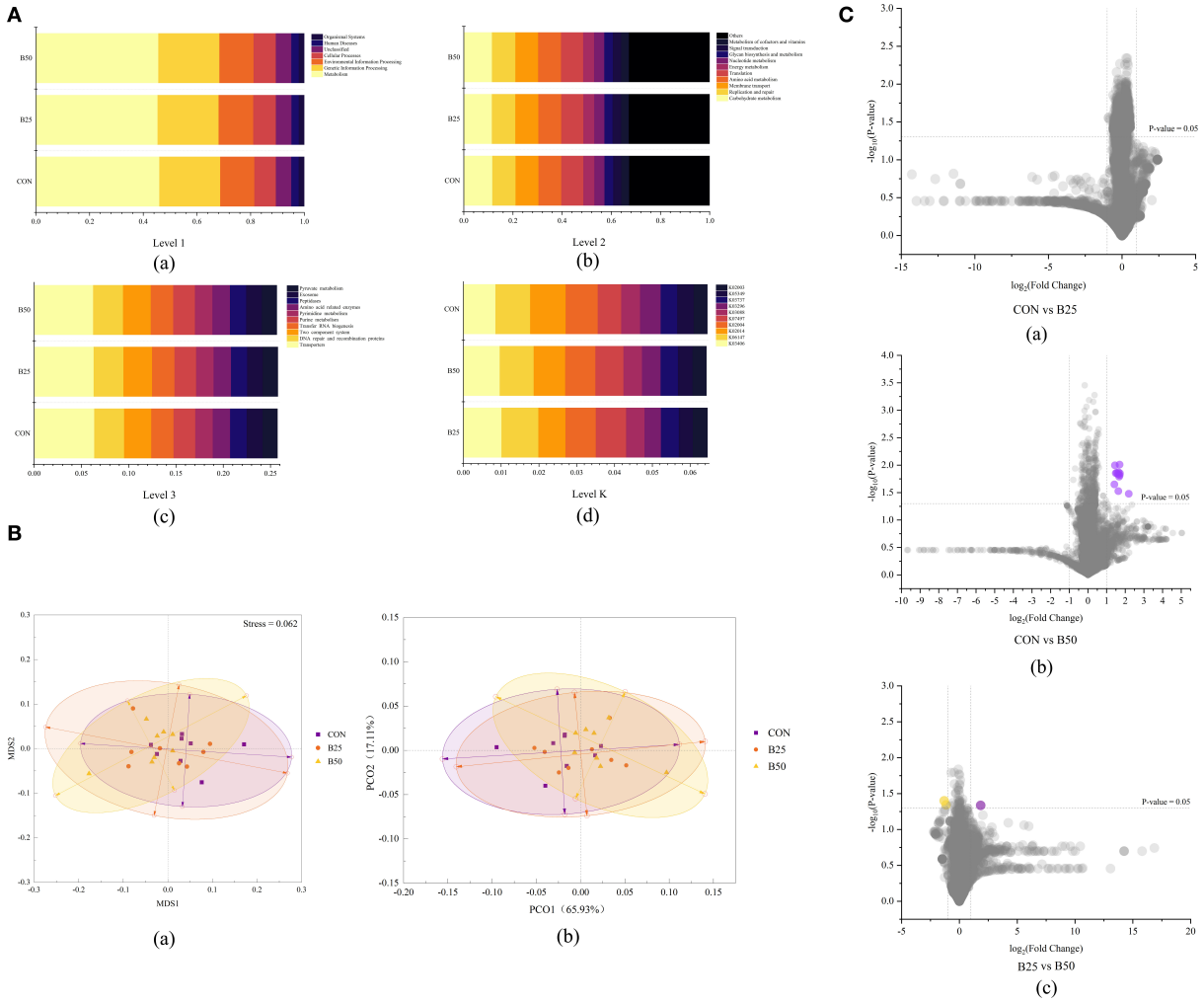


FIGURE 7
Functional prediction on the composition and difference for fecal microflora in the three treatments. **(A)** Functional prediction on the fecal microflora: (a–d) bar plot of the relative abundance of the predicted function on levels 1, 2, 3, and K; **(B)** (a) NMDS plot; (b) PCoA plot; and **(C)** pairwise comparison of different functions between the groups based on the *t*-test.

function of fecal microflora. The difference analysis results of functional prediction are provided in Figure 7C. Volcano plots ($p < 0.05$, fold-change = 2) showed 12 upregulated points in CON–B25 and one upregulated and two downregulated points in B25–B50. Different KEGG orthologs are given in Table 5.

Tax4Fun detected 6,278 KEGG orthologs in rumen fluid. The first level (Figure 8Aa) that was related to rumen fluid microflora included cellular processes, environmental information processing, genetic information processing, human diseases, metabolism, organismal systems, and unclassified. In the second level (Figure 8Ab), the primary orthologs in rumen fluid were related to carbohydrate metabolism, replication and repair, membrane transport, translation, and amino acid metabolism. Figure 8Ac shows that at the third level, the dominant orthologs were transporters, DNA repair and recombination proteins, a two-component system, transfer RNA biogenesis, and purine metabolism. The main orthologs at the K level (Figure 8Ad) were K06147, K02014, K03406, K02004, and K03296. The β-diversity results (Figure 8B) from PCoA and NMDS analyses revealed no noticeable separation between CON and B50. The ANOSIM results given in Table 4 further illustrated the lack of differentiation ($p > 0.05$). In addition, LEfse analysis showed that 27 differential pathways were found in rumen fluid microflora (Figure 8C). Of these pathways, 14 and 13 were significantly enriched in CON and B50, respectively.

## Discussion

Fermented pineapple peel residue is an unconventional feedstuff (UCF) with the characteristics of unique fragrance,

TABLE 5 Significant differential KEGG orthologs of functional prediction in fecal microorganisms.

| | KEGG orthologs | Fold change | *p*-value |
|---|---|---|---|
| CON vs. B50 | K00204 | 3.11 | 0.01 |
| | K03231 | 3.24 | 0.01 |
| | K03573 | 3.09 | 0.03 |
| | K03626 | 3.28 | 0.01 |
| | K05576 | 3.26 | 0.02 |
| | K06011 | 2.9 | 0.01 |
| | K07730 | 3.06 | 0.02 |
| | K09835 | 2.67 | 0.02 |
| | K11005 | 2.81 | 0.01 |
| | K11058 | 2.72 | 0.01 |
| | K14121 | 4.55 | 0.03 |
| | K15896 | 3.19 | 0.02 |
| B25 vs. B50 | K07445 | 0.45 | 0.05 |
| | K08097 | 0.4 | 0.04 |
| | K02077 | 3.6 | 0.05 |

sufficient water content, soft texture, and good palatability. It can considerably stimulate the appetite of black goats. This study showed that FPPR had an obvious effect on the weight gain of black goats. In addition, as the experimental period proceeded, the regularity of the feed intake of the goats in the FPPR group increased, indicating that FPPR might lessen the susceptibility of goats to the adverse influence of external factors.

Various biochemical components in the blood are the material basis of life activities, and their contents and changes reflect the basic situation of nutrition and metabolism in the body. UREA is the decomposition product of protein and fat and plays an important role in renal function (Liu et al., 2020; Lu et al., 2020). A previous research showed that high UREA reflects the high decomposition rates of proteins and amino acids and indicates the levels of fatigue in the body (Wang et al., 2020). High levels of NEFA in the blood are often used as a sign of negative energy balance or fat mobilization (Hendriks et al., 2022). Compared with WCS without FPPR, the partially replaced feeds contained more energy that can be provided to goats and were more beneficial to goat fat deposition during production. In this study, other biochemical indicators did not show no significant differences between the two treatments, indicating that the replacement of WCS with FPPR had no adverse effect on the physical status of black goats.

The α-diversity analysis is a single-sample microflora diversity test based on OTU clustering results. Biodiversity can be estimated by calculating species richness indexes, such as the Chao1 index, and diversity indexes, such as Shannon index. In this study, FPPR did not appear to cause significant changes in the abundance or diversity of microorganisms in rumen fluid or feces ($p > 0.05$). PCoA and NMDS can reflect the variance within and between the groups based on the distance between dots. Furthermore, ANOSIM is a nonparametric test that is performed to determine the presence of significant differences in the community structure between the groups and to compare between- and within-group differences. Using a combination of the three analysis methods mentioned earlier can provide a great indication of the β-diversity of the microbiota. In this study, the different levels of FPPR addition did not cause significant differences in fecal microflora ($p > 0.05$). However, LEfse analysis showed that *g_Blautia* and c_Alphaproteobacteria that differed significantly in each treatment were enriched in B50 and CON, respectively. Previous studies have demonstrated that *Blautia* is a group of beneficial microorganisms that can be beneficial to glucose metabolism and obesity-associated inflammation (Ding et al., 2022). Functional prediction of the microflora did not significantly differ between the treatments with or without FPPR addition, indicating that FPPR presumably did not cause changes in microbial functions.

Furthermore, goats that were highly affected by FPPR in terms of ADG and feed intake were selected to further explore the effects of partially replacing WCS with FPPR on Chuanzhong black goats. Then, the effects of FPPR on rumen microflora and meat quality were studied after the goats were slaughtered.

Muscle pH is related to the glycolysis of glycogen and plays an important role in meat quality (Beauclercq et al., 2016). Studies have reported that when animals are in a highly stressed state before slaughter, the pH of the muscles increases significantly (El Otmani et al., 2021; Zhang J. Y. et al., 2021). The results indicated that in black goats, FPPR might reduce preslaughter stress, but had no effect on the flesh color, tenderness, and WHC of longissimus dorsi. For amino acid composition, the content of phenylalanine under the FPPR treatment was lower than that under CON. However, TAAs, EAAs, and DAA did not significantly differ between CON and B50. Thus, FPPR had little effect on the amino acid composition.

The rumen is vital for ruminant digestion and health. It contains microbes that enable ruminants to digest plant fiber for energy, and this environment is a natural home for many anaerobic bacteria. Moreover, rumen fermentation provides 70% of a ruminant's energy (Fregulia et al., 2021). *s_Butyrivibrio fibrisolvens*, *s_Ruminococcus albus*, *g_Ralstonia*, *o_Rhizobiales*, *c_Mollicutes*, and *p_Tenericutes* were affected by FPPR and became biomarkers and enriched in B50. *s_Butyrivibrio fibrisolvens* and *s_Ruminococcus albus* are bacteria that are good for ruminants. *s_Butyrivibrio fibrisolvens* can increase the content of conjugated linoleic acid in the intestine and adipose tissue (Srivastava et al., 2021) and produce diverse carbohydrate-active enzymes that hydrolyze cellulose and other plant-derived macromolecular polymers (Sengupta et al., 2022).

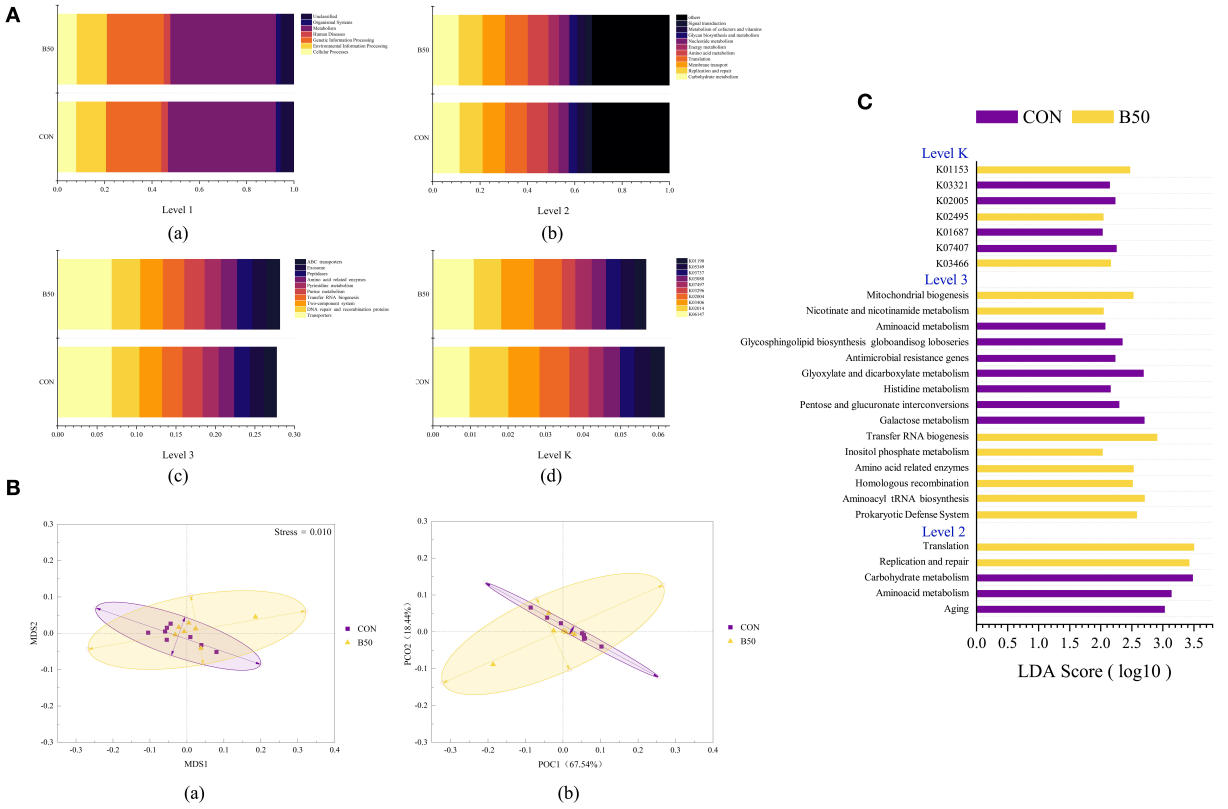


FIGURE 8
Functional prediction on the composition and LEfSe analyses for rumen fluid microflora affected by FPPR: **(A)** Functional prediction on fecal microflora: (a–d) Bar plot of the relative abundance of the predicted function on Levels 1, 2, 3, and K; **(B)** (a) NMDS plot; (b) PCoA plot; and **(C)** LAD bar plot for the predicted function of rumen microflora.

This bacterium is similar to *s_Ruminococcus albus*, which can also synthesize some types of hydrolytic enzymes that help ruminants digest plant-based feed (Storani et al., 2020; Ortiz-Chura et al., 2021). Firmicutes and Bacteroidetes are essential for ruminant digestion. Bacteroidetes plays an important role in the degradation of nonfibrous and protein materials, whereas Firmicutes is mainly involved in the degradation of fibrous materials (Zhang X. et al., 2021). These two phyla were the most abundant phyla in the rumen of goats in this experiment, but were not significantly enriched in either group, likely because the partial substitution of Water Soluble Carbohydrates (WSC) by FPPR did not result in an obvious difference in fiber and protein content and, therefore, did not cause any significant differences between the two bacterial phyla. Similar to this experiment, a previous work found that Tenericutes, the third most abundant phylum in the rumen and the phylum that was enriched in the group under FPPR treatment, has a significant association with feed intake (Zhang Y. K. et al., 2021). Nevertheless, other studies have shown that Tenericutes is abundant in animals at high risk of subacute ruminal acidosis (Li et al., 2017). However, no symptomatic goats were found in this study. This finding will be the focus of further research on the use of FPPR to replace WCS. The results shown above indicated that although replacing WCS with FPPR can increase the number of bacteria that are capable of degrading and increasing the ADG of goats, it did not cause differences between the two main phyla in the rumen and can likely improve the digestion ability of the rumen to some extent in Chuanzhong black goats. Additionally, *g_Ralstonia* can be used to degrade monofluoroactetate (MFA) contained in plants, and in goats, the inoculation of *g_Ralstonia* into the rumen under artificial conditions can effectively relieve the symptoms of MFA poisoning (da Silva et al., 2016). Among the predicted functions affected by FPPR, pathways related to replication and repair, translation, and K03466 (ftsK, DNA segregation ATPase FtsK/SpoIIIE) were enriched. Metabolism-related functions, such as inositol phosphate metabolism, amino-acid-related enzymes, nicotinate, and nicotinamide metabolism, were also enriched by FPPR replacement. The abovementioned phenomena indicated that the mixed feed might have promoted the proliferation and metabolic activity of bacteria.

## Conclusion

This study revealed that FPPR has potential as a substitute for WCS. Like UCF, FPPR had no harmful effects on the physical status of Chuanzhong black goats. In contrast, feed intake and ADG improved. Replacing WCS with a certain ratio of FPPR might play a positive role in the hindgut and rumen health manifested as the upregulation of probiotics, such as *Blautia*, *B. fibrisolvens*, and *R. albus*. FPPR could exert significant effects on the composition of fecal and rumen microflora. However, the mechanism underlying these effects is unclear and requires deep research.

## Data availability statement

The raw sequence data reported in this paper have been deposited in the Genome Sequence Archive (Genomics, Proteomics & Bioinformatics 2021) in National Genomics Data Center (Nucleic Acids Res 2022), China National Center for Bioinformation / Beijing Institute of Genomics, Chinese Academy of Sciences (GSA: CRA007078 and CRA007079) that are publicly accessible at https://ngdc.cncb.ac.cn/gsa.

## Ethics statement

The animal study was reviewed and approved by the Committee of Animal Experiments of South China Agricultural University.

## Author contributions

CY, WZ, BS, CG, and MW conceived and designed the study. CY, HT, WZ, YG, MW, and CG performed the experiments. MW and YG organized the database and performed the statistical analysis. CY and HT wrote the manuscript. HT and WZ visualized the results. YG and BS revised this 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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## References

Abdel-Wareth, A. A. A., Kehraus, S., and Suedekum, K. H. (2019). Peppermint and its respective active component in diets of broiler chickens: growth performance, viability, economics, meat physicochemical properties, and carcass characteristics. *Poultry Sci.* 98, 3850–3859. doi: 10.3382/ps/pez099

Beauclercq, S., Nadal-Desbarats, L., Hennequet-Antier, C., Collin, A., Tesseraud, S., Bourin, M., et al. (2016). Serum and muscle metabolomics for the prediction of ultimate ph, a key factor for chicken-meat quality. *J. Prot. Res.* 15, 1168–1178. doi: 10.1021/acs.jproteome.5b01050

Brito, T. B. N., Pereira, A. P. A., Pastore, G. M., Moreira, R. F. A., Ferreira, M. S. L., Fai, A. E. C., et al. (2020). Chemical composition and physicochemical characterization for cabbage and pineapple by-products flour valorization. *Lwt-Food Sci. Technol.* 124, 4–11. doi: 10.1016/j.lwt.2020.109028

Brulc, J. M., Antonopoulos, D. A., Miller, M. E. B., Wilson, M. K., Yannarell, A. C., Dinsdale, E. A., et al. (2009). Gene-centric metagenomics of the fiber-adherent bovine rumen microbiome reveals forage specific glycoside hydrolases. *Proc. Nat. Acad. Sci. USA* 106, 1948–1953. doi: 10.1073/pnas.0806191105

Cadena, S., Leopoldina Aguirre-Macedo, M., Cerqueda-Garcia, D., Cervantes, F. J., Herrera-Silveira, J. A., Garcia-Maldonado, J. Q., et al. (2019). Community structure and distribution of benthic Bacteria and Archaea in a stratified coastal lagoon in the Southern Gulf of Mexico. *Estuarine Coastal Shelf Sci.* 230, 106433. doi: 10.1016/j.ecss.2019.106433

da Silva, L. C., Pessoa, D. A., Lopes, J. R., Laio, G., da Silva, L. S., Junior, F. G., and Riet-Correa, F. (2016). Protection against Amorimia septentrionalis poisoning in goats by the continuous administration of sodium monofluoroacetate-degrading bacteria. *Toxicon* 111, 65–68. doi: 10.1016/j.toxicon.2015.12.016

De Filippis, F., Parente, E., Zotta, T., and Ercolini, D. (2018). A comparison of bioinformatic approaches for 16S rRNA gene profiling of food bacterial microbiota. *Int. J. Food Microbiol.* 265, 9–17. doi: 10.1016/j.ijfoodmicro.2017.10.028

Ding, Q., Cao, F., Lai, S., Zhuge, H., Chang, K., Valencak, T. G., et al. (2022). Lactobacillus plantarum ZY08 relieves chronic alcohol-induced hepatic steatosis and liver injury in mice via restoring intestinal flora homeostasis. *Food Res. Int.* 157, 111259. doi: 10.1016/j.foodres.2022.111259

El Otmani, S., Chebli, Y., Hornick, J. L., Cabaraux, J. F., and Chentouf, M. (2021). Growth performance, carcass characteristics and meat quality of male goat kids supplemented by alternative feed resources: olive cake and cactus cladodes. *Anim. Feed Sci. Technol.* 272, 114746. doi: 10.1016/j.anifeedsci.2020.114746

Fregulia, P., Neves, A. L. A., Dias, R. J. P., and Campos, M. M. (2021). A review of rumen parameters in bovines with divergent feed efficiencies: what do these parameters tell us about improving animal productivity and sustainability? *Livestock Sci.* 254, 104761. doi: 10.1016/j.livsci.2021.104761

Fu, Q., Leng, Z. X., Ding, L. R., Wang, T., Wen, C., Zhou, Y. M., et al. (2016). Complete replacement of supplemental DL-methionine by betaine affects meat

quality and amino acid contents in broilers. *Anim. Feed Sci. Technol.* 212, 63–69. doi: 10.1016/j.anifeedsci.2015.12.004

Gasmi Benahmed, A., Gasmi, A., Dosa, A., Chirumbolo, S., Mujawdiya, P. K., Aaseth, J., et al. (2021). Association between the gut and oral microbiome with obesity. *Anaerobe* 70, 102248–102248. doi: 10.1016/j.anaerobe.2020.102248

Hendriks, S. J., Phyn, C. V. C., Turner, S. A., Mueller, K. R., Kuhn-Sherlock, B., Donaghy, D. J., et al. (2022). Associations between peripartum lying and activity behaviour and blood non-esterified fatty acids and beta-hydroxybutyrate in grazing dairy cows. *Anim. Int. J. Anim. Biosci.* 16, 100470. doi: 10.1016/j.animal.2022.100470

Hu, H., Zhao, Q., Xie, J., and Sun, D. (2019). Polysaccharides from pineapple pomace: new insight into ultrasonic-cellulase synergistic extraction and hypoglycemic activities. *Int. J. Biol. Macromol.* 121, 1213–1226. doi: 10.1016/j.ijbiomac.2018.10.054

Jami, E., Israel, A., Kotser, A., and Mizrahi, I. (2013). Exploring the bovine rumen bacterial community from birth to adulthood. *ISME J.* 7, 1069–1079. doi: 10.1038/ismej.2013.2

Jin, H., Mo, L., Pan, L., Hou, Q., Li, C., Darima, I., et al. (2018). Using PacBio sequencing to investigate the bacterial microbiota of traditional Buryatian cottage cheese and comparison with Italian and Kazakhstan artisanal cheeses. *J. Dairy Sci.* 101, 6885–6896. doi: 10.3168/jds.2018-14403

Kiggundu, M., and Kabi, F. (2019). Effect of wilting organic pineapple by-products and jack bean (*Canavalia ensiformis*) foliage inclusion on silage fermentation and its nutritive value. *Anim. Feed Sci. Technol.* 258, 114303. doi: 10.1016/j.anifeedsci.2019.114303

Kunz, I. G. Z., Reed, K. J., Metcalf, J. L., Hassel, D. M., Coleman, R. J., Hess, T. M., et al. (2019). Equine fecal microbiota changes associated with anthelmintic administration. *J. Equine Vet. Sci.* 77, 98–106. doi: 10.1016/j.jevs.2019.01.018

Lee, C., Copelin, J. E., Dieter, P. A., and Berry, E. A. (2020). Effects of trace mineral supply from rumen boluses on performance, carcass characteristics, and fecal bacterial profile in beef cattle. *Anim. Feed Sci. Technol.* 269. doi: 10.1016/j.anifeedsci.2020.114626

Li, F., Wang, Z. L., Dong, C. X., Li, F. D., Wang, W. M., Yuan, Z. H., et al. (2017). Rumen bacteria communities and performances of fattening lambs with a lower or greater subacute ruminal acidosis risk. *Front. Microbiol.* 8, 2506. doi: 10.3389/fmicb.2017.02506

Liu, C., Asano, S., Ogata, H., Ito, S., Nakase, T., Takeda, S., et al. (2021). Digestive, fermentative, and physical properties of pineapple residue as a feed for cattle. *Anim. Sci. J.* 92, e13535. doi: 10.1111/asj.13535

Liu, G., Chen, X. F., Lu, X., Zhao, J. Y., and Li, X. L. (2020). Sunflower head enzymatic hydrolysate relives hyperuricemia by inhibiting crucial proteins (xanthine oxidase, adenosine deaminase, uric acid transporter1) and restoring gut microbiota in mice. *J. Funct. Foods* 72, 104055. doi: 10.1016/j.jff.2020.104055

Lu, W. G., Sun, H. L., Xu, M. H., Luo, Y. H., Jin, J. D., Shao, H. Z., et al. (2020). Blood urea nitrogen may serve as a predictive indicator of retained placenta in dairy cows. *Anim. Rep. Sci.* 218, 106481. doi: 10.1016/j.anireprosci.2020.106481

Ortiz-Chura, A., Gere, J., Marcoppido, G., Depetris, G., Cravero, S., Faverín, C., et al. (2021). Dynamics of the ruminal microbial ecosystem, and inhibition of methanogenesis and propiogenesis in response to nitrate feeding to Holstein calves. *Anim. Nutr.* 7, 1205–1218. doi: 10.1016/j.aninu.2021.07.005

Padrón-Mederos, M., Rodríguez-Galdón, B., Díaz-Romero, C., Gloria Lobo-Rodrigo, M., and Rodríguez-Rodríguez, E. M. (2020). Quality evaluation of minimally fresh-cut processed pineapples. *Lwt-Food Sci. Technol.* 129, 109607. doi: 10.1016/j.lwt.2020.109607

Roda, A., and Lambri, M. (2019). Food uses of pineapple waste and by-products: a review. *Int. J. Food Sci. Technol.* 54, 1009–1017. doi: 10.1111/ijfs.14128

Sanya, C. A. K., Chadare, F. J., Hounhouigan, M. H., Hotegni, N. V. F., Gbaguidi, M. A., Dekpemadoha, J. E., et al. (2020). Effects of plant density and fertilizer formula on physicochemical and sensorial characteristics of pasteurized juice from Perolera sugarloaf pineapples grown in the long rainy season. *Njas-Wageningen J. Life Sci.* 92, 100320. doi: 10.1016/j.njas.2019.100320

Sengupta, K., Hivarkar, S. S., Palevich, N., Chaudhary, P. P., Dhakephalkar, P. K., Dagar, S. S., et al. (2022). Genomic architecture of three newly isolated unclassified Butyrivibrio species elucidate their potential role in the rumen ecosystem. *Genomics* 114, 110281. doi: 10.1016/j.ygeno.2022.110281

Sharma, S., Lee, M., Reinmann, C. S., Pumneo, J., Cutright, T. J., Senko, J. M., et al. (2020). Impact of acidmine drainage chemistry and microbiology on the development of efficient Fe removal activities. *Chemosphere* 249, 126117. doi: 10.1016/j.chemosphere.2020.126117

Shivakumar Gowda, N. K., Vallesha, N. C., Awachat, V. B., Anandan, S., Pal, D. T., Prasad, C. S., et al. (2015). Study on evaluation of silage from pineapple (*Ananas comosus*) fruit residue as livestock feed. *Trop. Anim. Health Prod.* 47, 557–561. doi: 10.1007/s11250-015-0762-2

Silva, T. B. P., Del Valle, T. A., Ghizzi, L. G., Silva, G. G., Gheller, L. S., Marques, J. A., et al. (2021). Partial replacement of corn silage with whole-plant soybean and black oat silages for dairy cows. *J. Dairy Sci.* 104, 9842–9852. doi: 10.3168/jds.2021-20200

Song, W., Thomas, T., and Edwards, R. J. (2019). Complete genome sequences of pooled genomic DNA from 10 marine bacteria using PacBio long-read sequencing. *Marine Genomics* 48, 35–43. doi: 10.1016/j.margen.2019.05.002

Srivastava, A., Kumar, S., Tyagi, A., Shrivastava, N., Varma, A., Tyagi, A. K., et al. (2021). Butyrivibrio fibrisolvens F7 dietary supplementation increases levels of cis 9-trans 11 conjugated linoleic acid in gut and adipose tissue in mice. *Curr. Res. Biotechnol.* 3, 300–307. doi: 10.1016/j.crbiot.2021.11.001

Storani, A., Guerrero, S. A., and Iglesias, A. A. (2020). On the functionality of the N-terminal domain in xylanase 10A from Ruminococcus albus 8. *Enzyme Microb. Technol.* 142, 109673. doi: 10.1016/j.enzmictec.2020.109673

Sun, J., Zeng, B., Chen, Z., Yan, S., Huang, W., Sun, B., et al. (2017). Characterization of faecal microbial communities of dairy cows fed diets containing ensiled Moringa oleifera fodder. *Sci. Rep.* 7, 1–9. doi: 10.1038/srep41403

Tafaj, M., Zebeli, Q., Maulbetsch, A., Steingass, H., and Drochner, W. (2006). Effects of fibre concentration of diets consisting of hay and slowly degradable concentrate on ruminal fermentation and digesta particle size in mid-lactation dairy cows. *Archives Anim. Nutr.* 60, 254–266. doi: 10.1080/17450390600679322

Tian, H., Chen, Y., Zhu, N., Guo, Y., Deng, M., Liu, G., et al. (2020). Effect of Broussonetia papyrifera silage on the serum indicators, hindgut parameters and fecal bacterial community of Holstein heifers. *Amb Exp.* 10. doi: 10.1186/s13568-020-01135-y

Van Soest, P. J., Robertson, J. B., and Lewis, B. A. (1991). Methods for dietary fiber, neutral detergent fiber, and nonstarch polysaccharides in relation to animal nutrition. *J. Dairy Sci.* 74, 3583–3597. doi: 10.3168/jds.S0022-0302(91)78551-2

Wang, C. T., Yang, C. M., Chen, Z. S., and Lee, Y. C. (2013). Performance of straw-fed microbial fuel cells with mixed rumen microorganisms by using different catholytes. *Biomass Bioenergy* 59, 412–417. doi: 10.1016/j.biombioe.2013.08.033

Wang, P. X., Zeng, H. L., Lin, S. L., Zhang, Z. G., Zhang, Y., Hu, J. M., et al. (2020). Anti-fatigue activities of hairtail (*Trichiurus lepturus*) hydrolysate in an endurance swimming mice model. *J. Funct. Foods* 74, 104207. doi: 10.1016/j.jff.2020.104207

Wang, T., Jiao, J., Wang, H., Degen, A. A., Gou, N., Li, S., et al. (2021). The effects of supplementing sweet sorghum with grapeseeds on dry matter intake, average daily gain, feed digestibility and rumen parameters and microbiota in lambs. *Anim. Feed Sci. Technol.* 272, 114750. doi: 10.1016/j.anifeedsci.2020.114750

Wassie, T., Zeng, F., Jiang, X., Liu, G., Kasimu, H., Ling, S., et al. (2020). Effect of Kisspeptin-54 immunization on performance, carcass characteristics, meat quality and safety of Yiling goats. *Meat Sci.* 166, 108139. doi: 10.1016/j.meatsci.2020.108139

Xie, Y., Chen, Z., Wang, D., Chen, G., Sun, X., He, Q., et al. (2020). Effects of fermented herbal tea residues on the intestinal microbiota characteristics of holstein heifers under heat stress. *Front. Microbiol.* 11, 1014. doi: 10.3389/fmicb.2020.01014

Yang, C., Zhao, F., Hou, Q., Wang, J., Li, M., Sun, Z., et al. (2020). PacBio sequencing reveals bacterial community diversity in cheeses collected from different regions. *J. Dairy Sci.* 103, 1238–1249. doi: 10.3168/jds.2019-17496

Zhang, J. Y., Qian, S. H., Chen, J. H., Ding, L. Y., Wang, M. Z., Maloney, S. K., et al. (2021). Calm Hu ram lambs assigned by temperament classification are healthier and have better meat quality than nervous Hu ram lambs. *Meat Sci.* 175, 108436. doi: 10.1016/j.meatsci.2021.108436

Zhang, X., Wang, H., and Guo, X. (2021). Comparative analysis of rumen fermentation parameters and bacterial profiles during adaption to different fattening stages in beef cattle fed TMR with various forage silage. *Animal Feed Sci. Technol.* 278, 115006. doi: 10.1016/j.anifeedsci.2021.115006

Zhang, Y. K., Zhang, X. X., Li, F. D., Li, C., Li, G. Z., Zhang, D. Y., et al. (2021). Characterization of the rumen microbiota and its relationship with residual feed intake in sheep. *Animal* 15, 100161. doi: 10.1016/j.animal.2020.100161

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# Effects of supplementation of sodium acetate on rumen fermentation and microbiota in postpartum dairy cows

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The primary product of rumen fermentation is acetic acid, and its sodium salt is an excellent energy source for post-partum cows to manage negative energy balance (NEB). However, it is unknown how adding sodium acetate (NAc) may affect the rumen bacterial population of post-partum cows. Using the identical nutritional total mixed ration (TMR), this research sought to characterize the impact of NAc supplementation on rumen fermentation and the composition of bacterial communities in post-partum cows. After calving, 24 cows were randomly assigned to two groups of 12 cows each: a control group (CON) and a NAc group (ACE). All cows were fed the same basal TMR with 468g/d NaCl added to the TMR for the CON group and 656g/d NAc added to the TMR for the ACE group for 21 days after calving. Ruminal fluid was collected before morning feeding on the last day of the feeding period and analyzed for rumen bacterial community composition by 16S rRNA gene sequencing. Under the identical TMR diet conditions, NAc supplementation did not change rumen pH but increased ammonia nitrogen ($NH_3$-N) levels and microbial crude protein (MCP) concentrations. The administration of NAc to the feed upregulated rumen concentrations of total volatile fatty acids (TVFA), acetic, propionic, isovaleric and isobutyric acids without affecting the molar ratio of VFAs. In the two experimental groups, the *Bacteroidota*, *Firmicutes*, *Patescibacteria* and *Proteobacteria* were the dominant rumen phylum, and *Prevotella* was the dominant rumen genus. The administration of NAc had no significant influence on the α-diversity of the rumen bacterial community but upregulated the relative abundance of *Prevotella* and downregulated the relative abundance of *RF39* and *Clostridia_UCG_014*. In conclusion, the NAc supplementation in the post-peripartum period altered rumen flora structure and thus improved rumen fermentation in dairy cows. Our findings provide a reference for the addition of sodium acetate to alleviate NEB in cows during the late perinatal period.

KEYWORDS

sodium acetate, bacterial community composition, rumen fermentation, negative energy balance, *Prevotella*

## Introduction

After calving, dairy cows in the transition period are subjected to various physiological stress shocks that cause low dry matter intake, hormonal changes, and high morbidity (Contreras et al., 2018). Especially in high-performance dairy cows, decreased feed intake makes it impossible to fulfill the nutritional needs of rapidly producing milk, resulting in development of NEB (Lopreiato et al., 2020). Adipose tissue is catabolized into considerable amounts of non-esterified fatty acids (NEFA), which subsequently enter the liver. This will swiftly lead to the development of ketosis or fatty liver, which will reduce liver and immune function in post-partum cows (Gao et al., 2018; White, 2020). Additionally, the body's metabolism is boosted, and a significant amount of reactive oxygen species and lipid peroxides are created in the NEB state to adjust to breastfeeding requirements. This puts cows in a condition of oxidative stress that surpasses the body's antioxidant system's capacity for scavenging, which causes immunosuppression and in turn causes metabolic illnesses such as mastitis, metritis, retained placenta, and reduced fertility (Ingvartsen and Moyes, 2013). Therefore, the intensity and length of NEB have a significant impact on cow health, making them more prone to illness and less effective in terms of milk output and quality, which in turn has a negative impact on farm economics.

As a frequent treatment for NEB, exogenous nutritional addition to modulating dairy cow metabolism during the perinatal period can help dairy cows produce more milk, eliminate energy metabolism problems, and enhance breeding effectiveness (Sordillo and Raphael, 2013; Lopreiato et al., 2020). Acetic acid, which typically makes up around 60% of fermented acid products in ruminants, is crucial for the synthesis of milk fat and energy supply as it serves as a primary precursor to milk fat and a basic energy source (Aschenbach et al., 2010). Sodium acetate (NAc) can be utilized to treat NEB issues in dairy cows by hydrolyzing into lactic acid and sodium ions in the rumen (Bampidis et al., 2021). According to the experimental results of injecting 0, 5, 10, and 15 mol/d of acetic acid into the rumen, dairy cows' ability to produce milk and milk fat increased linearly (Urrutia and Harvatine, 2017). In addition, reports have been made using intraruminal infusion of acetic acid or NAc to test its effects on milk production and milk composition and feed intake in lactating dairy cows (Rook and Balch, 1961; Urrutia and Harvatine, 2017). Although intraruminal infusion of acetic acid has been proven to positively impact energy balance, body health, milk output, and milk composition in nursing dairy cows. The effectiveness of acetic acid to treat NEB of post-partum cows as a result of inadequate feed intake has not been documented, although the putative role of acetic acid in the energy metabolism of cows is well recognized. We postulated that adding acetic acid to TMR after calving may help cows to produce more milk, boost immunity, and successfully treat NEB. By including an additional 8 mol/d of NAc in the feed of post-calving cows, we confirmed this theory (unpublished data). Whereas, uncertainty persists about the part rumen microbes play in the supplementary NAc-induced NEB reduction.

A wide range of microorganisms are engaged in the deconstruction and use of feed to supply the nutritional needs of ruminants for maintenance, development, and lactation through fermentation of feed in the rumen of ruminants, which is a sizable "fermenter" (Bickhart and Weimer, 2018). The rumen microbiome, which is sensitive to changes in feed type and diet structure, is substantially connected with dairy cow performance (Liu et al., 2019). The health and productivity of dairy cows can be controlled by modifying the makeup of the rumen's microbial population and fermentation products. It is critical to understand the alterations in the makeup of the rumen microbial population of dairy cows in order to explore the influence of NAc feed additives on the rumen microorganisms of dairy cows. Because acetate is one of the principal fermentation products of the rumen microflora, it may modify rumen fermentation indicators and rumen bacterial community composition, lowering NEB in post-partum cows. Based on this, we hypothesize that NAc may improve post-partum cows performance by altering rumen fermentation and microbiota. As a result, the purpose of this study was to use 16S rRNA sequencing to investigate the impact of NAc supplementation on rumen fermentation and rumen bacterial community composition in dairy cows following calving.

## Materials and methods

All Holstein dairy bovines utilized in this think were entirely cared for in understanding the standards of Yangzhou University, the Institutional Animal Care and Use Committee (SYXK (Su) IACUC 2016-0019).

### Experimental design, animals, diets, and management

The exploration was conducted from September to December 2021 at the test base of The Animal Nutrition and Feed Engineering Research Center of Yangzhou University (Gaoyou, China; yoke stall). Twenty-four Holstein cows in

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

| Ingredients | Content |
|---|---|
| Alfalfa | 8.39 |
| Oat grass | 6.43 |
| Whole corn silage | 52.18 |
| Corn | 8.1 |
| Soya bean meal | 10.8 |
| Cotton seed meal | 6.8 |
| DDGS | 4.8 |
| Premix[1] | 2.5 |
| Total | 100 |
| *Nutrient levels* | |
| CP | 16.47 |
| EE | 3.84 |
| NDF | 33.06 |
| ADF | 20.25 |
| Ash | 7.28 |
| Calcium | 0.74 |
| Phosphorus | 0.35 |
| NEL(MJ/kg)[2] | 3.25 |

DDGS, distiller dried grains with soluble; CP, crude protein; EE, ether extract; NDF, neutral detergent fiber; ADF, acid detergent fiber.
[1]Per kilogram of premix provide 55 mg of Mn as $MnSO_4$; 62.5 mg of Zn as $ZnSO_4$; 0.55 mg of Se as $Na_2SeO_3$; 75 mg of Fe as $Fe_2(SO_4)_3$; 32 mg of Cu as $CuSO_2$; 1.00 mg of I as KI; 0.40 mg of Co as $CoCl_2$; 1,100,000 IU of Vitamin A; 270,000 IU of Vitamin D; 200,000 mg of Vitamin E; 4,200 mg of Vitamin $K_3$.
[2]Predicted values from NRC (2001) model.

TABLE 2 Effect of NAc addition on rumen pH, $NH_3$-N and volatile fatty acids (VFA).

| Items | Treatments[1] | | *p*-value |
|---|---|---|---|
| | CON | ACE | |
| pH | 6.90 ± 0.06 | 6.77 ± 0.05 | 0.110 |
| $NH_3$-N, mg/dL | 10.21 ± 0.76 | 11.07 ± 0.0.71 | 0.418 |
| MCP, mg/dL | 44.33 ± 1.64 | 55.07 ± 0.92 | <0.001 |
| **Total VFA, mmol/L** | 82.48 ± 3.34 | 103.47 ± 4.02 | 0.001 |
| Acetate, mmol/L | 57.61 ± 2.10 | 72.90 ± 2.79 | <0.001 |
| Propionate, mmol/L | 13.98 ± 0.47 | 17.12 ± 0.71 | 0.001 |
| Iso-butyrate, mmol/L | 0.29 ± 0.01 | 0.35 ± 0.02 | 0.008 |
| Butyrate, mmol/L | 8.64 ± 0.87 | 10.67 ± 0.58 | 0.066 |
| Iso-valerate, mmol/L | 0.97 ± 0.10 | 1.26 ± 0.07 | 0.025 |
| Valerate, mmol/L | 0.98 ± 0.08 | 1.16 ± 0.05 | 0.059 |
| Acetate/propionate | 4.14 ± 0.13 | 4.28 ± 0.10 | 0.430 |

$NH_3$-N = ammonia-N, MCP = microbial crude protein.[1]Treatments: CON = control group, basal diet plus 468 g/day oral sodium chloride; ACE = NAc group, basal diet plus 656 g/day oral NAc.

postpartum were randomly divided into two blocks ($n = 12$ per treatment) based on parity (2, $n = 24$), weight (640.1 ± 20.9 kg), and anticipated calving date. All cows were nourished the equal basal total mixed ration diet (TMR), formulated based on the nutritional requirement of the National Research Council (NRC, 2001). The composition and nutrient level of the diets are given in Table 1. Similar to previous research (Urrutia et al., 2019), the control and NAc groups were given 468 g/day sodium chloride (Jiangsu Baimei Chemical Co., Ltd.) and 656 g/day sodium acetate (Shandong Yutian Food Science & Technology Co., Ltd.) with >99% effective constituent content from calving to 21 days postpartum, respectively. The control group (sodium chloride) was designed to provide equal moles of sodium as the sodium acetate group. The oral drenching was achieved by separately taking an equal portion from the offered TMR (Ensure 5% ~ 10% orts daily), mixing it with sodium chloride or sodium acetate using a mixer and then orally delivering (drenched) to each cow within the experimental groups (control or NAc group). Afterwards, the cows had access to the remaining portion of the TMR diet offered. Cows were drenched and fed TMR three times a day at 08:00, 14: 00 and 21: 00. The cows had free access to water throughout the experimental period. The daily offered TMR feed throughout the experimental period was adjusted based on the daily leftover from each cow. The feed samples were collected from the trough on three consecutive days per week, and one sample was mixed each week. The nutrient content was assayed in previous studies (Jiang et al., 2022).

## Ruminal sample collection and analyses

Ruminal contents samples were collected with an oral collector (A1141K, Colibri Pastoral Technology Co., Wuhan, Hubei, China) before morning feeding (At 07: 30) on day 21 of parturition, and ruminal fluid was collected from a total of 24 cows. Among them, the insertion length of the rumen fluid collector is about two meters. The first 150 ml of rumen contents were discarded to avoid saliva contamination. Then 150 ml of rumen contents were collected after filtering through 4 layers of sterile gauze and dispensed into ten 15 ml plastic cryotubes. Ruminal pH was immediately with a mettler-toledo pH meter (Model FE20, Shanghai, China). The $NH_3$-N, VFA and MCP concentrations were determined using the methods of previous studies (Zhang et al., 2022). The rumen content samples were kept in liquid nitrogen to quickly cool down and then stored at −80°C for later microbiome analysis (Tables 2, 3).

## 16S rRNA gene sequencing and data processing

The total DNA of rumen bacteria were extracted by the cetyltrimethylammonium bromide (CTAB) and then assessed for DNA quality by 1% agarose gel electrophoresis. The V4 high variant region of the bacteria was amplified using primers 515F (5′-GTGCCAGCMGCCGCGGTAA-3′) and 806R (5′-GGACT ACNNGGGTATCTAAT-3′; Lin et al., 2021). The PCR products

TABLE 3 Effects of dietary NAc supplementation on α-diversity indexes of rumen bacteria.

| Items | Treatments[1] | | *p*-value |
|---|---|---|---|
| | CON | ACE | |
| chao1 | 1233.93 ± 40.98 | 1174.37 ± 29.38 | 0.250 |
| observed_otus | 1229.25 ± 40.97 | 1171.08 ± 29.75 | 0.263 |
| shannon | 8.94 ± 0.24 | 7.91 ± 0.12 | 0.487 |
| simpson | 0.973 ± 0.013 | 0.981 ± 0.003 | 0.594 |
| pielou_e | 0.789 ± 0.021 | 0.776 ± 0.010 | 0.581 |

[1]Treatments: CON = control group, basal diet plus 468 g/day oral sodium chloride; ACE = NAc group, basal diet plus 656 g/day oral NAc.

were sent to Novogene Co., Ltd. (Beijing) for high-throughput sequencing analysis.

Data read from both ends of the raw DNA fragments were processed using FASTP (Version 0.14.1; Chen et al., 2018). The final amplicon sequence variants (ASVs), as well as feature tables, were obtained by noise reduction using the DADA2 module in QIIME2 (Version 1.9.1) software or deblur based on the quality-controlled Effective Tags and filtering out sequences with abundance less than 5 (Bolyen et al., 2019). Subsequently, the ASVs were compared with the SILVA (version 138.1) database to determine species information (Quast et al., 2012). Based on the ASVs information, α-diversity analysis (chao1, observed_otus, shannon, simpson, pielou_e) was calculated using QIIME2. Principal coordinate analysis (PCoA), non-metric multidimensional scale analysis (NMSD) and LDA Effect Size (Lefse) were analyzed using online resources.[1] The 16S sequence data have been submitted to the GSA database under the PRJCA010405 program.

## Statistical analysis

The correlation and differences between rumen fermentation parameters and bacterial abundance were analyzed with Spearman's correlation analysis and independent samples t-tests in SPSS 26 (IBM, New York, USA) and results were expressed as mean ± SEM. Correlation coefficients with absolute values greater than 0.5 are considered relevant. Significant differences were declared at $p < 0.05$. And $p < 0.01$ was considered a highly significant difference.

# Results

## Ruminal fermentation parameters

The NAc supplementation significantly increased rumen microbial crude protein (MCP) concentration ($p < 0.001$), but had no significance on pH ($p = 0.110$) and $NH_3$-N concentration ($p = 0.418$). Dietary addition of NAc significantly increased the concentrations of acetate ($p < 0.001$), propionate ($p = 0.001$), isobutyrate ($p = 0.008$), isovalerate ($p = 0.025$) and TVFA ($p = 0.001$) in the rumen fluid, but did not affect the ratio of acetate to propionate (Table 2). NAc supplementation induced an increasing trend in the concentration of both butyrate ($p = 0.066$) and valerate ($p = 0.059$). The molar ratios of individual rumen fluid VFA are shown in Supplementary Table S1. The addition of NAc did not alter the molar percentage of rumen fluid VFA.

## Sequencing and diversity measures

A total of 1,927,895 high-quality V4 16S rRNA sequences were generated in 24 rumen samples, with an average of 80,329 per sample (minimum 71,936; maximum 88,726). A total of 5,499 ASVs were detected in the samples, with 4,364 and 3,613 detected in the CON and ACE groups, respectively. Venn diagrams (Supplementary Figure S1) show the number of common and unique rumen bacterial ASVs in the two groups. The sparsity curves of the rumen samples (Supplementary Figure S2) indicate that the sequencing depth was sufficient to assess the different bacterial communities accurately. The supplementation of NAc did not affect ($p > 0.05$) the α-diversity (Table 3) measurement index (chao1, observed_otus, shannon, simpson, pielou_e). As shown in the PCoA and NMDS plots, the distribution of samples from the ACE group was relatively concentrated, and the two groups crossed and overlapped each other (Figure 1). This indicates that the samples from the ACE group had better reproducibility and did not differ significantly from the rumen microbial community of the CON group in terms of β-diversity.

## Microbiota composition

The effect of NAc addition on bacterial composition of the rumen was compared by taxonomic analysis. A total of 28 bacterial phyla were obtained from 24 rumen samples. Figure 2A shows that the *Bacteroidota* (CON: 43.54%; ACE: 65.82%), *Firmicutes* (CON: 46.47%; ACE: 28.43%), *Patescibacteria* (CON: 4.02%; ACE: 1.25%) and *Proteobacteria* (CON: 3.41%; ACE: 3.29%) were the major clades in all groups. The addition of NAc increased the relative abundance of *Bacteroidota* in the rumen ($p = 0.001$) but decreased the abundance of *Firmicutes* ($p = 0.001$) and *Patescibacteria* ($p < 0.01$; Table 4). At the genus level, a total of 388 genera were detected in the rumen. As shown in Figure 2B and Table 5, the unclassified sequences in the CON and ACE groups were 9.82 and 7.48%, respectively, with *Prevotella* (CON: 30.73%; ACE: 49.71%) being the most abundant genus in the rumen. The results of this experiment showed that the addition of NAc to the diet increased the abundance of *Prevotella* ($p < 0.01$) and *Rikenellaceae_RC9_gut_group* ($p = 0.001$) in *Bacteroidota*. However, NAc supplementation reduced the

1 https://magic.novogene.com/

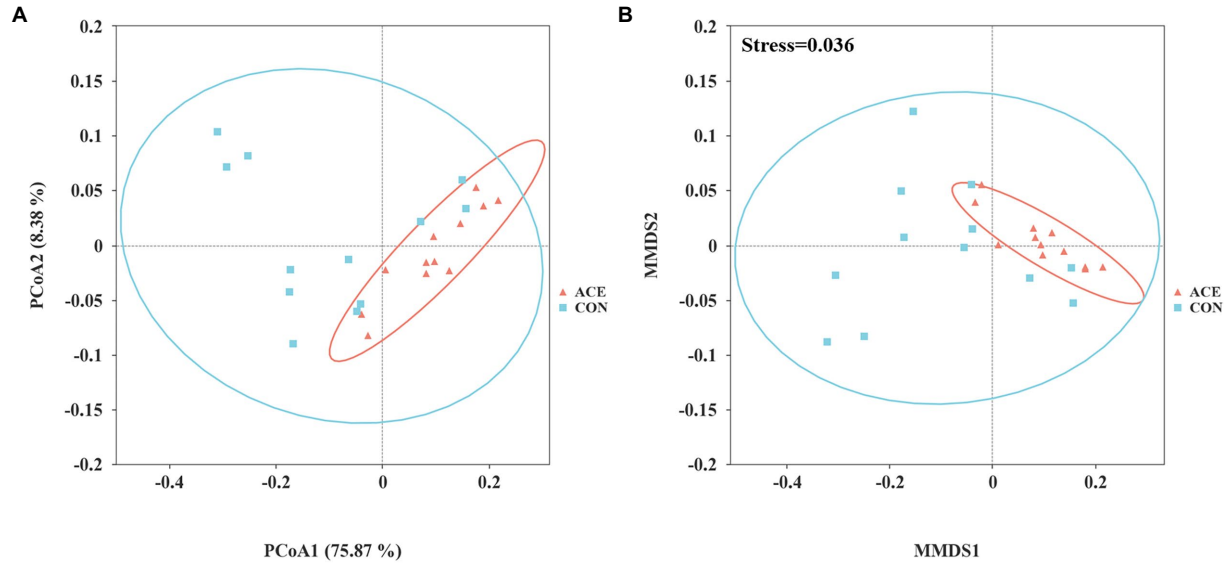


FIGURE 1
Beta community diversity analysis of the CON and ACE groups of rumen microbiota. **(A)** Principal coordinate analysis (PCoA) and **(B)** non-metric multidimensional scale analysis (NMDS) CON=control group, basal diet plus 468g/day oral sodium chloride; ACE=NAc group, basal diet plus 656g/day oral NAc.

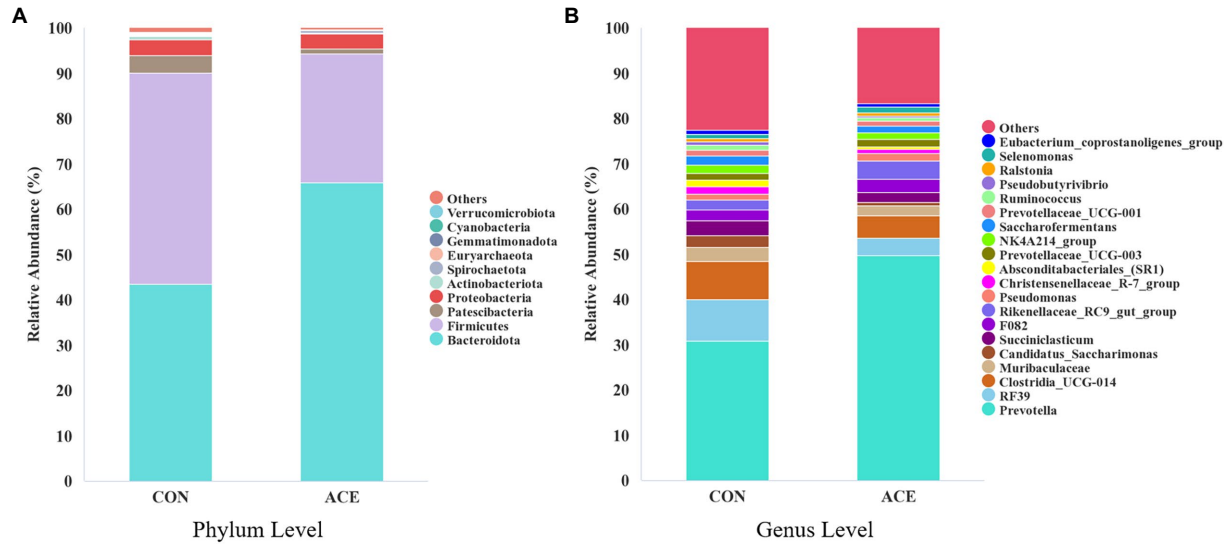


FIGURE 2
Distribution of rumen bacterial community composition in CON and ACE groups. **(A)** Phylum level; **(B)** genus level. CON=control group, basal diet plus 468g/day oral sodium chloride; ACE=NAc group, basal diet plus 656g/day oral NAc.

abundance of *RF39* ($p < 0.05$), *Clostridia_UCG-014* ($p < 0.05$), *Saccharofermentans* ($p < 0.05$), *Pseudobutyrivibrio* ($p < 0.05$), *Lachnospira* ($p < 0.05$), *Eubacterium_ruminantium_group* ($p < 0.05$), *Butyrivibrio* ($p < 0.05$) and *Anaerovibrio* ($p < 0.05$) in *Firmicutes*. Besides, the abundance of *Candidatus_Saccharimonas* ($p < 0.05$) and *Absconditabacteriales_(SR1)* ($p < 0.05$) in the *Patescibacteria* of the ACE group of rumen microorganisms were reduced.

LEfSe analysis was performed using all microbial data to identify key bacterial groups associated with the diets and NAc addition. Figure 3 depicts a representative structural branched map of the major microbial groups, showing the most significant

TABLE 4 Effects of dietary supplementation with NAc on the relative abundance of ruminal bacterial communities at phylum level (average relative abundance ≥0.5% for at least one treatment) of dairy cows.

| Phylum | Treatments[1] | | *P*-value |
|---|---|---|---|
| | CON | ACE | |
| *Bacteroidota* | 43.54 ± 5.09 | 65.82 ± 9.47 | 0.001 |
| *Firmicutes* | 46.47 ± 3.95 | 28.43 ± 7.54 | 0.001 |
| *Patescibacteria* | 4.02 ± 0.85 | 1.25 ± 0.15 | 0.008 |
| *Proteobacteria* | 3.41 ± 0.71 | 3.29 ± 0.86 | 0.918 |
| *Actinobacteriota* | 0.68 ± 0.16 | 0.20 ± 0.03 | 0.014 |

[1]Treatments: CON = control group, basal diet plus 468 g/day oral sodium chloride; ACE = NAc group, basal diet plus 656 g/day oral NAc.

differences in taxa between treatments. Figure 4 shows the differences in the abundance of the different bacterial groups in CON and ACE. Among them, the bacterial genera identified as biomarkers in the CON group were *RF39* and *Clostridia_UCG-014*, and the bacterial genus with the greatest difference in the ACE group was *Prevotella*.

## Correlation analysis of rumen fermentation parameters and major bacteria

Spearman correlation analysis was performed to assess the relationship between rumen fermentation parameters and rumen microflora structure in the presence of NAc addition. At the genus level, pH was negatively correlated with the relative abundance of *F082* ($r = -0.56$; $p < 0.01$) and *Rikenellaceae_RC9_gut_group* ($r = -0.51$; $p < 0.05$). The relative abundance of *Prevotella* in rumen fluid was positively correlated with the concentrations of MCP ($r = 0.65$; $p < 0.001$), Acetate ($r = 0.56$; $p = 0.005$), and Butyrate ($r = 0.53$; $p = 0.008$). The relative abundance of *Rikenellaceae_RC9_gut_group* had a positive correlation with the concentrations of MCP ($r = 0.55$; $p < 0.01$) and Acetate ($r = 0.55$; $p < 0.01$) similar to that of *Prevotella*. In contrast, the relative abundance of *RF39* was negatively correlated with the concentrations of MCP ($r = -0.66$; $p < 0.001$), Acetate ($r = -0.64$; $p < 0.001$), and Butyrate ($r = -0.51$; $p < 0.05$). Besides, the relative abundance of *Saccharofermentans* was negatively correlated with the concentrations of MCP ($r = -0.67$; $p < 0.001$), Acetate ($r = -0.68$; $p < 0.001$), Propionate ($r = -0.59$; $p < 0.01$) and Butyrate ($r = -0.52$; $p < 0.01$) concentrations were also negatively correlated. But the concentration of MCP was positively correlated with the concentrations of *Clostridia_UCG_014* ($r = -0.57$; $p < 0.01$), *Candidatus_Saccharimonas* ($r = -0.52$; $p < 0.01$), *Christensenellaceae_ R7_group* ($r = -0.52$; $p < 0.01$) and *Pseudobutyrivibrio* ($r = -0.67$; $p < 0.001$) were negatively correlated in relative abundance. Other negative correlations included between *Butyrivibrio* and MCP ($r = -0.61$; $p < 0.01$), acetate ($r = -0.61$; $p < 0.01$), and propionate ($r = -0.56$; $p < 0.01$; Figure 5.)

TABLE 5 Effects of dietary supplementation with NAc on the relative abundance of ruminal bacterial communities at genus level (average relative abundance ≥0.5% for at least one treatment) of dairy cows.

| Phylum | Genus | Treatments[1] | | *P*-value |
|---|---|---|---|---|
| | | CON | ACE | |
| *Bacteroidota* | *Prevotella* | 30.73 ± 5.10 | 49.71 ± 3.21 | 0.005 |
| | *Muribaculaceae* | 3.09 ± 0.81 | 2.18 ± 0.32 | 0.311 |
| | *F082* | 2.23 ± 0.50 | 2.99 ± 0.24 | 0.18 |
| | *Rikenellaceae_RC9_gut_group* | 2.27 ± 0.24 | 3.94 ± 0.34 | 0.001 |
| | *Prevotellaceae_UCG-003* | 1.41 ± 0.21 | 1.69 ± 0.16 | 0.287 |
| | *Prevotellaceae_UCG-001* | 1.24 ± 0.22 | 1.22 ± 0.14 | 0.927 |
| *Firmicutes* | *RF39* | 9.25 ± 1.78 | 3.94 ± 0.31 | 0.013 |
| | *Clostridia_UCG-014* | 8.48 ± 1.40 | 4.92 ± 0.61 | 0.035 |
| | *Succiniclasticum* | 3.46 ± 0.58 | 2.19 ± 0.40 | 0.087 |
| | *Christensenellaceae_R-7_group* | 1.66 ± 0.32 | 1.01 ± 0.12 | 0.077 |
| | *NK4A214_group* | 1.92 ± 0.26 | 1.39 ± 0.23 | 0.135 |
| | *Saccharofermentans* | 2.03 ± 0.16 | 1.40 ± 0.17 | 0.015 |
| | *Ruminococcus* | 1.09 ± 0.19 | 0.75 ± 0.10 | 0.122 |
| | *Pseudobutyrivibrio* | 0.82 ± 0.19 | 0.35 ± 0.04 | 0.034 |
| | *Selenomonas* | 0.93 ± 0.18 | 1.32 ± 0.16 | 0.123 |
| | *Eubacterium_coprostanoligenes_group* | 0.95 ± 0.14 | 0.63 ± 0.08 | 0.054 |
| | *Lachnospira* | 0.64 ± 0.12 | 0.34 ± 0.03 | 0.03 |
| | *Eubacterium_ruminantium_group* | 0.80 ± 0.09 | 0.56 ± 0.07 | 0.048 |
| | *Butyrivibrio* | 0.61 ± 0.11 | 0.28 ± 0.04 | 0.012 |
| | *Anaerovibrio* | 0.49 ± 0.07 | 0.57 ± 0.07 | 0.468 |
| *Patescibacteria* | *Candidatus_Saccharimonas* | 2.52 ± 0.67 | 0.73 ± 0.15 | 0.023 |
| | *Absconditabacteriales_(SR1)* | 1.50 ± 0.37 | 0.51 ± 0.12 | 0.026 |
| *Proteobacteria* | *Pseudomonas* | 1.23 ± 0.37 | 1.66 ± 0.50 | 0.489 |
| | *Ralstonia* | 0.69 ± 0.22 | 0.69 ± 0.24 | 0.991 |

[1]Treatments: CON = control group, basal diet plus 468 g/day oral sodium chloride; ACE = NAc group, basal diet plus 656 g/day oral NAc.

# Discussion

Acetate is the main VFA produced in the rumen, providing 50% of the energy produced by VFA metabolism and is the main source of energy and substrate for milk fat synthesis in dairy cows (Baldwin and Smith, 1971; Bergman, 1990; Urrutia et al., 2019). Numerous studies have shown that rumen infusion of acetic acid or NAc has a positive effect on milk production, milk composition and especially milk fat synthesis in lactating cows (Rook and Balch, 1961; Reynolds et al., 1979; Urrutia and Harvatine, 2017; Matamoros et al., 2021). However, there is little research on the combined effects of NAc as a supplement to diets on cows in the post-peripartum period. This study was part of a larger experiment to investigate the effects of NAc supplementation in the diet on feed intake, body weight changes, rumen fermentation, rumen

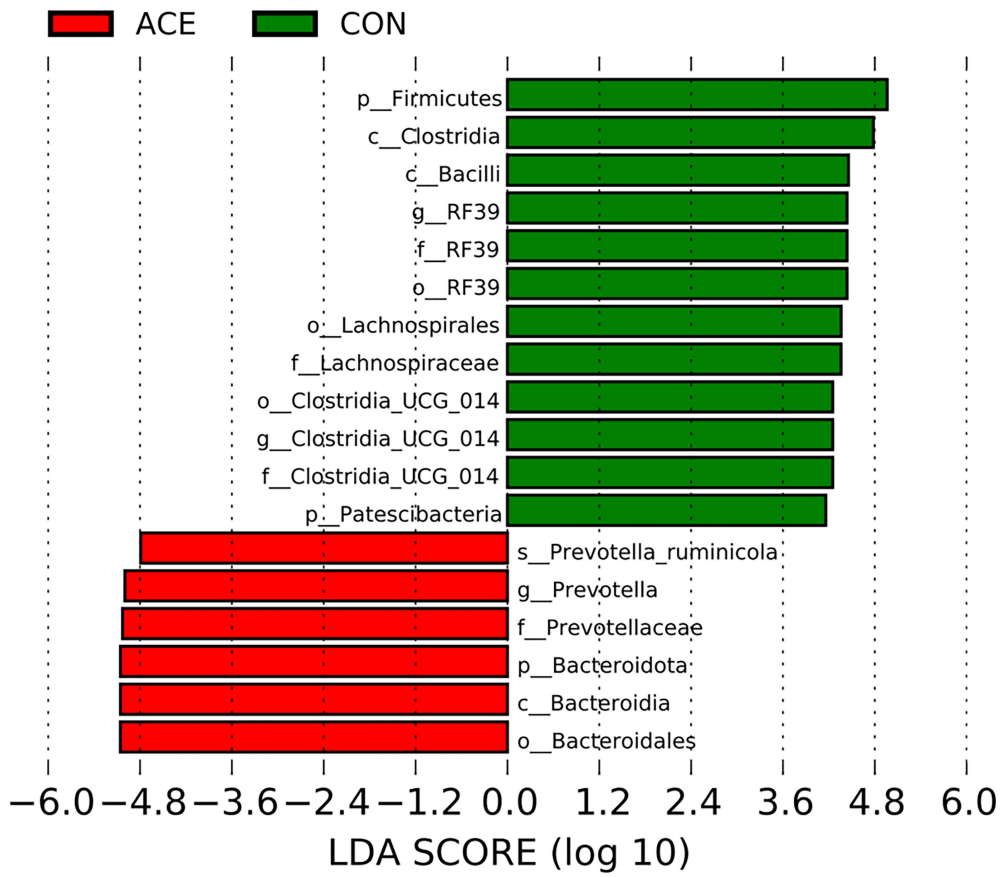


FIGURE 3
Histograms of LDA scores >4 were calculated for each taxonomic unit from phylum to genus. LDA scores indicate differences in relative abundance between two communities, with an exponential multiplicative change of 10 indicating significant differences between taxa.

microbiome, lipid metabolism, innate immunity, milk production performance, and plasma physiological and biochemical parameters in the post-peripartum period (unpublished). The objective of this study was to assess the effect of NAc supplementation on rumen fermentation parameters and bacterial community composition of Chinese Holstein cows in the post-peripartum period under the same TMR conditions, using high-throughput sequencing techniques.

The fermentation profile of rumen fluid pH, $NH_3$-N, MCP and VFA concentrations reflects the function and stability of the internal rumen environment (Zhang et al., 2022). Rumen pH is influenced by the composition of the diet and can reflect rumen fermentation and health (Wang et al., 2020). The increased concentrations of total VFA often lead to a lower rumen pH (Aschenbach et al., 2011; Fregulia et al., 2021). However, in this trial, the increase in total VFA did not change the rumen fluid pH. We speculate that the stability of rumen pH may be related to the weak alkalinity of the aqueous NAc solution, as NAc is a strong base and weak acid salt (Neavyn et al., 2013). Another significant influencing factor is the higher level of $NH_3$-N concentration in the rumen, which readily associates with protons to increase pH (Wang and Fung, 1996). The stable rumen fluid pH also suggests that NAc supplementation did not affect the normal function of rumen microorganisms.

In ruminants, nitrogen is shuttled between the portal excretory viscera (particularly the rumen) and the liver in the form of $NH_3$-N and urea, a physiological mechanism known as urea-N recycling, which is vital for the utilization of nitrogen in the diet (Lapierre and Lobley, 2001). Ruminal fluid $NH_3$-N concentration is influenced by the amount and degradability of dietary and endogenous sources of nitrogen and dietary carbohydrate sources, which reflects changes in the degree of ruminal crude protein degradation (Reynolds and Kristensen, 2008; Li et al., 2019). The use of $NH_3$-N for MCP synthesis is energy-dependent (Reynolds, 2006). Therefore, the metabolite of carbohydrates, short-chain fatty acids, can influence the concentration of $NH_3$-N. In this experiment, the addition of NAc did not affect $NH_3$-N levels but increased the concentration of MCP in the rumen fluid. The MCP is synthesized by microorganisms with $NH_3$-N and amino acids using energy to provide endogenous rumen protein, which allows cows to improve crude protein availability (Xie et al., 2019). Furthermore, the results of our additional trial (unpublished) showed that NAc supplementation increased feed intake in cows in the

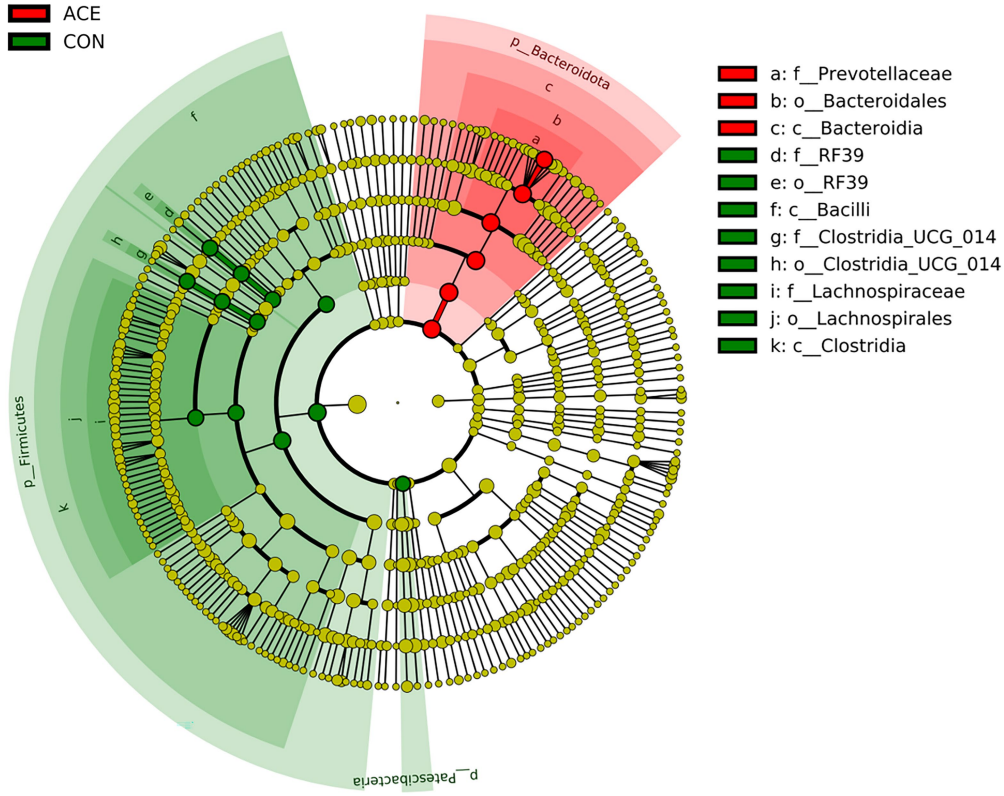


FIGURE 4
Linear discriminant analysis effect size (LEfSe) analysis to identify biomarker genera within the rumen microbial community of diets supplemented with NAc. Nodes indicate the position of microbial groups within taxa, and circles and axes indicate the number of taxa at the level of the taxon and at lower levels. Letters provided prior to the microbial taxa associated with the taxonomic level (p_: phylum; c_: class; o_: order; f_: family; g_: genus).

post-peripartum period. Hence, we suggest that NAc supplementation provides more crude protein and carbohydrates to the rumen by promoting feed intake, which increases $NH_3$-N production. Microorganisms use the $NH_3$-N, peptides and amino acids produced to synthesize MCP, which ultimately leads to increased concentrations of MCP in the rumen. However, whether this process increases the apparent digestibility of crude protein is something we need to verify further.

The VFAs produced by microbial fermentation of carbohydrates and endogenous substrates are an essential source of energy for cows, providing approximately 70% of the total energy required (Bergman, 1990). In this trial, NAc supplementation increased the concentration of rumen TVFA. More TVFA production was able to stimulate an expansion of the rumen absorption area while improving VFA absorption, which was beneficial in alleviating NEB in cows in the post-peripartum period (Melo et al., 2013; Schären et al., 2016; Dieho et al., 2017; Zhang et al., 2022). Moreover, in the NAc supplemented group, the concentrations of acetic, propionic, isobutyric and isovaleric acids were increasing, and there was a trend towards increasing concentrations of butyric and valeric acids. Similarly, a previous study reported that infusion of 36 mol/d acetic acid into cows in the post-peripartum period increased ruminal acetic, isobutyric and isovaleric acid concentrations. The molar ratios of rumen acetic, propionic and butyric acids were all unaltered in this study, unlike the previous report (Sheperd and Combs, 1998). This may be due to the fact that Sheperd et al. collected rumen fluid 2 h after the afternoon feeding when the VFAs concentrations had not reached equilibrium values for fermentation and absorption. In this experiment, the rumen was sampled in the morning before feeding, when rumen VFAs concentrations and molar ratios were in equilibrium. In addition, the addition of NAc did not alter the molar ratio of VFAs in the rumen, which may be related to the increased absorption capacity of the rumen epithelium (Zhang et al., 2022). Acetate is the primary substrate for the *ab initio* synthesis of milk fat in cows and is the main VFA produced by the rumen, which provides 45% of the energy generated by VFA metabolism (Baldwin and Smith, 1971; Bergman, 1990). Recent studies have suggested that acetate supplementation increases milk fat production primarily by increasing mammary gland neonatal lipogenesis (Urrutia and Harvatine, 2017; Urrutia et al.,

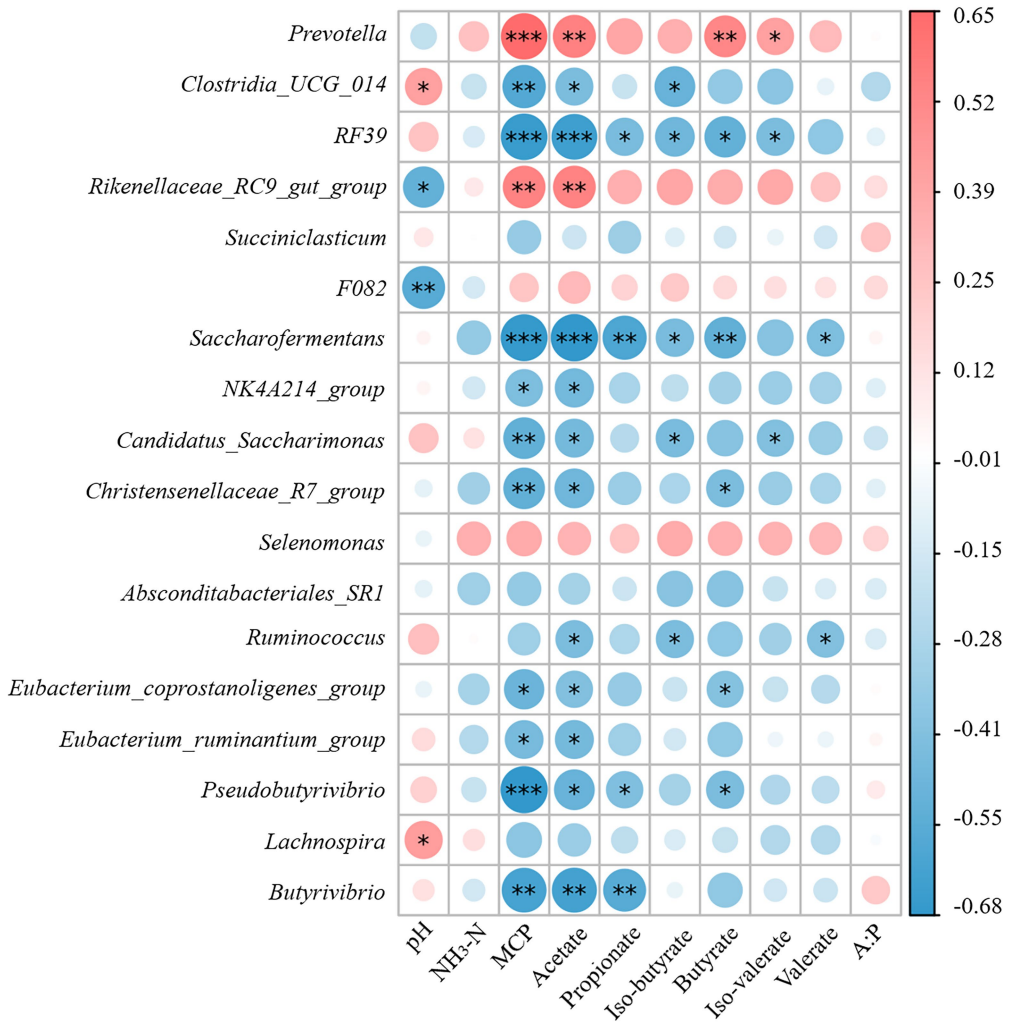


FIGURE 5
Spearman correlation between rumen fermentation indicators and bacterial populations. The area of the circle indicates the magnitude of the correlation, and the different colors indicate positive correlation (red) or negative correlation (blue). * indicates $0.01<p\leq0.05$, ** indicates $p\leq0.01$ and *** indicates $p\leq0.001$.

2019; Matamoros et al., 2021). However, it is unclear whether the use of acetate affects rumen fermentation patterns in dairy cows. Acetic acid is rapidly absorbed in the rumen, and the molar ratio of acetic acid may increase slightly after feeding, but there is no change at the time of sampling. Our results showed that supplementation with NAc increased rumen fluid TVFA concentration and kept the molar ratio constant, suggesting that supplementation with NAc promotes rumen fermentation and increases rumen VFA production.

Differences in microbial community composition were mainly attributed to diets (Ley et al., 2008; Henderson et al., 2015). However, there were few previous reports on the effects of NAc supplementation on rumen bacterial communities in dairy cows in the post-peripartum period. The results showed that NAc supplementation did not alter the α-diversity indices (chao1, observed_otus, shannon, simpson, pielou_e), indicating bacterial diversity and richness in the rumen were not affected. The influence of the relative abundance of rumen bacteria is crucial to the apparent digestibility of nutrients and rumen fermentation characteristics, which may significantly affect ruminants' productive performance and health status (Ran et al., 2021; Zhou et al., 2022). Feeding NAc did not affect the α-diversity of the rumen bacterial community, which explains the unaltered rumen $NH_3$-N and VFA molar percentages in the NAc treated group.

In contrast, the PCoA and NMDS results showed that NAc supplementation altered the structure of the rumen bacterial community. In the current results, NAc supplementation changed the relative abundance of the dominant phylum and most genera with relative abundance ≥0.5%. Although evidence that NAc supplementation affects rumen microbial communities in the post-peripartum period is lacking, it has been shown that short-chain fatty acid salt supplementation reduces bacterial diversity and alters rumen microbiota before and after the weaning of calves (Cao et al., 2020). Dietary variation has an important effect

on rumen microbial community composition. Still, the ratio of forage to concentrate and the processing method of the diet are the main factors affecting rumen microbial population structure on the same diet basis (Henderson et al., 2015; Bi et al., 2018; Zhou et al., 2022). More studies have shown that high grain diets can alter the structure of the rumen microbiota (Mao et al., 2015; Seddik et al., 2019; Plaizier et al., 2021). Ruminal hydrogenotrophic acetogens (e.g., *Acetitomaculum*) can use both inorganic ($H_2$/$CO_2$) and organic substrates (e.g., cellobiose and glucose) in the diet as energy and carbon sources to produce acetate. And the molar percentage of acetate and the molar ratio of acetate to propionate is higher in cattle adapted to fibre-rich diets (Joblin, 1999; Kim et al., 2020; Li Q. S. et al., 2022). Our results suggest that the relative abundance of rumen microbiota composition at the genus level is influenced by NAc supplementation. More often than not, the molar ratio of fermentable acids in the rumen fluid remains relatively balanced after acetic acid, a precursor substance for milk fat synthesis, is rapidly absorbed by the cow's rumen for milk fat synthesis. This study showed that supplementation of NAc in the diet altered the composition of the rumen bacterial community but not the α-diversity, which may have contributed to the increase in the concentration of VFAs in the rumen. At the same time, the molar ratio of VFAs remained unchanged.

The *Bacteroidota*, *Firmicutes*, *Patescibacteria* and *Proteobacteria* were the main gates in the rumen in this study, similar to previous studies (Seddik et al., 2019; Plaizier et al., 2021). The sequence analysis of 16S rRNA showed that *Bacteroidota* was the dominant bacterium after NAc supplementation and consisted mainly of *Prevotella*. Previous reports have demonstrated that *Prevotella* was the dominant genus in the rumen of dairy cows which was similar to the present study results (Mu et al., 2021; Plaizier et al., 2021). *Prevotella* is known to degrade starch and protein and is usually positively correlated with the levels of carbohydrate, resistant starch and fiber in the diet, and has a number of OTUs strongly correlated with feed efficiency (Fregulia et al., 2021; Tett et al., 2021). Furthermore, $NH_3$-N concentrations were significantly positively correlated with the relative abundance of *Prevotella* (Cao et al., 2020). In this study, supplementation with NAc increased the relative abundance of *Prevotella*, which explained the greater $NH_3$-N production in the rumen. Previous studies have reported that high-fat diets can increase the relative abundance of the *Bacteroidota* (Wan et al., 2019), thereby improving lipid metabolism by regulating acetate production, of which the genus *Rikenellaceae_RC9_gut_group* also promotes lipid metabolism (Yin et al., 2018; Zhou et al., 2018). In the current study, supplementation with NAc increased the relative abundance of *Rikenellaceae_RC9_gut_group*, suggesting a potential role in rumen lipid metabolism. However, the results also showed that the relative abundance of *RF39* and *Clostridia_UCG-014* was lower in the NAc group than in the control group. The *Clostridia_UCG-014* is a pro-inflammatory bacterium, and its high abundance may lead to the production of more pro-inflammatory metabolites, which are detrimental to the organism's health (Wang et al., 2021; Li M. et al., 2022). Previous studies have shown that the relative abundance of *RF39* and *Clostridia_UCG-014* is negatively correlated with milk protein production and that these bacteria have a negative role in improving rumen nitrogen metabolism. This may indicate that the addition of NAc improves rumen health, promotes nitrogen metabolism and regulates milk fat production by inhibiting *RF39* and *Clostridia_UCG-014* in *Bacteroidota*. Overall, supplementing the same TMR diet with NAc changed the composition of the microbial community at the phylum and genus level but not the α-diversity, which may improve lipid metabolism, nitrogen metabolism and rumen health.

VFAs produced by microbial fermentation of organic matter in the diet cause pH changes that affect the microbial ecosystem. And the change in pH value affects the growth of rumen microorganisms and the type and quantity of fermentation products (Aschenbach et al., 2011). By comparing Spearman correlations between rumen fermentation indicators and bacterial populations, we determined the changes in rumen microbial genus levels induced by NAc supplementation and its effect on rumen fermentation. The main fermentation products of *Prevotella* are acetic and succinic acids and, to a lesser extent, isobutyric, isovaleric and lactic acids, which are usually closely related to feed efficiency (Roager et al., 2019; Fregulia et al., 2021). Previous studies have suggested that significant increases in *Prevotella* are positively associated with voluntary feed intake, growth performance and high energy use efficiency (Zhang et al., 2019; Wu et al., 2022). It has been shown that the supplementation of acetic acid promotes the growth of *Prevotella* by promoting the synthesis of outer membrane proteins and lipid membranes (Trautmann et al., 2020). This may explain why NAc supplementation increased the relative abundance of *Prevotella* in this study, implying a positive feeding effect of NAc supplementation in the post-peripartum period cows. In addition, *RF39* and *Clostridia_UCG_014* were negatively correlated with rumen fluid VFAs and MCP concentrations, which negatively contribute to rumen nitrogen metabolism and can lead to low feed efficiency and milk protein production (Wang et al., 2021; Li M. et al., 2022). Thus, NAc supplementation may improve NEB status in late-periparturient cows by promoting the growth of *Prevotella*, which is associated with high energy efficiency and inhibiting the relative abundance of *RF39* and *Clostridia_UCG_014*, which are negatively associated with feed efficiency, thereby increasing ruminal VFAs concentrations.

In general, there was no significant change in microbial α-diversity, but microbial community composition at the phylum and genus level was altered; no change in the molar ratio of VFAs, but an increase in the concentration of VFAs; this may be due to NAc supplementation promoting rumen fermentation by altering the structure of the flora, on an identical diet TMR basis. However, it is undeniable that rumen microbial community composition does not fully represent the actual effects of NAc supplementation on the rumen of post-partum cows. Therefore, future studies involving metabolomics and *in vitro* trial validation should

be considered, and additional directions are needed to explore the specific effects of acetic acid supplementation.

## Conclusion

This study showed that supplementation of NAc to late periparturient cows on the same basal TMR diet altered microbiota and promoted ruminal fermentation. NAc supplementation had no significant effect on the α-diversity of the rumen bacterial community but upregulate the relative abundance of *Prevotella* and downregulate the relative abundance of *RF39* and *Clostridia_UCG_014*. This study will help guide future research into using NAc in alleviating NEB in post-partum period cows.

## 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://ngdc.cncb.ac.cn/gsa/, PRJCA010405.

## Ethics statement

The animal study was reviewed and approved by the Institutional Animal Care and Use Committee (SYXK (Su) IACUC 2016-0019), Yangzhou University. Written informed consent was obtained from the owners for the participation of their animals in this study.

## Author contributions

ZC and KZ designed the whole experiment and verified the validity of experiment and checked the results. ZC, ZM, and DT performed the experiment, including chemical analysis, and statistical analysis. GZ, OD, and ML worked on the manuscript. ZM, DT, and ML participated in the experiment design and gave valuable advice. All of the authors have read and approved the final version 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

The Supplementary material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2022.1053503/full#supplementary-material

## References

Aschenbach, J. R., Kristensen, N. B., Donkin, S. S., Hammon, H. M., and Penner, G. B. (2010). Gluconeogenesis in dairy cows: the secret of making sweet milk from sour dough. *IUBMB Life* 62, 869–877. doi: 10.1002/iub.400

Aschenbach, J. R., Penner, G. B., Stumpff, F., and Gäbel, G. (2011). RUMINANT NUTRITION SYMPOSIUM: role of fermentation acid absorption in the regulation of ruminal pH12. *J. Anim. Sci.* 89, 1092–1107. doi: 10.2527/jas.2010-3301

Baldwin, R. L., and Smith, N. E. (1971). Intermediary aspects and tissue interactions of ruminant fat metabolism. *J. Dairy Sci.* 54, 583–595. doi: 10.3168/jds.S0022-0302(71)85889-7

Bampidis, V., Azimonti, G., Bastos, M. D. L., Christensen, H., Dusemund, B., Fašmon Durjava, M., et al. (2021). Safety and efficacy of a feed additive consisting of acetic acid for all animal species. *EFS2* 19:6615. doi: 10.2903/j.efsa.2021.6615

Bergman, E. N. (1990). Energy contributions of volatile fatty acids from the gastrointestinal tract in various species. *Physiol. Rev.* 70, 567–590. doi: 10.1152/physrev.1990.70.2.567

Bi, Y., Zeng, S., Zhang, R., Diao, Q., and Tu, Y. (2018). Effects of dietary energy levels on rumen bacterial community composition in Holstein heifers under the same forage to concentrate ratio condition. *BMC Microbiol.* 18:69. doi: 10.1186/s12866-018-1213-9

Bickhart, D. M., and Weimer, P. J. (2018). Symposium review: host–rumen microbe interactions may be leveraged to improve the productivity of dairy cows. *J. Dairy Sci.* 101, 7680–7689. doi: 10.3168/jds.2017-13328

Bolyen, E., Rideout, J. R., Dillon, M. R., Bokulich, N. A., Abnet, C. C., Al-Ghalith, G. A., et al. (2019). Reproducible, interactive, scalable and extensible microbiome data science using QIIME 2. *Nat. Biotechnol.* 37, 852–857. doi: 10.1038/s41587-019-0209-9

Cao, N., Wu, H., Zhang, X. Z., Meng, Q. X., and Zhou, Z. M. (2020). Calcium propionate supplementation alters the ruminal bacterial and archaeal communities in pre-and postweaning calves. *J. Dairy Sci.* 103, 3204–3218. doi: 10.3168/jds.2019-16964

Chen, S., Zhou, Y., Chen, Y., and Gu, J. (2018). fastp: an ultra-fast all-in-one FASTQ preprocessor. *Bioinformatics* 34, i884–i890. doi: 10.1093/bioinformatics/bty560

Contreras, G. A., Strieder-Barboza, C., and De Koster, J. (2018). Symposium review: modulating adipose tissue lipolysis and remodeling to improve immune function during the transition period and early lactation of dairy cows. *J. Dairy Sci.* 101, 2737–2752. doi: 10.3168/jds.2017-13340

Dieho, K., van Baal, J., Kruijt, L., Bannink, A., Schonewille, J. T., Carreño, D., et al. (2017). Effect of supplemental concentrate during the dry period or early lactation on rumen epithelium gene and protein expression in dairy cattle during the transition period. *J. Dairy Sci.* 100, 7227–7245. doi: 10.3168/jds.2016-12403

Fregulia, P., Neves, A. L. A., Dias, R. J. P., and Campos, M. M. (2021). A review of rumen parameters in bovines with divergent feed efficiencies: what do these parameters tell us about improving animal productivity and sustainability? *Livest. Sci.* 254:104761. doi: 10.1016/j.livsci.2021.104761

Gao, W., Du, X., Lei, L., Wang, H., Zhang, M., Wang, Z., et al. (2018). NEFA-induced ROS impaired insulin signalling through the JNK and p38MAPK pathways in non-alcoholic steatohepatitis. *J. Cell. Mol. Med.* 22, 3408–3422. doi: 10.1111/jcmm.13617

Henderson, G., Cox, F., Ganesh, S., Jonker, A., Young, W., and Janssen, P. H. (2015). Rumen microbial community composition varies with diet and host, but a core microbiome is found across a wide geographical range. *Sci. Rep.* 5:14567. doi: 10.1038/srep14567

Ingvartsen, K. L., and Moyes, K. (2013). Nutrition, immune function and health of dairy cattle. *Animal* 7, 112–122. doi: 10.1017/S175173111200170X

Jiang, M., Datsomor, O., Cheng, Z., Meng, Z., Zhan, K., Yang, T., et al. (2022). Partial substitution of Alfalfa Hay by Stevia (*Stevia rebaudiana*) Hay can improve lactation performance, rumen fermentation, and nitrogen utilization of dairy cows. *Front. Vet. Sci.* 9:899148. doi: 10.3389/fvets.2022.899148

Joblin, K. N. (1999). Ruminal acetogens and their potential to lower ruminant methane emissions. *Aust. J. Agric. Res.* 50:1307. doi: 10.1071/AR99004

Kim, S.-H., Mamuad, L. L., Islam, M., and Lee, S.-S. (2020). Reductive acetogens isolated from ruminants and their effect on in vitro methane mitigation and milk performance in Holstein cows. *J. Anim. Sci. Technol.* 62, 1–13. doi: 10.5187/jast.2020.62.1.1

Lapierre, H., and Lobley, G. E. (2001). Nitrogen recycling in the ruminant: a review. *J. Dairy Sci.* 84, E223–E236. doi: 10.3168/jds.S0022-0302(01)70222-6

Ley, R. E., Hamady, M., Lozupone, C., Turnbaugh, P. J., Ramey, R. R., Bircher, J. S., et al. (2008). Evolution of mammals and their gut microbes. *Science* 320, 1647–1651. doi: 10.1126/science.1155725

Li, M. M., Titgemeyer, E. C., and Hanigan, M. D. (2019). A revised representation of urea and ammonia nitrogen recycling and use in the Molly cow model. *J. Dairy Sci.* 102, 5109–5129. doi: 10.3168/jds.2018-15947

Li, Q. S., Wang, R., Ma, Z. Y., Zhang, X. M., Jiao, J. Z., Zhang, Z. G., et al. (2022). Dietary selection of metabolically distinct microorganisms drives hydrogen metabolism in ruminants. *ISME J.* 16, 2535–2546. doi: 10.1038/s41396-022-01294-9

Li, M., Zhong, H., Li, M., Zheng, N., Wang, J., and Zhao, S. (2022). Contribution of ruminal bacteriome to the individual variation of nitrogen utilization efficiency of dairy cows. *Front. Microbiol.* 13:815225. doi: 10.3389/fmicb.2022.815225

Lin, M., Feng, L., Cheng, Z., and Wang, K. (2021). Effect of ethanol or lactic acid on volatile fatty acid profile and microbial community in short-term sequentially transfers by ruminal fermented with wheat straw in vitro. *Process Biochem.* 102, 369–375. doi: 10.1016/j.procbio.2020.12.018

Liu, C., Wu, H., Liu, S., Chai, S., Meng, Q., and Zhou, Z. (2019). Dynamic alterations in yak rumen bacteria community and metabolome characteristics in response to feed type. *Front. Microbiol.* 10:1116. doi: 10.3389/fmicb.2019.01116

Lopreiato, V., Mezzetti, M., Cattaneo, L., Ferronato, G., Minuti, A., and Trevisi, E. (2020). Role of nutraceuticals during the transition period of dairy cows: a review. *J. Animal. Sci. Biotechnol.* 11:96. doi: 10.1186/s40104-020-00501-x

Mao, S., Zhang, M., Liu, J., and Zhu, W. (2015). Characterising the bacterial microbiota across the gastrointestinal tracts of dairy cattle: membership and potential function. *Sci. Rep.* 5:16116. doi: 10.1038/srep16116

Matamoros, C., Cai, J., Patterson, A. D., and Harvatine, K. J. (2021). Comparison of the effects of short-term feeding of sodium acetate and sodium bicarbonate on milk fat production. *J. Dairy Sci.* 104, 7572–7582. doi: 10.3168/jds.2020-19526

Melo, L. Q., Costa, S. F., Lopes, F., Guerreiro, M. C., Armentano, L. E., and Pereira, M. N. (2013). Rumen morphometrics and the effect of digesta pH and volume on volatile fatty acid absorption 1. *J. Anim. Sci.* 91, 1775–1783. doi: 10.2527/jas.2011-4999

Mu, Y. Y., Qi, W. P., Zhang, T., Zhang, J. Y., Mei, S. J., and Mao, S. Y. (2021). Changes in rumen fermentation and bacterial community in lactating dairy cows with subacute rumen acidosis following rumen content transplantation. *J. Dairy Sci.* 104, 10780–10795. doi: 10.3168/jds.2021-20490

Neavyn, M. J., Boyer, E. W., Bird, S. B., and Babu, K. M. (2013). Sodium acetate as a replacement for sodium bicarbonate in medical toxicology: a review. *J. Med. Toxicol.* 9, 250–254. doi: 10.1007/s13181-013-0304-0

Plaizier, J. C., Danscher, A.-M., Azevedo, P. A., Derakhshani, H., Andersen, P. H., and Khafipour, E. (2021). A grain-based SARA challenge affects the composition of epimural and mucosa-associated bacterial communities throughout the digestive tract of dairy cows. *Animals* 11:1658. doi: 10.3390/ani11061658

Quast, C., Pruesse, E., Yilmaz, P., Gerken, J., Schweer, T., Yarza, P., et al. (2012). The SILVA ribosomal RNA gene database project: improved data processing and web-based tools. *Nucleic Acids Res.* 41, D590–D596. doi: 10.1093/nar/gks1219

Ran, T., Tang, S. X., Yu, X., Hou, Z. P., Hou, F. J., Beauchemin, K. A., et al. (2021). Diets varying in ratio of sweet sorghum silage to corn silage for lactating dairy cows: feed intake, milk production, blood biochemistry, ruminal fermentation, and ruminal microbial community. *J. Dairy Sci.* 104, 12600–12615. doi: 10.3168/jds.2021-20408

Reynolds, C. K. (2006). Production and metabolic effects of site of starch digestion in dairy cattle. *Anim. Feed Sci. Technol.* 130, 78–94. doi: 10.1016/j.anifeedsci.2006.01.019

Reynolds, C. K., and Kristensen, N. B. (2008). Nitrogen recycling through the gut and the nitrogen economy of ruminants: an asynchronous symbiosis 1. *J. Anim. Sci.* 86, E293–E305. doi: 10.2527/jas.2007-0475

Reynolds, P. J., Tyrrell, H. F., and Moe, P. W. (1979). Effects of ruminal infusion of acetic acid onto three Hay to concentrate ratios on apparent digestibilities and rumen parameters in cattle 1. *J. Anim. Sci.* 48, 1491–1500. doi: 10.2527/jas1979.4861491x

Roager, H. M., Vogt, J. K., Kristensen, M., Hansen, L. B. S., Ibrügger, S., Mærkedahl, R. B., et al. (2019). Whole grain-rich diet reduces body weight and systemic low-grade inflammation without inducing major changes of the gut microbiome: a randomised cross-over trial. *Gut* 68, 83–93. doi: 10.1136/gutjnl-2017-314786

Rook, J. A. F., and Balch, C. C. (1961). The effects of intraruminal infusions of acetic, propionic and butyric acids on the yield and composition of the milk of the cow. *Br. J. Nutr.* 15, 361–369. doi: 10.1079/BJN19610046

Schären, M., Seyfang, G. M., Steingass, H., Dieho, K., Dijkstra, J., Hüther, L., et al. (2016). The effects of a ration change from a total mixed ration to pasture on rumen fermentation, volatile fatty acid absorption characteristics, and morphology of dairy cows. *J. Dairy Sci.* 99, 3549–3565. doi: 10.3168/jds.2015-10450

Seddik, H., Xu, L., Wang, Y., and Mao, S. Y. (2019). A rapid shift to high-grain diet results in dynamic changes in rumen epimural microbiome in sheep. *Animal* 13, 1614–1622. doi: 10.1017/S1751731118003269

Sheperd, A. C., and Combs, D. K. (1998). Long-term effects of acetate and propionate on voluntary feed intake by midlactation cows. *J. Dairy Sci.* 81, 2240–2250. doi: 10.3168/jds.S0022-0302(98)75803-5

Sordillo, L. M., and Raphael, W. (2013). Significance of metabolic stress, lipid mobilization, and inflammation on transition cow disorders. *Vet. Clin. N. Am. Food Anim. Pract.* 29, 267–278. doi: 10.1016/j.cvfa.2013.03.002

Tett, A., Pasolli, E., Masetti, G., Ercolini, D., and Segata, N. (2021). *Prevotella* diversity, niches and interactions with the human host. *Nat. Rev. Microbiol.* 19, 585–599. doi: 10.1038/s41579-021-00559-y

Trautmann, A., Schleicher, L., Deusch, S., Gätgens, J., Steuber, J., and Seifert, J. (2020). Short-chain fatty acids modulate metabolic pathways and membrane lipids in *Prevotella bryantii* B14. *Proteomes* 8:28. doi: 10.3390/proteomes8040028

Urrutia, N., Bomberger, R., Matamoros, C., and Harvatine, K. J. (2019). Effect of dietary supplementation of sodium acetate and calcium butyrate on milk fat synthesis in lactating dairy cows. *J. Dairy Sci.* 102, 5172–5181. doi: 10.3168/jds.2018-16024

Urrutia, N. L., and Harvatine, K. J. (2017). Acetate dose-dependently stimulates Milk fat synthesis in lactating dairy cows. *J. Nutr.* 147, 763–769. doi: 10.3945/jn.116.245001

Wan, Y., Wang, F., Yuan, J., Li, J., Jiang, D., Zhang, J., et al. (2019). Effects of dietary fat on gut microbiota and faecal metabolites, and their relationship with cardiometabolic risk factors: a 6-month randomised controlled-feeding trial. *Gut* 68, 1417–1429. doi: 10.1136/gutjnl-2018-317609

Wang, J., and Fung, D. Y. C. (1996). Alkaline-fermented foods: a review with emphasis on pidan fermentation. *Crit. Rev. Microbiol.* 22, 101–138. doi: 10.3109/10408419609106457

Wang, Y., Nan, X., Zhao, Y., Jiang, L., Wang, H., Zhang, F., et al. (2021). Dietary supplementation of inulin ameliorates subclinical mastitis via regulation of rumen microbial community and metabolites in dairy cows. *Microbiol. Spectr.* 9, e00105–e00121. doi: 10.1128/Spectrum.00105-21

Wang, L., Zhang, G., Li, Y., and Zhang, Y. (2020). Effects of high forage/concentrate diet on volatile fatty acid production and the microorganisms involved in VFA production in cow rumen. *Animals* 10:223. doi: 10.3390/ani10020223

White, H. M. (2020). ADSA foundation scholar award: influencing hepatic metabolism: can nutrient partitioning be modulated to optimize metabolic health in the transition dairy cow? *J. Dairy Sci.* 103, 6741–6750. doi: 10.3168/jds.2019-18119

Wu, Q.-C., Wang, W.-K., Zhang, F., Li, W.-J., Wang, Y.-L., Lv, L.-K., et al. (2022). Dietary cysteamine supplementation remarkably increased feed efficiency and shifted rumen fermentation toward glucogenic propionate production via enrichment of *Prevotella* in feedlot lambs. *Microorganisms* 10:1105. doi: 10.3390/microorganisms10061105

Xie, Y., Wu, Z., Wang, D., and Liu, J. (2019). Nitrogen partitioning and microbial protein synthesis in lactating dairy cows with different phenotypic residual feed intake. *J. Animal. Sci. Biotechnol.* 10:54. doi: 10.1186/s40104-019-0356-3

Yin, J., Li, Y., Han, H., Chen, S., Gao, J., Liu, G., et al. (2018). Melatonin reprogramming of gut microbiota improves lipid dysmetabolism in high-fat diet-fed mice. *J. Pineal Res.* 65:e12524. doi: 10.1111/jpi.12524

Zhang, L., Jiang, X., Liu, X., Zhao, X., Liu, S., Li, Y., et al. (2019). Growth, health, rumen fermentation, and bacterial community of Holstein calves fed *Lactobacillus rhamnosus* GG during the preweaning stage 1. *J. Anim. Sci.* 97, 2598–2608. doi: 10.1093/jas/skz126

Zhang, F., Wang, Y., Wang, H., Nan, X., Guo, Y., and Xiong, B. (2022). Calcium propionate supplementation has minor effects on major ruminal bacterial community composition of early lactation dairy cows. *Front. Microbiol.* 13:847488. doi: 10.3389/fmicb.2022.847488

Zhou, L., Xiao, X., Zhang, Q., Zheng, J., Li, M., Yu, M., et al. (2018). Improved glucose and lipid metabolism in the early life of female offspring by maternal dietary genistein is associated with alterations in the gut microbiota. *Front. Endocrinol.* 9:516. doi: 10.3389/fendo.2018.00516

Zhou, J., Xue, B., Hu, A., Yue, S., Wu, M., Hong, Q., et al. (2022). Effect of dietary pe NDF levels on digestibility and rumen fermentation, and microbial community in growing goats. *Front. Microbiol.* 13:950587. doi: 10.3389/fmicb.2022.950587

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# Effects of black soldier fly larvae as protein or fat sources on apparent nutrient digestibility, fecal microbiota, and metabolic profiles in beagle dogs

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Black soldier fly (*Hermetia illucens*) larvae (BSFL) act as a biological system converting organic waste into protein and fat with great potential application as pet food. To evaluate the feasibility of BSFL as a protein and fat source, 20 healthy beagle dogs were fed three dietary treatments for 65 days, including (1) a basal diet group (CON group), (2) a basal diet that replaced 20% chicken meal with defatted black soldier fly larvae protein group (DBP group), and (3) a basal diet that replaced 8% mixed oil with black soldier fly larvae fat group (BF group). This study demonstrated that the serum biochemical parameters among the three groups were within the normal range. No difference ($p>0.05$) was observed in body weight, body condition score, or antioxidant capacity among the three groups. The mean IFN-γ level in the BF group was lower than that in the CON group, but there was no significant difference ($p>0.05$). Compared with the CON group, the DBP group had decreasing ($p<0.05$) apparent crude protein and organic matter digestibility. Furthermore, the DBP group had decreasing ($p<0.05$) fecal propionate, butyrate, total short-chain fatty acids (SCFAs), isobutyrate, isovalerate, and total branched-chain fatty acids (BCFAs) and increased ($p<0.05$) fecal pH. Nevertheless, there was no difference ($p>0.05$) in SCFAs or BCFAs between the CON and BF groups. The fecal microbiota revealed that *Lachnoclostridium*, *Clostridioides*, *Blautia*, and *Enterococcus* were significantly enriched in the DBP group, and *Terrisporobacter* and *Ralstonia* were significantly enriched in the BF group. The fecal metabolome showed that the DBP group significantly influenced 18 metabolic pathways. Integrating biological and statistical correlation analysis on differential fecal microbiota and metabolites between the CON and DBP groups found that *Lachnoclostridium*, *Clostridioides*, and *Enterococcus* were positively associated with biotin. In addition, *Lachnoclostridium*, *Clostridioides*, *Blautia*, and *Enterococcus* were positively associated with niacinamide, phenylalanine acid, fumaric acid, and citrulline and negatively

associated with cadavrine, putrescine, saccharopine, and butyrate. In all, 20% DBP restrained the apparent CP and OM digestibility, thereby affecting hindgut microbial metabolism. In contrast, 8% BF in the dog diet showed no adverse effects on body condition, apparent nutrient digestibility, fecal microbiota, or metabolic profiles. Our findings are conducive to opening a new avenue for the exploitation of DBP and BF as protein and fat resources in dog food.

KEYWORDS

**black soldier fly larvae, pet food, beagle dog, protein and fat, apparent nutrient digestibility, microbiota, metabolomics**

## Introduction

With the increase in the population of humans and animals, the shortages of protein and fat, mainly referring to fishmeal (Dawood and Koshio, 2020; Luthada-Raswiswi et al., 2021) and soybean (Herrera et al., 2022), is an urgent problem. Apart from traditional economic animals, the amount of pets, mainly dogs and cats, is also in a booming development stage, and the demand for pet food has further increased (De Marchi et al., 2018). In addition, meats, such as chicken, swine, bovine, fish, and exotic meats, and meat and bone byproducts are the major protein sources of dog food, which also fail to meet the increasing demands of the pet industry (Swanson et al., 2013). It is well known that dogs are omnivorous animals, cats are carnivorous animals, and the levels of animal protein and fat are high in their diets, which has promoted the development of new high-quality and sustainable protein and fat sources for pet food (Bosch et al., 2016). Hence, it is necessary to develop novel protein and fat sources.

Insects, which have been successfully introduced in animal diets (poultry, swine, rabbits, fish, and pets) in recent years, have bright prospects as alternate protein and fat sources (Benzertiha et al., 2020). Protein from insects shows high biological value, and fat may replace palm (i.e., kernel) fat and hence contribute to the conservation of tropical forests (Müller et al., 2017). The three most evaluated insects, black soldier fly (*Hermetia illucens*) larvae (BSFL), mealworm, and adult cricket, have high protein contents (dry matter basis) and are similar to soybean meal and meat meal (Valdés et al., 2022). Moreover, these insects are rich in essential amino acids (Bosch et al., 2014), such as aspartic acid, glutamic acid, valine, leucine, and alanine, which are similar to those of animals (Churchward-Venne et al., 2017) and have a high digestibility (76–98%; Ramos-Elorduy et al., 1997). Particularly, among these insects, BSFL is a highly investigated insect due to its strong fecundity, high conversion rate, high nutrition, low cost, and easy management (Yildirim Aksoy et al., 2020), and it is most commonly used in pet food (Valdés et al., 2022). In detail, BSFL promote environmental sustainability by converting a vast amount of low-value organic wastes, such as vegetables, fruits, and garbage, into protein and fat (Kim et al., 2011; Kelemu et al., 2015), leaving behind a compost-like residue that can be used as a soil conditioner (Somroo et al., 2019). Moreover, BSFL is rich in fatty acids (Tschirner and Simon, 2015; Ewald et al., 2020), including lauric acid, palmitic acid, oleic acid, linoleic acid, and linolenic acid, and contains abundant amino acids (Do et al., 2020), such as arginine, histidine, isoleucine, leucine, and lysine, which suggests that BSFL is an excellent raw feed material. Early studies have demonstrated that BSFL showed accelerative effects on the growth performance and nutrient digestibility of finishing pigs (Hong and Kim, 2022), was a suitable substitute for soybean meal in the diet of poultry (Józefiak et al., 2016; Mwaniki et al., 2018; Secci et al., 2018), and acted as a complementary protein source in dog diets with characteristics comparable to fish meal (Freel et al., 2021). Recently, a similar study was conducted to assess the digestibility and safety, including dry matter, protein, fat, energy, and hematology parameters of BSFL and BSFL fat, in beagle dogs, and some referential results were obtained (Freel et al., 2021). However, the investigation of BSFL as a protein or fat material in pets is highly limited and not profound enough to explore the relationship between intestinal health (Bruno et al., 2019) and metabolic variation.

Thus, the purpose of this study was to explore the effects of protein and fat isolated from BSFL on apparent nutrient digestibility, serum biochemistry, antioxidant and anti-inflammatory properties, and fecal SCFAs in dogs. In addition, we further detected fecal microbiota and metabolic profiles through 16S rRNA amplicon sequencing and ultra-performance liquid chromatography-Orbitrap-tandem mass spectrometry (UPLC-Orbitrap-MS/MS) and mined the potential relationships between microbiota and metabolites. This study is conducive to providing a new understanding of the exploitation of defatted black soldier fly larvae protein (DBP) and black soldier fly larvae (BF) as protein and fat resources.

## Materials and methods

### Preparation of defatted black soldier fly larvae protein and black soldier fly larvae fat

The DBP and BF were purchased from Guangzhou Unique Biotechnology Co., Ltd., and BSFL aged 10–12 days were reared

with kitchen waste under constant temperature and humidity (28°C, 80%). The BSFL were killed by the refrigeration technique by keeping the temperature under −20°C for 24 h and then oven drying under 65°C for 24 h. The dried BSFL were boiled in a steamer for 20–30 min and then stir-fried in a wok for 10–20 min. Afterward, the stir-fried BSFL was finely pulverized and prepared using n-hexane as the solvent in the press, and the leach solution was collected as the BF. Next, the DBP was extracted by alkaline solution and acid precipitation, dialysis desalting, and lyophilization.

## Animals, diets, and experimental design

The animal experimental procedures mentioned in this study were reviewed and approved by the Experimental Animal Ethics Committee of South China Agricultural University (approval code 2021E028).

After a month of adaptation, 20 beagle dogs [mean age: 10 months; mean body weight (BW): 12.67 ± 1.48 kg; mean body condition score (BCS): 5.84 ± 0.71] were randomly allotted to three dietary treatments according to their gender and BW. The dietary treatments included the following: (1) a basal diet group (CON group; $n = 6$, 2 male and 4 female); (2) a basal diet that replaced 20% chicken meal with 20% defatted black soldier fly larvae protein group (DBP group; $n = 7$, 3 male and 4 female); and (3) a basal diet that replaced 8% mixed oil with 8% black soldier fly larvae fat group (BF group; $n = 7$, 3 male and 4 female). These extruded diets exceeded the nutrient requirements of adult dogs recommended by the Association of American Feed Control Officials (AFFCO; AAFCO Official Publication, 2022). Table 1 shows the ingredients and nutrient levels of the experimental diets, and Supplementary Table 1 presents the proximate analysis data of chicken meal and DBP. The three kinds of dog food were made at Guangzhou Qingke Biotechnology Co., Ltd., and the experimental period lasted for 65 days, including a 5-day preliminary trial period and a 60-day formal trial period.

All dogs were housed individually in custom-made stainless steel metabolism cages (1.2 × 1.0 × 1.1 m kennels) under a constant temperature and humidity (23°C, 70%) with a 12 h light/dark cycle. A restricted diet of 130 g per dog was offered at each of the two daily meals at 8:00 am and 5:00 pm. All dogs were dewormed and vaccinated before the experiment, and no drugs were used throughout the entire experiment. All dogs were always given fresh water and toys and socialized with humans at least once a day. Final BW and BCS (Cline et al., 2021) were performed on day 65 before the morning feeding.

TABLE 1 Ingredients and nutrient levels of the experimental diets (as-fed basis, %).

| Items | CON | DBP | BF |
|---|---|---|---|
| Ingredients | | as-fed basis, % | |
| Corn | 25.00 | 25.00 | 25.00 |
| Sweet potato flour | 12.00 | 12.00 | 12.00 |
| Wheat flour | 10.00 | 10.00 | 10.00 |
| Corn gluten meal | 5.00 | 5.00 | 5. 00 |
| Beet pulp | 2.50 | 2.50 | 2.50 |
| Duck meal | 8.00 | 8.00 | 8.00 |
| Fish meal | 2.50 | 2.50 | 2.50 |
| Meat and bone meal | 3.00 | 3.00 | 3.00 |
| Calcium bicarbonate | 1.00 | 1.00 | 1.00 |
| Solid flavor enhancer | 2.00 | 2.00 | 2.00 |
| Vitamin and mineral premix[1] | 1.00 | 1.00 | 1.00 |
| Chicken meal | 20.00 | 0 | 20.00 |
| Mixed oil[2] | 8.00 | 8.00 | 0 |
| Defatted black soldier fly protein | 0 | 20.00 | 0 |
| Black solider fly fat | 0 | 0 | 8.00 |
| Nutrient levels[3] | | | |
| DM (%) | 91.20 | 91.29 | 92.66 |
| OM (%) | 93.68 | 92.49 | 93.25 |
| CP (%) | 32.72 | 30.21 | 32.55 |
| EE (%) | 14.30 | 16.15 | 15.99 |
| GE (kcal/kg) | 4759.86 | 4560.91 | 4598.08 |
| Calcium (%) | 0.57 | 0.53 | 0.50 |
| Phosphorus (%) | 0.46 | 0.42 | 0.41 |
| Calcium and phosphorus ratio | 1.24 | 1.26 | 1.22 |

CON: basal diet group; DBP: defatted black soldier fly larvae protein group; BF: black soldier fly larvae fat group; DM: dry matter; OM: organic matter; CP: crude protein; EE: ether extract; GE: gross energy.
[1]Vitamin and mineral premix provided the following per kilogram of diet: vitamin A, 2,260,000 IU; vitamin D3, 50,000 IU; vitamin E, 5,400 mg; vitamin K3, 10 mg; vitamin B1 (thiamine), 1,680 mg; vitamin B2 (riboflavin), 740 mg; vitamin B6, 840 mg; vitamin B12, 3 mg; niacin, 9,800 mg; calcium pantothenate 948 mg; biotin, 11 mg; folacin, 90 mg; choline chloride, 264,180 mg; Fe, 8,000 mg; Cu, 1,500 mg; Mn, 780 mg; Zn, 7,520 mg; I, 180 mg; Se, 30 mg.
[2]Mixed oil contained 2% fish oil and 6% chicken oil.
[3]Measured values in dry matter basis.

## Diets, feces collection and analysis

Three diet samples (100 g) were collected when each bag of the three kinds of dog food was opened throughout the experimental period. Whole feces were collected, and 10% HCl was added to the nitrogen fixation on days 62–65. Diet and feces samples were stored at −20°C, oven-dried at 65°C for 48 h and finely ground to pass through a 1-mm mesh screen for subsequent analysis. The dry matter (DM) and organic matter (OM) contents of the diet and feces samples were determined according to the methods of the Association of Official Analytical Chemists (AOAC, 2000; Hortwitz and Latimer, 2007). Based on AOAC, crude protein (CP), ether extract (EE), and gross energy (GE) were determined with a semiautomatic Kjeldahl apparatus (VAPODEST 200, C. Gerhardt GmbH & Co. KG, Germany), fatty analyzer (FT640, Grand Analytical Instrument Co., Ltd., Guangzhou, China), and oxygen bomb calorimeter [IKA C 200, IKA (Guangzhou)

Instrument Equipment Co., Ltd., Guangzhou, China], respectively. Finally, the apparent digestibility of nutrients was calculated by referencing the following formula:

Apparent nutrient digestibility (%) = (Nutrient intake – Nutrient in feces)/Nutrient intake*100 (g/d, DM basis).

## Serum sample collection and analysis

On day 65, fasting blood was collected and placed for 30 min and centrifuged at 1,811 × *g* at room temperature for 15 min. Finally, aliquots for serum biochemical, antioxidant, inflammatory parameters and metabolomics analysis were snap-frozen in liquid $N_2$ and stored at −80°C until analysis. The serum biochemical parameters, including albumin (ALB), total protein (TP), globulin (GLO), albumin/globulin (ALB/GLO), aspartate aminotransferase (AST), alanine transaminase (ALT), amylase (AMY), creatine kinase (CK), creatinine (CRE), urea nitrogen (BUN), glucose (GLU), calcium (Ca), and inorganic phosphorus (IP), were detected using an automatic biochemical analyzer (SMT-120VP, Chengdu Seamaty Technology Co., Ltd., Chengdu, China). According to the manufacturer's protocol of commercial kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, China), the serum contents of total antioxidant capacity (T-AOC), glutathione peroxidase (GSH-Px), malondialdehyde (MDA), catalase (CAT), and superoxide dismutase (SOD) were detected. Serum tumor necrosis factor-α (TNF-α), interferon-γ (IFN-γ), interleukin 6 (IL-6), IL-8, IL-10, IL-1β, immunoglobulin A (IgA), IgG, and IgM were measured using canine enzyme-linked immunosorbent assay (ELISA) kits (MEIMIAN, Jiangsu Meimian Industrial Co., Ltd., Jiangsu, China).

## Fecal 16S rRNA high-throughput sequencing analysis

On day 65, fresh fecal samples of each dog were collected within 15 min of defecation. A total of 0.5 g of each fecal sample was taken for the extraction of total fecal DNA using the CTAB method according to the manufacturer's instructions. Determination of DNA concentration and purity using a NanoDrop2000. The 16S rRNA genes of V3–V4 were amplified with the barcode using the primers 515F (5′-GTGYCAGCMGCC GCGGTAA-3′) and 805R (5′-GGACTACHVGGGTW CTAAT-3′). PCRs were carried out with approximately 10 ng of template DNA and 15 μl of Phusion® High-Fidelity PCR Master Mix (New England Biolabs) with 2 μM forward and reverse primers. The cycling parameters consisted of 98°C for 30 s, followed by 32 cycles of denaturation at 98°C for 10 s, annealing at 54°C for 30 s, and elongation at 72°C for 45 s, followed by 72°C for 10 min. PCR amplification products were detected by 2% agarose gel electrophoresis, and the target fragments were recovered using the AxyPrep PCR Cleanup Kit. The purified PCR products were quantified by a Quant-iT PicoGreen dsDNA Assay Kit on a Qbit fluorescence quantitative system, and the qualified library concentration was above 2 nM. The qualified libraries (index sequence could not be repeated) were gradient diluted, mixed according to the required amount of sequencing in proportion, and denatured by NaOH into a single chain for on-machine sequencing. A NovaSeq 6000 sequence analyzer was used for 2 × 250 bp double-end sequencing, and the corresponding reagent was a NovaSeq 6000 SP Reagent Kit (500 cycles).

After the on-board sequencing was completed, we obtained the original off-board data RawData, used overlap to splice the dual-end data, and performed quality control chimaism filtering to obtain the high-quality CleanData. DADA2 (Divisive Amplicon Denoising Algorithm; Callahan et al., 2016) no longer clusters in sequence similarity but instead clusters by dereplication (Dereplication, equivalent to clustering with 100% similarity) to obtain representative sequences with single base accuracy, which greatly improves data accuracy and species resolution. The core of DADA2 was denoised, and then amplicon sequence variants (ASVs) were constructed (Blaxter et al., 2005) to obtain the final ASV feature table and feature sequence and to further conduct diversity analysis, species classification annotation, difference analysis, etc. Based on these output-normalized data, subsequent analyses of alpha diversity and beta diversity were performed. Alpha diversity, including Observed_species, Chao1, Shannon, Simpson, and Pielou_e, was applied to analyze species diversity and richness. All these indices were calculated using QIIME 2 (Version QIIME2-202006). We calculated the linear discriminant analysis (LDA) effect size (LEfSe) using LEfSe software[1] with the default setting of LDA score ≥ 3.

## Fecal fermentation metabolite analysis

Fresh fecal samples from each dog were collected within 15 min of defecation at the end of the 65-day intervention, and pH was measured immediately after mixing the 10% fecal suspension with ultrapure water using a portable pH meter (Starter 3,100, Ohaus Instruments Co., Ltd., Shanghai, China). Fecal samples were snap-frozen in liquid $N_2$ and stored at −80°C for further analysis. The fecal short-chain fatty acids (SCFAs) and branched-chain fatty acids (BCFAs) were measured by gas chromatography–mass spectrometry (GC–MS; Shimadzu, Tokyo, Japan) with a DB-FFAP capillary column (30 m × 0.25 mm × 0.25 μm, Onlysci, China). The instrument parameters and sample processing procedures were performed according to Yang et al. (2021).

1 http://huttenhower.sph.harvard.edu/lefse/

## Fecal and serum untargeted metabolomics analysis

### Fecal untargeted metabolomics analysis

Frozen fecal samples were thawed at 4°C, and approximately 60 mg of sample was put into 2-ml round-bottom centrifuge tubes. Magnetic beads and 600 μl of methanol:water (1:1, *v*/*v*) were added to the centrifuge tubes for homogenization to extract the fecal metabolites. Ultrasonic crushing was performed at a low temperature for 10 min and placed at −20°C for 30 min. The samples were then centrifuged at 19,745 × *g* and 4°C for 15 min, and 200 μl of supernatant was dried in a vacuum centrifuge. Then, the samples were redissolved with 200 μl of methanol (chromatographic grade) water and vortexed for 2 min. After ice bath ultrasonication for 10 min at low temperature, the microcentrifuge tube was centrifuged again at 19,745 × *g* and 4°C for 15 min. The supernatant was placed in a sample bottle with a lined tube and stored at −80°C. Fecal untargeted metabolomic analysis was performed using the UPLC-Orbitrap-MS/MS system from Thermo Fisher Scientific (Q-Exactive Focus, United States).

### Serum untargeted metabolomics processing

Frozen serum samples were thawed at 4°C and vortexed for 2 min. Then, 200 μl of each serum sample was added to 800 μl of methanol (chromatographic grade). Then, the mixed solution was sequentially vortexed for 2 min and centrifuged at 19745 × *g* and 4°C for 15 min (Eppendorf, Centrifuge 5,424, Germany), after which 800 μl of the supernatant was dried in a vacuum centrifuge and processed immediately. The detection procedure was similar to that for the fecal samples.

### Ultra-performance liquid chromatography-Orbitrap-MS/MS analysis and metabolite profiling analysis

The UPLC-Orbitrap-MS/MS analysis method was described in a previous work (Xin et al., 2018). Briefly, the raw data were processed by Compound Discoverer 2.1 software (Thermo Fisher Scientific, USA) to produce a data matrix including retention time (RT), mass spectrometry (m/z), and peak intensity. Meanwhile, metabolic features with a relative standard deviation greater than 30% were excluded. Then, we searched the mzCloud and mzVault libraries to identify metabolites from these data.

Principal component analysis (PCA), orthogonal partial least squares discriminant analysis (OPLS-DA) and response permutation testing (RPT) were performed using SIMCA-P 14.1 software (Umetrics, Umea, Sweden). OPLS-DA was applied to better understand the different metabolic patterns, and RPT was conducted to examine the accuracy of the OPLS-DA models.

## MetOrigin analysis

MetOrigin is a web server analysis system that integrates microbiome and metabolome data by providing the quick identification of microbiota-related metabolites and their metabolic functions in metabolomics studies (Yu et al., 2022). We performed the origin analysis, function analysis, correlation analysis, and network summary using MetOrigin analysis. Functional analysis was performed to perform metabolic pathway enrichment analysis according to different categories of metabolites: metabolites belonging to the host, bacteria, or both. Correlation analysis was performed to determine the correlation between microbiota at different levels and metabolites by Spearman analysis. Network summary highlights the interactions with both biological and statistical signification. MetOrigin analysis is freely available at http://metorigin.met-bioinformatics.cn/.

## Statistical analysis

All data were analyzed by SPSS 26.0, graphical presentation was performed using GraphPad Prism 8.0 software, and the results were expressed as the mean ± standard error (mean ± SE). *p*-values were determined using an unpaired Student's *t test* for comparisons between two groups. $p < 0.05$ and $p < 0.10$ indicated significant differences and tendencies, respectively. Furthermore, variable importance in the projection (VIP) was calculated in the OPLS-DA model. The metabolites with VIP > 1 and $p < 0.05$ were deemed differential metabolites. The KEGG database was applied to functionally annotate these differential metabolites, which were further mapped to the KEGG pathway database using MetaboAnalyst 5.0.[2]

# Results

## Effects of DBP and BF on BW, BCS, and apparent nutrient digestibility in dogs

The effects of DBP and BF on BW, BCS, and the apparent nutrient digestibility of dogs are shown in Table 2. At the end of the experiment, BW and BCS among the three groups showed no difference ($p > 0.05$). Compared to the CON group, the apparent CP and OM digestibility in the DBP group were significantly decreased ($p < 0.05$), while apparent DM, EE, and GE digestibility were not different ($p > 0.05$). All apparent nutrient digestibilities in the BF group showed no differences compared with the CON group ($p > 0.05$).

2 https://www.metaboanalyst.ca

TABLE 2 Effects of DBP and BF on BW, BCS, and apparent nutrient digestibility in dogs.

| Items[1] | CON | DBP | BF | *p*-Value (CON/DBP) | *p*-Value (CON/BF) |
|---|---|---|---|---|---|
| BW | 13.70 ± 2.07 | 13.47 ± 2.02 | 13.14 ± 1.71 | 0.847 | 0.602 |
| BCS | 6.33 ± 1.25 | 6.21 ± 1.11 | 5.86 ± 0.56 | 0.860 | 0.381 |
| DM (%) | 82.2 ± 0.01 | 79.86 ± 0.03 | 78.91 ± 0.61 | 0.106 | 0.281 |
| OM (% DM) | 87.01 ± 0.20 | 83.93 ± 0.71 | 85.90 ± 0.98 | 0.046 | 0.203 |
| CP (% DM) | 82.46 ± 0.37 | 72.70 ± 0.85 | 79.64 ± 0.56 | 0.001 | 0.132 |
| EE (% DM) | 95.47 ± 0.81 | 95.95 ± 0.68 | 93.92 ± 0.51 | 0.226 | 0.254 |
| GE (% DM) | 87.80 ± 0.51 | 86.63 ± 0.02 | 86.01 ± 0.21 | 0.180 | 0.135 |

[1]CON: basal diet group; DBP: defatted black soldier fly larvae protein group; BF: black soldier fly larvae fat group; BW: body weight; BCS: body condition score; DM: dry matter; CP: crude protein; EE: ether extract; OM: organic matter; GE: gross energy.

## Effects of DBP and BF on serum biochemistry, antioxidant, and inflammatory parameters in dogs

The effects of DBP and BF on serum biochemistry, antioxidant, and inflammatory parameters are presented in Supplementary Table 2. Neither the DBP group nor the BF group affected the serum biochemical parameters ALB, GLO, ALB/GLO, AST, ALT, AMY, CK, CRE, BUN, GLU, Ca, and IP compared with the CON group ($p > 0.05$).

The effects of DBP and BF on serum antioxidant and inflammatory parameters are presented in Table 3. Neither the DBP group nor the BF group affected the serum antioxidant parameters GSH-Px, MDA, T-AOC, CAT, and SOD and serum inflammatory parameters TNF-α, IL-6, IL-8, IL-10, IL-1β, IgA, IgG, and IgM compared with the CON group ($p > 0.05$). However, the mean IFN-γ level in the BF group was lower than that in the CON group but showed no significant difference ($p > 0.05$).

## Effects of DBP and BF on serum metabolomics in dogs

To further explore the influence of DBP and BF on host metabolic profiles in dogs, the serum metabolome among the three groups was monitored. The PCA score plots showed no obvious separation after DBP and BF administration (Figure 1A). Ultimately, the OPLS-DA score plot demonstrated that the DBP and BF groups were separated from the CON group (Figures 1B,D). Additionally, RPT models showed that the models were reliable and had good accuracy and fitness (Figures 1C, E).

Differential serum metabolites were screened out using the standard of VIP (threshold >1) combined with the *p* value (threshold <0.05), which was applied to select the significant differential metabolites. We found no differential metabolites between the CON and DBP or BF groups, indicating that feeding 20% DBP or 8% BF had no effect on serum metabolic profiles.

## Effects of DBP and BF on the fecal microbiota in dogs

As demonstrated in Supplementary Figure 1, the Venn diagram revealed 393 shared features between the CON and DBP groups and 864 and 703 in the CON and DBP groups, respectively. The Venn analysis identified 479 shared features between the CON and BF groups and 778 and 617 in the BF and CON groups, respectively. The Venn analysis identified 423 shared features between the DBP and BF groups and 673 and 673 in the DBP and BF groups, respectively. As presented in Supplementary Figure 2A, the alpha diversity, including Observed_species, Shannon, Simpson, Chao1, Goods_coverage, and Pielou_e, of the fecal microbiota showed no difference between the CON and DBP or BF groups ($p > 0.05$). Principal component analysis revealed distinct separation among the three groups (Supplementary Figure 2B), indicating that environment and DBP or BF exhibited no influence on gut microbiota composition or diversity in dogs.

At the phylum level, Firmicutes, Bacteroidetes, Fusobacteria, Actinobacteria, and Proteobacteria were the dominant bacteria and showed no difference among the three groups (Figure 2A). At the genus level, *Fusobacterium*, *Faecalibacterium*, *Collinsella*, *Bacteroides*, *Ligilactobacillus*, *Alloprevotella*, *Blautia*, and *Phascolarctobacterium* constituted the dominant genera in the top 20 among the three groups (Figure 2B). We identified ASV biomarkers using the LEfSe algorithm. A cladogram representing the fecal microbiota and the predominant species is shown in Figures 2C,D. Compared with the CON group, the DBP group had an elevated relative abundance of *Blautia*, *Allobaculum*, *Prevotellaceae_Ga6A1_group*, *Escherichia_shigella*, *Enterococcus*, *Holdemanella*, *Lachnoclostridium*, *Erysipelotrichaceae_unclassified*, *Flavonifractor*, *Erysipelotrichaceae_UCG_003*, *Clostridia_UCG_014_unclassified*, and *Clostridium_innocuum_group* (Figure 2C). Meanwhile, the BF group was more enriched in *Terrisporobacter* and *Ralstonia* than the CON group (Figure 2D).

## Effects of DBP and BF on the fecal metabolomics in dogs

The effects of DBP and BF on fecal fermentation metabolites are shown in Figure 3. Fecal pH was markedly elevated ($p < 0.05$), and propionate, butyrate, total SCFAs, isobutyrate, isovalerate, and total BCFAs were significantly lowered in the DBP group compared to in the CON group ($p < 0.05$), while all the SCFAs and BCFAs showed no difference between the BF and CON groups ($p > 0.05$).

TABLE 3 Effects of DBP and BF on serum biochemical parameters in dogs.

| Items[1] | CON | DBP | BF | *P*-value (CON/DBP) | *P*-value (CON/BF) |
|---|---|---|---|---|---|
| GSH-Px (U) | 922.17 ± 158.94 | 952.97 ± 234.50 | 991.57 ± 189.98 | 0.792 | 0.508 |
| SOD (U/ml) | 134.59 ± 12.70 | 132.82 ± 6.91 | 121.20 ± 15.34 | 0.756 | 0.113 |
| T-AOC (mM) | 0.542 ± 0.04 | 0.545 ± 0.06 | 0.550 ± 0.10 | 0.943 | 0.861 |
| CAT (U/ml) | 2.43 ± 1.00 | 2.36 ± 1.37 | 2.29 ± 1.13 | 0.911 | 0.809 |
| MDA (nmol/ml) | 7.44 ± 5.81 | 6.04 ± 5.40 | 4.25 ± 2.12 | 0.646 | 0.593 |
| TNF-α (ng/l) | 149.82 ± 17.27 | 158.77 ± 18.28 | 159.84 ± 9.62 | 0.384 | 0.244 |
| IFN-γ (ng/l) | 39.16 ± 2.44 | 40.06 ± 3.62 | 36.45 ± 2.85 | 0.605 | 0.092[#] |
| IL-6 (g/l) | 268.10 ± 32.42 | 246.46 ± 22.73 | 268.61 ± 25.82 | 0.204 | 0.976 |
| IL-8 (ng/l) | 90.42 ± 8.19 | 94.69 ± 12.30 | 95.32 ± 9.11 | 0.472 | 0.329 |
| IL-10 (ng/l) | 47.48 ± 3.41 | 47.32 ± 4.22 | 48.06 ± 1.95 | 0.943 | 0.721 |
| IL-1β (ng/l) | 78.89 ± 7.63 | 79.13 ± 7.18 | 74.68 ± 7.75 | 0.954 | 0.346 |
| IgA (μg/ml) | 9.54 ± 1.15 | 9.69 ± 0.75 | 9.09 ± 1.03 | 0.795 | 0.473 |
| IgG (ng/l) | 46.88 ± 4.07 | 51.66 ± 2.47 | 50.18 ± 2.93 | 0.461 | 0.133 |
| IgM (ng/l) | 5.10 ± 0.42 | 5.02 ± 0.47 | 4.92 ± 0.57 | 0.748 | 0.525 |

[1]CON: basal diet group; DBP: defatted black soldier fly larvae protein group; BF: black soldier fly larvae fat group; GSH-Px: glutathione peroxidase; MDA: malondialdehyde; T-AOC: total antioxidant capacity; CAT: catalase; SOD: superoxide dismutase; IFN-γ: interferon-γ; IL-6: interleukin 6; IL-8: interleukin 8; IL-10: interleukin 10; IL-1β: interleukin 1β; IgA: immunoglobulin A; IgG: immunoglobulin G; IgM: immunoglobulin M; TNF-α: tumour necrosis factor-α. The symbol (#) indicates $p < 0.10$ calculated by Student's *t*-test.

To further investigate the effects of DBP and BF on intestinal microbiota, untargeted metabolomics techniques were used to analyze the contents of metabolites in feces. The PCA score plots showed obvious separation after DBP and BF administration (Figure 4A). In addition, the OPLS-DA model (Figures 4B,E) further separately distinguished the separation in the fecal metabolites between the CON and DBP or BF groups. The quality of the resulting discriminant models between the CON and DBP or BF groups is shown in Figures 4C,F, demonstrating that the models were reliable and predicted.

Differential fecal metabolites were screened out using the standard of VIP > 1 and $p < 0.05$. There were 54 identified potential markers between the CON and DBP groups (Supplementary Table 3), while there were no differential metabolites between the CON and BF groups. In addition, the KEGG analysis further revealed that DBP mainly impacted 18 metabolic pathways, the most dominant of which were arachidonic acid metabolism, arginine biosynthesis, pentose and glucuronate interconversions, biotin metabolism, nicotinate and nicotinamide metabolism, arginine and proline metabolism, alanine, aspartate and glutamate metabolism, and lysine degradation (Figure 4D).

## Effects of DBP and BF on the MetOrigin analysis in dogs

To further understand the association between the gut microbiota and metabolic changes, we employed MetOrigin analysis on the differential fecal microbiota and metabolites between the CON and DBP groups. A total of 49 identified metabolites were initially classified into three groups: 10 bacteria-specific metabolites, 17 bacteria-host cometabolites, and 22 others (drug, food, and unknown; Figure 5A). Origin-based metabolic pathway enrichment analysis identified 8 bacteria-specific metabolites and 25 bacteria-host metabolites (Figure 5B). Spearman analysis indicated a strong correlation between the fecal microbiota and metabolites (Figure 5C). The microbiota network of biotin metabolism illustrated that *Clostridioides, Lachnoclostridium*, and *Enterococcus* were positively associated with biotin in the microbiota network ($p < 0.05$), which had been validated by both biological and statistical correlation analysis (Figure 5D). The cometabolism network of nicotinate and nicotinamide metabolism, glutathione metabolism, lysine degradation, arginine biosynthesis, phenylalanine metabolism, styrene degradation, butanoate metabolism, and alanine, aspartate and glutamate metabolism showed that eight metabolites were biologically and statistically associated with eight differential bacteria ($p < 0.05$). Among them, six bacteria (i.e., *Lachnoclostridium*, *Clostridioides*, *Blautia*, *Enterococcus*, *Gordonibacter*, and *Flavonifractor*) and four metabolites (i.e., niacinamide, fumaric acid, citrulline, and phenylacetic acid) were upregulated, while two bacteria (i.e., *Romboutsia* and *Turicibacter*) and another four metabolites (i.e., cadaverine, putrescine, saccharopine, and butyrate) were downregulated by DBF (Figure 5E).

# Discussion

Meat and byproduct meals of poultry, cattle, pig, lamb, and fish are most commonly used as the main protein and fat sources in pet formula (Bosch and Swanson, 2021). These traditional sources of protein and fat are not sufficient to meet the demand for additional feed production; thus, there is an urgent need for alternative protein and fat sources for pet diets (Benzertiha et al.,

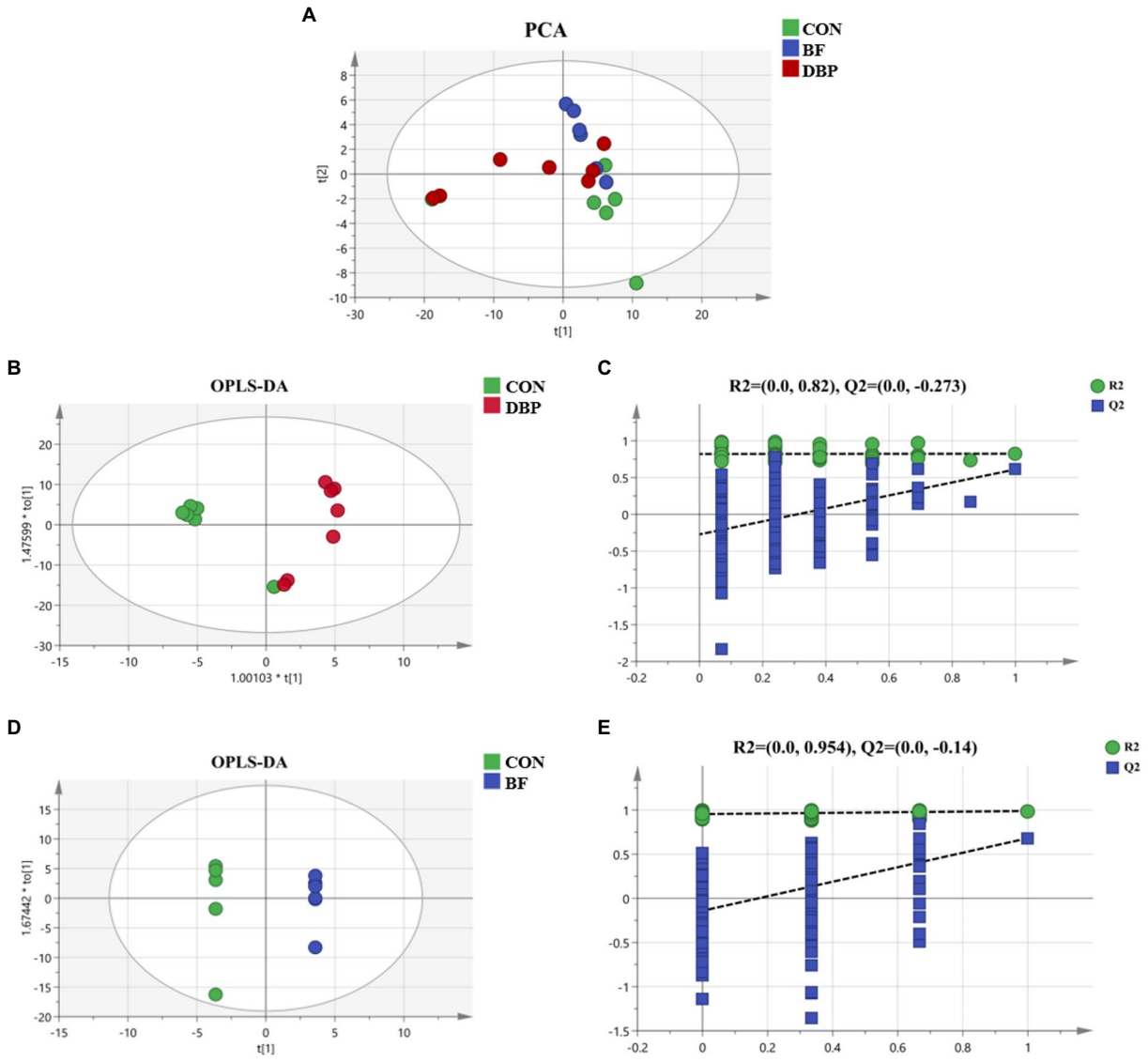


FIGURE 1
Effects of defatted black soldier fly larvae protein (DBP) and black soldier fly larvae fat (BF) on serum metabolites in dogs. Principal component analysis (PCA) of serum metabolites among the three groups **(A)**. Orthogonal partial least squares discriminant analysis (OPLS-DA) plot of serum metabolites after DBP **(B)**, and BF **(D)**, administration in dogs. Response permutation testing (RPT) derived from the DBP group **(C)**, or the BF group **(E)**, compared with the CON group.

2020). The amount of protein and fat in insects is comparable to meat (Baiano, 2020), and several novel insect protein and fat sources have been proposed since the last decade (Feng et al., 2018; Hua, 2021). Among them, BSFL has gained substantial attention worldwide as protein (Lalander et al., 2019) and fat (Spranghers et al., 2018; Pinotti et al., 2019; Kim et al., 2020; Li et al., 2022a) substitutes in pet food in recent years due to its economic, nutritional, and environmental advantages (Do et al., 2020). To our knowledge, previous studies have not systematically explored the fungibility of the protein or fat in BSFL. Hence, a 65-day randomized controlled-feeding trial among healthy dogs was implemented. We evaluated apparent nutrient digestibility, serum biochemistry, antioxidant and anti-inflammatory properties, and metabolomics, as well as fecal microbiota and metabolic profiles in the DBP and BF groups compared to the CON group.

The apparent nutrient digestibility reflects the degree of absorption and utilization of the dietary nutrient. In poultry research, DBP meals have been assessed as an excellent source of apparent metabolizable energy and ileal amino acid digestibility (Schiavone et al., 2017). A recent report demonstrated that 1% and 2% DBP elevated CP digestibility in beagle dogs (Lei et al., 2019). In contrast, our findings suggested that 20% DBP reduced the apparent CP and OM digestibility, while 8% BF had no effect on apparent nutrient digestibility. Similar to 20% DBP, previous studies in beagle dogs and cats showed that feeding 20% or 5%

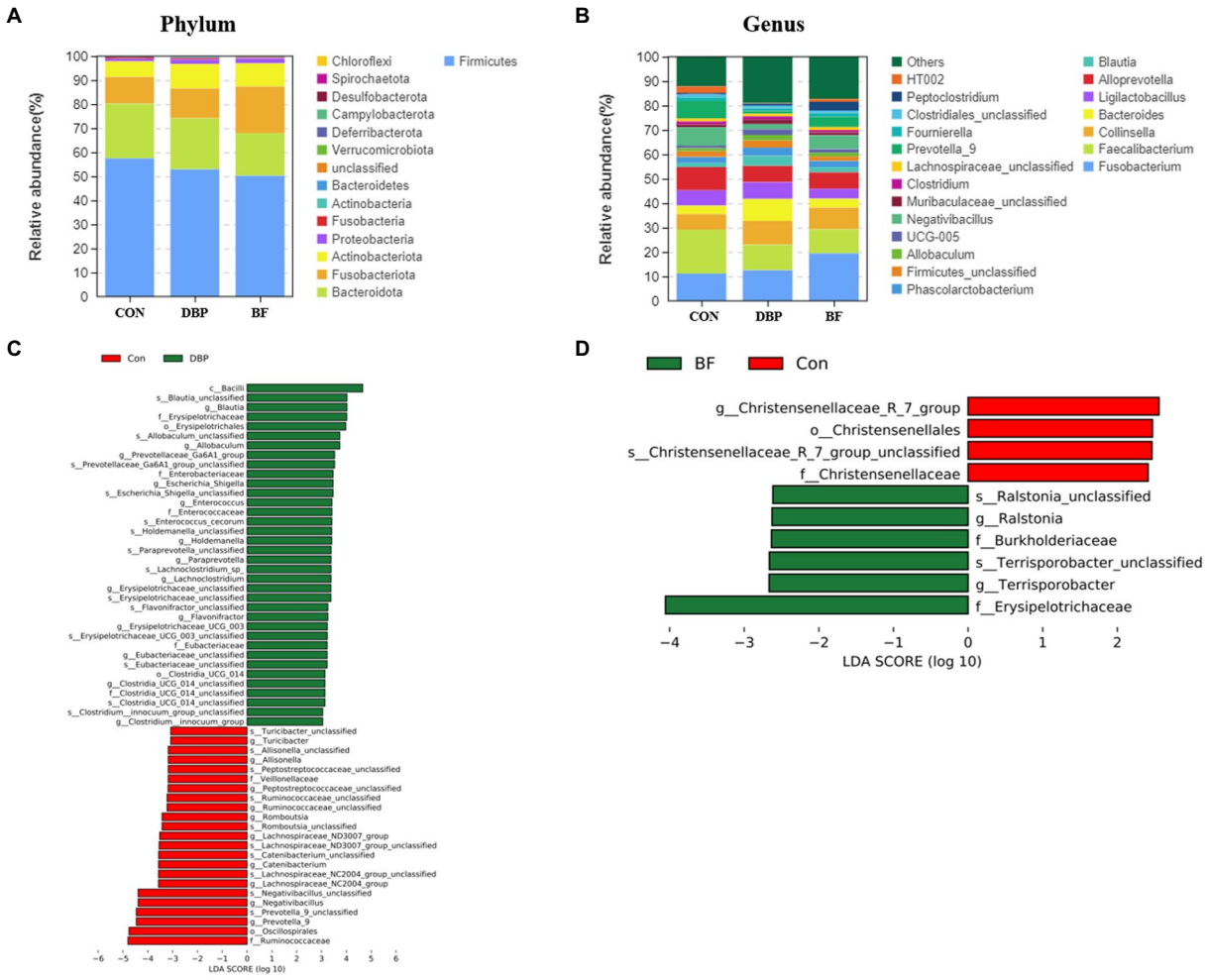


FIGURE 2
Effects of DBP and BF on gut microbiota and structure in dogs. Histogram of abundance distribution at phylum **(A)**, and genus **(B)**. The LEfSe analysis between the CON group and the DBP **(C)**, or the BF groups **(D)**.

BSFL also decreased the apparent CP digestibility (Kröger et al., 2020; Do et al., 2022). Moreover, several studies of BSFL in economic animals (Cullere et al., 2016; Hartinger et al., 2021) and cricket meal in beagle dogs (Kilburn et al., 2020) yielded similar results. Thus, we speculate that the reduction in apparent CP and OM digestibility may be related to the 4.65 ~ 6.43% concentration of chitin in the BSFL (Gariglio et al., 2019; Caimi et al., 2020). Chitin, a linear polymer of β-(1–4) N-acetyl-D-glucosamine units, has high molecular weight, poor water solubility, and protein-binding activity, which makes it difficult to be digested by monogastric animals, has an anti-nutritional effect and has a negative effect on protein digestibility (Longvah et al., 2011). Taking into account the increased proportion of chitin caused by the defatted process, about 5.0 ~ 7.21% (Schiavone et al., 2017; Traksele et al., 2021), the anti-nutritional effect of chitin could explain the reduction of apparent CP and OM digestibility. However, the underlying mechanisms remain to be elucidated (Penazzi et al., 2021).

The serum biochemistry, antioxidant, and anti-inflammatory properties and metabolomics were analyzed to confirm the safety of feeding DBP and BF. Our results revealed that the serum biochemistry and metabolomics were within the normal range with no difference among the three groups, illustrating that neither 20% DBP nor 8% BF affected the heath of all dogs in the experiment. A study by Kröger et al. (2020) reached a similar conclusion that 20% BSFL had no effect on serum biochemistry in adult dogs, and a cricket meal evaluation on beagle dogs also found that all blood values remained within desired reference intervals (Kilburn et al., 2020). In weaning piglets, 2% BF regulated serum GLO, TP, and TG, decreased IFN-γ levels and increased IL-10 and IgA levels (Yu et al., 2020). Likewise, in this study, a lower level but no significant difference in serum IFN-γ level was observed in the BF group, indicating that the anti-inflammatory potential of BF may be due to the anti-inflammatory effects of lauric acid and linoleic acid in BSFL (Kim et al., 2020; Darwish et al., 2021). In addition, a study on beagle dogs found a decreasing

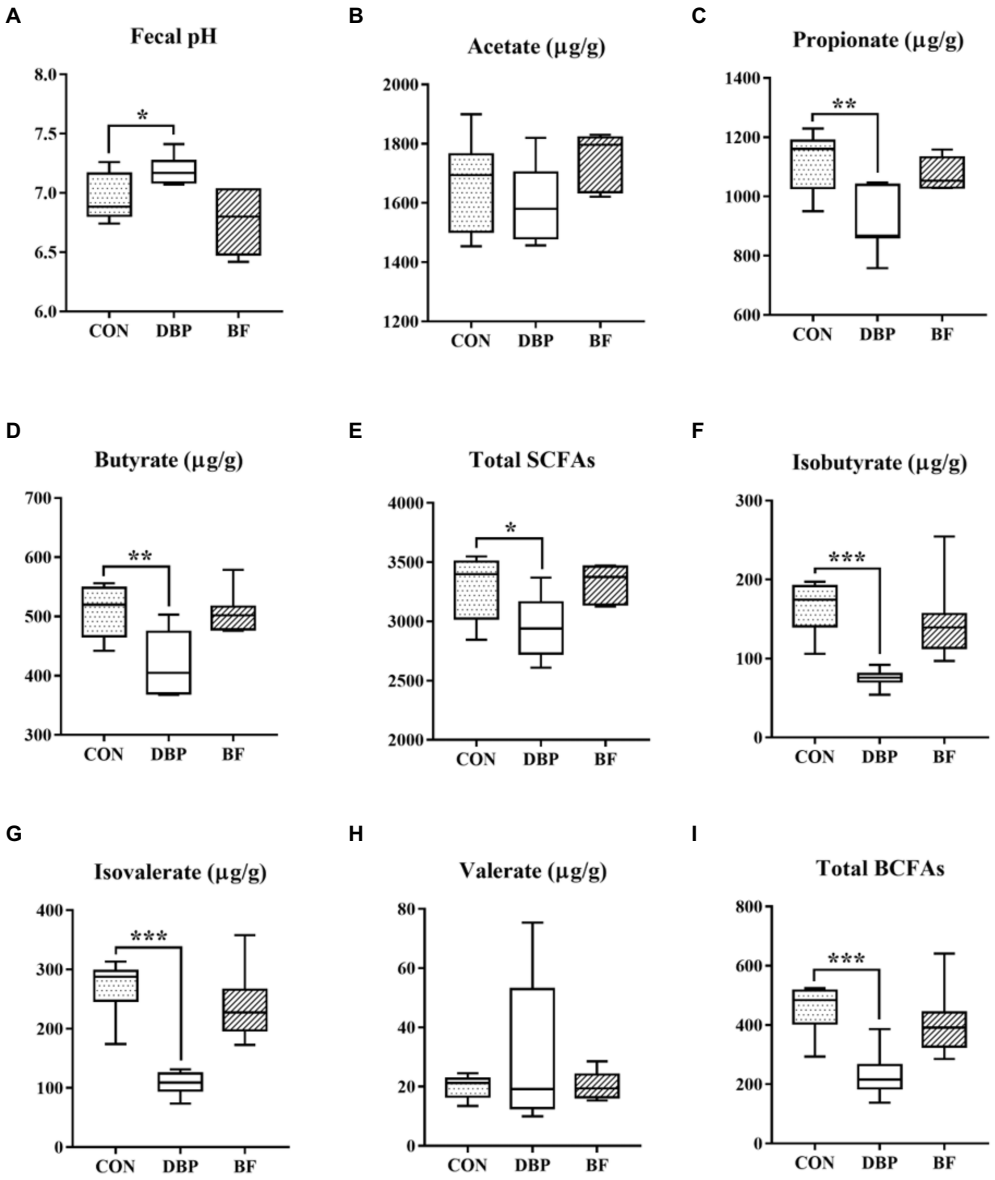


FIGURE 3
Effects of DBP and BF on fecal pH **(A)**, acetate **(B)**, propionate **(C)**, butyrate **(D)**, total SCFAs **(E)**, isobutyrate **(F)**, isovalerate **(G)**, valerate **(H)**, and total BCFAs **(I)** in dogs. Data are presented as mean±SE ($n$=6, 7, or 7). The symbol (*) indicates a significant correlation (*$p$<0.05, **$p$<0.01, and ***$p$<0.001). Total SCFAs=acetate+propionate+butyrate; Total BCFAs=isobutyrate+isovalerate+valerate.

serum TNF-α level and an increasing GSH-Px level that linearly altered with increasing DBP (0, 1, and 2%; Lei et al., 2019), and beagle dogs treated with house flies revealed a lower serum MDA level (Hong et al., 2020). However, no difference was observed between the CON and DBP groups in our study, indicating that a high proportion of DBP (20%) did not exert antioxidant or anti-inflammatory effects.

Insects are known to contain nondigestible components that are important fermentable substrates for the colonic microbiota (Bosch et al., 2016). Thus, we further explored the changes in gut microbial composition of the DBP and BF groups. No differences were noted in the α- and β-diversity of the fecal microbiota among the three groups, indicating that 20% DBP and 8% BF had no obvious effect on the gut microbial richness or diversity in adult dogs. Consistent with previous studies (Pilla and Suchodolski, 2020; Yang K. et al., 2022a,b), Firmicutes, Bacteroidetes, Fusobacteria, Actinobacteria, and Proteobacteria were the dominant bacterial phyla. Moreover, *Fusobacterium*, *Faecalibacterium*, *Collinsella*, and *Bacteroides* were the dominant bacterial genera among the three groups and showed no difference between the CON and DBP or BF groups. Upon further analysis of bacterial genera, we found that 20% DBP decreased the relative abundance of SCFA-producing bacteria, including *Prevotella_9* (Li et al., 2022b), *Lachnospiraceae_NC2004_group* (Egerton et al., 2022), *Catenibacterium* (Liu et al., 2021), *Allisonella* (Zhao et al., 2021), *Turicibacter*, and *Romboutsia* (Li et al., 2021), and a

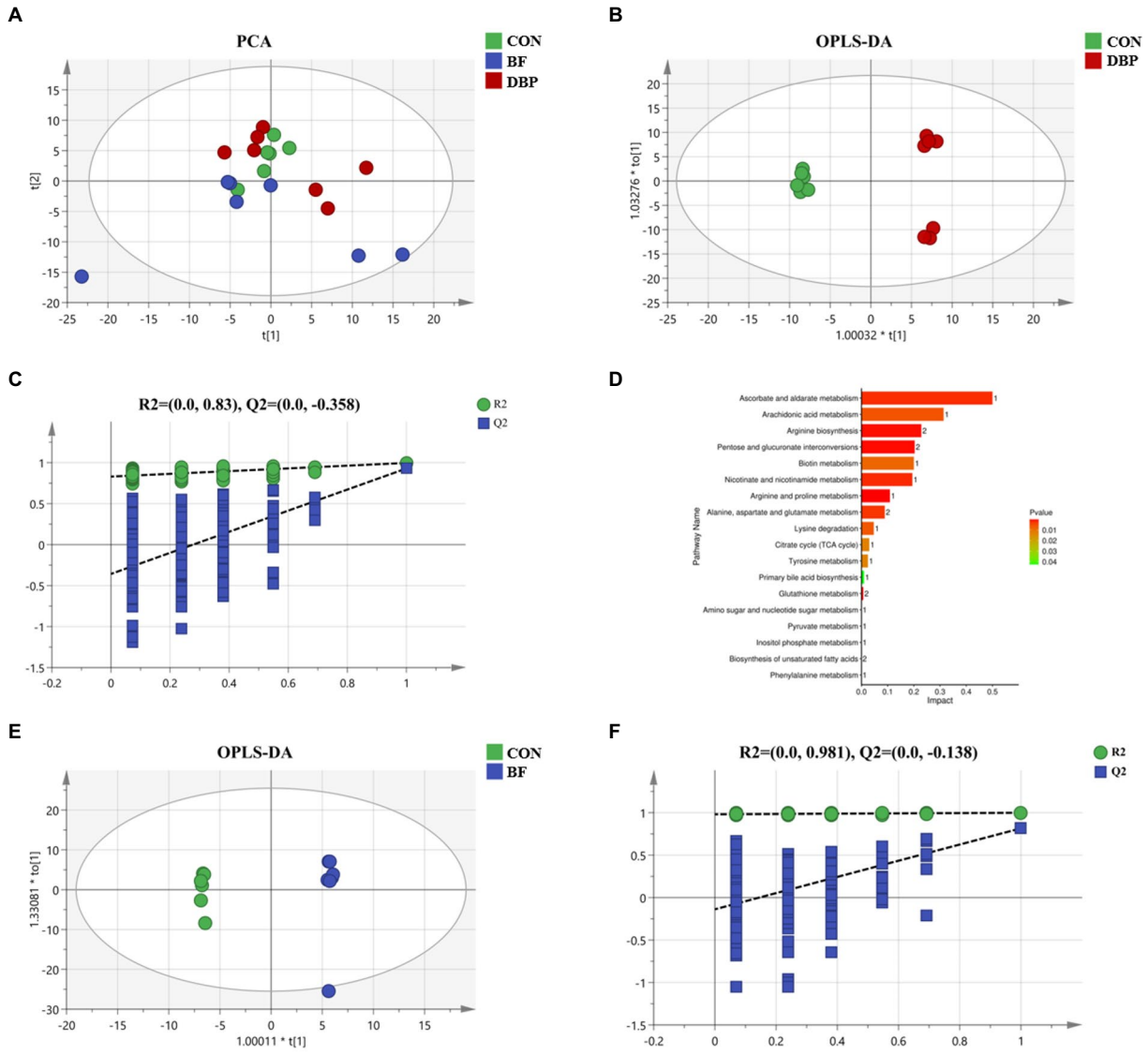


FIGURE 4
Effects of DBP and BF on fecal metabolites in dogs. PCA of fecal metabolites among the three groups **(A)**. OPLS-DA plot of fecal metabolites between the CON group and DBP group **(B)**, or the BF group **(E)**, in dogs. RPT between the CON group and the DBP group **(C)**, or the BF group **(F)**, in dogs. KEGG metabolic pathways enrichment analysis based on differential fecal metabolites after DBP treatment compared with group **(D)**.

previous study confirmed that *Flavonifractor* (Hong et al., 2021), *Blautia*, and *Enterococcus* (Kellingray et al., 2018) acted as inhibitors of butyrate, resulting in a reduction in butyrate in feces, thereby increasing fecal pH. SCFAs are produced by the microbial fermentation of undigestible carbohydrates (Kawauchi et al., 2011). Numerous studies have demonstrated the beneficial roles of SCFAs, including maintaining host immunity and nutritional metabolism (Song et al., 2021) and positively affecting the regulation of inflammation and intestinal barrier function (Liu et al., 2021). In addition, we found that 20% DBP increased the relative abundance of *Blautia*, which exhibited a negative correlation with the levels of fecal SCFAs (Pérez-Burillo et al., 2019; Lin et al., 2021). Meanwhile, increasing *Escherichia_shigella* (Cattaneo et al., 2017; Yang L. et al., 2022), *Enterococcus*, *Holdemanella* (Xu et al., 2021), *Lachnoclostridium* (Chen et al., 2021), and *Flavonifractor* (Straub et al., 2021) in the DBP group have potentially negative consequences for gut health (Sekirov et al., 2010). Unlike our results, a study of cricket on the gut microbiota in beagle dogs demonstrated that cricket decreased the abundance of *Faecalibacterium* and *Bacteroides* (Jarett et al., 2019). It is known that the gut microbiota generates BCFAs as a result of proteolysis of undigested proteins and deamination of branched-chain amino acids (Badri et al., 2021). We found that the microbial alterations obviously reduced the concentrations of isobutyrate, isovalerate, and total BCFAs in the DBP group. One of the key reasons for this was that the high proportion of chitin constrained the utilizability of protein (Gariglio et al., 2019), thereby affecting BCFA production. In brief, long-term feeding with 20% DBP may

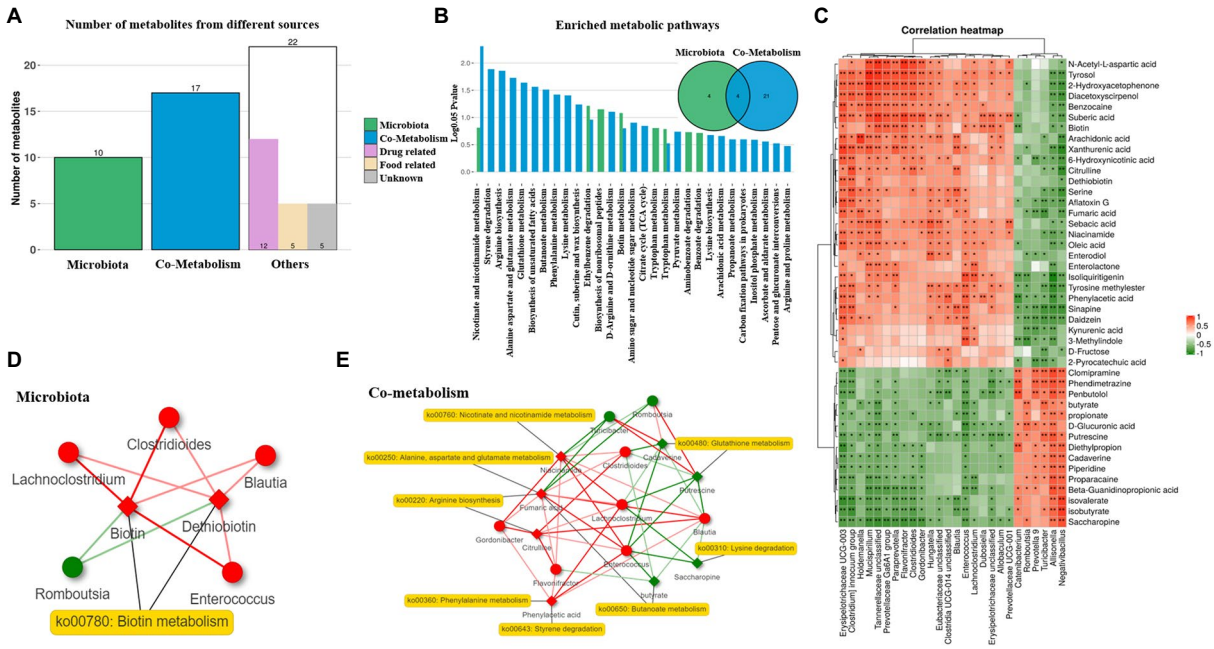


FIGURE 5
MetOrigin analysis on the differential fecal microbiota and metabolites between the CON and DBP groups. Bar plot of the number of metabolites in different categories **(A)**. Venn diagram and bar plot of the number of enriched metabolic pathways from origin-based MPEA analysis **(B)**. Correlation analysis between the differential fecal microbiota and metabolites using Spearman **(C)**. Network summary of DBP on beagle dogs for microbiota **(D)**, and co-metabolism **(E)**. Diamond and dot shapes indicate correlate metabolites and bacteria, correspondingly. The red/green color of nodes indicates up/down regulation. The red/green lines indicate the positive/negative correlations between microbes and metabolites. The symbol (*) indicates a significant correlation (*$p$<0.05 and **$p$<0.01).

have adverse effects on canine health. The present study also found that the BF group recruited more *Terrisporobacter,* which played a key role as a beneficial intestinal bacterium (Lin et al., 2020), indicating that 8% BF may act as an anti-inflammatory (a lower level of serum IFN-γ) effect by enhancing the production of beneficial bacteria.

Gut microbial metabolites are closely associated with nutritional status, metabolism, and stress response (Yang et al., 2021). Therefore, untargeted metabolomics was performed to determine the effects of DBP and BF on fecal metabolic profiles. The PCA and OPLS-DA plots showed that the DBP group rather than the BF group had a distinct separation of fecal metabolites from the CON group. To further confirm the biological relationships of metabolites to gut microbiota, MetOrigin, an interactive web server that discriminates metabolites originating from the microbiome, was applied to explore their relationship (Yu et al., 2022). From the outcome of the microbiota network *via* MetOrigin analysis, we found that feeding 20% DBP increased the relative abundances of *Lachnoclostridium*, *Clostridioides*, and *Enterococcus*, which metabolized desthiobiotin to produce biotin by secreting biotin synthase, in turn modulating the biotin metabolic pathway. Biotin, a cofactor of intermediary metabolism, is covalently attached to enzymes (Sirithanakorn and Cronan, 2021), which may have therapeutic potential for patients with inflammatory bowel disease (Skupsky et al., 2020). Moreover, in the cometabolism network, we found that *Lachnoclostridium*, *Blautia*, *Enterococcus*, and *Clostridioides* upregulated niacinamide, phenylalanine acid, fumaric acid, and citrulline and downregulated cadavrine, putrescine, saccharopine, and butyrate. Among them, glutathione metabolism and nicotinate and nicotinamide metabolism participate in antioxidant activity (Seo et al., 2018) and protect against aging (Si et al., 2019), respectively, which may benefit gut health in dogs. Moreover, 20% DBP increased the relative abundances of *Flavonifractor*, *Blautia*, and *Enterococcus*, which inhibited the secretion of acetoacetate CoA-transferase, thereby reducing the production of butyrate (Kellingray et al., 2018; Hong et al., 2021). This further verified our conclusion.

Overall, our findings confirmed that 20% DBP restrained the apparent CP and OM digestibility, thereby affecting hindgut microbial metabolism, while 8% BF had no negative effect on canine gut health. Specifically, taking into account the increased proportion of chitin caused by the defatted process, continued efforts are warranted in understanding the chitin effects. We suggest that adding high-quality chitinase to dog food may be an effective way to improve the apparent nutrient digestibility of DBP.

## Conclusion

The current findings suggested that neither 20% DBP nor 8% BF affected the body condition of all dogs in this experiment. 20%

DBP had decreasing apparent CP and OM digestibility on day 65, while 8% BF had no effect on apparent nutrient digestibility. Furthermore, the DBP group had decreasing fecal propionate, butyrate, total SCFAs, isobutyrate, isovalerate, and total BCFAs and increased fecal pH. Nevertheless, there was no difference in SCFAs and BCFAs between the CON and BF groups. The fecal microbiota revealed that *Lachnoclostridium*, *Clostridioides*, *Blautia*, and *Enterococcus* were enriched in the DBP group, and *Terrisporobacter* and *Ralstonia* were enriched in the BF group. The fecal metabolome further showed that the DBP group significantly influenced 18 metabolic pathways. Additionally, MetOrigin analysis between the CON and DBP groups found that *Lachnoclostridium*, *Clostridioides*, and *Enterococcus* were positively associated with biotin. In addition, *Lachnoclostridium*, *Clostridioides*, *Blautia*, and *Enterococcus* were positively associated with niacinamide, phenylalanine acid, fumaric acid, and citrulline and negatively associated with cadavrine, putrescine, saccharopine, and butyrate. Overall, 20% DBP restrained the apparent CP and OM digestibility, thereby affecting hindgut microbial metabolism. In contrast, 8% BF in the dog diet showed no adverse effects on body condition, apparent nutrient digestibility, fecal microbiota, or metabolic profiles. Our findings are conducive to opening a new avenue for the exploitation of DBP and BF as protein and fat resources in dog food.

## Data availability statement

The 16S rRNA data presented in the study are deposited in the NCBI repository, accession number: https://www.ncbi.nlm.nih.gov/, PRJNA865813. The untargeted metabolomic data presented in the study are deposited in the EMBL-EBI MetaboLights repository with the identifier MTBLS6097 (serum metabolomics) and MTBLS6102 (fecal metabolomics), accession numbers corresponding to the following: https://www.ebi.ac.uk/metabolights/MTBLS6097 and https://www.ebi.ac.uk/metabolights/MTBLS6102.

## Ethics statement

The animal study was reviewed and approved by Experimental Animal Ethics Committee of South China Agricultural University (approval code 2021E028).

## Author contributions

SJ and LZ designed the study and performed the experiments. SJ detected the samples, analyzed the data, and prepared the manuscript. ND, KY and ZX carried out the assays described in the study. MH, ZdZ and ZhZ contributed to the data analysis. KY and BD revised the manuscript. JD and BD provided the funding and resources. All authors contributed to the article and approved the submitted version.

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## Conflict of interest

LZ is employed by Guangzhou Qingke Biotechnology Co., Ltd., ND is employed by Guangzhou Customs Technology Center, and MH, ZdZ, and ZhZ are employed by Guangzhou GeneralPharmaceutical Research Institute Co., Ltd. (National CanineLaboratory Animal Resources Center).

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/fmicb.2022.1044986/full#supplementary-material

# References


AAFCO Official Publication (2022). *Association of American Feed Control Official*. AAFCO Official Publication: Oxford, IN.

Badri, D. V., Jackson, M. I., and Jewell, D. E. (2021). Dietary protein and carbohydrate levels affect the gut microbiota and clinical assessment in healthy adult cats. *J. Nutr.* 151, 3637–3650. doi: 10.1093/jn/nxab308

Baiano, A. (2020). Edible insects: an overview on nutritional characteristics, safety, farming, production technologies, regulatory framework, and socio-economic and ethical implications. *Trends Food Sci. Tech.* 100, 35–50. doi: 10.1016/j.tifs.2020.03.040

Benzertiha, A., Kierończyk, B., Rawski, M., Mikołajczak, Z., Urbański, A., Nogowski, L., et al. (2020). Insect fat in animal nutrition-a review. *Ann. Anim. Sci.* 20, 1217–1240. doi: 10.2478/aoas-2020-0076

Blaxter, M., Mann, J., Chapman, T., Thomas, F., Whitton, C., Floyd, R., et al. (2005). Defining operational taxonomic units using DNA barcode data. *Philos. T. R. Soc. B.* 360, 1935–1943. doi: 10.1098/rstb.2005.1725

Bosch, G., and Swanson, K. S. (2021). Effect of using insects as feed on animals: pet dogs and cats. *J. Insects Food Feed* 7, 795–805. doi: 10.3920/JIFF2020.0084

Bosch, G., Vervoort, J. J. M., and Hendriks, W. H. (2016). In vitro digestibility and fermentability of selected insects for dog foods. *Anim. Feed Sci. Tech.* 221, 174–184. doi: 10.1016/j.anifeedsci.2016.08.018

Bosch, G., Zhang, S., Oonincx, D. G., and Hendriks, W. H. (2014). Protein quality of insects as potential ingredients for dog and cat foods. *J. Nutr. Sci.* 3:e29. doi: 10.1017/jns.2014.23

Bruno, D., Bonelli, M., De Filippis, F., Di Lelio, I., Tettamanti, G., Casartelli, M., et al. (2019). The intestinal microbiota of *Hermetia illucens* larvae is affected by diet and shows a diverse composition in the different midgut regions. *Appl. Environ. Microb.* 85, e1818–e1864. doi: 10.1128/AEM.01864-18

Caimi, C., Renna, M., Lussiana, C., Bonaldo, A., Gariglio, M., Meneguz, M., et al. (2020). First insights on black soldier fly (*Hermetia illucens* L.) larvae meal dietary administration in Siberian sturgeon (*Acipenser baerii* Brandt) juveniles. *Aquaculture* 515:734539. doi: 10.1016/j.aquaculture.2019.734539

Callahan, B. J., McMurdie, P. J., Rosen, M. J., Han, A. W., Johnson, A. J. A., and Holmes, S. P. (2016). DADA2: high-resolution sample inference from Illumina amplicon data. *Nat. Methods* 13, 581–583. doi: 10.1038/nmeth.3869

Cattaneo, A., Cattane, N., Galluzzi, S., Provasi, S., Lopizzo, N., Festari, C., et al. (2017). Association of brain amyloidosis with pro-inflammatory gut bacterial taxa and peripheral inflammation markers in cognitively impaired elderly. *Neurobiol. Aging* 49, 60–68. doi: 10.1016/j.neurobiolaging.2016.08.019

Chen, W., Zhang, M., Guo, Y., Wang, Z., Liu, Q., Yan, R., et al. (2021). The profile and function of gut microbiota in diabetic nephropathy. *Diabet. Metab. Synd Ob.*, 4283–4296. doi: 10.2147/DMSO.S320169

Churchward-Venne, T. A., Pinckaers, P., van Loon, J., and van Loon, L. (2017). Consideration of insects as a source of dietary protein for human consumption. *Nutr. Rev.* 75, 1035–1045. doi: 10.1093/nutrit/nux057

Cline, M. G., Burns, K. M., Coe, J. B., Downing, R., Durzi, T., Murphy, M., et al. (2021). 2021 AAHA nutrition and weight management guidelines for dogs and cats. *J. Am. Anim. Hosp. Assoc.* 57, 153–178. doi: 10.5326/JAAHA-MS-7232

Cullere, M., Tasoniero, G., Giaccone, V., Miotti-Scapin, R., Claeys, E., De Smet, S., et al. (2016). Black soldier fly as dietary protein source for broiler quails: apparent digestibility, excreta microbial load, feed choice, performance, carcass and meat traits. *Animal* 10, 1923–1930. doi: 10.1017/S1751731116001270

Darwish, R. S., Shawky, E., Nassar, K. M., Rashad ElSayed, R. M., Hussein, D. E., Ghareeb, D. A., et al. (2021). Differential anti-inflammatory biomarkers of the desert truffles *Terfezia claveryi* and *Tirmania nivea* revealed via UPLC-QqQ-MS-based metabolomics combined to chemometrics. *LWT-Food Sci. Technol.* 150:111965. doi: 10.1016/j.lwt.2021.111965

Dawood, M. A. O., and Koshio, S. (2020). Application of fermentation strategy in aquafeed for sustainable aquaculture. *Rev. Aquacult.* 12, 987–1002. doi: 10.1111/raq.12368

De Marchi, M., Righi, F., Meneghesso, M., Manfrin, D., and Ricci, R. (2018). Prediction of chemical composition and peroxide value in unground pet foods by near-infrared spectroscopy. *J. Anim. Physiol. An. N.* 102, 337–342. doi: 10.1111/jpn.12663

Do, S., Koutsos, E. A., McComb, A., Phungviwatnikul, T., de Godoy, M. R. C., and Swanson, K. S. (2022). Palatability and apparent total tract macronutrient digestibility of retorted black soldier fly larvae-containing diets and their effects on the fecal characteristics of cats consuming them. *J. Anim. Sci.* 100:c68. doi: 10.1093/jas/skac068

Do, S., Koutsos, L., Utterback, P. L., Parsons, C. M., de Godoy, M. R. C., and Swanson, K. S. (2020). Nutrient and AA digestibility of black soldier fly larvae differing in age using the precision-fed cecectomized rooster assay1. *J. Anim. Sci.* 98:z363. doi: 10.1093/jas/skz363

Egerton, S., Donoso, F., Fitzgerald, P., Gite, S., Fouhy, F., Whooley, J., et al. (2022). Investigating the potential of fish oil as a nutraceutical in an animal model of early life stress. *Nutr. Neurosci.* 25, 356–378. doi: 10.1080/1028415X.2020.1753322

Ewald, N., Vidakovic, A., Langeland, M., Kiessling, A., Sampels, S., and Lalander, C. (2020). Fatty acid composition of black soldier fly larvae (*Hermetia illucens*)-possibilities and limitations for modification through diet. *Waste Manag.* 102, 40–47. doi: 10.1016/j.wasman.2019.10.014

Feng, Y., Chen, X., Zhao, M., He, Z., Sun, L., Wang, C., et al. (2018). Edible insects in China: utilization and prospects. *Insect Sci.* 25, 184–198. doi: 10.1111/1744-7917.12449

Freel, T. A., McComb, A., and Koutsos, E. A. (2021). Digestibility and safety of dry black soldier fly larvae meal and black soldier fly larvae oil in dogs. *J. Anim. Sci.* 99:b47. doi: 10.1093/jas/skab047

Gariglio, M., Dabbou, S., Biasato, I., Capucchio, M. T., Colombino, E., Hernandez, F., et al. (2019). Nutritional effects of the dietary inclusion of partially defatted Hermetia illucens larva meal in Muscovy duck. *J Anim Sci Biotechnol.* 10:37. doi: 10.1186/s40104-019-0344-7

Hartinger, K., Greinix, J., Thaler, N., Ebbing, M. A., Yacoubi, N., Schedle, K., et al. (2021). Effect of graded substitution of soybean meal by *Hermetia illucens* larvae meal on animal performance, apparent ileal digestibility, gut histology and microbial metabolites of broilers. *Animals* 11:1628. doi: 10.3390/ani11061628

Herrera, E., Petrusan, J., Salvá-Ruiz, B., Novak, A., Cavalcanti, K., Aguilar, V., et al. (2022). Meat quality of Guinea pig (*Cavia porcellus*) fed with black soldier fly larvae meal (*Hermetia illucens*) as a protein source. *Sustainability-Basel.* 14:1292. doi: 10.3390/su14031292

Hong, J., and Kim, Y. Y. (2022). Insect as feed ingredients for pigs. *Anim. Biosci.* 35, 347–355. doi: 10.5713/ab.21.0475

Hong, D., Yoo, M., Heo, K., Shim, J., and Lee, J. (2021). Effects of L. Plantarum HY7715 on the gut microbial community and riboflavin production in a three-stage semi-continuous simulated gut system. *Microorganisms.* 9:2478. doi: 10.3390/microorganisms9122478

Hong, Y., Zhou, J., Yuan, M., Dong, H., Cheng, G., Wang, Y., et al. (2020). Dietary supplementation with housefly (Musca domestica) maggot meal in growing beagles: hematology, serum biochemistry, immune responses and oxidative damage. *Ann. Anim. Sci.* 20, 1351–1364. doi: 10.2478/aoas-2020-0045

Hortwitz, W., and Latimer, G. (2007). *Official methods of analysis of AOAC international*. AOAC International: Gaithersburg, MD.

Hua, K. (2021). A meta-analysis of the effects of replacing fish meals with insect meals on growth performance of fish. *Aquaculture* 530:735732. doi: 10.1016/j.aquaculture.2020.735732

Jarett, J. K., Carlson, A., Rossoni Serao, M., Strickland, J., Serfilippi, L., and Ganz, H. H. (2019). Diets with and without edible cricket support a similar level of diversity in the gut microbiome of dogs. *PeerJ.* 7:e7661. doi: 10.7717/peerj.7661

Józefiak, D., Józefiak, A., Kierończyk, B., Rawski, M., Świątkiewicz, S., Długosz, J., et al. (2016). Insects-a natural nutrient source for poultry-a review. *Ann. Anim. Science.* 16, 297–313. doi: 10.1515/aoas-2016-0010

Kawauchi, I. M., Sakomura, N. K., Vasconcellos, R. S., De-Oliveira, L. D., Gomes, M. O. S., Loureiro, B. A., et al. (2011). Digestibility and metabolizable energy of maize gluten feed for dogs as measured by two different techniques. *Anim. Feed Sci. Tech.* 169, 96–103. doi: 10.1016/j.anifeedsci.2011.05.005

Kelemu, S., Niassy, S., Torto, B., Fiaboe, K., Affognon, H., Tonnang, H., et al. (2015). African edible insects for food and feed: inventory, diversity, commonalities and contribution to food security. *J. Insects Food Feed* 1, 103–119. doi: 10.3920/JIFF2014.0016

Kellingray, L., Gall, G. L., Defernez, M., Beales, I. L. P., Franslem-Elumogo, N., and Narbad, A. (2018). Microbial taxonomic and metabolic alterations during faecal microbiota transplantation to treat infection. *J. Infection.* 77, 107–118. doi: 10.1016/j.jinf.2018.04.012

Kilburn, L. R., Carlson, A. T., Lewis, E., and Serao, M. C. R. (2020). Cricket (Gryllodes sigillatus) meal fed to healthy adult dogs does not affect general health and minimally impacts apparent total tract digestibility. *J. Anim. Sci.* 98:a83. doi: 10.1093/jas/skaa083

Kim, W., Bae, S., Park, K., Lee, S., Choi, Y., Han, S., et al. (2011). Biochemical characterization of digestive enzymes in the black soldier fly, *Hermetia illucens* (Diptera: Stratiomyidae). *J. Asia Pac. Entomol.* 14, 11–14. doi: 10.1016/j.aspen.2010.11.003

Kim, B., Bang, H. T., Kim, K. H., Kim, M. J., Jeong, J. Y., Chun, J. L., et al. (2020). Evaluation of black soldier fly larvae oil as a dietary fat source in broiler chicken diets. *J. Anim. Sci. Technol.* 62, 187–197. doi: 10.5187/jast.2020.62.2.187

Kim, S. M., Park, E., Kim, J., Choi, J., and Lee, H. (2020). Anti-inflammatory effects of fermented lotus root and linoleic acid in lipopolysaccharide-induced RAW 264.7 cells. *Life Basel.* 10:293. doi: 10.3390/life10110293

Kröger, S., Heide, C., and Zentek, J. (2020). Evaluation of an extruded diet for adult dogs containing larvae meal from the black soldier fly (*Hermetia illucens*). *Anim. Feed Sci. Tech.* 270:114699. doi: 10.1016/j.anifeedsci.2020.114699

Lalander, C., Diener, S., Zurbrügg, C., and Vinneras, B. (2019). Effects of feedstock on larval development and process efficiency in waste treatment with black soldier fly (*Hermetia illucens*). *J. Clean. Prod.* 208, 211–219. doi: 10.1016/j.jclepro.2018.10.017

Lei, X. J., Kim, T. H., Park, J. H., and Kim, I. H. (2019). Evaluation of supplementation of defatted black soldier fly (*Hermetia illucens*) larvae meal in beagle dogs. *Ann. Anim. Sci.* 19, 767–777. doi: 10.2478/aoas-2019-0021

Li, X., Dong, Y., Sun, Q., Tan, X., You, C., Huang, Y., et al. (2022a). Growth and fatty acid composition of black soldier fly *Hermetia illucens* (Diptera: Stratiomyidae) larvae are influenced by dietary fat sources and levels. *Animals* 12:486. doi: 10.3390/ani12040486

Li, X., Wang, Q., Wu, D., Zhang, D. W., Li, S. C., Zhang, S. W., et al. (2022b). The effect of a novel anticonvulsant chemical Q808 on gut microbiota and hippocampus neurotransmitters in pentylenetetrazole-induced seizures in rats. *BMC Neurosci.* 23:7. doi: 10.1186/s12868-022-00690-3

Li, Y., Zafar, S., Salih Ibrahim, R. M., Chi, H., Xiao, T., Xia, W., et al. (2021). Exercise and food supplement of vitamin C ameliorate hypertension through improvement of gut microflora in the spontaneously hypertensive rats. *Life Sci.* 269:119097. doi: 10.1016/j.lfs.2021.119097

Lin, C., Wei, Z., Yi, Z., Tingting, T., Huamao, D., and Lichun, F. (2020). Analysis of the effects of nanosilver on bacterial community in the intestinal fluid of silkworms using high-throughput sequencing. *B. Entomol. Res.* 110, 309–320. doi: 10.1017/S0007485319000634

Lin, W., Wen, L., Wen, J., and Xiang, G. (2021). Effects of sleeve gastrectomy on fecal gut microbiota and short-chain fatty acid content in a rat model of polycystic ovary syndrome. *Front. Endocrinol.* 12:747888. doi: 10.3389/fendo.2021.747888

Liu, C., Du, P., Cheng, Y., Guo, Y., Hu, B., Yao, W., et al. (2021). Study on fecal fermentation characteristics of aloe polysaccharides in vitro and their predictive modeling. *Carbohyd. Polym.* 256:117571. doi: 10.1016/j.carbpol.2020.117571

Liu, P., Wang, Y., Yang, G., Zhang, Q., Meng, L., Xin, Y., et al. (2021). The role of short-chain fatty acids in intestinal barrier function, inflammation, oxidative stress, and colonic carcinogenesis. *Pharmacol. Res.* 165:105420. doi: 10.1016/j.phrs.2021.105420

Longvah, T., Mangthya, K., and Ramulu, P. (2011). Nutrient composition and protein quality evaluation of eri silkworm (*Samia ricinii*) prepupae and pupae. *Food Chem.* 128, 400–403. doi: 10.1016/j.foodchem.2011.03.041

Luthada-Raswiswi, R., Mukaratirwa, S., and O'Brien, G. (2021). Animal protein sources as a substitute for fishmeal in aquaculture diets: a systematic review and meta-analysis. *Appl. Sci.* 11:3854. doi: 10.3390/app11093854

Müller, A., Wolf, D., and Gutzeit, H. O. (2017). The black soldier fly, *Hermetia illucens*-a promising source for sustainable production of proteins, lipids and bioactive substances. *Z. Naturforsch. C* 72, 351–363. doi: 10.1515/znc-2017-0030

Mwaniki, Z., Neijat, M., and Kiarie, E. (2018). Egg production and quality responses of adding up to 7.5% defatted black soldier fly larvae meal in a corn-soybean meal diet fed to shaver white leghorns from wk 19 to 27 of age. *Poultry Sci.* 97, 2829–2835. doi: 10.3382/ps/pey118

Penazzi, L., Schiavone, A., Russo, N., Nery, J., Valle, E., Madrid, J., et al. (2021). In vivo and *in vitro* digestibility of an extruded complete dog food containing black soldier fly (Hermetia illucens) larvae meal as protein source. *Front. Vet. Sci.* 8:653411. doi: 10.3389/fvets.2021.653411

Pérez-Burillo, S., Pastoriza, S., Fernández-Arteaga, A., Luzón, G., Jiménez-Hernández, N., D'Auria, G., et al. (2019). Spent coffee grounds extract, rich in mannooligosaccharides, promotes a healthier gut microbial community in a dose-dependent manner. *J. Agr. Food Chem.* 67, 2500–2509. doi: 10.1021/acs.jafc.8b06604

Pilla, R., and Suchodolski, J. S. (2020). The role of the canine gut microbiome and metabolome in health and gastrointestinal disease. *Front. Vet. Sci.* 6:498. doi: 10.3389/fvets.2019.00498

Pinotti, L., Giromini, C., Ottoboni, M., Tretola, M., and Marchis, D. (2019). Review: insects and former foodstuffs for upgrading food waste biomasses/streams to feed ingredients for farm animals. *Animal* 13, 1365–1375. doi: 10.1017/S1751731118003622

Ramos-Elorduy, J., Moreno, J. M. P., Prado, E. E., Perez, M. A., Otero, J. L., and de Guevara, O. L. (1997). Nutritional value of edible insects from the state of Oaxaca. *Mexico. J. Food Compos. Anal.* 10, 142–157. doi: 10.1006/jfca.1997.0530

Schiavone, A., De Marco, M., Martínez, S., Dabbou, S., Renna, M., Madrid, J., et al. (2017). Nutritional value of a partially defatted and a highly defatted black soldier fly larvae (*Hermetia illucens* L.) meal for broiler chickens: apparent nutrient digestibility, apparent metabolizable energy and apparent ileal amino acid digestibility. *J. Anim. Sci. Biotechno.* 8:51. doi: 10.1186/s40104-017-0181-5

Secci, G., Bovera, F., Nizza, S., Baronti, N., Gasco, L., Conte, G., et al. (2018). Quality of eggs from Lohmann Brown classic laying hens fed black soldier fly meal as substitute for soya bean. *Animal* 12, 2191–2197. doi: 10.1017/S1751731117003603

Sekirov, I., Russell, S. L., Antunes, L. C. M., and Finlay, B. B. (2010). Gut microbiota in health and disease. *Physiol. Rev.* 90, 859–904. doi: 10.1152/physrev.00045.2009

Seo, J. H., Pyo, S., Shin, Y. K., Nam, B. G., Kang, J. W., Kim, K. P., et al. (2018). The effect of environmental enrichment on glutathione-mediated xenobiotic metabolism and Antioxidation in Normal adult mice. *Front. Neurol.* 9:425. doi: 10.3389/fneur.2018.00425

Si, H., Wang, X., Zhang, L., Parnell, L. D., Ahmed, B., LeRoith, T., et al. (2019). Dietary epicatechin improves survival and delays skeletal muscle degeneration in aged mice. *FASEB J.* 33, 965–977. doi: 10.1096/fj.201800554RR

Sirithanakorn, C., and Cronan, J. E. (2021). Biotin, a universal and essential cofactor: synthesis, ligation and regulation. *FEMS Microbiol. Rev.* 45:1. doi: 10.1093/femsre/fuab003

Skupsky, J., Sabui, S., Hwang, M., Nakasaki, M., Cahalan, M. D., and Said, H. M. (2020). Biotin supplementation ameliorates murine colitis by preventing NF-κB activation. *Cell Mol. Gastroenter.* 9, 557–567. doi: 10.1016/j.jcmgh.2019.11.011

Somroo, A. A., Ur Rehman, K., Zheng, L., Cai, M., Xiao, X., Hu, S., et al. (2019). Influence of lactobacillus buchneri on soybean curd residue co-conversion by black soldier fly larvae (Hermetia illucens) for food and feedstock production. *Waste Manag.* 86, 114–122. doi: 10.1016/j.wasman.2019.01.022

Song, Q., Wang, Y., Huang, L., Shen, M., Yu, Y., Yu, Q., et al. (2021). Review of the relationships among polysaccharides, gut microbiota, and human health. *Food Res. Int.* 140:109858. doi: 10.1016/j.foodres.2020.109858

Spranghers, T., Michiels, J., Vrancx, J., Ovyn, A., Eeckhout, M., De Clercq, P., et al. (2018). Gut antimicrobial effects and nutritional value of black soldier fly (*Hermetia illucens* L.) prepupae for weaned piglets. *Anim. Feed Sci. Tech.* 235, 33–42. doi: 10.1016/j.anifeedsci.2017.08.012

Straub, T. J., Chou, W., Manson, A. L., Schreiber, H. L., Walker, B. J., Desjardins, C. A., et al. (2021). Limited effects of long-term daily cranberry consumption on the gut microbiome in a placebo-controlled study of women with recurrent urinary tract infections. *BMC Microbiol.* 21:53. doi: 10.1186/s12866-021-02106-4

Swanson, K. S., Carter, R. A., Yount, T. P., Aretz, J., and Buff, P. R. (2013). Nutritional sustainability of pet foods. *Adv. Nutr.* 4, 141–150. doi: 10.3945/an.112.003335

Traksele, L., Speiciene, V., Smicius, R., Alencikiene, G., Salaseviciene, A., Garmiene, G., et al. (2021). Investigation of in vitro and in vivo digestibility of black soldier fly (Hermetia illucens L.) larvae protein. *J. Funct. Foods.* 79:104402. doi: 10.1016/j.jff.2021.104402

Tschirner, M., and Simon, A. (2015). Influence of different growing substrates and processing on the nutrient composition of black soldier fly larvae destined for animal feed. *J. Insects Food Feed* 1, 249–259. doi: 10.3920/JIFF2014.0008

Valdés, F., Villanueva, V., Durán, E., Campos, F., Avendaño, C., Sánchez, M., et al. (2022). Insects as feed for companion and exotic pets: a current trend. *Animals* 12:1450. doi: 10.3390/ani12111450

Xin, Z., Ma, S., Ren, D., Liu, W., Han, B., Zhang, Y., et al. (2018). UPLC-Orbitrap-MS/MS combined with chemometrics establishes variations in chemical components in green tea from Yunnan and Hunan origins. *Food Chem.* 266, 534–544. doi: 10.1016/j.foodchem.2018.06.056

Xu, T., Ge, Y., Du, H., Li, Q., Xu, X., Yi, H., et al. (2021). *Berberis kansuensis* extract alleviates type 2 diabetes in rats by regulating gut microbiota composition. *J. Ethnopharmacol.* 273:113995. doi: 10.1016/j.jep.2021.113995

Yang, K., Deng, X., Jian, S., Zhang, M., Wen, C., Xin, Z., et al. (2021). Gallic acid alleviates gut dysfunction and boosts immune and antioxidant activities in puppies under environmental stress based on microbiome-metabolomics analysis. *Front. Immunol.* 12:813890. doi: 10.3389/fimmu.2021.813890

Yang, K., Jian, S., Guo, D., Wen, C., Xin, Z., Zhang, L., et al. (2022a). Fecal microbiota and metabolomics revealed the effect of long-term consumption of gallic acid on canine lipid metabolism and gut health. *Food Chem X.* 15:100377. doi: 10.1016/j.fochx.2022.100377

Yang, K., Jian, S., Wen, C., Guo, D., Liao, P., Wen, J., et al. (2022b). Gallnut tannic acid exerts anti-stress effects on stress-induced inflammatory response, dysbiotic gut microbiota, and alterations of serum metabolic profile in beagle dogs. *Front. Nutr.* 9:847966. doi: 10.3389/fnut.2022.847966

Yang, L., Xiang, Z., Zou, J., Zhang, Y., Ni, Y., and Yang, J. (2022). Comprehensive analysis of the relationships between the gut microbiota and fecal metabolome in individuals with primary sjogren's syndrome by 16S rRNA sequencing and LC-MS-based metabolomics. *Front. Immunol.* 13:874021. doi: 10.3389/fimmu.2022.874021

Yildirim Aksoy, M., Eljack, R., and Beck, B. H. (2020). Nutritional value of frass from black soldier fly larvae, *Hermetia illucens*, in a channel catfish, *Ictalurus punctatus*, diet. *Aquac. Nutr.* 26, 812–819. doi: 10.1111/anu.13040

Yu, M., Li, Z., Chen, W., Rong, T., Wang, G., Wang, F., et al. (2020). Evaluation of full-fat *Hermetia illucens* larvae meal as a fishmeal replacement for weanling piglets: effects on the growth performance, apparent nutrient digestibility, blood parameters and gut morphology. *Anim. Feed Sci. Tech.* 264:114431. doi: 10.1016/j.anifeedsci.2020.114431

Yu, G., Xu, C., Zhang, D., Ju, F., and Ni, Y. (2022). MetOrigin: discriminating the origins of microbial metabolites for integrative analysis of the gut microbiome and metabolome. *iMeta.* 1:e10. doi: 10.1002/imt2.10

Zhao, Y., Bi, J., Yi, J., Wu, X., Ma, Y., and Li, R. (2021). Pectin and homogalacturonan with small molecular mass modulate microbial community and generate high SCFAs via *in vitro* gut fermentation. *Carbohyd. Polym.* 269:118326. doi: 10.1016/j.carbpol.2021.118326

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# Effects of heat stress on growth performance, carcass traits, serum metabolism, and intestinal microflora of meat rabbits

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To investigate the effects of heat stress on meat rabbits, we assigned 80 rabbits to the moderate temperature group (24±1°C; Control group) and the continuous high-temperature group (HT group), then monitored the effects using growth performance, carcass characteristics, biochemical assays, UPLC–MS/MS-based metabolomics, and microbiome. The results showed that after continuous high-temperature exposure, the average daily gain, average daily feed intake, and thymus index were significantly decreased ($p<0.05$). Contents of HSP70, ALP, and Cortisol in serum were significantly increased, while TP, GLU, T3, and T4 were significantly decreased ($p<0.05$). Nine kinds of differential metabolites were screened by serum metabolomics, which can be used as biomarkers of heat stress in meat rabbits. The selected differential metabolites were analyzed by KEGG annotation and enrichment analysis. The results showed that 14 pathways affected by heat stress were identified by KEGG pathway enrichment analysis, including Sphingolipid metabolism, Pyrimidine metabolism, Citrate cycle (TCA cycle)), aminoacyl-tRNA biosynthesis, and so on. The analysis of the effect of heat stress on the cecal microflora of meat rabbits showed that the abundance of cecal Proteus in the HT group was significantly higher than that in the moderate Control group. The number of Candidatus-saccharimonas in the cecum microflora was significantly higher than that in the moderate temperature group ($p<0.05$) which may be related to inflammatory diseases in the heat stress group. These findings indicated that the heat-stressed rabbits were in negative energy balance, which affected protein metabolism, and subsequently affected growth performance and carcass characteristics.

KEYWORDS

**rabbits, heat stress, growth performance, metabolomics, microbiome**

# Introduction

With the continuous acceleration of global warming and intensive breeding environment, heat stress has gradually become a global issue of common concern in modern livestock and poultry production, and heat stress is considered to be one of the most important stresses in livestock and poultry breeding. Heat stress refers to the sum of non-specific responses produced by animals when they are stimulated by excessive temperature beyond their thermoregulatory capacity (Mount, 1975). Heat stress causes serious economic losses to the livestock industry by changing various physiological and biochemical reactions of the body, reducing feed intake, daily gain, feed conversion rate, and product quality (West, 2003; Franco, 2004; Beckford et al., 2020).

Climate warming is unequivocal, and will greatly impact animal health and growth, especially during the summer months, either directly or secondarily (Leon et al., 2005). Heat stress seriously restricts the development of the rabbit industry. Rabbits belong to constant temperature animals, and the normal body temperature is 38.5–39.9°C, while the upper limit of the moderate temperature of rabbits is lower than 30°C in all current studies. The suitable growth temperature of meat rabbits is 15–25°C. The heat insulation layer formed by a few sweat glands and thick hair on the body surface of rabbits leads to poor heat dissipation ability (Lee, 1965). Moreover, the rabbit is very sensitive to temperature change and is easy to produce stress reactions. When the ambient temperature is higher than the body surface temperature of rabbits, non-evaporative heat dissipation methods such as radiation, convection and conduction fail, and respiratory heat dissipation becomes the main way of heat dissipation in rabbits. However, after all, the ability of breathing and heat dissipation is limited, and high temperature for a long time will increase the respiratory frequency of rabbits, wheeze, increase body temperature, and then appear heat stress reaction, resulting in changes in rabbit behavior and physiology. Heat stress can cause changes in some of the physiological indicators of meat rabbits such as rectal temperature increase, feed intake and daily gain decreased, and water consumption increased, thus heat stress will increase energy consumption, reduce production performance, and reproductive performance. The thermoregulation characteristics of rabbits make them have poor tolerance to a high heat environment, which is related to breed, age, sex, stress intensity, and duration (Yang et al., 2011; Li et al., 2019; Zheng et al., 2022). Heat stress resulted in a decrease in feed intake, daily gain, and meat quality in rabbits (Marai et al., 2002). Research has found that, heat stress regulates stress response through the endocrine system (Mete et al., 2012).

The present study aimed to characterize the serum metabolic profile of heat-stressed broilers and to investigate the change rule and related regulatory mechanisms using Ultra Performance Liquid Chromatography Tandem Mass Spectrometry (UPLC–MS/MS) metabolomics, biochemical validation, microbiomics, and multivariate data analysis. The study also aimed to elucidate the relationships between growth performance, serum biochemical and hormonal parameters, serum metabolism, and gut microflora changes in heat-stressed rabbits.

# Materials and method

## Animal and experiment

In the experiment, eighty 35-day-old Hyla meat rabbits (half male and half female) were pre-fed at 24 ± 1°C for 7 days, and then sixty 42-day-old meat rabbits were randomly divided into two groups: moderate temperature group (24 ± 1°C) and continuous high-temperature group (high-temperature feeding, 34 ± 2°C). The temperature and humidity were adjusted by the air conditioner and humidifier (ZDR-41). Hangzhou Zeda Instrument Co., Ltd.) automatically records room temperature every 30 min, the experimental period is 14 days (Figure 1). Rabbits were housed in homemade plastic cages (60 × 40 × 40 cm) in couples. The relative humidity and photoperiod were maintained according to commercial conditions (55°C to 65°C, 12 light/12 dark). Diets were pelleted using pressure, and the diameter of the pellets was 4 mm. All rabbits had free access to feed and water during the rearing period. At the end of the experimental period, 8 rabbits in each group were randomly selected to fast for 12 h, and the blood was collected from the heart. The blood was placed for 30 min and centrifuged at 3,000 rpm for 10 min. The serum was separated and stored at-80°C for blood biochemical index and metabonomics determination. After blood collection, the rabbits were killed after breaking their necks and weighed after peeling off the fur, thymus, spleen, liver, kidney, shoulder fat, and perirenal fat. Samples of cecal contents were quickly frozen in liquid nitrogen and stored at-80°C for 16 s analysis of microbial diversity and metabonomics analysis.

## Growth performance and sample collection

Rabbits in every replicate from each treatment group were weighed both on the transfer days (experimental 8-day) and at the end of the experiment (experimental 21-day). Average daily gain (ADG), Average daily feed intake (ADFI), Feed conversion ratio (FCR), Liver index, Spleen index, Thymus index, Kidney index, Shoulder fat rate, and Kidney fat rate were calculated according to the formula:

ADG = body increase (g)/number of trial days.

ADFI = (feed intake-leftover feed) /number of trial days.

FCR = ADFI/ADG.

Fully eviscerated slaughtering rate (%) = full eviscerated weight/live weight before slaughter * 100.

Liver index (%) = liver weight/live weight before slaughter * 100.

Spleen index (%) = spleen weight / live weight before slaughter * 100

Thymus index (%) = thymus weight / live weight before slaughter * 100

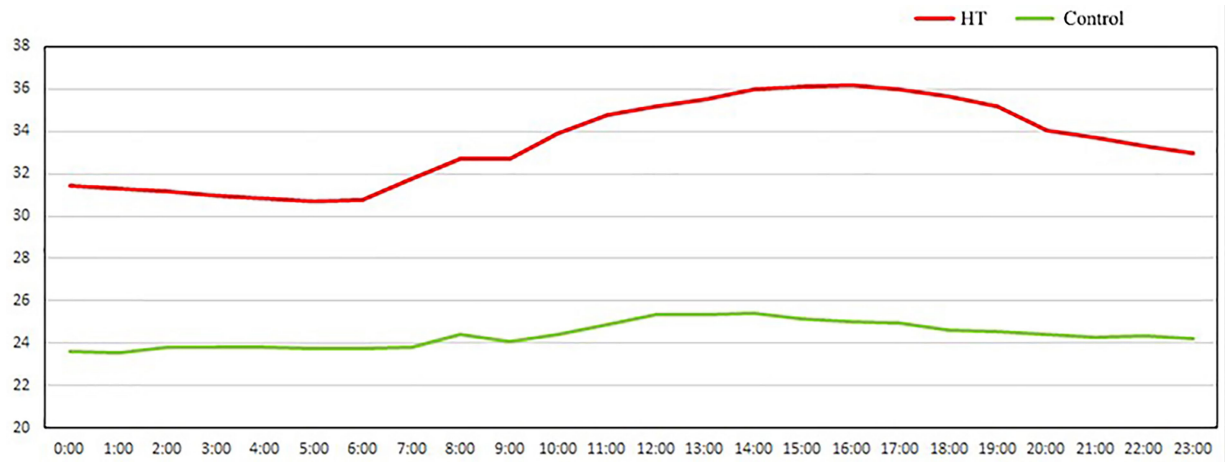


FIGURE 1
The actual average temperature in the room every day.

Kidney index (%) = kidney weight / live weight before slaughter * 100

Shoulder fat rate (%) = shoulder fat weight/live weight before slaughter * 100.

Kidney fat rate (%) = perirenal fat weight/live weight before slaughter * 100.

## Serum biochemical and hormonal parameters

Serum concentrations of total protein (TP), albumin (ALB), glucose (GLU), triglyceride (TG), cholesterol (CHO), urea (UREA), high-density lipoprotein (HDL), low-density lipoprotein (LDL), calcium (Ca), phosphorus (P), glutamic pyruvic transaminase (ALT), and glutamic-oxalacetic transaminase (AST) were measured using a Hitachi 7,020 automatic biochemical analyzer (Hitachi Ltd., Tokyo, Japan). Serum growth hormone (GH), thyroid-stimulating hormone (TSH), triiodothyronine (T3), and tetraiodothyronine (T4) were determined by the ELISA method with the corresponding ELISA kits (Ybio Bio-tech Co., Ltd., Shanghai, China).

## Metabonomic analysis based on UPLC–MS/MS

The serum samples were taken from the refrigerator at-80°C and thawed in the refrigerator at 4°C. Six samples were selected for each group. 100 and 400 μl pre-cooled 80% methanol containing 0.1% formic acid were mixed by rotation method, and the samples were incubated for 5 min on ice. Under the condition of 15,000 g 10 min at 4°C, the supernatant was injected into the sample by centrifugation and analyzed by LC–MS/MS system. The materials used for LC-MS/MS analyses were given in Supplementary material S1 (Liu et al., 2022b).

## Gut microbiomics

Microbial community genomic DNA was extracted from cecal contents samples using the E.Z.N.A.® soil DNA Kit (Omega Bio-tek, Norcross, GA, U.S.) according to the manufacturer's instructions. The materials used for DNA extraction, PCR amplification of 16S rRNA gene, and 16S rRNA gene sequencing reads were given in Supplementary material S2 (Liu et al., 2022a).

The DNA extract was checked on 1% agarose gel, and DNA concentration and purity were determined with NanoDrop 2000 UV–vis spectrophotometer (Thermo Scientific, Wilmington, United States). The hypervariable region V3-V4 of the bacterial 16S rRNA gene was amplified with primer pairs 338F (5′-ACTCCTACGGGAGGCAGCAG-3′) and 806R (5′-GGACTA CHVGGGTWTCTAAT-3′) by an ABI GeneAmp® 9,700 PCR thermocycler (ABI, CA, United States).

The PCR amplification of 16S rRNA gene was performed as follows: initial denaturation at 95°C for 3 min, followed by 27 cycles of denaturing at 95°C for 30 s, annealing at 55°C for 30 s and extension at 72°C for 45 s, and single extension at 72°C for 10 min, and end at 4°C. The PCR mixtures contain 5 × TransStart FastPfu buffer 4 μl, 2.5 mM dNTPs 2 μl, forward primer (5 μM) 0.8 μl, reverse primer (5 μM) 0.8 μl, TransStart FastPfu DNA Polymerase 0.4 μl, template DNA 10 ng, and finally ddH2O up to 20 μl. PCR reactions were performed in triplicate. The PCR product was extracted from 2% agarose gel and purified using the AxyPrep DNA Gel Extraction Kit (Axygen Biosciences, Union City, CA, United States) according to the manufacturer's instructions and quantified using Quantus™ Fluorometer (Promega, United States).

Purified amplicons were pooled in equimolar and paired-end sequenced on an Illumina NovaSeq PE250 platform (Illumina, San Diego, United States) according to the standard protocols by Majorbio Bio-Pharm Technology Co. Ltd. (Shanghai, China). The raw reads were deposited into the NCBI Sequence Read Archive (SRA) database.

The raw 16S rRNA gene sequencing reads were demultiplexed, quality-filtered by fastp version 0.20.0 and merged by FLASH version 1.2.7 with the following criteria: (i) the 300 bp reads were truncated at any site receiving an average quality score of <20 over a 50 bp sliding window, and the truncated reads shorter than 50 bp were discarded, reads containing ambiguous characters were also discarded; (ii) only overlapping sequences longer than 10 bp were assembled according to their overlapped sequence. The maximum mismatch ratio of overlap region is 0.2. Reads that could not be assembled were discarded; (iii) Samples were distinguished according to the barcode and primers, and the sequence direction was adjusted, exact barcode matching, 2 nucleotide mismatch in primer matching.

Operational taxonomic units (OTUs) with 97% similarity cutoff were clustered using UPARSE version 7.1, and chimeric sequences were identified and removed. The taxonomy of each OTU representative sequence was analyzed by RDP Classifier version 2.2 against the 16S rRNA database (eg. Silva v138) using the confidence threshold of 0.7.

# Data processing and statistical analysis

## Production performance and serum biochemical data

The raw data were statistically processed using Excel and the data were analyzed using the One-Way ANOVA model in SAS 8.2 software and compared using Duncan's multiple tests. $p$-values were obtained, with $p < 0.05$ indicating significant differences, and results are presented as means ± SEs.

## Metabolomics data processing and analysis

Statistical analyses were performed using the statistical software R (R version R-3.4.3), Python (Python version 2.7.6) and CentOS (CentOS version 6.6), and when the data were not normally distributed, they were transformed normally using the area normalization method. The metabolites were annotated using the KEGG database[1] and the HMDB database[2] and Lipidmap database.[3] Principal component analysis (PCA) and partial least squares discriminant analysis (PLS-DA) were performed on MetaX. Statistical significance was calculated using a univariate analysis of variance (T-test) $p < 0.05$.

1 http://www.genome.jp/kegg/

2 http://www.hmdb.ca/

3 http://www.lipidmaps.org/

## Microbial diversity data processing and analysis

Quality control of raw sequenced sequences using fastp (https://github.com/OpenGene/fastp, version 0.20.0) software and FLASH (http://www.cbcb.umd.edu/software/flash, version 1.2.7) software for splicing. OTU clustering of sequences based on 97% similarity using UPARSE software (http://drive5.com/uparse/, version 7.1). Each sequence was annotated for species classification using the RDP classifier (http://rdp.cme.msu.edu/, version 2.2), compared to the Silva 16S rRNA database (v132), and a comparison threshold of 70% was set.

# Results

## Effect of heat stress on production performance of meat rabbits

Table 1 showed that under the condition of no difference in initial body weight, the final weight, ADFI and ADG of meat rabbits in the heat stress group were significantly lower than those in the control group ($p < 0.05$). At the same time, the thymus index and liver index in the heat stress group were significantly lower than those in the control group ($p < 0.05$), while the shoulder fat rate and kidney fat rate in the heat stress group were significantly higher than those in the control group ($p < 0.05$).

## Effects of heat stress on serum biochemical indexes of meat rabbits

The effect of heat stress on serum biochemical indexes of meat rabbits is shown in Table 2. Compared with the control group, the contents of HSP70, ALP and cortisol in the serum of the heat stress group increased significantly ($p < 0.05$), while the contents of TP, GLU, T3 and T4 decreased significantly ($p < 0.05$), and other biochemical indexes did not change significantly.

TABLE 1 Effect of heat stress on meat rabbit's carcass trait.

| Items | Control | HT | $p$-value |
|---|---|---|---|
| Initial weigh (kg) | 1.19 ± 0.02 | 1.21 ± 0.03 | 0.5679 |
| Final weight of test (kg) | 1.62 ± 0.02$^{a}$ | 1.54 ± 0.03$^{b}$ | 0.0240 |
| ADFI (g) | 133.28 ± 2.76$^{a}$ | 107.21 ± 2.30$^{b}$ | <0.001 |
| ADG (g) | 31.65 ± 1.32$^{a}$ | 26.10 ± 1.26$^{b}$ | 0.0036 |
| FCR | 4.43 ± 0.17 | 4.30 ± 0.22 | 0.6322 |
| Eviscerated rate (%) | 46.03 ± 0.51 | 47.18 ± 0.84 | 0.2521 |
| Thymus index (%) | 0.21 ± 0.01$^{a}$ | 0.17 ± 0.01$^{b}$ | 0.0282 |
| Spleen index (%) | 0.08 ± 0.01 | 0.07 ± 0.01 | 0.0834 |
| Liver index (%) | 2.94 ± 0.08$^{a}$ | 2.66 ± 0.09$^{b}$ | 0.0326 |
| Kidney index (%) | 0.67 ± 0.02 | 0.64 ± 0.02 | 0.2505 |
| Shoulder fat percentage (%) | 0.15 ± 0.01$^{b}$ | 0.20 ± 0.02$^{a}$ | 0.0482 |
| Kidney fat rate (%) | 0.25 ± 0.01$^{b}$ | 0.37 ± 0.05$^{a}$ | 0.0270 |

Values are means ± SEMs; $n = 8$. $^{ab}$Values with different superscripts differ significantly ($p < 0$ 0.05).

## Effects of heat stress on serum metabolomics in meat rabbits

Metabolome refers to a collection of small molecule compounds that are involved in the metabolism of organisms and maintain normal growth and development of organisms. These are mainly referred to as endogenous small molecules with relative molecular weight of less than 1,000. We used LC–MS/MS method combined with positive ion mode (POS) and negative ion mode (NEG) for serum metabolite analysis, making metabolite coverage higher and the detection effect better. In POS mode, a total of 5,456 peaks were obtained, and 343 substances of these were identified while the number of metabolites annotated to the public database was 399. In NEG mode, a total of 4,809 peaks were obtained, and 496 substances of these were identified and 464 were annotated to the public database (see Table 3).

According to the expression of metabolites in different samples, PCA principal component analysis was carried out to evaluate the similarity of samples within groups and the differences between samples, and PLS-DA analysis was carried out. The results are shown in Figure 2. The PCA and PLS-DA score maps show that the QC samples are gathered together, and PCA shows that the confidence ellipses of the two groups of samples overlap, but there are no outliers, indicating that the degree of variability between the two groups of samples is small, and the results of PLS-DA analysis show that the two groups of samples are separated greatly, indicating that the classification effect is significant.

TABLE 2 Effects of Heat stress on serum biochemical of meat rabbits.

| Items | Control | HT | *p*-value |
|---|---|---|---|
| HSP70 (pg/ml) | 1403.61 ± 32.10$^{b}$ | 1569.05 ± 14.48$^{a}$ | 0.0015 |
| Cortisol (ng/L) | 19.48 ± 1.60$^{b}$ | 52.39 ± 5.81$^{a}$ | 0.0003 |
| TP (g/L) | 64.50 ± 1.83$^{a}$ | 58.63 ± 1.05$^{b}$ | 0.0194 |
| ALB (g/L) | 30.60 ± 0.47 | 29.37 ± 1.03 | 0.3029 |
| UREA (mmol/L) | 7.37 ± 0.44 | 8.25 ± 0.18 | 0.0967 |
| GLU (mmol/L) | 7.45 ± 0.21$^{a}$ | 6.84 ± 0.1$^{b}$ | 0.0269 |
| TG (mmol/L) | 1.38 ± 0.32 | 0.72 ± 0.10 | 0.0816 |
| Ca (mmol/L) | 4.26 ± 0.08 | 4.53 ± 0.12 | 0.0918 |
| P (mmol/L) | 3.28 ± 0.12 | 2.85 ± 0.20 | 0.0938 |
| HDL (mmol/L) | 0.43 ± 0.04 | 0.61 ± 0.09 | 0.0875 |
| LDL (mmol/L) | 0.46 ± 0.08 | 0.50 ± 0.02 | 0.5943 |
| T3 (ng/ml) | 0.41 ± 0.06$^{a}$ | 0.13 ± 0.03$^{b}$ | 0.0014 |
| T4 (μg/dl) | 1.39 ± 0.21$^{a}$ | 0.29 ± 0.12$^{b}$ | 0.0011 |

Values are means ± SEMs; $n = 8$. $^{ab}$Values with different superscripts differ significantly ($p < 0.05$).

TABLE 3 TOTAL ion count and identification statistics table.

| Ion mode | All peaks | Identified metabolites | Metabolites in Library |
|---|---|---|---|
| POS | 5,456 | 343 | 299 |
| NEG | 4,809 | 496 | 464 |

Multidimensional statistical analysis of the identified metabolites was carried out by PCA, PLS-DA and OPLS-DA statistical analysis (Figure 3) to screen out the most important and noteworthy differential metabolites. In the PCA model, the sample distances between the two sample groups overlap, indicating that the intra-and inter-group differences between the two groups are small. The PLS-DA score chart shows that the separation degree of the two groups of samples is high, indicating that the classification effect is significant. The OPLS-DA score map filters out the information that has nothing to do with the grouping through orthogonal rotation so that it can better distinguish the differences between groups and improve the efficiency of the model. It can be seen from the chart that the classification effect between the two groups of samples is significant, which shows that the construction of the experimental model is reliable and the data is effective.

Multidimensional statistical analysis showed that VIP > 1 and FC > 1.2 or FC < 0.83 were used as screening criteria for differential metabolites. A total of 126 differential metabolites were screened, including 48 up-regulated differential metabolites and 78 down-regulated differential metabolites, while there are 9 different metabolites with specific names (volcanic diagram see Figure 4, results see Table 4). For all the identified metabolites, FC and T-tests were combined to measure the influence and explanatory ability of the expression patterns of metabolites on the classification and discrimination of each group of samples according to the VIP values obtained by the PLS-DA model. The differential metabolites with biological significance were mined and selected at the same time.

The differential metabolites were analyzed by KEGG database and significantly enriched to 14 pathways affected by heat stress (Figure 5), including Phospholipid metabolism pathway, Glyoxylate and dicarboxylate metabolism, Pyrimidine metabolism, Pyruvate metabolism, Alanine, aspartate and glutamate metabolism, Lysine biosynthesis, Citrate cycle (TCA cycle), Aminoacyl-tRNA biosynthesis, etc.

## Effects of heat stress on cecal microflora of meat rabbits

Tags were clustered at 97% similarity level to obtain OTU, and taxonomic annotation of OTU based on Silva (bacteria) taxonomic database, and the number of OTU of each sample was obtained. A total of 1,113 different OTU, were obtained from the two groups of samples. The Venn map (Figure 6) showed that there were 907 OTU in the moderate temperature group and heat stress group, 128 in the cecum of meat rabbits in the appropriate temperature group, and 78 OTU in the heat stress group.

The α diversity of the samples was analyzed by Chao1, Shannon, and Simpson by T-tests. The results show that there is no significant difference between each index in Figure 7, indicating that there is no significant difference in flora diversity, flora abundance and community coverage between the two groups.

As shown in Figure 8, PCA uses the analysis of variance of the OTUs of different samples to present the calculation results

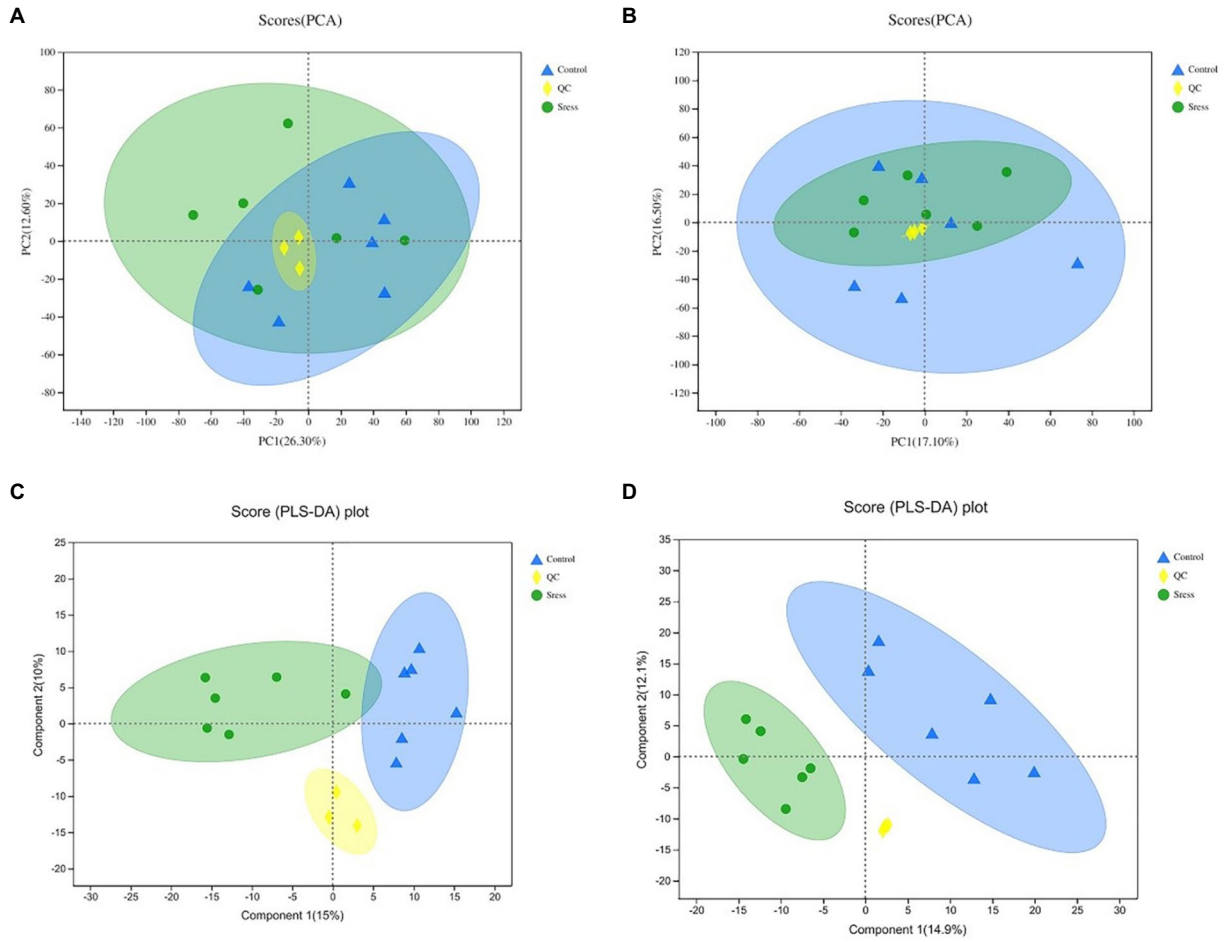


FIGURE 2
PCA and PLS-D Aanalysis score of quality control under the positive ion mode **(A,C)** and negative ion mode **(B,D)**. In the figure, Control represents the appropriate temperature group, and Stress represents the heat stress group, $n$=6.

on a two-dimensional coordinate graph, and the distance between the samples reflects the differences between the samples. It can be seen that the relative aggregation of the two groups of samples in the picture, PC1 = 14.27%, PC2 = 11.99%, shows that the species composition of the two groups of samples is similar, there is no significant difference.

As shown in Figure 9, the dominant flora in the cecum of meat rabbits was Firmicutes, bacteroidota and verrucomicobiota, while the Proteobacteri of Proteus in cecum of meat rabbits under heat stress was significantly higher than that in the temperature group ($p < 0.05$). Further refinement of the taxonomy to genus level, Muribaculaceae unnamed genera, Ackermania are the dominant genera. At the species level, the abundance of akkermansia_muciniphila increased. The results of the T-test in the cecum of heat-stressed meat rabbits showed that the number of Lachnospiraceae-UGG-010 in the intestines of heat-stressed meat rabbits was significantly lower than that in the normal temperature group, while the unnamed genera of Lachnospiraceae, Ruminococcaceae and Candidatus-saccharimonas in the heat-stressed meat rabbits were significantly higher than those in the cecum samples of the heat-stressed meat rabbits.

LEfSe (LDA Effect Size) analysis showed that the genera of Spirillaceae and Rumen Fungi played an important role in the heat stress group, while Colidextribacter, Eubacterium_nodatum_group and Anaeroplasma played an important role in the moderate temperature group, and the heat stress group played an important role in the heat stress group (Figure 10), while in the moderate temperature group, colonic bacilli, Escherichia coli and anaerobic mycoplasma played an important role in the heat stress group.

# Discussion

## Effects of heat stress on production performance, serum biochemical and hormonal parameters of meat rabbits

In recent years, the environmental problem has become an important factor affecting the development of the rabbit industry,

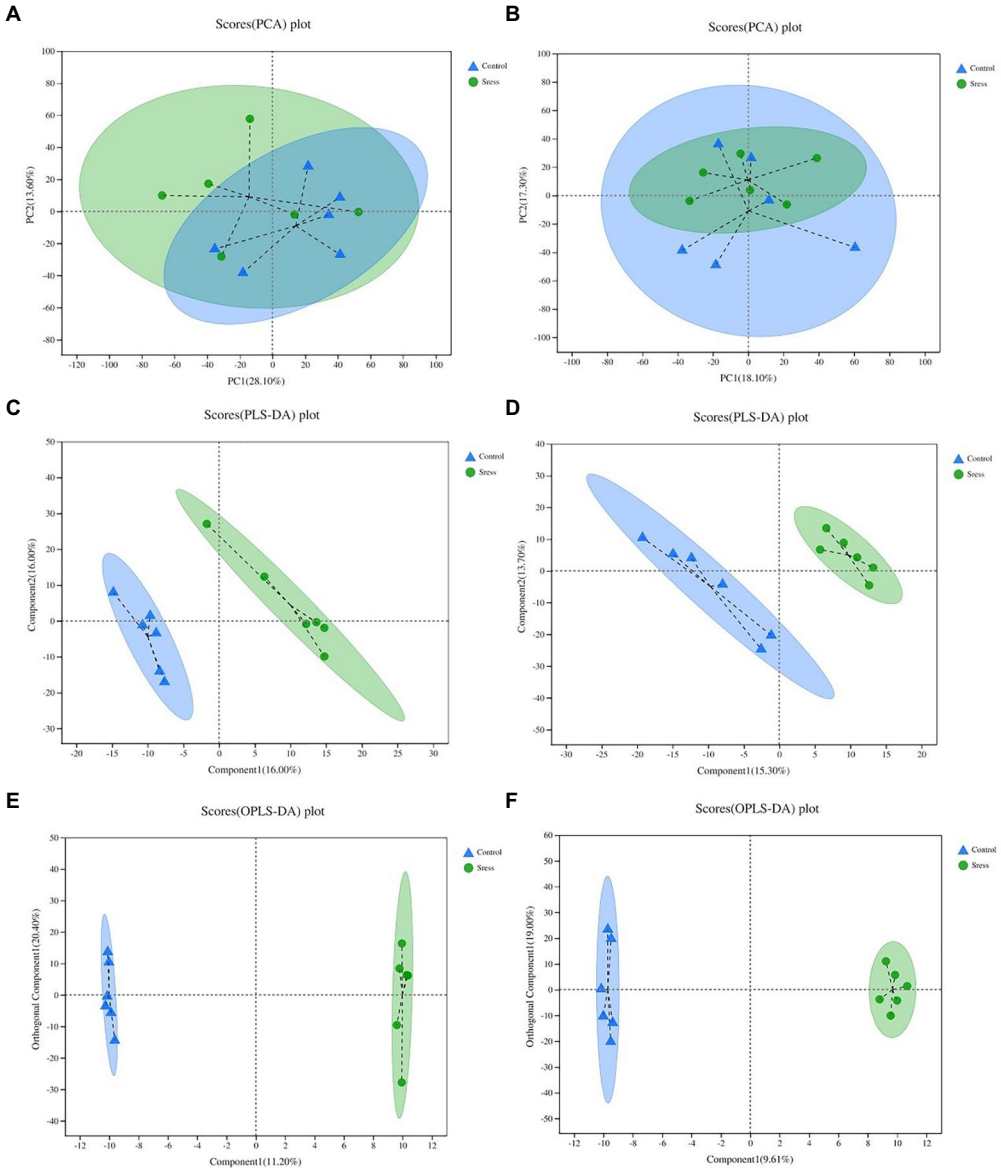


FIGURE 3
PCA, PLS-DA, OPL-DA analysis under the positive ion mode **(A,C,E)** and negative ion mode **(B,D,F)**. In the figure, Control represents the appropriate temperature group, and Stress represents the HT group, *n*=6.

especially the temperature problem. Studies have pointed out that meat rabbits exposed to high temperatures for a long time will inhibit the excitability of their feeding centers and decrease their food intake, resulting in insufficient supply of nutrients in the body. Heat stress reduces feed utilization and body weight gain by reducing feed intake and nutrient metabolism, which affects the production performance of meat rabbits. This is a typical manifestation of heat stress. In this study, it was found that under the condition of continuous high temperature, the average daily gain and average daily feed intake of meat rabbits decreased significantly. This is consistent with the results of previous studies (Liu et al., 2010; Anoh et al., 2020). A high-temperature environment has a great influence on the change of immune index, the increase in temperature will significantly inhibit the immune function (Thaxton et al., 1968), and its immune organ will change in response, and the index of the immune organ is an important index to reflect the development of the immune organ (Tang and Chen, 2016). In this study, it was found that the immune organ index of meat rabbits decreased significantly under high temperatures, which was consistent with the results of chicken research.

The changes in serum biochemical and hormonal parameters are important characteristics that reflect the changes in material metabolism and functional state of tissues

and organs during heat stress, and it is found that serum biochemical indexes are related to productivity. HSP70 plays an important role in protecting antioxidant cells and reducing inflammation in animals (Farid et al., 2006; Zhou and Zhou, 2010). It is the main sign of biological heat shock response, and can still maintain a high measured value after the disappearance of the stressor, so the detection of HSP70 content can reflect the level of heat stress (Boyko, 2013). In this study, it was found that the content of HSP70 in the serum of meat rabbits increased under high temperatures, which was consistent with previous studies. The results showed that under the condition of high temperature, the content of alkaline phosphatase in meat rabbits increased, while the contents of total protein and serum glucose decreased, which was similar to the results of previous studies of Other species (Li et al., 2016). Under the condition of heat stress, meat rabbits produce available energy through glycogen decomposition to enhance their resistance to heat stress, so the supply of serum glucose can not be guaranteed, so the serum glucose concentration is reduced. When the meat rabbit is under heat stress, the weakening of thyroid activity leads to the obstruction of protein synthesis, which leads to the decrease of protein content in the blood. Heat stress caused cell damage, increased cell membrane permeability and increased enzyme content. It is the energy supply during stress, and the serum glucose decreases during chronic stress (Jia, 2012).

The results showed that when the ambient temperature was 34°C, the contents of T3 and T4 in the serum of meat rabbits decreased significantly, while the content of cortisol increased significantly (You et al., 1999). Thyroid hormone acts on almost all organs and tissues of the body, affecting growth, development, metabolism and other aspects. A large number of studies have shown that the increase in body temperature will reduce the secretory function of thyroid gland, reduce the level of thyroid hormone in blood during heat stress, inhibit the metabolic function of the body, and reduce thermogenesis (Cui and Gu, 2015). Heat stress modes significantly decreased the ADG, liver index and the contents of TP and GLU in serum, and increased the contents of cortisol and ALP in the

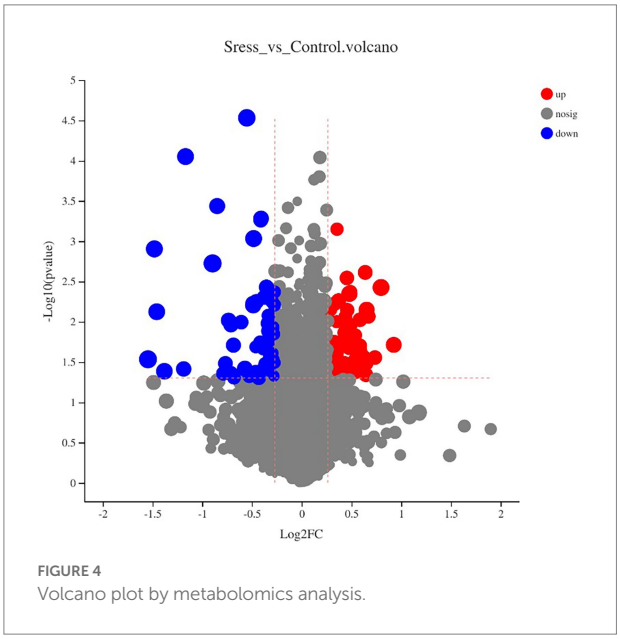


FIGURE 4
Volcano plot by metabolomics analysis.

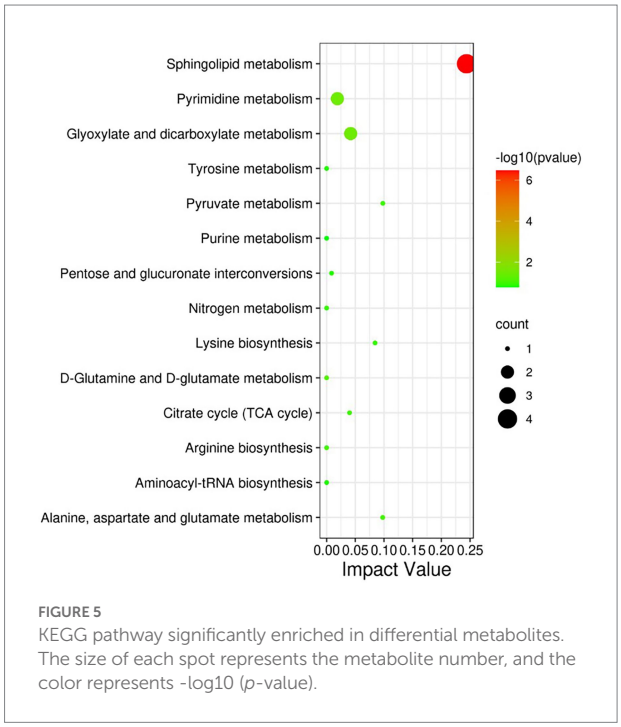


FIGURE 5
KEGG pathway significantly enriched in differential metabolites. The size of each spot represents the metabolite number, and the color represents -log10 (*p*-value).

TABLE 4 Different metabolites in the meat rabbit serum after heat stress.

| Metabolite name | VIP | FC | *p*-value | Regulated |
|---|---|---|---|---|
| Diaminopimelic acid | 2.8278 | 0.5879 | 0.0330 | Down |
| Sphinganine | 2.3686 | 0.7955 | 0.0396 | Down |
| Dihydronaringenin-O-sulphate | 3.2487 | 1.8992 | 0.01937 | Up |
| Erinacine P | 2.0796 | 1.323 | 0.03228 | Up |
| Cis-4,10,13,16-Docosatetraenoic Acid | 3.2878 | 0.555 | 0.000367 | Down |
| 3-keto-Digoxigenin | 2.1074 | 0.7263 | 0.02053 | Down |
| Glycinoeclepin B | 3.7207 | 0.3584 | 0.001247 | Down |
| Ganoderic acid H | 3.6173 | 0.3643 | 0.00752 | Down |
| 2-Methoxyacetaminophen glucuronide | 2.1419 | 0.8201 | 0.02961 | Down |

serum of meat rabbits, which is similar to the research on broiler (Wang et al., 2003).

## Effects of heat stress on serum metabonomics in meat rabbits

In the state of heat stress, the body needs to use reserves to deal with heat stress, leading to metabolic disorders or the re-establishment of homeostasis. In this study, 39 kinds of differential metabolites were screened by multidimensional statistical analysis, which can effectively distinguish meat rabbits from the heat stress group and moderate temperature group. These potential biomarkers are involved in the phospholipid metabolism pathway, glyoxylic acid, and dicarboxylic acid metabolism, pyrimidine metabolism pathway, tricarboxylic acid cycle, nitrogen metabolism and other pathways, involving glucose metabolism, lipid metabolism, nucleic acid metabolism, amino acid metabolism, energy metabolism and translation process. The results of heat stress are consistent with those of broilers (Tang, 2015) and mice (Straadt et al., 2010; Ippolito et al., 2014). In this study, it was found that heat stress significantly decreased the levels of L-glutamine and L-malic acid in the blood of meat rabbits. Glutamine can produce glucose through gluconeogenesis (Anders, 1981; Mccauley et al., 1998; Shanware et al., 2011), and malic acid is an important intermediate in the tricarboxylic acid cycle (Jun-Lin et al., 2014; Wei et al., 2021). Glutamine is a necessary nutrient for intestinal mucosal cell metabolism, which plays a very important role in maintaining the integrity of the intestinal mucosal epithelial structure, improving the antioxidant capacity of the body and promoting protein synthesis (Zhangjun-Min, 2002).In addition, several recent reports have highlighted the functions of Gln metabolism in regulating oxidative stress resistance (Amoressánchez, 1999; Gao et al., 2009, p. 23).

Sphingosine and sphingosine-1-phosphate, which are involved in the pathway of phospholipid metabolism, were significantly down-regulated under heat stress. Sphingosine, also known as sphingosine, is one of the components of the cell membrane, while sphingosine-1-phosphate. Sphingosine-1-phosphate is a powerful signaling lipid molecule, which can regulate cell proliferation, regeneration, migration and intracellular calcium movement, expression of adhesion molecules and activation of monocytes adhering to endothelial cells (Spiegel and Milstien, 2003). Heat

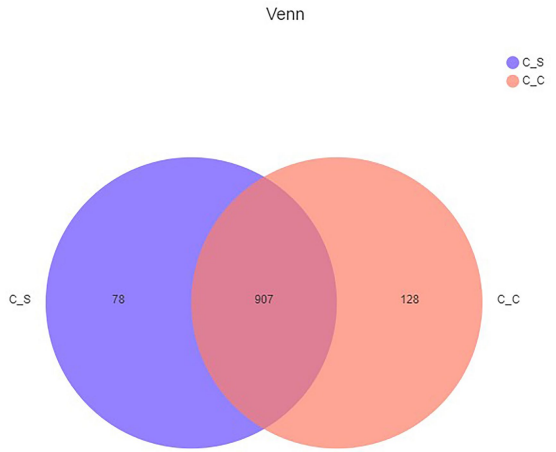


FIGURE 6
Shared OTU across different groups. In the figure, C■S represents the HT group and C■C represents the appropriate temperature group, *n*=6.

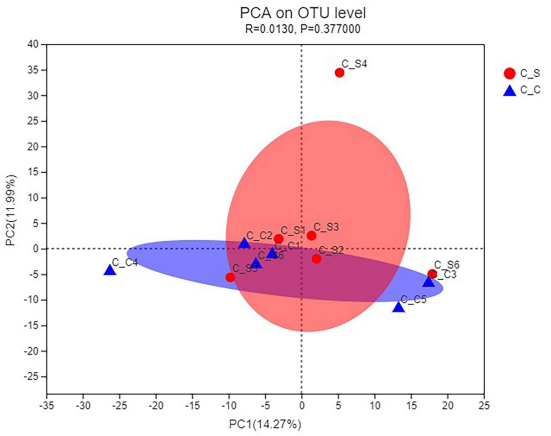


FIGURE 8
PCA of the cecal microflora of rabbits. In the figure, C■S represents the HT group and C■C represents the appropriate temperature group, *n*=6.

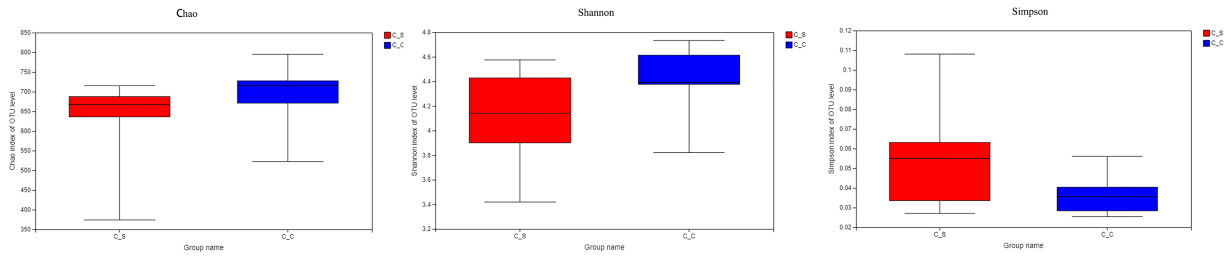


FIGURE 7
Alpha diversity.

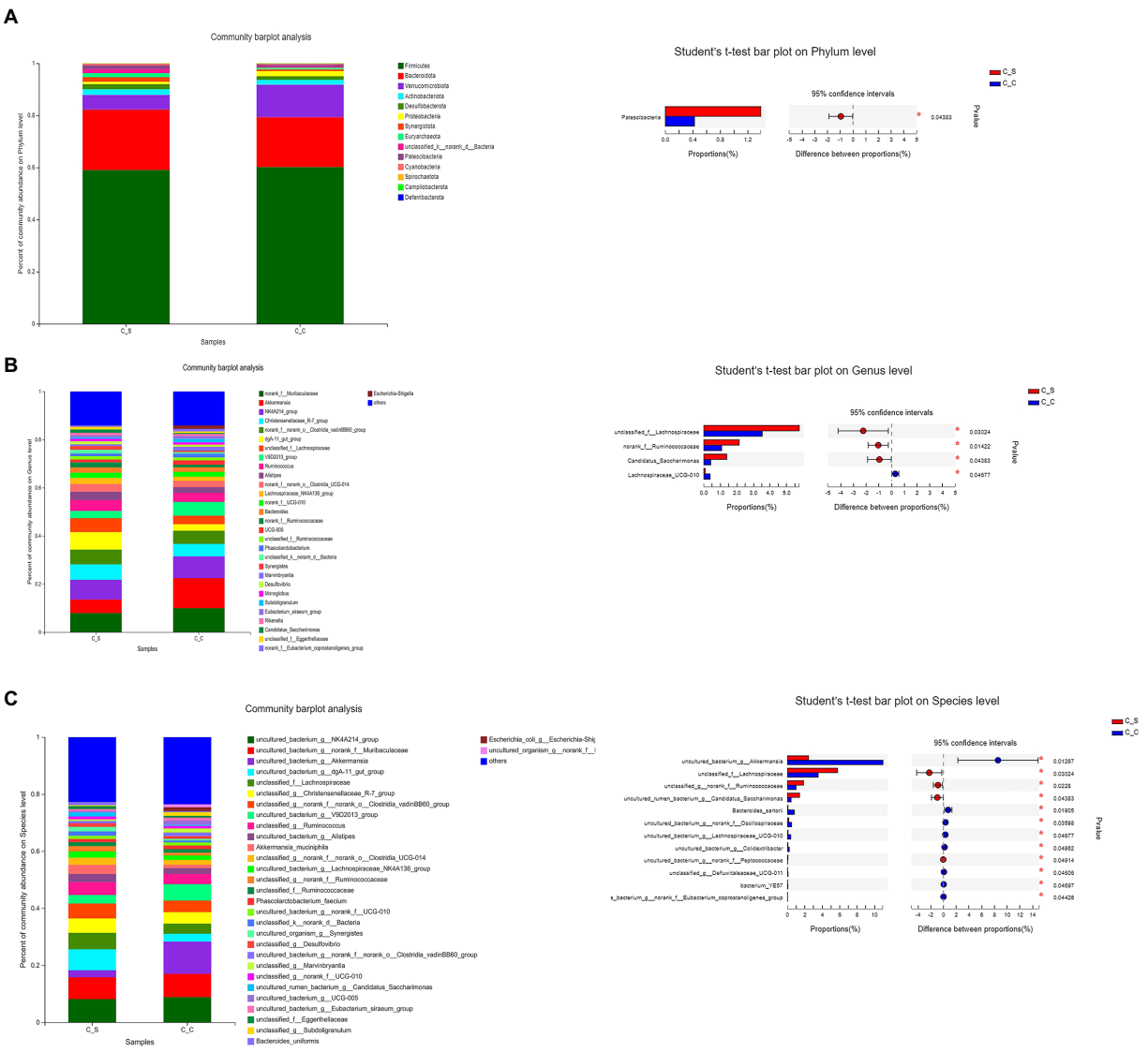


FIGURE 9
Effects of stress on cecum bacterial in meat rabbits. Relative abundance of species and between-group difference tests at different species taxonomy levels (**A**:Phylum level, **B**:Genus level, **C**:Species level). In the figure, C▬S represents the HT group and C▬C represents the appropriate temperature group, *n*=6.

stress decreased the level of cytosine nucleosides involved in pyrimidine metabolism in the blood of meat rabbits, and L-glutamine was also involved in pyrimidine metabolism.

## Effects of heat stress on cecal microflora of meat rabbits

When the animals are in a healthy state, the species and number of intestinal microorganisms are relatively balanced, and there is a balance between the intestinal microflora host and the intestinal microenvironment. However, many external factors can break this balance, and it has been reported that diet, age and environmental factors can affect the intestinal flora structure of meat rabbits (Blas, 2013; Li et al., 2022). At present, it has been found that heat stress can cause changes in the structure of intestinal microflora in chickens and dairy cows (Song et al., 2014; Koch et al., 2019). The dominant bacteria in the cecum of meat rabbits were thick-walled bacteria and Bacteroides. The phylum of Proteus in the cecum of meat rabbit in the heat stress group was significantly higher than that in the temperature group, and it was gram-negative bacteria, including Escherichia coli, Salmonella, Helicobacter and other pathogenic bacteria. This shows that heat stress may increase the number of harmful bacteria in the intestinal tract of meat rabbits.

The dominant bacteria in the cecum were Ackermann (AKK) and Muri. Akk is a strictly anaerobic, non-mobile,

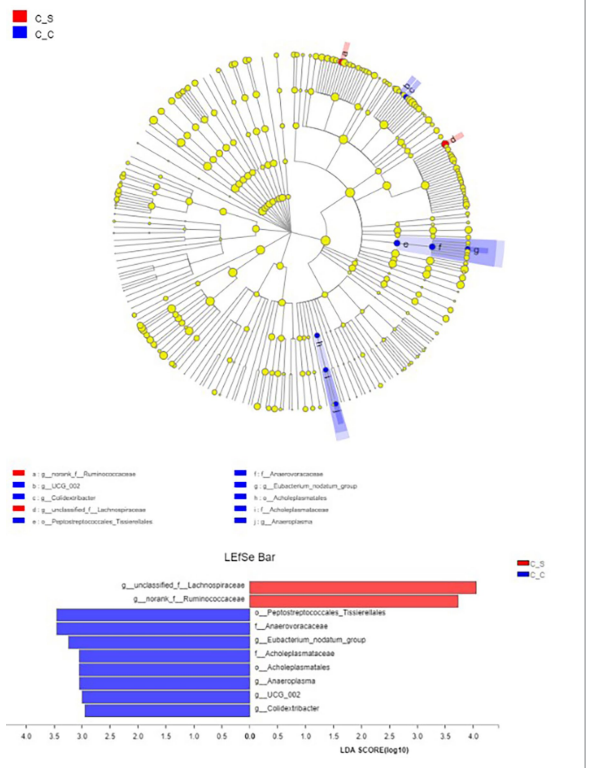


FIGURE 10
Column diagram of LDA value distribution (default set to 4) and evolutionary branch diagram. In the Figure, C■S represents the HT group and C■C represents the appropriate temperature group, *n*=6.

non-spore-producing oval Gram-negative bacteria that can grow in the intestinal mucus layer and grow well using gastrointestinal mucin as the only carbon and nitrogen source. It can grow individually or in pairs, and can also grow in clusters in a medium containing mucin, thus settling in the intestines by competitive rejection and protecting the intestines from pathogens (Derrien et al., 2004). Although Akk bacteria use mucin as an energy source, a large number of observations have confirmed that Akk bacteria have a positive regulatory effect on intestinal mucus layer thickness and intestinal barrier integrity, which is closely related to energy metabolism, immune response and intestinal mucin secretion (Zhang et al., 2009; Png et al., 2010). Muribaculaceae, formerly known as S24-7, is a dominant family of Bacteroides in the intestinal tract, which has functional diversity in the degradation of complex carbohydrates. The Candidatus-saccharimonas microflora in the cecal flora of heat-stressed meat rabbits was significantly higher than that of the cecal samples in the moderate temperature group. Studies have found that Candidatus-saccharimonas may be associated with inflammatory diseases (Cruz et al., 2020). In this study, it was found that heat stress had a certain effect on the structure of cecal flora in meat rabbits, which may cause intestinal inflammation and increase the abundance of harmful bacteria.

## Conclusion

In summary,combined physiological, UHPLC–MS/MS-based metabolomics, and 16S rRNA microbiome analyses were performed to investigate metabolic and gut microbiota responses in the meat rabbits to continuous high-temperature exposure for 14 days. The results revealed that heat stress significantly reduced the production performance of meat rabbits and destroyed the blood biochemical balance of meat rabbits. There were 9 kinds of differential metabolites could be used as biomarkers of heat stress in meat rabbits. Heat stress may affect glucose and fat metabolism in meat rabbits by modulating the gut microflora. These investigations provide new insights into the metabolism mechanisms of meat rabbits to heat stress and contribute to a better understanding of the adverse effects of heat stress on rabbits.

## Data availability statement

The datasets presented in this study can be found in the Supplementary material and online repositories. The names of the repository/repositories and accession number(s) of 16s sequencing data contained in our article can be found at: https://www.ncbi.nlm.nih.gov/, BioProject PRJNA899341 with corresponding BioSample Accessions SAMN31652990-SAMN31653001.

## Ethics statement

All study procedures were approved by the Shandong Agricultural University Animal Care and Use Committee (SDAUA-2017-057) and were following the Guidelines for Experimental Animals established by the Ministry of Science and Technology (Beijing, China).

## Author contributions

HL and FL planned the experiment and finalized the draft of the manuscript with consent from all contributing authors. HL, BZ, and FL carried out the experiment, finalized, and analyzed the collected data. TY, HZ, and LL assisted with manuscript editing and language calibration. 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/fmicb.2022.998095/full#supplementary-material

## References

Amoressánchez, MI, and Medina, M. A. (1999). Glutamine, as a precursor of glutathione, and oxidative stress. *Mol. Genet. Metab.* 67, 100–105. doi: 10.1006/mgme.1999.2857

Anders, H. (1981). Glutamine: metabolism, enzymology, and regulation. Jaime Mora, Rafael Palacios. *Q. Rev. Biol.* 56, 459–460. doi: 10.1086/412449

Anoh, K. U., Barje, P. P., Iyeghe-Erakpotobor, G. T., and Akpa, G. N. (2020). Growth performance of heat stressed rabbits fed diets supplemented with synthetic and organic antioxidants. *Niger. J. Anim. Prod.* 44, 177–180. doi: 10.51791/njap.v44i5.1349

Beckford, R. C., Ellestad, L. E., Proszkowiec-Weglarz, M., Farley, L., Brady, K., Angel, R., et al. (2020). Effects of heat stress on performance, blood chemistry, and hypothalamic and pituitary mRNA expression in broiler chickens. *Poult. Sci.* 99, 6317–6325. doi: 10.1016/j.psj.2020.09.052

Blas, J. C. D. (2013). Nutritional impact on health and performance in intensively reared rabbits. *Animal* 7, 102–111. doi: 10.1017/S1751731112000213

Boyko, A. (2013). Different anti-HSP70 antibodies detect different dynamics of intracellular HSP70 content in heat shocked human neutrophils. *Front. Immunol.* 4:104. doi: 10.3389/conf.fimmu.2013.02.00104

Cruz, B. C. Dos S., Conceição, L. L.Da, Mendes, T. A. De O., Ferreira, C. L. De L. F., Gonçalves, R. V., and Peluzio, M. Do C. G. (2020). Use of the synbiotic VSL#3 and yacon-based concentrate attenuates intestinal damage and reduces the abundance of Candidatus Saccharimonas in a colitis-associated carcinogenesis model. *Food Res. Int.* 137:109721. doi: 10.1016/j.foodres.2020.109721.

Cui, Y., and Gu, X. (2015). Proteomic changes of the porcine small intestine in response to chronic heat stress. *J. Mol. Endocrinol.* 55, 277–293. doi: 10.1530/JME-15-0161

Derrien, M., Vaughan, E. E., Plugge, C. M., and de Vos, W. M. (2004). Akkermansia muciniphila gen. nov., sp. nov., a human intestinal mucin-degrading bacterium. *Int. J. Syst. Evol. Microbiol.* 54, 1469–1476. doi: 10.1099/ijs.0.02873-0

Farid, B., Hamdy, H., Mona, E. N., and Hassan, S. (2006). Heat shockprotein 70 [HSP70]: A novel biomarker for narcotic drugs and hepatotoxic agents. 138–148.

Franco, D. J. (2004). Effect of heat stress on production, physiological, and metabolic parameters in three varieties of laying hens. Available at: https://scholar.google.com/scholar_lookup?title=Effect+of+heat+stress+on+production%2C+physiological%2C+and+metabolic+parameters+in+three+varieties+of+laying+hens&author=Franco%2C+Danilo+Jose.&publication_year=2004 [Accessed March 9, 2022].

Gao, P., Tchernyshyov, I., Chang, T., Lee, Y., and Kita, K. (2009). C-Myc suppression of miR-23 enhances mitochondrial glutaminase and glutamine metabolism. *Nature* 458, 762–765. doi: 10.1038/nature07823

Ippolito, D. L., Lewis, J. A., Yu, C., Leon, L. R., and Stallings, J. D. (2014). Alteration in circulating metabolites during and after heat stress in the conscious rat: potential biomarkers of exposure and organ-specific injury. *BMC Physiol.* 14:14. doi: 10.1186/s12899-014-0014-0

Jia, Y. J. (2012). *Effect of dietary supplemention of vitamin on alleviating heat stress in anhui strain angora rabbit in summer*. [master's thesis]. [Anhui]: Anhui agricultural university.

Jun-Lin, W. U., Qing-Ping, W. U., Zhang, J. M., Zhang, W., Shu-Ping, M. O., and Bai, J. L. (2014). Progress in L-malic acid biosynthesis. Food Sci. Available at: http://en.cnki.com.cn/Article_en/CJFDTOTAL-SPKX201403051.htm [Accessed March 7, 2022].

Koch, F., Thom, U., Albrecht, E., Weikard, R., Nolte, W., Kuhla, B., et al. (2019). Heat stress directly impairs gut integrity and recruits distinct immune cell populations into the bovine intestine. *Proc. Natl. Acad. Sci.* 116, 10333–10338. doi: 10.1073/pnas.1820130116

Lee, D. H. K. (1965). Climatic stress indices for domestic animals. *Int. J. Biometeorol.* 9, 29–35. doi: 10.1007/BF02187306

Leon, L. R., DuBose, D. A., and Mason, C. W. (2005). Heat stress induces a biphasic thermoregulatory response in mice. *Am. J. Phys. Regul. Integr. Comp. Phys.* 288, R197–R204. doi: 10.1152/ajpregu.00046.2004

Li, C., Chen, J., Zhao, M., Liu, M., Yue, Z., Liu, L., et al. (2022). Effect of sodium butyrate on slaughter performance, serum indexes and intestinal barrier of rabbits. *J. Anim. Physiol. Anim. Nutr.* 106, 156–166. doi: 10.1111/jpn.13571

Li, Z., Liu, Z., Wang, Y. N., Kang, Y. J., Wang, J. F., Shi, H. N., et al. (2016). Effects of heat stress on serum cortisol, alkaline phosphatase activity and heat shock protein 40 and 90β mRNA expression in rainbow trout Oncorhynchus mykiss. *Biologia* 71, 109–115. doi: 10.1515/biolog-2016-0013

Li, Y. J., Xu, L., Sun, G. Z., Sun, R., Zhao, W., Qi, H. Y., et al. (2019). Effects of acute short-term heat stress on behavioral indicators, physiological constant and serum antioxidant indicators of rabbits. *Animal Husbandry & Veterinary Medicine* 9, 64–68.

Liu, H., Dong, X., Tong, J., and Zhang, Q. (2010). Alfalfa polysaccharides improve the growth performance and antioxidant status of heat-stressed rabbits. *Livest. Sci.* 131, 88–93. doi: 10.1016/j.livsci.2010.03.004

Liu, H., Zhang, B., Li, F., Liu, L., and Li, F. (2022a). Shifts in the intestinal microflora of meat rabbits in response to glucocorticoids. *J. Sci. Food Agric.* 102, 5422–5428. doi: 10.1002/jsfa.11895

Liu, H., Zhang, B., Liu, L., and Li, F. (2022b). Metabolomics research on the effect of dexamethasone-induced chronic stress in serum of rabbits. *Ital. J. Anim. Sci.* 21, 188–197. doi: 10.1080/1828051X.2021.2004937

Marai, I. F. M., Habeeb, A. A. M., and Gad, A. E. (2002). Rabbits' productive, reproductive and physiological performance traits as affected by heat stress: a review | onAcademic. *Livest. Prod. Sci.* 78, 71–90. doi: 10.1016/s0301-6226(02)00091-x

Mccauley, R., Kong, S., and Hall, J. (1998). Review: glutamine and nucleotide metabolism within enterocytes. *JPEN J. Parenter. Enteral Nutr.* 22, 105–111. doi: 10.1177/014860719802200210

Mete, F., Kilic, E., Somay, A., and Yilmaz, B. (2012). Effects of heat stress on endocrine functions & behaviour in the pre-pubertal rat. *Indian J. Med. Res.* 135, 233–239. doi:http://dx.doi.org/.

Mount, L. E. (1975). The assessment of thermal environment in relation to pig production. *Livest. Prod. Sci.* 2, 381–392. doi: 10.1016/0301-6226(75)90121-9

Png, C. W., Lindén, S. K., Gilshenan, K. S., Zoetendal, E. G., McSweeney, C. S., Sly, L. I., et al. (2010). Mucolytic bacteria with increased prevalence in IBD mucosa AugmentIn VitroUtilization of mucin by other bacteria. *Off. J. Am. Coll. Gastroenterol. ACG* 105, 2420–2428. doi: 10.1038/ajg.2010.281

Shanware, N. P., Mullen, A. R., Deberardinis, R. J., and Abraham, R. T. (2011). Glutamine: pleiotropic roles in tumor growth and stress resistance. *J. Mol. Med.* 89, 229–236. doi: 10.1007/s00109-011-0731-9

Song, J., Xiao, K., Ke, Y. L., Jiao, L. F., Hu, C. H., Diao, Q. Y., et al. (2014). Effect of a probiotic mixture on intestinal microflora, morphology, and barrier integrity of broilers subjected to heat stress. *Poult. Sci.* 93, 581–588. doi: 10.3382/ps.2013-03455

Spiegel, S., and Milstien, S. (2003). Sphingosine-1-phosphate: an enigmatic signalling lipid. *Nat. Rev. Mol. Cell Biol.* 4, 397–407. doi: 10.1038/nrm1103

Straadt, I. K., Young, J. F., Bross, P., Gregersen, N., Oksbjerg, N., Theil, P. K., et al. (2010). NMR-based metabonomic investigation of heat stress in myotubes reveals a time-dependent change in the metabolites. *J. Agric. Food Chem.* 58, 6376–6386. doi: 10.1021/jf904197u

Tang, X. (2015). Proteomics and metabolomics analysis on the molecular mechanism of heat stress in broilers.

Tang, J., and Chen, Z. (2016). The protective effect of γ-aminobutyric acid on the development of immune function in chickens under heat stress. *J. Anim. Physiol. Anim. Nutr.* 100, 768–777. doi: 10.1111/jpn.12385

Thaxton, P., Sadler, C. R., and Glick, B. (1968). Immune response of chickens following heat exposure or injections with ACTH. *Poult. Sci.* 47, 264–266. doi: 10.3382/ps.0470264

Wang, G., Luo, X. G., Liu, B., Shao, G. Z., Su-Fen, L. I., and Shun-Xiang, Y. U. (2003). Effect of dietary chromium on Growth,Serum biochemical traits and carcass quality of heat stressed broilers. Chin. J. Anim. Vet Sci. Available at: http://en.cnki.com.cn/Article_en/CJFDTOTAL-XMSY200302002.htm (Accessed March 7, 2022).

Wei, Z., Xu, Y., Xu, Q., Cao, W., Huang, H., and Liu, H. (2021). Microbial biosynthesis of L-malic acid and related metabolic engineering strategies: advances and prospects. *Front. Bioeng. Biotechnol.* 9:765685. doi: 10.3389/fbioe.2021.765685

West, J. W. (2003). Effects of heat-stress on production in dairy cattle. *J. Dairy Sci.* 86, 2131–2144. doi: 10.3168/jds.S0022-0302(03)73803-X

Yang, F. Z., Xie, Z. Q., Xie, X. H., Yi, J., and Lei, M. (2011). Research progress on nutritional regulation of rabbit heat stress. *China Herbivores* 4, 68–71.

You, L., Lin, H., Luo, D., and Liu, Y. F. (1999). Effect of heat stress on biochemical indices in serum and Endocrinic function of broilers. J. ZHANJIANG OCEAN Univ. Available at: http://en.cnki.com.cn/article_en/cjfdtotal-shdx199901012.htm [Accessed March 7, 2022].

Zhang, H., DiBaise, J. K., Zuccolo, A., Kudrna, D., Braidotti, M., Yu, Y., et al. (2009). Human gut microbiota in obesity and after gastric bypass. *Proc. Natl. Acad. Sci.* 106, 2365–2370. doi: 10.1073/pnas.0812600106

Zhangjun-Min, G. C. (2002). Effect of glutamine supplementation on intestinal mucosal protein, DNA and mucosal morphology of early-weaned piglets. J. Integr. Agric.. Available at: https://d.wanfangdata.com.cn/Periodical/zgnykx-e200212012 [Accessed March 7, 2022].

Zheng, J., Yang, C., Lei, M., Tang, L., Zhang, X. Y., Xie, X. H., et al. (2022). Effect of heat stress on reproductive performance of rabbits and research progress of heat shock proteins. *Chinese Journal of Rabbit Farming* 1, 19–22.

Zhou, J., and Zhou, X. (2010). HSP70 and its role in airway chronic inflammation. Int. J. Pathol. Clin. Med. Available at: http://en.cnki.com.cn/Article_en/CJFDTOTAL-WYSB201005015.htm (Accessed March 7, 2022).

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# Rumen-protected glucose supplementation alters fecal microbiota and its metabolic profiles in early lactation dairy cows

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**Introduction:** Negative energy balance (NEB) is the pathological basis of metabolic disorders in early lactation dairy cows. Rumen-protected glucose (RPG) is a feed additive to relieve NEB of cows in early lactation. The aims of the current study were to evaluate the impact of different doses of RPG supply on fecal microbiota and metabolome in early lactation dairy cows, and their correlation with each other.

**Methods:** A total of 24 multiparous Holstein dairy cows in early lactation were randomly assigned to one of four treatments for the first 35 days of the early lactation period, as follows: control group, a basal diet without RPG (CON); low RPG, a basal diet plus 200 g/d RPG (LRPG); medium RPG, a basal diet plus 350 g/d RPG (MRPG); or HRPG, high RPG, a basal diet plus 500 g/d RPG (HRPG). After 35 days, fecal samples were obtained from cows in all groups individually and using 16S rRNA gene sequencing to evaluate their microbiotas, while their metabolites were evaluated through metabolomics.

**Results:** As expected, *Firmicutes* and *Bacteroidetes* were the core bacteria phyla. After RPG supplementation, there were an increase in *Firmicutes* and a decrease in *Bacteroidetes*. MRPG increased the relative abundance of cellulolytic bacteria, including *Ruminococcaceae_UCG-005*, *Lachnospiraceae_UCG-008*, *Lachnospiraceae_FCS020_group*, and *Ruminiclostridium_9*, while it decreased the relative abundance of *Alistipes*, *Prevotellaceae_UCG-003*, and *Dorea*. RPG supplementation could regulate the carbohydrate metabolism and amino acid metabolism pathway significantly and relieve lipolysis in dairy cows. Correlation analysis of fecal

microbiome and metabolome showed that some major differential bacteria were the crucial contributors to differential metabolites.

**Conclusion:** In conclusion, RPG supplementation can affect the fecal microbial components and microbial metabolism, and 350 g RPG might be the ideal dose as a daily supplement.

KEYWORDS

**negative energy balance, rumen-protected glucose, fecal microbiota, metabolite, dairy cows**

## Introduction

Accompanied by the decrease in dry matter intake (DMI) around parturition and the increased energy requirements for milk in early lactation, cows are prone to negative energy balance (NEB), leading to a series of metabolic disorders such as ketosis and fatty liver (Pérez-Báez et al., 2019a,b). The essence of NEB is the lack of glucose, while the bacterial transformation of dietary components may play key roles in host health and disease (Zhang and Davies, 2016), so the potential of manipulating the microbiome and meeting livestock challenges through dietary interventions have recently emerged as promising new technologies (Huws et al., 2018). Extensive degradation of directly supplemented glucose in the rumen via microbial degradation substantially lowers the post-ruminal availability of dietary glucose. Rumen-protected glucose (RPG) can escape rumen fermentation partly and play a role in the intestine under the coating of hydrogenated fat.

The gut microbiota produces a large number of bioactive metabolites through fermented feed, which plays a crucial role in regulating key physiological processes, like host metabolism and immune response (Thursby and Juge, 2017). These coordinated processes facilitate the growth and development of dairy cows (Malmuthuge and Guan, 2017). Recently, some research revealed that diet changes greatly influence the intestinal microbiota of cattle (Shanks et al., 2011; Faulkner et al., 2017; Lourenco et al., 2020), and the fecal microbiome may serve as a means for assessing intestinal (Faulkner et al., 2017). The fecal microbiome of cows plays an important part not only in animal development and performance but also in food safety, pathogen shedding, and the performance of fecal contamination detection methods (Shanks et al., 2011). Diet is considered as one of the main drivers that shape the gut microbiota throughout life time. Intestinal bacteria play an important part in maintaining immune and metabolic homeostasis and protecting against pathogens. Altered gut bacterial composition and even dysregulation are implicated in the pathogenesis of many inflammatory diseases and infections (Thursby and Juge, 2017). A previous study uncovered that dietary RPG supplementation not only promoted epithelial metabolism but also improved immune homeostasis in the ileum (Zhang et al., 2019). However, as a feed additive that plays a role in the hindgut, whether and how RPG supplementation has an effect on the flora and metabolism of the hindgut microorganisms remains unknown.

Metabolomics constitutes a powerful avenue for the functional characterization of the gut microbiota and its interactions with the host (Marcobal et al., 2013). The application of metabolomics approaches has greatly advanced our understanding of the mechanisms that link the composition and activity of the gut microbiote to health and disease phenotypes (Thursby and Juge, 2017). Our previous work used metabolomics technology to reveal that RPG had a positive effect on dairy cattle serum metabolism and conventional serum biochemical indicators of energy balance. As far as we know, no studies were performed to investigate the effects of different doses of RPG supplementation on fecal microbiota and metabolites in early lactation dairy cows. Therefore, it was our objective to evaluate the dynamic profile changes and interactions of fecal microbiota and metabolites in early lactation dairy cows by supplementing different doses of RPG. We hypothesized that RPG supplementation can improve the structure of intestinal microbiota, alleviate lipid metabolism of intestinal microbiota, and provide more glucose for intestinal absorption and utilization. To address this hypothesis, we employed 16S rRNA gene sequencing along with metabolomics approach to exhaustively characterize the structural and metabolic changes in the gut microbiota elicited by experimental treatments.

## Materials and methods

Protocols for this experiment were reviewed and approved by the Animal Ethics Committee of the Chinese Academy of Agricultural Sciences (Beijing, China) (Approval Number: IAS2019-54) and were under the academy's guidelines for animal research.

### Animals, diets, and experimental design

The dairy farm is located in Beijing, China, and is affiliated with Beijing Sunlon Livestock Development Ltd. All diets were

formulated to meet National Research Council [NRC] (2001) dairy predicted requirements. Nutrient composition of the basal diet during the experiment is shown in **Supplementary Table 1**. During the experiment, cows were milked three times daily at 06:00, 13:00, and 20:00 and fed three times daily at 07:00, 14:00, and 21:00, respectively. Cows were housed in individual stalls, where water was available at all times.

Multiparous Holstein cows in early lactation ($n$ = 24) were grouped according to their parity (2.92 ± 1.19) and milk yield (35.78 ± 7.90 kg/d) and assigned randomly to receive control diet (CON, $n$ = 6), low RPG diet (LRPG, $n$ = 6), medium RPG diet (MRPG, $n$ = 6), or high RPG diet (HRPG, $n$ = 6) group in a randomized block design. Supplemental RPG was fed twice daily as a top dress on the basal diet from the calving date to 35 days postpartum with the first 7 days for adaptation. The dosage of RPG was based on the manufacturer's recommendation (200–500 g/cow/day) and a previous study which reported 200 g of supplemented RPG was insufficient to meet the elevated energy requirements associated with increased milk production which reported 200 g of supplemented RPG was insufficient to meet the elevated energy requirements associated with increased milk production (Li et al., 2019; Wang et al., 2020). Therefore, cows were supplemented with 0, 200, 350, or 500 g RPG per cow per day, respectively. The commercially sourced RPG is in the form of small beads made by mass with 45% glucose as the core, 45% hydrogenated fat as the coating, and 10% water. As such, 0, 90, 157.5, or 225 g of glucose supplement per day was received by cows from CON, LRPG, MRPG, or HRPG group, respectively.

## Fecal sampling and storage

At the end of the experiment (day 35), fecal samples were collected by digital rectal palpation before midday feeding according to institutional animal care guidelines. Immediately after collection, a subset of the fecal samples were immersed in liquid nitrogen for freezing and then stored at −80°C for further DNA extraction, and part of the samples used for metabolomics analysis were first filtered through four layers of cheesecloth, followed by the same procedures. Unfortunately, a fecal sample used to determine the 16S rRNA was damaged.

## DNA extraction and sequencing

Using the E.Z.N.A.® soil DNA Kit (Omega Bio-tek, Norcross, GA, USA) to extract microbial genomic DNA from fecal samples according to manufacturer's instructions. The DNA extract was checked on 1% agarose gel, and DNA concentration and purity were determined with NanoDrop 2000 UV-vis spectrophotometer (Thermo Fisher Scientific, Wilmington, USA). After extracting the total DNA of the sample, primers were designed according to the conserved region, the hypervariable region V3-V4 of the bacterial 16S rRNA gene was amplified with primer pairs 338F (5′-ACTCCTACGGGAGGCAGCAG-3′) and 806R (5′-GGACTACHVGGGTWTCTAAT-3′) by an ABI Gene Amp® 9700 PCR thermocycler (ABI, CA, USA) (Zhang et al., 2017), and sequencing adaptors were added to the end of primers. The amplicons were purified, quantified, and homogenized to obtain a sequencing library; then library QC was performed and the qualified library was sequenced on Illumina HiSeq 2500. The original image data files got by high-throughput sequencing of sequencing platforms, such as Illumina MiSeq were converted into Sequenced Reads by Base Calling analysis, and the results were stored in FASTQ referred to as fq format file, which contains sequence information of reads and their corresponding sequencing quality information.

## Bioinformatics workflow

Data preprocessing: According to the overlap relation between PE reads, merge the paired-ends sequence data obtained from Miseq sequencing into tags of one sequence, then perform quality control and filtering for reads quality and Merge effect. There are three main steps as follows: (1) PE reads merge: FLASH (Version 1.2.7) software is used to merge reads through overlap, the obtained merged sequences are Raw Tags; (2) Tags filtering: Use Trimmomatic (Version 0.33) software to filter merged Raw Tags to get high quality processed reads; (3) Remove Chimera: Use UCHIME (Version 4.2) software to identify and remove chimera sequences to get Effective Tags. Bioinformatics content: Divide operational taxonomic units (OTU), diversity, and difference analysis. All the raw reads were submitted to the NCBI Sequence Read Archive (SRA)[1] database (Accession Number: PRJNA657329).

OTU is Operational Taxonomic Unit. Perform OTU classification for all sequences according to different similarity levels. Generally speaking, when the similarity between sequences is higher than 97%, it can be defined as an OTU, and each OTU corresponds to a representative sequence. Use Usearch software (Edgar, 2013) then perform taxonomic annotation for OTU on the basis of SILVA (Bacteria) and UNITEUNITE (fungi) taxonomic database. The count of OTU of each sample was obtained at 97% similarity level. Combined with the species represented by OTU, shared microorganisms in different environments can be found. In order to obtain the species classification information of each OTU, the representative sequences of the OTU can be aligned to the microbial reference database, and then the community composition of each sample at each level (phylum, class, order, family, genus, and species) was counted.

1 http://www.ncbi.nlm.nih.gov/Traces/sra/

Use QIIME software to generate the composition of the microbial community, at varying taxonomic levels, which was graphed in R (detail the package used and the version of R used). Alpha diversity reflects the species richness and the species diversity of the individual sample. Use Mothur (Version 1.30) software to investigate the Alpha diversity index of samples. The Shannon diversity rarefaction curve is made by Mothur software and R, Shannon indexes of sequencing amount under different sequencing depths are also used. Use QIIME software to perform Beta diversity analysis to compare the similarity of species diversity between different samples. Non-metric multi-dimensional scaling (NMDS) can simplify the research objects from multi-dimensional space to low-dimensional space for positioning, analysis, and classification. Significant difference analysis between groups is mainly used to find biomarkers with statistical differences between different groups. According to the established Biomarker screening criteria [line discriminant analysis (LDA) score $>$ 2], the eligible biomarkers are identified and presented as icons. Line discriminant analysis effect size (LEfSe) can find Biomarkers with statistical difference between different groups (Segata et al., 2011), by combining the statistical significance of the Kruskal-Wallis tests with additional tests to assess biological consistency and effect relevance.

## Metabolomics processing

Fecal samples were layered for 1 h at room temperature and then centrifuged at 3,000 g for 10 min at 4°C. Before analysis, the supernatant was added with 400 μL of ice-cold methanol/water (4:1, v/v) and then ground with high-throughput tissue grinder at a low temperature. After vertexing, samples were sonicated three times for 10 min on ice, followed by treated at −20°C for 30 min. Subsequently, after a centrifugation at 13,000 *g*, 4°C for 15 min, all the samples supernatant were collected and transferred to injection vials for further liquid chromatography-mass spectrometry analysis (LC-MS).

Fecal metabolites were analyzed using Ultra-performance liquid chromatography (UPLC) coupled with a Triple time-of-flight (TOF) system (AB SCIEX, Framingham, USA). The chromatographic separation was performed on a BEH C18 column at 40°C (100 mm × 2.1 mm, 1.7 μm, Waters, Milford, USA). The mobile phase consisted of 0.1% formic acid aqueous solution (solvent A) and acetonitrile/isopropanol (v/v, 1:1) solution with 0.1% formic acid (solvent B). The injection volume was 20 μL and the elution gradient was shown in **Supplementary Table 2**.

Mass spectrometry was performed on a Triple TOF mass spectrometer, and the electrospray ionization source operated simultaneously in positive and negative-ion modes. The capillary voltage was 1.0 kV, the injection voltage was 40 V, and the collision energy was 6 eV. Source temperature was 120°C, and desolvation temperature was 500°C. Gas flow rate was 900 L/h. Mass data were collected between 50 and 1,000 m/z and the instrument resolution was 30,000.

## Metabolomics data analysis

Metabolomics data files were processed to multivariate statistical analysis using SIMCA-P 14.0 software (Umetrics, Umeå, Sweden). The preprocessing results were imported into the software package for log-transformation and pareto-scaling prior to performing multivariate analysis. The score plot of the unsupervised multivariate statistical principal component analysis (PCA) model was used to display the overall metabolic profile of all samples, which was used to reveal the similarities and dissimilarities among the inter-groups. Then partial least squares discriminant analysis (PLS-DA) was used to identify the overall differences in metabolic profiles between groups and to find metabolites with different abundance. PLS-DA is a widely used supervised multivariate classification model. Samples were specified and grouped during analysis, and the model will automatically add another data set Y. This method of model calculation forcibly classifies each component to facilitate the discovery of similarities and differences between different groups. For samples with insufficient differences between groups, the PLS-DA model may be more effective. In PLS-DA analysis, the value of variable importance in projection (VIP) score was greater than 1, which was the typical rule for selecting differential variables. The permutation test was a random sort method to evaluate the accuracy of the PLS-DA model. In order to prevent the model from over-fitting, 200 permutation tests were used to examine the fitting effect of the model. T-test combined with PLS-DA multivariate analysis was used to screen the metabolites with differential abundance between groups. The screening criteria were: VIP $>$ 2 and $P < 0.05$.

## Correlations between microbial communities and fecal metabolites

By Spearman's correlation analysis in R (version 3.2.4), correlation analysis of different metabolites with VIP $>$ 2, $P < 0.05$, and differentially abundant microbial genera ($P < 0.05$ and relative abundance $>$ 0.05% in at least one of the samples) were assessed. Spearman's rank correlations were calculated using the Psych packages (FDR correct was embedded in the package) in R. Only connections with *p*-value below 0.05 and $|r| > 0.4$ were considered as statistically significant. These correlations were visualized using the Heatmap package in R and Cytoscape (version 2.8.2).

# Results

## Changes of fecal bacteria

Following Illumina sequencing, a total of the 1,807,144 raw reads were obtained in the four group samples. After paired-end reads were spliced and filtered, 1,760,224 processed reads were generated, accounting for 97% of the raw reads.

At least 44,129 processed reads were obtained from each sample, with an average of 76,531 processed reads. Rarefaction curves indicated that a high level of microbial diversity was obtained for subsequent analysis of treatments (**Supplementary Figure 1**). When the curve tends to be flat, it means the sequencing data are sufficient and the OTU species won't grow with sequencing data growing (**Supplementary Figure 1**). In addition, OTU Coverage is counted, the higher its value is, the higher the probability of species measured in the sample is. The index reflects whether the sequencing result represents the actual situation of the microorganism in the sample. Similarly, the Good's coverage value of all samples exceeded 99.7%, demonstrating the accuracy and reproducibility of the sequencing in this study.

The curve explains both the species richness and species evenness in the sample (**Supplementary Figure 2**). Rank abundance curves for all treatments were usually overlapping, which revealed that overall microbial diversity was similar. NMDS analysis result was shown as follows: the closer the samples are on the coordinate graph, the higher the similarity is. The treatment groups were separated from the control group (**Figure 1**). Chao1 and Ace indexes measure species richness, while larger Shannon index and smaller Simpson index indicate that the species diversity of the sample is higher (Grice et al., 2009). Alpha diversity analysis showed that there were no significant differences in diversity and richness between groups (**Table 1**).

In total, 12 bacterial phyla were identified in the fecal samples. Independent of the dose of RPG, *Firmicutes* and *Bacteroidetes* were the two predominant phyla in the dairy cow's fecal samples. The combined abundance of these two phyla accounts for 90.46% of the entire microbial community. As shown in **Figure 2A**, the mean abundance levels of *Firmicutes* and *Bacteroidetes* are 55.9 ± 4.4% (mean ± standard error of

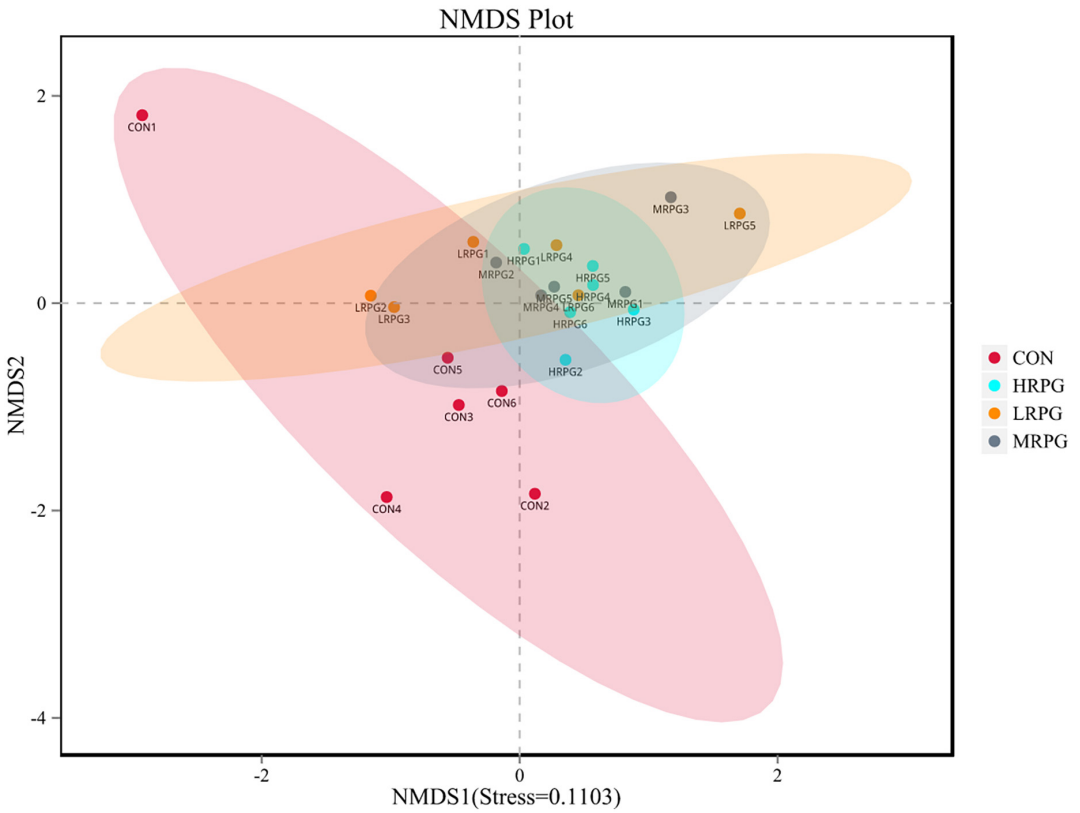


FIGURE 1
Non-metric multi-dimensional scaling (NMDS) analysis graph. Dots represent all samples; different colors represent different groups, and the distance between dots presents the difference; when stress is less than 0.2, it indicates that NMDS analysis has certain reliability. The closer the samples are on the coordinate diagram, the higher the similarity is.

TABLE 1 Alpha diversity index statistics of fecal microbiota.

| Item | Treatments[a] | | | | SEM[b] | *P* |
|---|---|---|---|---|---|---|
| | CON | LRPG | MRPG | HRPG | | |
| OTU | 689.67 | 678.83 | 681.60 | 693.50 | 28.50 | 0.793 |
| ACE | 703.62 | 697.07 | 691.68 | 707.51 | 20.22 | 0.552 |
| Chao1 | 706.37 | 701.61 | 694.35 | 711.28 | 22.18 | 0.601 |
| Simpson | 0.03 | 0.05 | 0.058 | 0.06 | 0.02 | 0.140 |
| Shannon | 4.80 | 4.63 | 4.71 | 4.64 | 0.29 | 0.715 |

[a] Treatments: CON, control group, a basal diet; LRPG, low RPG, a basal diet plus 200 g/d RPG; MRPG, medium RPG, a basal diet plus 350 g/d RPG; HRPG, high RPG, a basal diet plus 500 g/d RPG. [b] SEM, standard error of the mean.

the mean) and 34.6 ± 3.2%, respectively. Between them, the relative abundance of *Firmicutes* in CON was significantly lower than that in the three RPG supplementation groups ($P = 0.001$), while the *Bacteroidetes* were the opposite ($P = 0.0004$). The remaining bacterial phyla (e.g., *Proteobacteria*, *Spirochetes*, *Verrucomicrobia*, etc.) accounted for < 4% of the total sequences (Supplementary Table 3).

152 bacterial taxa were identified at the genus level through the analysis of microbiota compositions. Among these genus, *Ruminococcaceae_UCG-005* (28.6 ± 4.7%), *Rikenellaceae_RC9_gut_group* (16.2 ± 2.3%), *Ruminococcaceae_UCG-010* (6.75 ± 0.4%), *uncultured_bacterium_f_Bacteroidales_RF16_group* (5.2 ± 0.5%), *Bacteroides* (5.0 ± 1.27%), *Treponema_2* (3.2 ± 1.5%), and *Alistipes* (2.3 ± 0.4%) were considered as relatively high abundance genus (Figure 2B). Table 2 compared the fecal bacteria composition among the four groups. Among them, seven different genera bacteria were identified. *Ruminococcaceae_UCG-005* ($P = 0.007$), *Lachnospiraceae_UCG-008* ($P = 0.005$), *Lachnospiraceae_FCS020_group* ($P = 0.020$), and *Ruminiclostridium_9* ($P = 0.012$) were greater in MRPG and HRPG compared to CON. However, the relative abundance of *Alistipes* ($P = 0.045$), *Prevotellaceae_UCG-003* ($P = 0.003$), and *Dorea* ($P = 0.023$) was higher in CON than RPG supplementary groups (Table 2).

Line discriminant analysis effect size analysis identified numerous bacteria with significantly different abundances between CON, LRPG, MRPG, and HRPG (Figure 3). Bacteria with LDA scores greater than 3 were speculated to have different abundance across the four dose groups. Here, MRPG cows had a greater abundance of *Lachnospiraceae_UCG-001*, *Lachnospiraceae_UCG-008*, *Escherichia_Shigella*, *Ruminiclostridium_9*, and *Helicobacter*. HRPG cows had a higher abundance of *Ruminococcaceae_UCG-005*, *Sphingomonas*. CON cows had a greater abundance of *Dorea*, *Prevotella_1*, *Prevotellaceae_UCG-003*. Furthermore, we found that the overall abundance of bacteria in the *Firmicutes* phylum was greater in MRPG, while that of *Bacteroidetes* was greater in CON group.

## Fecal metabolomics profiling

The PLS-DA score plot of fecal samples revealed significant separation between comparison groups, and the permutation testing $R^2$ between pairwise comparisons was all greater than 0.89 (Figure 4), which indicates that the model had satisfactory validity and could be used to identify the difference between the two groups.

As shown in Figure 5, a total of 295 metabolites ($P < 0.05$) were identified and quantified in the fecal samples (Supplementary Table 3) according to querying the Human Metabolome Database (HMDB).[2] Among them, 35.02, 19.41, and 16.88% of the differential metabolites were classified into lipids and lipid-like molecules, organic acids and derivatives, and organ heterocyclic compounds, respectively. Through further screening, a total of 45 differential metabolites ($P < 0.05$, VIP > 2) were obtained from the pairwise comparisons between the four groups. Among them, 13, 22, 17, 9, 6, and 11 metabolites ($P < 0.05$, VIP > 2) were significantly different between CON vs. LRPG, CON vs. MRPG, CON vs. HRPG, LRPG vs. MRPG, LRPG vs. HRPG, and MRPG vs. HRPG, respectively. Meanwhile, 11, 9, 5, 4, 3, 1, and 1 metabolites were classified into lipids and lipid-like molecules, organ heterocyclic compounds, organic acids and derivatives, phenylpropanoids and polyketides, organic oxygen compounds, nucleosides, nucleotides, and analogs, organooxygen compounds, respectively (Supplementary Table 4).

Here, we presented histograms of 11 major differential metabolites between each pair of comparison groups (Figure 6). CON cows had the greatest concentrations of KAPA, succinic acid, and some metabolites of lipids, like LysoPC(18:1(9Z)), LysoPE(16:1(9Z)/0:0), dioscoretine, and PE(18:1(9Z)/0:0). In contrast, MRPG cows had the highest concentrations of 2-isopropylmalic acid (2-iPMA) and 2-linoleoyl glycerol (2-LG). In addition, MRPG showed the highest concentrations of multiple metabolites in the paired comparison between the three RPG supplementation groups, like LysoPC(18:1(9Z)), LysoPE(16:1(9Z)/0:0), and PE(18:1(9Z)/0:0). While HRPG cows had a greater concentration of tetracycline than CON and the lowest concentrations of thymine and deoxyinosine.

## Correlations between the fecal microbiome and metabolome

A correlation analysis between the differential bacteria and major differential metabolites was conducted to investigate the potential co-occurrences as displayed with a correlation heat map. *Prevotallaceae_UCG-003* was strong positively associated

2 http://www.hmdb.ca

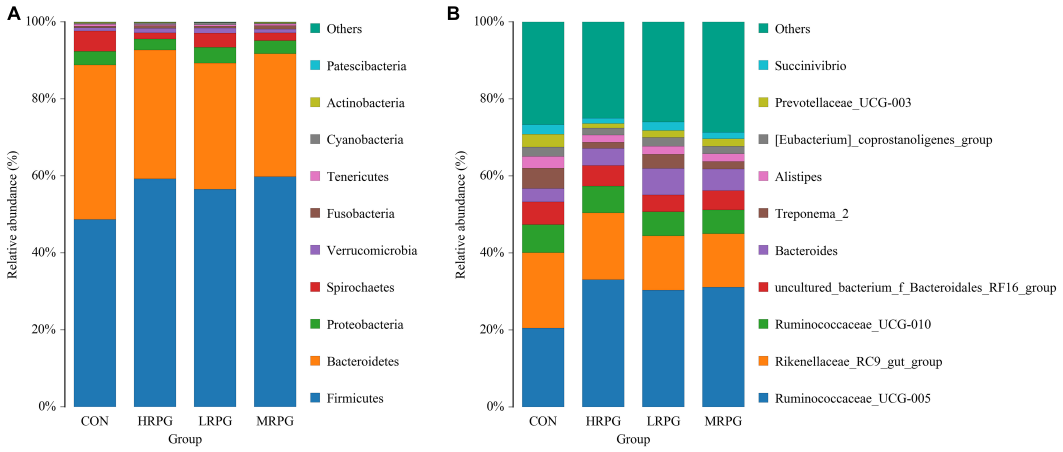


FIGURE 2
Histogram of species distribution of fecal microbiota. One color represents one specie, and the length of the color block presents the relative richness proportion of the species. For the best view, only species with the top 10 richness level will be shown, the rest species are combined as others in the chart, unclassified represents the species that has not been taxonomically annotated. The detailed species information can be found in the species richness table of the corresponding classification. **(A)** Phylum. **(B)** Genus.

TABLE 2 Main microbiota (accounting for ≥ 0.05% of the total sequences in at least one of the samples) in the four groups (abundance of the genera is expressed as a percentage).

| Phylum | Genus | Treatments[1] | | | | SEM[2] | *P* |
|---|---|---|---|---|---|---|---|
| | | CON | LRPG | MRPG | HRPG | | |
| *Firmicutes* | *Ruminococcaceae_UCG-005* | 20.60$^{b}$ | 29.77$^{ab}$ | 31.05$^{a}$ | 32.76$^{a}$ | 5.74 | 0.007 |
| *Bacteroidetes* | *Rikenellaceae_RC9_gut_group* | 19.61 | 14.11 | 13.95 | 17.15 | 3.51 | 0.033 |
| *Bacteroidetes* | *Bacteroides* | 3.40 | 6.76 | 5.60 | 4.37 | 2.54 | 0.147 |
| *Spirochetes* | *Treponema_2* | 5.41 | 3.72 | 2.01 | 1.63 | 3.87 | 0.333 |
| *Bacteroidetes* | *Alistipes* | 3.01$^{a}$ | 2.13$^{ab}$ | 2.03$^{ab}$ | 1.88$^{b}$ | 0.70 | 0.045 |
| *Bacteroidetes* | *Prevotellaceae_UCG-003* | 3.32$^{a}$ | 1.78$^{b}$ | 1.88$^{b}$ | 1.14$^{b}$ | 0.87 | 0.003 |
| *Firmicutes* | *Lachnospiraceae_UCG-008* | 0.24$^{b}$ | 0.32$^{ab}$ | 0.41$^{a}$ | 0.34$^{ab}$ | 0.07 | 0.005 |
| *Firmicutes* | *Dorea* | 0.20$^{a}$ | 0.10$^{ab}$ | 0.12$^{ab}$ | 0.08$^{b}$ | 0.07 | 0.023 |
| *Firmicutes* | *Lachnospiraceae_FCS020_group* | 0.08$^{b}$ | 0.08$^{b}$ | 0.11$^{a}$ | 0.09$^{ab}$ | 0.02 | 0.020 |
| *Firmicutes* | *Ruminiclostridium_9* | 0.06$^{ab}$ | 0.04$^{b}$ | 0.09$^{a}$ | 0.07$^{ab}$ | 0.02 | 0.012 |

$^{a,b,c}$ Means with a row with different superscripts differ. [1]Treatments: CON, control group, a basal diet; LRPG, low RPG, a basal diet plus 200 g/d RPG; MRPG, medium RPG, a basal diet plus 350 g/d RPG; HRPG, high RPG, a basal diet plus 500 g/d RPG. [2] SEM, standard error of the mean.

($r > 0.67$, $P < 0.001$) with thymine, deoxyinosine, and KAPA and positively associated ($r > 0.43$, $P < 0.04$) with succinic acid, dioscoretine, LysoPE(16:1(9Z)/0:0), and LysoPC(18:1(9Z)). *Dorea* was positively associated ($r > 0.48$, $P < 0.02$) with KAPA, deoxyinosine, thymine, succinic acid, dioscoretine, LysoPC(18:1(9Z)). *Treponema_2* was positively associated ($r > 0.47$, $P < 0.02$) with dioscoretine and thymine, but strong negatively associated ($r = -0.64$, $P = 0.001$) with tetracycline. *Alistipes* was positively associated ($r > 0.49$, $P < 0.02$) with dioscoretine, thymine, deoxyinosine, and KAPA and negatively associated ($r = -0.48$, $P = 0.02$) with tetracycline. *Ruminococcaceae_UCG-005* was negatively associated ($r < -0.42$, $P < 0.04$) with dioscoretine, succinic acid, and thymine. *Lachnospiraceae_UCG-008* was also negatively associated ($r < -0.47$, $P < 0.02$) with dioscoretine, succinic acid, and thymine, but positively associated ($r = 0.48$, $P = 0.02$) with 2-LG. *Lachnospiraceae_FCS020_group* was negatively associated ($r = -0.47$, $P = 0.02$) with succinic acid and positively associated ($r = 0.45$, $P = 0.03$) with 2-LG. *Ruminiclostridium_9* was also positively correlated ($r = 0.58$, $P = 0.004$) with 2-LG (Figure 7).

## Discussion

It is increasingly clear that the composition of mammalian gut microbial communities is essentially driven by diet. These microbiota form intricate symbiotic relationship with their hosts, which has profound implications for overall health

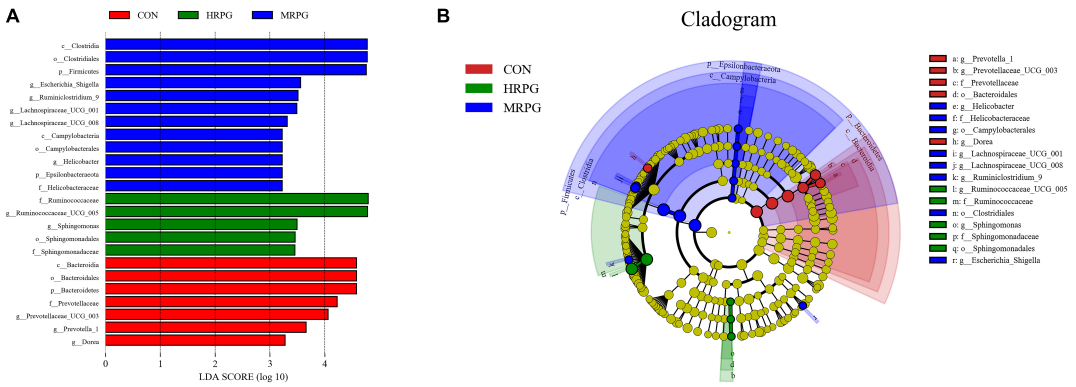


FIGURE 3
Line discriminant analysis effect size (LEfSe) analysis of samples between groups. **(A)** LDA value distribution histogram. The figure shows the species whose LDA Score is higher than the set value (the default is 3.0). The length of the histogram represents the impact of different species, and different colors represent species in different groups. **(B)** Evolutionary branch graph of LEfSe analysis. The circle of radiation from the inside to the outside represents the taxonomic rank from phylum to species. Each small circle at a different classification level represents a classification at that level. The diameter of the small circle is proportional to the relative abundance. The coloring principle is to color the species with no significant difference uniformly as yellow. Other different species are colored according to the group with the highest abundance of the species. Different colors represent different groups, and the nodes with different colors represent the microflora that plays an important role in the group represented by the color.

(La Reau et al., 2016). The variable presence of microorganisms exploiting diet-dependent metabolic pathways may be key to understanding the variable host response to specific dietary components and susceptibility to disease (Zeevi et al., 2015). Perturbations of gut microbiota composition or functions may play a crucial part in the development of health associated with altered metabolism (Velagapudi et al., 2010). The gut microbiota has recently been identified as an environmental factor that may promote metabolic diseases. The gut microbiota not only influences host energy and lipid metabolism but also highlights its role in the development of metabolic diseases (Velagapudi et al., 2010). Nevertheless, NEB is widely regarded as the pathological basis of metabolic disorders such as ketosis and fatty liver disease. Recent findings revealed that RPG significantly alleviated NEB in cows, significantly reduced NEB-related serum biochemical indexes such as NEFA and BHBA, and changed lipid metabolism in the body. However, to the best of our knowledge, no current data establishes associations between fecal microbiota shifts and doses in RPG. The theory about which (and how) bacteria affect gut metabolism after RPG supplementation is not fully clear yet (Lourenco et al., 2020). Although Zhang et al. (2019) have provided an overview of the ileal microflora under RPG supplementation, the effects of RPG on gut microbiota and metabolism need to be further studied. A more complete characterization of dairy cows' fecal bacterial community composition afforded by combined analysis of metabolome and 16S can also help address current research gaps concerning the effects of RPG on the changes of microflora and its metabolism in the hindgut of dairy cows. Inspired by Xue et al.'s 2020 research which assessed potential regulatory mechanisms of milk protein production at both the rumen microbiome and host levels by analyzing rumen metagenomics and metabolomics as well as serum metabolomics, we studied microbial changes in the hindgut at different doses of RPG supplementation by combining fecal 16S rRNA gene amplicon sequencing with metabolomics analysis. Because a massive outflow of fermentable materials from the rumen to the hindgut is considered to lead to post-rumen fermentation in the hindgut, we believe that the fecal microbiota not only partly reflects ruminal microbiota but also represents hindgut microbiota (Uchiyama et al., 2020). Furthermore, the method of collecting rumen contents is invasive and may cause damage to the health of dairy cows. Thus, considering animal welfare, the fecal microbiota is part of the final product of digestion, which could be used as an effective and non-invasive balance diagnosis of gastrointestinal tract health (Hagey et al., 2019). In this study, we used the Illumina-MiSeq sequencing method to study the changes in the microbial composition and metabolism of Holstein cow feces after supplementing RPG.

## Changes of gut microbes at the phylum level after rumen-protected glucose supplementation

*Firmicutes* and *Bacteroidetes* in particular play a critical role in the microbial ecology of the mammalian gut, including the bovine gut (Shanks et al., 2011). Clustering analysis showed that the *Firmicutes* and *Bacteroidetes* were the most dominant phyla in the total sequences, which was consistent with studies by Xie et al. (2020). Importantly, in this study, the composition and structure of the fecal microbial community were affected

by RPG. At the phylum level, the relative abundance of *Firmicutes* in the feces of dairy cows supplemented with RPG increased significantly, while the *Bacteroidetes* decreased significantly. The intestinal microflora is usually controlled by bacteria in Firmicutes and Bacteroidetes (La Reau et al., 2016). The changes in relative abundance of *Bacteroidetes* and the *Firmicutes* bacterial divisions are associated with obesity. It is shown that obesity is positively correlated with Firmicutes (Turnbaugh et al., 2006). The change of phylum level in this study indicates that RPG supplement may relieve lipolysis through the regulation of core microflora, so as to exert an effect on fat deposition in the body. Other bacterial phyla were not significantly different among the groups, a plausible reason for this was that the shared microflora was involved in housekeeping functions, thus masking the role of RPG.

## Changes of gut microbes at the genus level after rumen-protected glucose supplementation

*Ruminococcaceae_UCG-005*, *Rikenellaceae_RC9_gut_group*, *Ruminococcaceae_UCG-010*, and *Bacteroides* were the core bacteria, in line with the report by Li et al. (2020), which also revealed that the dominant genera were *Ruminococcaceae_UCG-005* and *Rikenellaceae_RC9_gut_group*

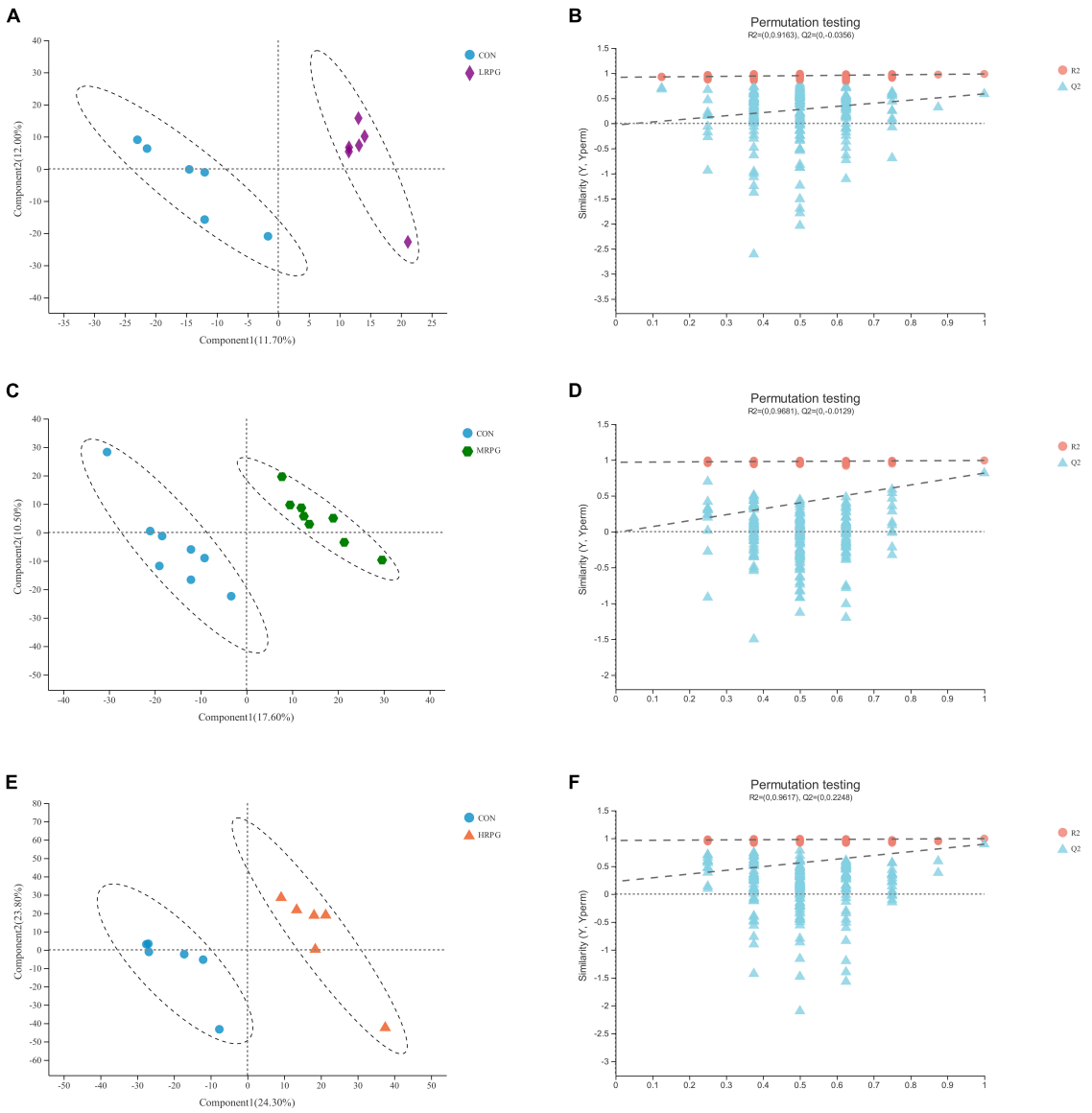


FIGURE 4
(Continued)

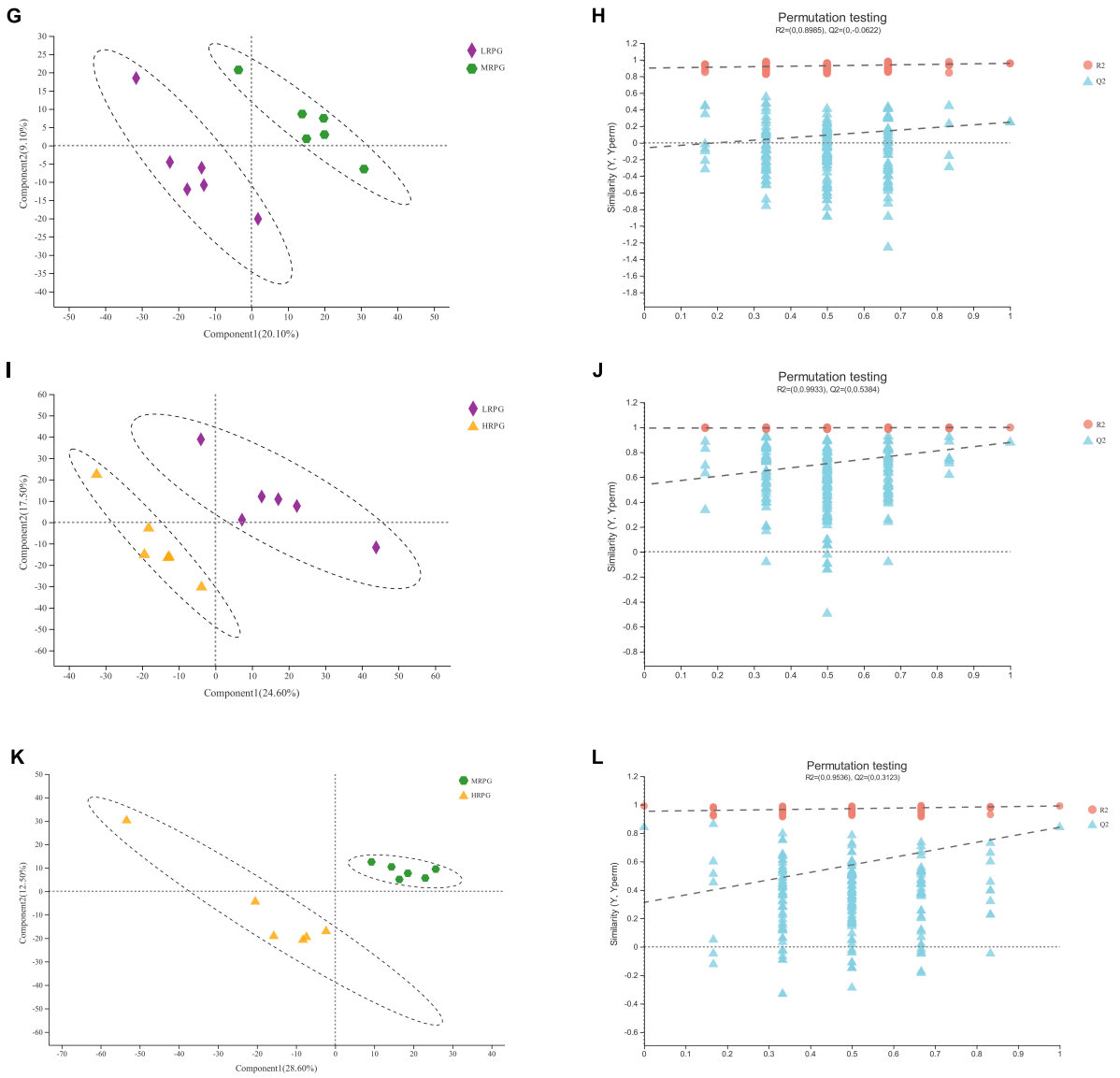


FIGURE 4
PLS-DA score plots and corresponding validation plots derived from the LC-MS metabolite profiles of rumen samples for cows fed increasing doses of RPG supplementary in their diets. PLS-DA score plots and corresponding validation plots (respectively) for: **(A,B)** the CON group vs. LRPG group, **(C,D)** the CON group vs. MRPG group, **(E,F)** the CON group vs. HRPG group, **(G,H)** the LRPG group vs. MRPG group, **(I,J)** the LRPG group vs. HRPG group, and **(K,L)** the MRPG group vs. HRPG group. CON, control group, a basal diet; LRPG, low RPG, a basal diet plus 200 g/d RPG; MRPG, medium RPG, a basal diet plus 350 g/d RPG; HRPG, high RPG, a basal diet plus 500 g/d RPG.

as well as *Bacteroides* observed in feces of dairy cows in the spring season, our study was conducted in the fall, similar to spring. After different doses of RPG supplementation, there were significant differences in the microbiota of feces between groups. The fecal abundances of *Ruminococcae_UCG-005*, *Ruminiclostridium_9*, *Lachnospiraceae_FCS020_group*, and *Lachnospiraceae_UCG-008* in MRPG group were significantly higher than that of CON or LRPG. They belong, respectively, to the *Ruminococcaceae* and *Lachnospiraceae* families. The *Lachnospiraceae* and *Ruminococcaceae* are two of the most abundant families from the order *Clostridiales* found in the mammalian gut environment (Biddle et al., 2013; Mancabelli et al., 2017); both families include cellulose- and hemicellulose-degrading bacteria and members contributing to butyrate production (Lourenco et al., 2020). Butyrate is absorbed by the gastrointestinal tract epithelium. Here, it is converted to β-hydroxybutyrate and acetoacetate and used as energy substrates for the epithelial cells. That could be beneficial to the energy metabolism and development of the gastrointestinal tract in cows (Kamke et al., 2016), that may protect healthy

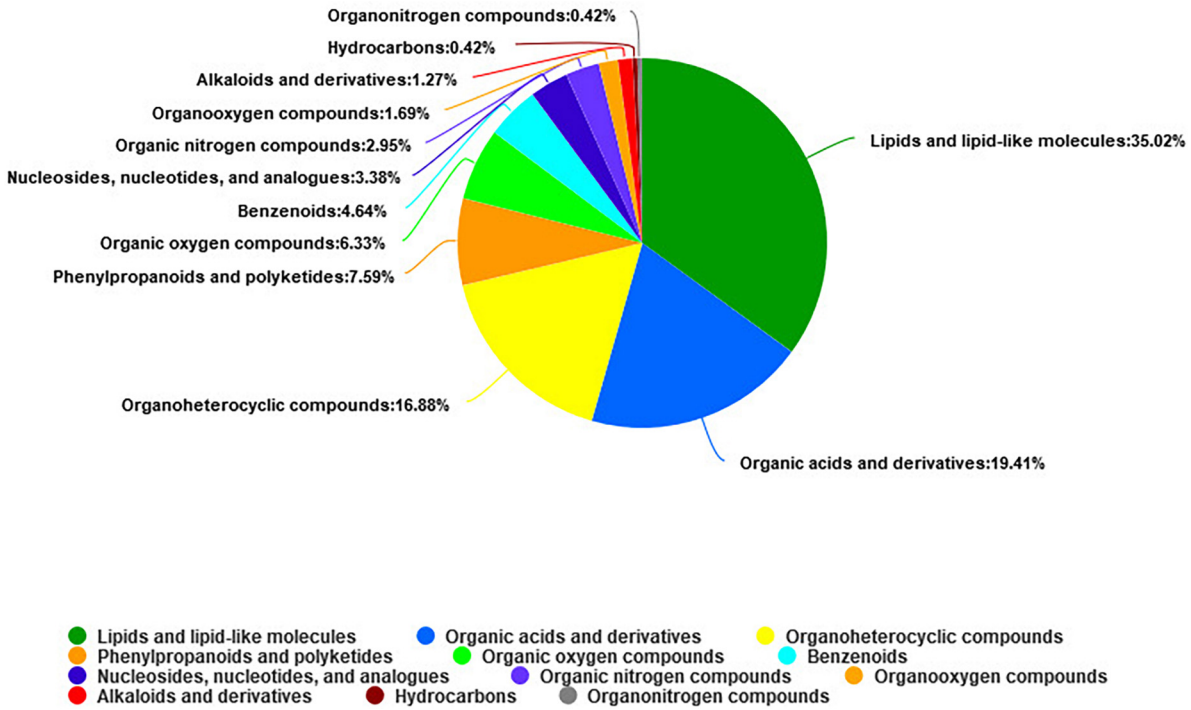


FIGURE 5
The HMDB compound classification of 295 identified metabolites. According to the number of metabolites, the name of selected HMDB hierarchy (superclass) and the percentage of metabolites are displayed from high to low. Different colors in each pie chart represent different HMDB classification, and the area represents the relative proportion of metabolites in the classification.

cows from chronic intestinal inflammation (Lepage et al., 2011) and have been associated with the maintenance of gut health and a healthy state of dairy cows (Biddle et al., 2013; Mancabelli et al., 2017). More specifically, *Ruminiclostridium_9* belongs to the *Ruminococcaceae* family and is associated with the release of inflammatory and cytotoxic factors from the gut for maintenance of a stable intestinal microecology (Xiao et al., 2019). The *Lachnospiraceae* family is known to contribute to volatile fatty acid production, especially butyrate, from diverse polysaccharides (Seshadri et al., 2018). Reduction in the abundance of butyrate-producing *Lachnospiraceae*, which is beneficial for the intestinal barrier, was involved in the formation of visceral hypersensitivity (Zhang et al., 2018). Furthermore, those short-chain fatty acids are known for their anti-inflammatory properties and may also prevent against the development of metabolic diseases (Nguyen et al., 2019). Together, the addition of RPG, especially MRPG, could regulate the relative abundances of intestinal health-related bacteria, and play a positive role in reducing intestinal inflammation and preventing related metabolic diseases.

Other abundant differential genera including *Prevotellaceae_UCG-003*, *Alistipes*, and *Dorea* showed the highest levels in CON group. Previous findings revealed that different *Prevotella spp.* can selectively utilize carbohydrates and proteins from diet to produce succinate and acetate (Stevenson and Weimer, 2007). However, although *Prevotella* have this capability of breaking down different carbohydrates, one of their most significant roles is in the protein and peptide breakdown (Lourenco et al., 2020). This genus plays an important role in normal intestinal metabolism and is also an important substance to maintain intestinal health (Xue et al., 2020). Similar evidence was observed in previous investigations, which highlighted that anaerobic organism such as the abnormal increase of *Prevotellaceae* abundance exacerbated the occurrence of inflammation (Elinav et al., 2011). *Alistipes* is a relatively new genus of bacteria, which was also classified as a member of the phylum *Bacteroidetes* (Kim et al., 2011). Although at a low rate compared to other genus members of the *Bacteroidetes* phylum, which are highly relevant in dysbiosis and disease (Parker et al., 2020). In a previous study, the percentage of genera

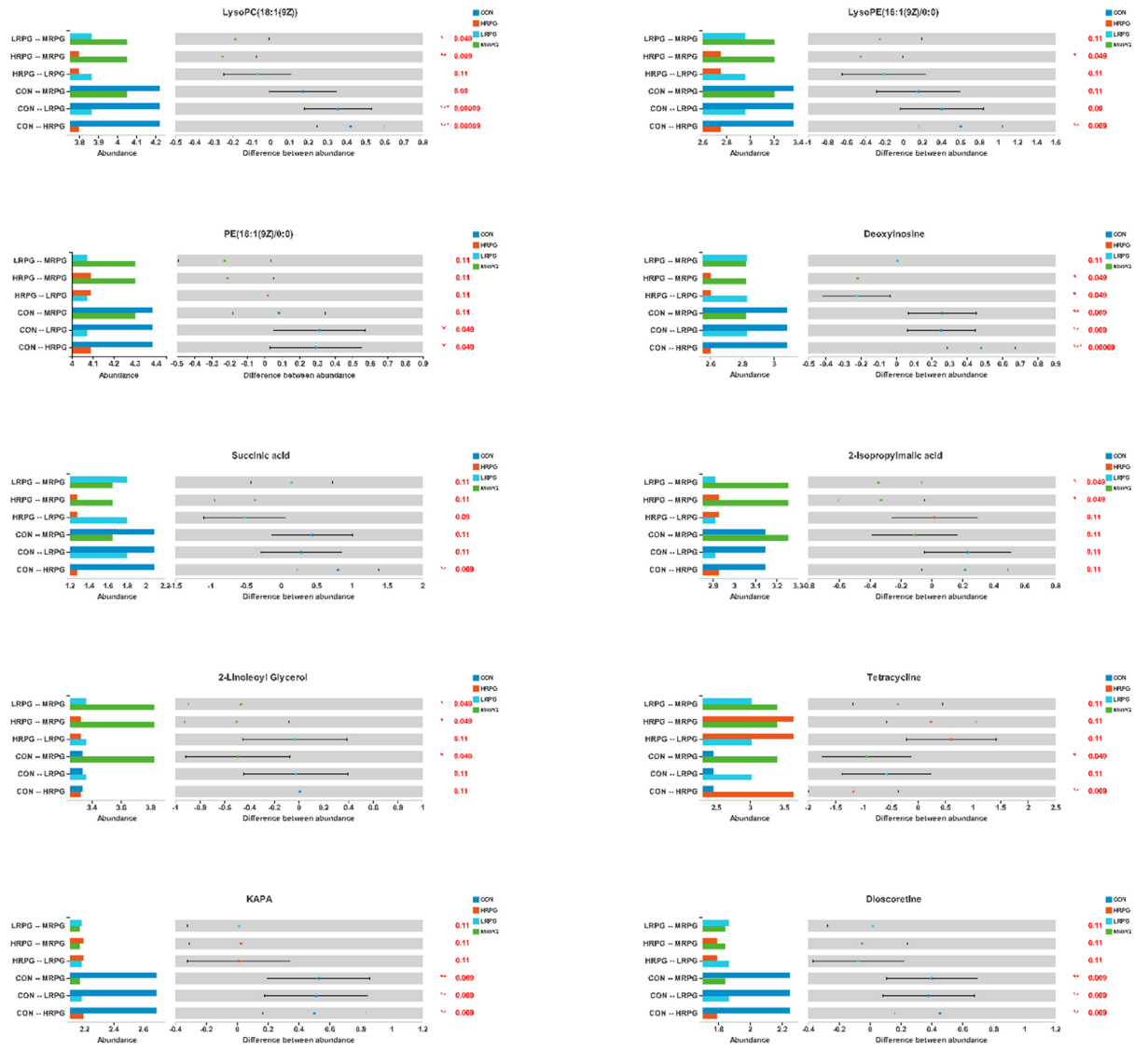


FIGURE 6
Differential bar graph of differential metabolites. The *X*-axis of the left histogram represents the average relative abundance of a certain metabolite in different groups, the *Y*-axis represents the group categories of pairwise comparison, and different colors represent different groups. The middle region is within the confidence interval set, and the value corresponding to the dot represents the difference of the average relative abundance of metabolites in the two groups. The color of the dot is the group color with a large proportion of metabolites. The interval of type I on the dot is the upper and lower limit of the difference value. The right-most value is *P*, *0.01 < *P* 0.05, **0.001 < *P* 0.01, ***$P < 0.001$.

*Alistipes* was significantly decreased in the fecal microbiota of colorectal cancer patients by 454 pyrosequencing (Li et al., 2020). In contrast, other studies indicate *Alistipes* is pathogenic in colorectal cancer and is associated with mental signs of depression (Parker et al., 2020). Meanwhile, dietary change affected the abundance of *Alistipes*, which is a bile-tolerant bacterium (David et al., 2014). Notably, in our study, there was a significant negative correlation between *Alistipes* and tetracycline concentration involved in bile synthesis, that may be related to the location of the farms and the feeding operation. The fecal microbiota of calves is rich, diverse, and potentially highly variable between farms (Weese and Jelinski, 2017). The feeding operation is a more important determinant of the cattle microbiome than in the geographic location of the feedlot (Shanks et al., 2011). *Dorea*, an intestinal bacterium that produces gas with carbohydrates, experienced a marked increase in irritable bowel syndrome. In the feces of patients with non-alcoholic fatty liver disease, the abundance of *Dorea* increased while that of *Ruminococcaceae* decreased (Raman et al., 2013). Similar to the change of bacteria in CON in this study, this indicated that cows in the control group may have risks such as impaired intestinal immunity and lipid metabolism

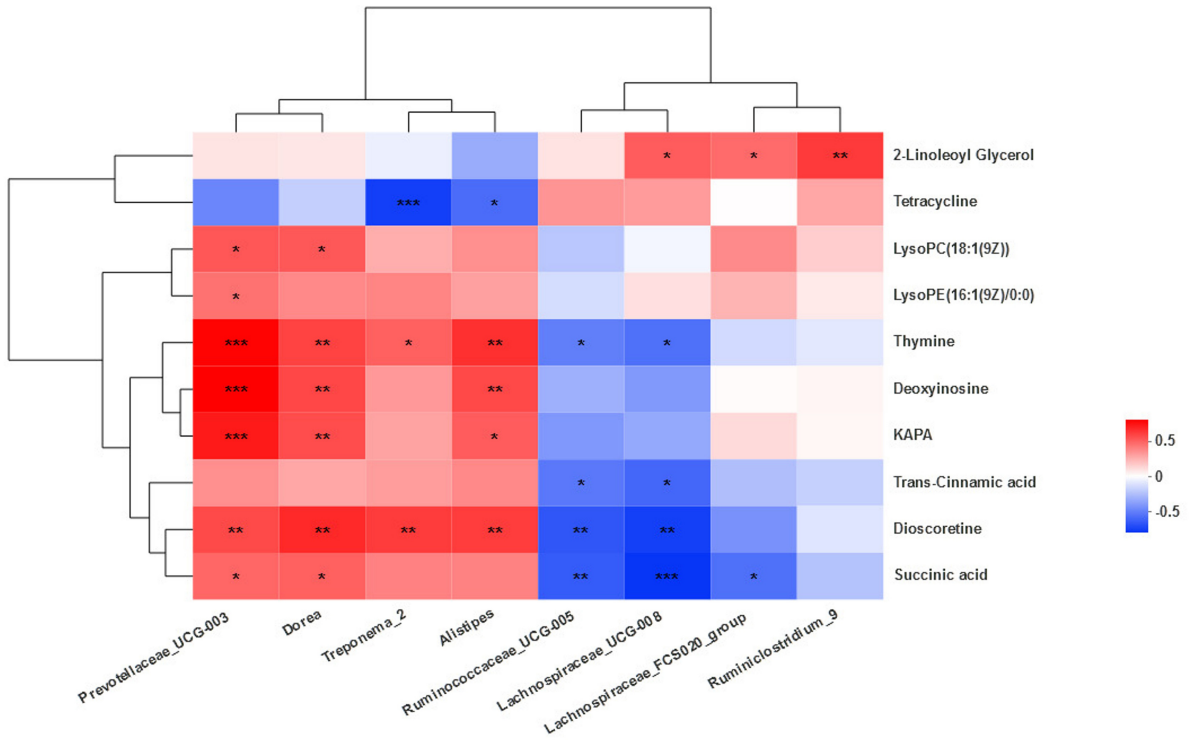


FIGURE 7
Correlation between the microbiota and metabolites. Cells are colored based on Spearman's correlation coefficient: positive correlations are shown in red and negative correlations in blue. ***Means strongly significant relationship ($P < 0.001$), **means extremely significant relationship ($0.001 < P < 0.01$), and *means significant relationship ($0.01 < P < 0.05$).

disorder. The three bacteria mentioned above had the highest levels in the control group, the result corresponded with the downregulation of amino acid metabolism and associated intestinal inflammation in dairy cows.

## Connection between microbe and metabolites

The change in the microbial community was probably caused by an altered metabolism of the host linked to a modified secretion of host metabolites into the gut lumen (Tilocca et al., 2017). A previous study suggested that the fecal bacterial community composition correlated significantly with fecal starch concentrations, largely reflected in changes in the *Bacteroidetes*, Proteobacteria, and *Firmicutes* populations (Shanks et al., 2011). As a result, a mechanism through which diet can influence immune responses is the gut microbiome, which is emerging as a critical contributor in numerous human diseases (Cignarella et al., 2018). The interactions between intestinal bacteria and the host immune system are mediated either via direct contact between bacteria and the innate immune system (e.g., toll-like receptors, NOD2 receptors) or through microbial metabolites. These metabolites can be produced directly by bacteria (e.g., vitamins, SCFA) or are primary host metabolites that are converted through bacterial enzymes into secondary metabolites (e.g., conversion of primary to secondary bile acids) (Suchodolski, 2016). The above contents suggest a higher concern of the response of fecal metabolites to RPG supplementation. Ultra-performance liquid chromatography (UPLC) coupled with high-resolution mass spectrometry (MS) allows reproducible measurements of a wide range of metabolites in a complex sample (Marcobal et al., 2013).

## Dominant gut microbial metabolites of control group, a basal diet without rumen-protected glucose, after rumen-protected glucose supplementation

LysoPC(18:1(9Z)), PE(18:1(9Z)/0:0), LysoPE(16:1(9Z)/0:0), and dioscoretine belong to lipids and lipid-like molecules. Compared with the three RPG groups, the three metabolites were at the highest levels in CON group. LysoPC(18:1(9Z)) is a kind of lysophosphatidylcholines (LysoPC) that belong to glycerophospholipids, resulting from the partial hydrolysis

of phosphatidylcholines, involved in choline metabolism in cancer and the glycerophospholipid metabolism pathway. LysoPC is a strong proinflammatory mediator, and it has been suggested to be mediated via the platelet-activating factor (PAF) receptor (Oestvang et al., 2011). LysoPC initiate and contribute to inflammation through the activation of phospholipase a2 enzymes and the consequent release of amino acid, which is a precursor for the proinflammatory hormones, the eicosanoids (Oestvang et al., 2011). LysoPCs are the major components of ox-low density lipoprotein which play dual functions in the cardiovascular disease (Nuli et al., 2019). LysoPC can activate endothelial cells during early atherosclerosis and can stimulate phagocyte recruitment when they were released by apoptotic cells (Li et al., 2018). Another glycerophosphate PE(18:1(9Z)/0:0) that positively regulate autophagy and longevity (Rockenfeller et al., 2015) is one kind of glycerophosphoethanolamines. Phosphatidylethanolamine (PE) is a class of phospholipids found in biological membranes; it can be found in all living organisms. PE is the second-most abundant phospholipid in mammalian membranes ranging from 20 to 50% (Nuli et al., 2019). LysoPE(16:1(9Z)/0:0) is a kind of lysophosphatidylethanolamine (LysoPE) that belongs to glycerophospholipids, resulting from the partial hydrolysis of PE. Metabolites such as LysoPE were related to the amount of fatty acid in feces (Lee et al., 2013). As stated before, Nuli explored the mechanisms underlying the potential characteristic metabolite changes during different glycemic stages and demonstrated that significantly changed metabolites LysoPC and PE between the normal group and the impaired glucose regulation patients were involved in glycerophospholipid metabolism (Nuli et al., 2019). Together, the results showed that the changes of glycerophosphate in the intestine could reflect the state of glucose metabolism in the body. RPG can down-regulate lipid metabolism and indirectly regulate the body's glucose metabolism.

## Dominant gut microbial metabolites of rumen-protected glucose groups after rumen-protected glucose supplementation

2-IPMA is mainly involved in carbohydrate metabolism and amino acid metabolism. 2-iPMA, an intermediate compound in the leucine biosynthesis pathway, is synthesized from α-ketoisovalerate and acetyl-coenzyme A, a reaction catalyzed by 2-iPMA synthase (Suzuki et al., 2007). Moreover, 2-iPMA possesses superior Al(III)-ion detoxification ability. 2-iPMA secreted from the yeast cells chelates Al ions and prevents them from entering the cells, resulting in Al tolerance (Suzuki et al., 2007). MRPG regulated amino acid metabolism and carbohydrate metabolism and may help alleviate heavy metal poisoning. MRPG interacted amino acid metabolism and carbohydrate metabolism and may help alleviate heavy metal poisoning. Similarly, MRPG significantly increases the concentration of 2-LG. In mammals, biosynthesis, degradation, and metabolism of these bio-active lipids like 2-arachidonoylglycerol (2-AG) and 2-LG intertwine and form a complicated biochemical pathway to affect the mammal neuromodulation of the central nervous system and also other physiological processes in most peripheral organs and non-nervous tissue cells (Yuan et al., 2016). For instance, 2-LG significantly inhibited the inactivation of 2-AG in neuronal and basophilic cells (Murataeva et al., 2016). After MRPG supplementation, some intestinal metabolites participated in neuromodulation. HRPG significantly down-regulated abnormal nucleotide metabolism. Deoxyinosine is an abnormal nucleoside and has hypoxanthine as its base moiety (Murataeva et al., 2016). Deoxyinosine occurs in DNA either by oxidative deamination of a previously incorporated deoxyadenosine residue or by misincorporation of deoxyinosine triphosphate from the nucleotide pool during replication (Yoneshima et al., 2016). Deoxyinosine triphosphate induces cell growth arrest and DNA instability in mammalian cells. The individual nucleobases in feces were analyzed by HPLC after hydrolysis using HClO, all the individual nucleobases were highly digestible. Uracil showed the highest digestibility (on average 96.8%), whereas thymine showed the lowest digestibility (Mydland et al., 2008). Considering that, it was not surprising that HRPG may also regulate related bacteria to improve the digestibility of thymine, for example, significantly negatively correlated *Ruminococcaceae_UCG-005* and *Lachonospiraceae_UCG-008*. However, additional targeted investigations are necessary to explore the mechanism. Collectively, MRPG improved the carbohydrate metabolism and amino acid metabolism pathway in dairy cows and changed the neuromodulation-related metabolites. HRPG supplementation down-regulated abnormal nucleotide metabolism and reduced the concentration of harmful metabolites in nucleotide metabolism pathway.

## Correlations between microbial communities and fecal metabolites

The results of the correlation analysis showed that *Prevotellaceae_UCG-003* and *Dorea* were positively correlated with most of the major differential metabolites. The interactions between microbial taxa and functions with microbial metabolites suggest that the *Prevotellaceae* family may be crucial contributors to microbial metabolites including amino acids and carbohydrates. This observation was consistent with the report by Xue et al. (2020). Gut microbiota is the predominant source of luminal succinate (Faith et al., 2014). Succinate is a gut microbiota-derived metabolite with a key

role in governing intestinal homeostasis and energy metabolism (Fernández-Veledo and Vendrell, 2019). The *Prevotella* species, along with *Succinimonas amilolytica*, act as a succinate-producing bacterium in the bovine rumen (Bryant et al., 1958). Therefore, we retain that the positive correlation could be explained. Even though, the available evidence suggested a link between dysbiosis, succinate accumulation in gut, and inflammation (Fernández-Veledo and Vendrell, 2019). In other words, succinic acid in the intestine was advantageous in certain concentrations. The human (mammalian) biotin cycle in humans is complex. Humans do not synthesize biotin but must obtain it from the diet or, perhaps, from gut bacteria (Beckett, 2018). 7-Keto-8-aminopelargonic acid synthase (KAPA synthase) is the enzyme that catalyzes the first committed step of the biotin synthesis pathway (Fan et al., 2015). Biotin is an essential cofactor for enzymes that function in the carboxylation, decarboxylation, and trans-carboxylation reactions found in processes such as fatty acid biosynthesis, gluconeogenesis, and amino acid metabolism (Fan et al., 2015; Beckett, 2018). *Prevotellaceae_UCG-003*, *Dorea*, and *Alistipes*, which were positively related, may regulate glucose metabolism and amino acid metabolism through the important role of this enzyme. Overall, *Prevotellaceae* and *Dorea* were important contributors of differential metabolites. Succinic acid and some lipids were important differential metabolites in this study. *Bacteroidetes* were positively correlated with major differential metabolites, while *Firmicutes* were negatively correlated with them.

## Conclusion

This study provides the first in-depth understanding of changes in hindgut microbiota, its metabolites and their relationships by combining high-throughput microbiomics and untargeted metabolomics after RPG supplementation in early lactation dairy cows. RPG supplement may relieve lipolysis through the regulation of core microflora, so as to exert an effect on fat deposition in the body, especially MRPG, could regulate the relative abundances of intestinal health-related bacteria, and play a positive role in reducing intestinal inflammation and preventing related metabolic diseases. MRPG improved the carbohydrate metabolism and amino acid metabolism pathway in dairy cows and changed the neuromodulation-related metabolites. HRPG supplementation down-regulated abnormal nucleotide metabolism and reduced the concentration of harmful metabolites in nucleotide metabolism pathway. Overall, our study contributes to an increased understanding of how the RPG supplementation affects the fecal microbiota functions. Our work also provides insights into the influence of RPG on gut metabolism. Further research is needed to understand how RPG influences fat metabolism and glucose metabolism by lipidomics and glycomics, so as to deeply reveal the metabolic mechanism of RPG alleviating NEB.

## 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 Animal Ethics Committee of the Chinese Academy of Agricultural Sciences (Beijing, China) (Approval Number: IAS2019-54).

## Author contributions

YPW conceived, designed, and performed the experiment and drafted the manuscript. XN and YZ participated in the editing of the manuscript. YUW and MC were involved in the animal experiment and sample analysis. QL, BX, and LJ supervised and provided continuous guidance for the experiment. All authors contributed to the article 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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# Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2022.1034675/full#supplementary-material

SUPPLEMENTARY FIGURE 1
Sample Shannon index curve.

SUPPLEMENTARY FIGURE 2
Sample rank-abundance curve.

SUPPLEMENTARY TABLE 1
Nutrition composition of the diet fed to early lactation dairy cows.

SUPPLEMENTARY TABLE 2
Ultra-performance liquid chromatography (UPLC) elution gradient.

SUPPLEMENTARY TABLE 3
Main microbiota (accounting for ≥ 0.05% of the total sequences in at least one of the samples) in the four groups (abundance of the phylum is expressed as a percentage).

SUPPLEMENTARY TABLE 4
List of differential metabolites.

# References

Beckett, D. (2018). Specificity and selectivity in post-translational biotin addition. *Biochem. Soc. Trans.* 46, 1577–1591. doi: 10.1042/BST20180425

Biddle, A., Stewart, L., Blanchard, J., and Leschine, S. (2013). Untangling the genetic basis of fibrolytic specialization by *Lachnospiraceae* and *Ruminococcaceae* in diverse gut communities. *Diversity* 5, 627–640. doi: 10.3390/d5030627

Bryant, M. P., Small, N., Bouma, C., and Chu, H. (1958). *Bacteroides Ruminicola* N. SP. and the new genus and species *Succinimonas amylolytica*. *J. Bacteriol.* 76:336.

Cignarella, F., Cantoni, C., Ghezzi, L., Salter, A., Dorsett, Y., Chen, L., et al. (2018). Intermittent fasting confers protection in CNS autoimmunity by altering the gut microbiota. *Cell Metab.* 27, 1222–1235. doi: 10.1016/j.cmet.2018.05.006

David, L. A., Maurice, C. F., Carmody, R. N., Gootenberg, D. B., Button, J. E., Wolfe, B. E., et al. (2014). Diet rapidly and reproducibly alters the human gut microbiome. *Nature* 505, 559–563. doi: 10.1038/nature12820

Edgar, R. C. (2013). UPARSE: Highly accurate OTU sequences from microbial amplicon reads. *Nat. Methods* 10, 996–998. doi: 10.1038/nmeth.2604

Elinav, E., Strowig, T., Kau, A. L., Henao-Mejia, J., Thaiss, C. A., Booth, C. J., et al. (2011). NLRP6 inflammasome regulates colonic microbial ecology and risk for colitis. *Cell* 145, 745–757. doi: 10.1016/j.cell.2011.04.022

Faith, J. J., Ahern, P. P., Ridaura, V. K., Cheng, J., and Gordon, J. I. (2014). Identifying gut microbe–host phenotype relationships using combinatorial communities in gnotobiotic mice. *Sci. Transl. Med.* 6, 211r–220r. doi: 10.1126/scitranslmed.3008051

Fan, S., Li, D., Wang, D., Fleming, J., Zhang, H., Zhou, Y., et al. (2015). Structure and function of *Mycobacterium smegmatis* 7-keto-8-aminopelargonic acid (KAPA) synthase. *Int. J. Biochem. Cell Biol.* 58, 71–80. doi: 10.1016/j.biocel.2014.11.006

Faulkner, M. J., Wenner, B. A., Solden, L. M., and Weiss, W. P. (2017). Source of supplemental dietary copper, zinc, and manganese affects fecal microbial relative abundance in lactating dairy cows. *J. Dairy Sci.* 100, 1037–1044. doi: 10.3168/jds.2016-11680

Fernández-Veledo, S., and Vendrell, J. (2019). Gut microbiota-derived succinate: Friend or foe in human metabolic diseases? *Rev. Endocr. Metab. Disord.* 20, 439–447. doi: 10.1007/s11154-019-09513-z

Grice, E. A., Kong, H. H., Conlan, S., Deming, C. B., Davis, J., Young, A. C., et al. (2009). Topographical and temporal diversity of the human skin microbiome. *Science* 324, 1190–11902. doi: 10.1126/science.1171700

Hagey, J. V., Bhatnagar, S., Heguy, J. M., Karle, B. M., Price, P. L., Meyer, D., et al. (2019). Fecal microbial communities in a large representative cohort of California dairy cows. *Front. Microbiol.* 10:1093. doi: 10.3389/fmicb.2019.01093

Huws, S. A., Creevey, C. J., Oyama, L. B., Mizrahi, I., Denman, S. E., Popova, M., et al. (2018). Addressing global ruminant agricultural challenges through understanding the rumen microbiome: Past, present, and future. *Front. Microbiol.* 9:2161. doi: 10.3389/fmicb.2018.02161

Kamke, J., Kittelmann, S., Soni, P., Li, Y., Tavendale, M., Ganesh, S., et al. (2016). Rumen metagenome and metatranscriptome analyses of low methane yield sheep reveals a Sharpea-enriched microbiome characterised by lactic acid formation and utilisation. *Microbiome* 4:56. doi: 10.1186/s40168-016-0201-2

Kim, M., Morrison, M., and Yu, Z. (2011). Status of the phylogenetic diversity census of ruminal microbiomes. *FEMS Microbiol. Ecol.* 76, 49–63. doi: 10.1111/j.1574-6941.2010.01029.x

La Reau, A. J., Meier-Kolthoff, J. P., and Suen, G. (2016). Sequence-based analysis of the genus *Ruminococcus* resolves its phylogeny and reveals strong host association. *Microb. Genom.* 2:e99. doi: 10.1099/mgen.0.000099

Lee, C. H., Kim, J., Ahn, S., and Lee, S. (2013). Metabolomic study of a diagnostic model for the metabolites of stool fat. *Korean J. Gastroenterol.* 61, 9–16. doi: 10.4166/kjg.2013.61.1.9

Lepage, P., Häsler, R., Spehlmann, M. E., Rehman, A., Zvirbliene, A., Begun, A., et al. (2011). Twin study indicates loss of interaction between microbiota and mucosa of patients with ulcerative colitis. *Gastroenterology* 141, 227–236. doi: 10.1053/j.gastro.2011.04.011

Li, H., Li, R., Chen, H., Gao, J., Wang, Y., Zhang, Y., et al. (2020). Effect of different seasons (spring vs summer) on the microbiota diversity in the feces of dairy cows. *Int. J. Biometeorol.* 64, 345–354. doi: 10.1007/s00484-019-01812-z

Li, X., Shao, Y., Sha, X., Fang, P., Kuo, Y., Andrews, A. J., et al. (2018). IL-35 (Interleukin-35) suppresses endothelial cell activation by inhibiting mitochondrial reactive oxygen species-mediated site-specific acetylation of H3K14 (Histone 3 Lysine 14). *Arterioscler. Thromb. Vasc. Biol.* 38, 599–609. doi: 10.1161/ATVBAHA.117.310626

Li, X. P., Tan, Z. L., Jiao, J. Z., Long, D. L., Zhou, C. S., Yi, K. L., et al. (2019). Supplementation with fat-coated rumen-protected glucose during the transition period enhances milk production and influences blood biochemical parameters of liver function and inflammation in dairy cows. *Anim. Feed Sci. Technol.* 252, 92–102. doi: 10.1016/j.anifeedsci.2019.04.010

Lourenco, J. M., Kieran, T. J., Seidel, D. S., Glenn, T. C., Silveira, M. F. D., Callaway, T. R., et al. (2020). Comparison of the ruminal and fecal microbiotas in beef calves supplemented or not with concentrate. *PLoS One* 15:e231533. doi: 10.1371/journal.pone.0231533

Malmuthuge, N., and Guan, L. L. (2017). Understanding the gut microbiome of dairy calves: Opportunities to improve early-life gut health1. *J. Dairy Sci.* 100, 5996–6005. doi: 10.3168/jds.2016-12239

Mancabelli, L., Milani, C., Lugli, G. A., Turroni, F., Cocconi, D., van Sinderen, D., et al. (2017). Identification of universal gut microbial biomarkers of common human intestinal diseases by meta-analysis. *FEMS Microbiol. Ecol.* 93:fix153. doi: 10.1093/femsec/fix153

Marcobal, A., Kashyap, P. C., Nelson, T. A., Aronov, P. A., Donia, M. S., Spormann, A., et al. (2013). A metabolomic view of how the human gut microbiota impacts the host metabolome using humanized and gnotobiotic mice. *ISME J.* 7, 1933–1943. doi: 10.1038/ismej.2013.89

Murataeva, N., Dhopeshwarkar, A., Yin, D., Mitjavila, J., Bradshaw, H., Straiker, A., et al. (2016). Where's my entourage? The curious case of 2-oleoylglycerol, 2-linolenoylglycerol, and 2-palmitoylglycerol. *Pharmacol. Res.* 110, 173–180. doi: 10.1016/j.phrs.2016.04.015

Mydland, L. T., Frøyland, J. R. K., and Skrede, A. (2008). Composition of individual nucleobases in diets containing different products from bacterial biomass grown on natural gas, and digestibility in mink (Mustela vison). *J. Anim. Physiol.* 92, 1–8. doi: 10.1111/j.1439-0396.2007.00674.x

National Research Council [NRC] (2001). *Nutrient requirements of dairy cattle: 2001*. Washington, DC: National Academies Press.

Nguyen, T. D., Prykhodko, O., Fåk Hållenius, F., and Nyman, M. (2019). Monovalerin and trivalerin increase brain acetic acid, decrease liver succinic acid, and alter gut microbiota in rats fed high-fat diets. *Eur. J. Nutr.* 58, 1545–1560. doi: 10.1007/s00394-018-1688-z

Nuli, R., Azhati, J., Cai, J., Kadeer, A., Zhang, B., and Mohemaiti, P. (2019). Metagenomics and faecal metabolomics integrative analysis towards the impaired glucose regulation and type 2 diabetes in uyghur-related omics. *J. Diabetes Res.* 2019:2893041. doi: 10.1155/2019/2893041

Oestvang, J., Anthonsen, M. W., and Johansen, B. (2011). LysoPC and PAF trigger arachidonic acid release by divergent signaling mechanisms in monocytes. *J. Lipids* 2011:532145. doi: 10.1155/2011/532145

Parker, B. J., Wearsch, P. A., Veloo, A. C. M., and Rodriguez-Palacios, A. (2020). The genus *Alistipes*: Gut bacteria with emerging implications to inflammation, cancer, and mental health. *Front. Immunol.* 11:906. doi: 10.3389/fimmu.2020.00906

Pérez-Báez, J., Risco, C. A., Chebel, R. C., Gomes, G. C., Greco, L. F., Tao, S., et al. (2019a). Association of dry matter intake and energy balance prepartum and postpartum with health disorders postpartum: Part I. Calving disorders and metritis. *J. Dairy Sci.* 102, 9138–9150. doi: 10.3168/jds.2018-15878

Pérez-Báez, J., Risco, C. A., Chebel, R. C., Gomes, G. C., Greco, L. F., Tao, S., et al. (2019b). Association of dry matter intake and energy balance prepartum and postpartum with health disorders postpartum: Part II. Ketosis and clinical mastitis. *J. Dairy Sci.* 102, 9151–9164. doi: 10.3168/jds.2018-15879

Raman, M., Ahmed, I., Gillevet, P. M., Probert, C. S., Ratcliffe, N. M., Smith, S., et al. (2013). Fecal microbiome and volatile organic compound metabolome in obese humans with nonalcoholic fatty liver disease. *Clin. Gastroenterol. Hepatol.* 11, 868–875. doi: 10.1016/j.cgh.2013.02.015

Rockenfeller, P., Koska, M., Pietrocola, F., Minois, N., Knittelfelder, O., Sica, V., et al. (2015). Phosphatidylethanolamine positively regulates autophagy and longevity. *Cell Death Differ.* 22, 499–508. doi: 10.1038/cdd.2014.219

Segata, N., Izard, J., Waldron, L., Gevers, D., Miropolsky, L., Garrett, W. S., et al. (2011). Metagenomic biomarker discovery and explanation. *Genome Biol.* 12:R60. doi: 10.1186/gb-2011-12-6-r60

Seshadri, R., Leahy, S. C., Attwood, G. T., Teh, K. H., Lambie, S. C., Cookson, A. L., et al. (2018). Cultivation and sequencing of rumen microbiome members from the Hungate1000 Collection. *Nat. Biotechnol.* 36, 359–367. doi: 10.1038/nbt.4110

Shanks, O. C., Kelty, C. A., Archibeque, S., Jenkins, M., Newton, R. J., McLellan, S. L., et al. (2011). Community structures of fecal bacteria in cattle from different animal feeding operations. *Appl. Environ. Microbiol.* 77, 2992–3001. doi: 10.1128/AEM.02988-10

Stevenson, D. M., and Weimer, P. J. (2007). Dominance of prevotella and low abundance of classical ruminal bacterial species in the bovine rumen revealed by relative quantification real-time PCR. *Appl. Microbiol. Biotechnol.* 75, 165–174. doi: 10.1007/s00253-006-0802-y

Suchodolski, J. S. (2016). Diagnosis and interpretation of intestinal dysbiosis in dogs and cats. *Vet. J.* 215, 30–37. doi: 10.1016/j.tvjl.2016.04.011

Suzuki, T., Tamura, S., Nakanishi, H., Tashiro, M., Nishizawa, N. K., and Yoshimura, E. (2007). Reduction of aluminum toxicity by 2-isopropylmalic acid in the budding yeast *Saccharomyces cerevisiae*. *Biol. Trace Elem. Res.* 120, 257–263. doi: 10.1007/s12011-007-8011-9

Thursby, E., and Juge, N. (2017). Introduction to the human gut microbiota. *Biochem. J.* 474, 1823–1836. doi: 10.1042/BCJ20160510

Tilocca, B., Burbach, K., Heyer, C. M. E., Hoelzle, L. E., Mosenthin, R., Stefanski, V., et al. (2017). Dietary changes in nutritional studies shape the structural and functional composition of the pigs' fecal microbiome—from days to weeks. *Microbiome* 5:144. doi: 10.1186/s40168-017-0362-7

Turnbaugh, P. J., Ley, R. E., Mahowald, M. A., Magrini, V., Mardis, E. R., and Gordon, J. I. (2006). An obesity-associated gut microbiome with increased capacity for energy harvest. *Nature* 444, 1027–1031. doi: 10.1038/nature05414

Uchiyama, J., Murakami, H., Sato, R., Mizukami, K., Suzuki, T., Shima, A., et al. (2020). Examination of the fecal microbiota in dairy cows infected with bovine leukemia virus. *Vet. Microbiol.* 240:108547. doi: 10.1016/j.vetmic.2019.108547

Velagapudi, V. R., Hezaveh, R., Reigstad, C. S., Gopalacharyulu, P., Yetukuri, L., Islam, S., et al. (2010). The gut microbiota modulates host energy and lipid metabolism in mice. *J. Lipid Res.* 51, 1101–1112. doi: 10.1194/jlr.M002774

Wang, Y. P., Cai, M., Hua, D. K., Zhang, F., Jiang, L. S., Zhao, Y. G., et al. (2020). Metabolomics reveals effects of rumen-protected glucose on metabolism of dairy cows in early lactation. *Anim. Feed Sci. Tech.* 269:114620. doi: 10.1016/j.anifeedsci.2020.114620

Weese, J. S., and Jelinski, M. (2017). Assessment of the fecal microbiota in beef calves. *J. Vet. Intern. Med.* 31, 176–185. doi: 10.1111/jvim.14611

Xiao, L., Chen, B., Feng, D., Yang, T., Li, T., and Chen, J. (2019). TLR4 may be involved in the regulation of colonic mucosal microbiota by vitamin A. *Front. Microbiol.* 10:268. doi: 10.3389/fmicb.2019.00268

Xie, Y., Chen, Z., Wang, D., Chen, G., Sun, X., He, Q., et al. (2020). Effects of fermented herbal tea residues on the intestinal microbiota characteristics of holstein heifers under heat stress. *Front. Microbiol.* 11:1014. doi: 10.3389/fmicb.2020.01014

Xue, M., Sun, H., Wu, X., Liu, J., and Guan, L. L. (2020). Multi-omics reveals that the rumen microbiome and its metabolome together with the host metabolome contribute to individualized dairy cow performance. *Microbiome* 8:64. doi: 10.1186/s40168-020-00819-8

Yoneshima, Y., Abolhassani, N., Iyama, T., Sakumi, K., Shiomi, N., Mori, M., et al. (2016). Deoxyinosine triphosphate induces MLH1/PMS2- and p53-dependent cell growth arrest and DNA instability in mammalian cells. *Sci. Rep.* 6:32849. doi: 10.1038/srep32849

Yuan, D., Wu, Z., and Wang, Y. (2016). Evolution of the diacylglycerol lipases. *Prog. Lipid Res.* 64, 85–97. doi: 10.1016/j.plipres.2016.08.004

Zeevi, D., Korem, T., Zmora, N., Israeli, D., Rothschild, D., Weinberger, A., et al. (2015). Personalized nutrition by prediction of glycemic responses. *Cell* 163, 1079–1094. doi: 10.1016/j.cell.2015.11.001

Zhang, J., Shi, H., Wang, Y., Li, S., Cao, Z., Ji, S., et al. (2017). Effect of dietary forage to concentrate ratios on dynamic profile changes and interactions of ruminal microbiota and metabolites in holstein heifers. *Front. Microbiol.* 8:2206. doi: 10.3389/fmicb.2017.02206

Zhang, J., Song, L., Wang, Y., Liu, C., Zhang, L., Zhu, S., et al. (2018). Beneficial effect of butyrate-producing *Lachnospiraceae* on stress-induced visceral hypersensitivity in rats. *J. Gastroenterol. Hepatol.* 34, 1368–1376. doi: 10.1111/jgh.14536

Zhang, L. S., and Davies, S. S. (2016). Microbial metabolism of dietary components to bioactive metabolites: Opportunities for new therapeutic interventions. *Genome Med.* 8:46. doi: 10.1186/s13073-016-0296-x

Zhang, X., Wu, J., Han, X., Tan, Z., and Jiao, J. (2019). Effects of rumen-protected glucose on ileal microbiota and genes involved in ileal epithelial metabolism and immune homeostasis in transition dairy cows. *Anim. Feed Sci. Technol.* 254:114199. doi: 10.1016/j.anifeedsci.2019.06.003

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# Influence of dietary n-3 long-chain fatty acids on microbial diversity and composition of sows' feces, colostrum, milk, and suckling piglets' feces

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**Introduction:** Very little is known about the impact of n-3 long-chain fatty acids (n-3 LCFAs) on the microbiota of sows and their piglets. The aim of this study was to evaluate the effect of n-3 LCFA in sow diets on the microbiota composition of sows' feces, colostrum, and milk as well as that of piglets' feces.

**Methods:** Twenty-two sows were randomly assigned to either a control or an n-3 LCFA diet from service to weaning. Sows' and piglets' performance was monitored. The gestating and lactating sows' microbiomes in feces, colostrum, and milk were characterized by 16s ribosomal RNA gene sequencing. The fecal microbiome from the two lowest (>800 g) and the two highest birth weight piglets per litter was also characterized, and the LPS levels in plasma were analyzed at weaning.

**Results and Discussion:** n-3 LCFA increased microbiota alpha diversity in suckling piglets' and gestating sows' feces. However, no effects were observed in colostrum, milk, or lactating sows' feces. Dietary n-3 LCFA modified the microbiota composition of gestating sows' feces, milk, and suckling piglets' feces, without affecting lactating sows' feces or colostrum. In gestating sows' feces and milk, the decrease in genus *Succinivibrio* and the increase of Proteobacteria phylum, due to the increased genera *Brenneria* and *Escherichia*, respectively, stand out. In the feces of suckling piglets, the higher abundance of the beneficial genus *Akkermansia* and *Bacteroides*, and different species of *Lactobacillus* are highlighted. In addition, positive correlations for families and genera were found between lactating sows' feces and milk, milk and suckling piglets' feces, and lactating sows' feces and suckling piglets' feces. To conclude, dietary n-3 LCFA had a positive impact on the microbiome of suckling piglet's feces by increasing microbial diversity and some beneficial

bacteria populations, had a few minor modifications on the microbiome of milk and gestating sows' feces and did not change the microbiome in lactating sows' feces or colostrum. Therefore, this study shows the effect of dietary n-3 LCFA on the microbiota of sows, colostrum, milk, and suckling piglets during the lactation period providing crucial information on the microbiota status at the early stages of life, which have an impact on the post-weaning.

KEYWORDS

**gestating and lactating sows, suckling piglets, n-3 long-chain fatty acids, microbial communities, colostrum, milk, microbial transference**

## Introduction

The microbiota of pigs is composed of hundreds of different microorganisms and their acquisition and establishment are influenced by different external factors. Concretely, the predominant immediate postnatal factors that determine initial microbial definition in newborn piglets are likely colostrum, milk, feed, oral-fecal transmission, and the neonatal environment (Nowland et al., 2019; Lauridsen, 2020). However, the exact time of initial colonization in pigs has not yet been determined, and some previous reports described prenatal microbial colonization driven by the mother, so it is reasonable to assume that sows' microbiota would also significantly influence the microbiota of their offspring before or during parturition (Nowland et al., 2021). Moreover, the establishment of the gut microbiota has become a key factor in piglet survival, as suggested by previous studies in humans showing that an appropriate intestinal microbiota resulting from optimal colonization may improve health and limit diseases (Nowland et al., 2019). After all, the microbiota is an important regulator of mammal physiology and could exert several beneficial roles in digestion, protection against pathogens, maintenance of normal function of intestinal villi, and regulation of the immune response (Gresse et al., 2017). In addition, optimal colonization during lactation could become a critical factor considering that the change from maternal milk to solid food during weaning entails microbial modifications that coincide with the morphological and functional maturation of the gut barrier, and with important changes in the gut immune system (Hooper, 2004; Beaumont et al., 2021).

Genetic selection for hyperprolific sows has resulted in a substantial increase in litter size, and this increase in the sows' prolificacy has resulted in an increased proportion of piglets born with a low weight (< 1.0 kg birth weight) (Quiniou et al., 2002). Low birth weight piglets present a greater energy requirement per kg of body weight (Noblet and Etienne, 1987), and a poorer thermoregulatory ability, which is combined with a limited colostrum intake (Edwards and Baxter, 2015) due to being less vigorous when competing for the limited number of teats in hyperprolific sows. As consequence, these animals present reduced growth rates (Lopez-Verge et al., 2018) and they are less likely to survive. Nutritional strategies applied to the sow may become an effective tool to shape the microbiota establishment of piglets to improve their growth and immune development.

Although under commercial conditions the ingredients in sow diets contain considerable amounts of n-6 long-chain fatty acids (**LCFAs**) and the fat sources used are rich in saturated fatty acids, over the last few years the study of the inclusion of n-3 LCFA sources in sow diets has gained interest (Tanghe and De Smet, 2013). Concretely, research on n-3 LCFA in sow nutrition has mainly focused on their influence on milk composition and offspring performance (Lauridsen, 2020; Llauradó-Calero et al., 2021). Moreover, due to their anti-inflammatory effects (Calder, 2010) and capability to influence the epithelial barrier functions (Liu, 2015), the impact of n-3 LCFA on immune status (Huber et al., 2018) and intestinal epithelium function (Liu et al., 2012) has also been evaluated. However, little is known about the influence of n-3 LCFA on microbiota diversity and composition in sows' feces, colostrum, milk, and piglets' feces.

Considering the sow as the first and most relevant factor for the establishment of an optimal microbiota in the newborn piglet, the aim of the current study was to evaluate the inclusion of n-3 LCFA, concretely a solid fish oil rich in eicosapentaenoic acid (**EPA**) (C20:5 n-3) and docosahexaenoic acid (**DHA**) (C22:6 n-3), in sow diets and their impact on the microbiota of gestating and lactating sows' feces, colostrum, milk and the feces of suckling piglets. The impact of n-3 LCFA on the sow to piglet microbial transference was also analyzed.

## Materials and methods

### Ethics statement

Institute for Food and Agricultural Research and Technology's (IRTA) Ethical Committee on Animal Experimentation approved the use of animals for this experiment in accordance with Directive 2010/63/EU of 22 September 2010 and according to the recommendation of

the European Commission 2007/526/CE, the Spanish guidelines for the care and use of animals in research (B.O.E. number 34, Real Decreto 53/2013) and the regional regulations on the use and handling of experimental animals (Decree 214/97, Generalitat de Catalunya) (project number: 10294).

## Animals, experimental design, and housing

Twenty-two sows in two batches (12 and 10 sows met the selection criteria of being between the third and the sixth parity, respectively) were fed one of the two experimental diets from service until the end of lactation (c.a. 28 days post-farrowing). Within each batch, sows were grouped regarding their body weight and their parity number into pairs, as similar as possible, and sows in each pair were randomly assigned to either a control or an n-3 long-chain fatty acid (n-3 LCFA) diet. At birth, the two piglets with the lowest (>800 g) (LBW) and the two piglets with the heaviest (HBW) body weight in each litter were selected for future microbiome analyses. Cross-fostering of piglets was performed only during the first 24 h of life to standardize litter size to 12 piglets per sow, whenever possible, solely among sows belonging to the same experimental treatment, and without involving selected piglets. Sow feeds were provided *ad libitum* in self-dispensing feeding hoppers. At 11 days of age, piglets were offered either a control or an n-3 LCFA creep feed according to the corresponding maternal diet, using floor-attached round feeders. Water was provided *ad libitum* from nipple drinkers.

Sows were allocated in individual stalls from service until pregnancy confirmation. Once confirmed, they were group-housed in a gestation barn until 1 week before farrowing. Sows were then relocated to individual farrowing crates (0.7 x 2 m) in pens equipped with partially slatted floors and a heated floor panel for piglets (set at 32–34°C). The farrowing room was lit with natural light from a window and with fluorescent artificial light (manually operated), and ventilated *via* single, variable-speed fans linked to temperature sensors. The inside temperature of the building was automatically controlled, and the target temperature of the stables at farrowing was set at 24°C and it was reduced by 0.5°C per week during the lactation period.

Unfortunately, one sow from the second batch (n-3 LCFA treatment) farrowed out of the scheduled time, without supervision, and it was not possible to record the litter characteristics at birth and sample colostrum, so the sow was removed from the trial.

## Experimental diets

Barley–corn-based gestation and lactation diets for sows and the creep feed for piglets were formulated in accordance with FEDNA specifications (de Blas et al., 2013), and their ingredient and nutrient composition were already described by Llauradó-Calero et al. (2021). Diets were formulated to contain the same level of nutrients (metabolizable energy, crude protein, digestive lysine, and ether extract) except for the fat content. In control diets, dietary fat was included using a common animal fat source (5 Sysfeed®; Sysfeed SLU, Granollers, Spain) at 15 and 30 g/kg in the gestation and lactation phases, respectively. In the test diets (n-3 LCFA), 15 g/kg of fat was replaced (totally during gestation and one-half during lactation) by solid fish oil (Lipomega®; V&S Asociados, Madrid, Spain). Creep feed diets for piglets contained 30 g/kg of animal fat or an equivalent amount of solid fish oil in the control or n-3 LCFA diet, respectively.

Feed intake of sows was restricted to a maximum of 3 kg/day during the gestation period and progressively increased after farrowing until reaching *ad libitum* feed intake. Piglets' creep feed was offered *ad libitum* from day 11 post-farrowing.

## Growth measurements and sampling

Sows were weighed at service, 1 week before farrowing (when moved to the farrowing crates), the day after farrowing, and at weaning. In addition, backfat thickness in the P2 position of sows was also measured at the same time points (except the day after farrowing) through ultrasound scanning (Piglog 105®; Frontmatec, Kolding, Denmark). Daily feed intake was monitored and recorded individually during gestation and lactation. The average daily gain of gestation period was calculated from service to day 107 of gestation (1 week before farrowing), and the average daily gain of lactation period from 1 day after farrowing to weaning (c.a. 28 days post-farrowing). At birth, the total number of piglets born, piglets born alive/dead, mummies, and their individual weights were recorded for each sow. During lactation, the weight of piglets was monitored at 24 h, at 20 days of age, and weaning. Cross-fostering was performed within 24 h after birth and the 24 h recordings were considered as the initial values for litter characteristics and growth performance of suckling piglets during lactation. In the same way, the average daily gain of litters and piglets was calculated from 24 h post-farrowing to each weighing time. Creep feed disappearance was monitored from day 11 of lactation until weaning. One sow from the n-3 LCFA group gave birth to less than six piglets and the corresponding data for litter characteristics at birth (obtained before performing cross-fostering) were excluded from the analysis.

Individual animals were selected for the microbiome studies. Fecal samples from all the sows were collected 1 week before parturition (day 107 of gestation) and at weaning after removing the piglets (c.a. day 28 post-farrowing). At birth, the two lowest (> 800 g) and the two highest birth-weight piglets in each litter were selected and their feces were sampled at weaning. In all

cases, samples were individually preserved with Real stock buffer (Durviz, Paterna, Spain) and stored at −80°C until analysis.

Colostrum samples from each sow were obtained immediately after the birth of the first piglet and milk samples were collected at weaning after the piglet's removal. Sows were milked from all mammary glands following i.v. injection of 1.0 ml of oxytocin (20 IU/ml) (Super's Diana S.L., Parets del Vallès, Spain). The samples from the different nipples in each sow were pooled and aliquots of at least 3 ml were immediately frozen and stored at −80°C until analysis for microbiome determination.

Blood samples from selected piglets were collected at weaning. Blood was obtained by jugular venipuncture in tubes with ethylenediaminetetraacetic acid (EDTA) and was kept at 4°C a maximum of 120 min until centrifugation (3,000 rpm, 10 min). Plasma aliquots for LPS measurement were obtained and stored at −80°C for a maximum of 30 min after centrifugation.

## Deoxyribonucleic acid extraction and bacterial 16S gene amplification and sequencing

For microbiome analysis, DNA from feces was isolated with the aid of QIAamp Power Fecal Pro DNA Kit (Qiagen, Hilden, Germany), with bead beating and enzymatic lysis steps prior to extraction to avoid bias in DNA purification toward the misrepresentation of gram-positive bacteria. For milk and colostrum, samples were processed with bead beating and enzymatic lysis steps followed by the Blood & Tissue Kit (Qiagen) and further DNA concentration. To evaluate the bacterial composition, massive genome sequencing of the hypervariable region V3–V4 of the bacterial 16s rRNA gene was conducted. Samples were amplified using key-tagged eubacterial primers (Klindworth et al., 2013) and sequenced on a MiSeq Illumina Platform, using a 2 × 300nt paired-end strategy, following Illumina Library preparation and sequencing for metagenomic studies protocol (Zhang et al., 2014; Marcel, 2011).

### Analysis of sequencing data

The resulting sequences were split considering the barcode introduced during the PCR reaction. PEAR program version 0.9.1 was used to overlap R1 and R2 reads (overlap of 50 nt and quality of the overlap with a minimum of Q20 (Zhang et al., 2014), providing a single FASTQ file for each of the samples. Cutadapt v2.6 (Marcel, 2011) was used to trim 16S rRNA PCR primers and sequences were treated with a quality filter, removing low-quality fragments (under Q20 in Phred scale) and short sequences (under 200nt). Chimeric sequences that potentially arise during the PCR amplification step were identified de novo using CD-HIT software v4.8.1 (Li et al., 2012) and removed. CD-HIT software was also used to create OTUs at 99.7% of identity. The BLAST tool was used to taxonomically identify each OTU against the National Center for Biotechnology Information (NCBI) 16S rRNA database (20th December 2020) using BLASTn version 2.10.0+.

## Lipopolysaccharides measurement in plasma of suckling piglets

The sandwich ELISA kit Porcine Lipopolysaccharides (LPSs) ELISA Kit (MBS269464; MyBioSource, San Diego, CA, USA) was employed according to the manufacturer's instructions for the quantitative measurement of LPS in all plasma samples from weaned piglets. Intra-assay precision and inter-assay precision of the kit were $\leq$ 8% and $\leq$ 12%, respectively.

## Statistical analysis

The analysis of variance of growth and performance data of gestating and lactating sows and suckling piglets and LPS concentration data of plasma from suckling piglets were performed through the GLIMMIX procedure of SAS software (SAS/STAT 14.1; SAS Institute Inc., Cary, NC, USA). For growth and performance measurements, dietary treatment was included in the model as the fixed effect and batch as the random effect. Sow body weight at the beginning of the trial and the parity number were initially introduced into the model as covariates. However, only the initial sow body weight was included in the statistical analysis since parity had no significant effect. In addition, the variable days of lactation were included in the model as a covariate for all the data at weaning. In terms of stillborn piglets, deaths, and mummies, data were normalized using a square root transformation ($\sqrt{X+0.5}$), but the means of the original data are presented in tables. For LPS concentrations, the model included dietary treatment as the fixed effect and sow as the random effect. All data were tested using Kolmogorov–Smirnov test to identify possible outliers and values were excluded if $P < 0.01$. Results were expressed as means ± SD. Significant differences were set at $P < 0.05$.

Microbiome analyses were done using R software (R Core Team, 2012). Alpha and beta diversity indexes were obtained using the vegan package, as implemented for R version 3.2.3 (Oksanen et al., 2020). Bray–Curtis distances were selected to analyze beta diversity, and their significance was studied with PERMANOVA tests. DESeq2 package from R (Love et al., 2014) was used to generate a generalized linear model with fixed effects (control vs. n-3 LCFA diet) with negative binomial family to compare operational taxonomic unit (OTU) counts between groups and select the potential bacterial biomarkers. *P*-values were corrected for multiple testing with Benjamini and Hochberg method, and the statistical significance cutoff

was set at $P < 0.05$. The ratios Firmicutes/Bacteroidetes and *Lactobacillus*/Proteobacteria were calculated through relative abundances and adjusted to a maximum value of 100. Higher values were considered outliers and were removed from the analysis. Significant differences were set at $P < 0.05$, while tendencies at $P < 0.1$ using Mann–Whitney–Wilcoxon test.

The PROC CORR procedure of SAS software was used to obtain the Pearson correlation coefficient of the relative abundances of differential microbial populations regardless of the dietary treatment between gestating sows' feces and colostrum, lactating sows' feces and milk, milk and sucking piglets' feces, and lactating sows' feces and suckling piglets' feces to study the sow-piglet microbial transference and between suckling piglets' feces and growth measurements of suckling piglets to identify possible microbial populations affecting the animal growth. A significant correlation level was set at $r > 0.5$ and $P < 0.05$.

# Results

## Sows' weight, feed Intake, litter characteristics, and piglets' weight

The body weight, backfat thickness, and feed intake of sows during gestation and lactation are presented in **Supplementary Table 1**. Sow's body weight at service ($P = 0.494$), 107 days of gestation ($P = 0.207$), one day after farrowing ($P = 0.336$), and at weaning ($P = 0.812$), and sow's average daily weight gain during gestation ($P = 0.202$) and lactation ($P = 0.278$) did not differ between dietary treatments. In the same way, backfat thickness in the P2 position at service ($P = 0.696$), 107 days of gestation ($P = 0.795$), and at weaning ($P = 0.502$), and the average daily feed intake during gestation ($P = 0.787$) or lactation ($P = 0.683$) did also not differ either between treatments.

The number of piglets born, piglets born alive/dead, mummies, piglet's weight, average daily gain during lactation, and creep feed intake are presented in **Supplementary Table 2**. Litter characteristics and the average piglet's body weight did not show any significant differences between treatments at birth, 24 h after birth, 20 days after birth, or at weaning (all $P \geq 0.166$). In the same way, no effects of dietary fish oil were observed for litter or piglet average daily weight gain during the first 20 days ($P = 0.339$ and $P = 0.553$, respectively) or during the whole lactation period ($P = 0.974$ and $P = 0.714$, respectively). Piglet creep feed disappearance did not differ between control and n-3 LCFA diets ($P = 0.599$) either.

## Microbiota diversity

**Table 1** shows richness and diversity values for each experimental group and type of sample at phylum, family, genera, and species levels. The largest differences in terms of alpha diversity were observed in the feces of suckling piglets. An increase in microbiota diversity calculated through Simpson and Shannon indices in all studied levels was detected in piglets from the n-3 LCFA sows compared with piglets from the control sows. In addition, microbiota diversity was significantly increased by dietary fish oil through the Simpson index in feces from n-3 LCFA gestating sows.

Alpha-diversity in terms of Richness, Simpson, and Shannon indices in colostrum, lactating sow's feces, or milk were not significantly affected by fish oil in the sows' diet (**Table 1**).

## Microbiota composition and differential microbial communities

The PCoA analysis of sows and piglets' feces, colostrum, and milk regardless of experimental dietary treatments is presented in **Figure 1**. PERMANOVA revealed distinct clusters for each sample type ($R^2 = 0.32$; $P = 0.001$). Beta-dispersion analysis showed differences among all the different types of samples analyzed (all $P \leq 0.037$) except between feces of gestating and lactating sows ($P = 0.235$).

The fecal microbiome of gestating sows was composed mainly of phylum Firmicutes, Families *Clostridiaceae*, *Peptostreptococcaceae*, *Lactobacillaceae*, *Streptococcaceae*, *Erysipelotrichaceae*, and *Lachnospiraceae*, and genera *Clostridium*, *Lactobacillus*, *Streptococcus*, *Terrisporobacter*, and *Turicibacter* (**Figure 2**). Lower abundances of the family *Succinivibrionaceae* ($P = 0.020$) and genus *Succinivibrio* ($P = 0.031$) were observed in the feces of sows from the n-3 LCFA group than those from the control group (**Figure 3**).

The core microbiome obtained in colostrum samples was represented by phyla Firmicutes, Actinobacteria, and Proteobacteria. *Staphylococcaceae*, *Lactobacillaceae*, *Clostridiaceae*, and *Moraxellaceae* were the dominant Families and *Staphylococcus*, *Lactobacillus*, *Clostridium*, and *Streptococcus* represented the most abundant genera (**Figure 2**). No differential bacterial communities were detected between control and n-3 LCFA treatments at any taxonomical level studied (**Figure 3**).

In the feces of lactating sows, Firmicutes was also the most abundant phylum. Families *Peptostreptococcaceae*, *Clostridiaceae*, *Erysipelotrichaceae*, *Lactobacillaceae*, and *Lachnospiraceae* and genera *Clostridium*, *Terrisporobacter*, *Turicibacter*, and *Lactobacillus* had the highest abundance (**Figure 2**). As for colostrum, no significant differences between the control and n-3 LCFA groups were observed for any taxa at any taxonomical level (**Figure 3**).

In milk, Firmicutes, Proteobacteria, and Actinobacteria were the dominant bacterial phyla followed by Bacteroidetes. At the family and genus level, the dominant taxa were *Lactobacillaceae*, *Streptococcaceae*, *Peptostreptococcaceae*,

TABLE 1 Microbiota alpha diversity indices, calculated at phylum, family, genera, and species level, of feces of gestating and lactating sows, colostrum, milk, and feces of suckling piglets from animals fed either control or n-3 LCFA diets.[1]

| | Alpha diversity indices | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | Richness | | | Simpson index | | | Shannon index | | |
| Taxonomy level | Control | n-3 LCFA | *P*-value | Control | n-3 LCFA | *P*-value | Control | n-3 LCFA | *P*-value |
| **Gestating sows' feces** | | | | | | | | | |
| Phylum | 7.65 ± 0.63 | 7.83 ± 0.55 | 0.48 | 0.57 ± 0.02 | 0.55 ± 0.02 | 0.11 | 1.01 ± 0.08 | 0.96 ± 0.09 | 0.21 |
| Family | 27.0 ± 1.28 | 27.1 ± 1.33 | 0.83 | 0.74 ± 0.04 | 0.77 ± 0.04 | 0.073 | 1.90 ± 0.10 | 1.94 ± 0.15 | 0.47 |
| Genera | 50.1 ± 1.44 | 49.6 ± 2.59 | 0.55 | 0.74 ± 0.04 | 0.77 ± 0.04 | 0.068 | 2.08 ± 0.11 | 2.12 ± 0.19 | 0.49 |
| Species | 80.1 ± 3.14 | 81.3 ± 4.96 | 0.52 | 0.75 ± 0.04 | 0.79 ± 0.05 | 0.037 | 2.31 ± 0.14 | 2.45 ± 0.25 | 0.14 |
| **Colostrum** | | | | | | | | | |
| Phylum | 8.04 ± 0.78 | 8.19 ± 0.35 | 0.58 | 0.54 ± 0.14 | 0.54 ± 0.08 | 0.93 | 1.08 ± 0.24 | 1.08 ± 0.14 | 0.94 |
| Family | 52.1 ± 4.98 | 52.8 ± 3.36 | 0.69 | 0.82 ± 0.17 | 0.83 ± 0.09 | 0.83 | 2.50 ± 0.53 | 2.47 ± 0.41 | 0.91 |
| Genera | 102 ± 19.5 | 102 ± 9.93 | 0.92 | 0.84 ± 0.16 | 0.85 ± 0.10 | 0.78 | 2.81 ± 0.60 | 2.81 ± 0.49 | 0.99 |
| Species | 206 ± 43.8 | 200 ± 29.4 | 0.72 | 0.91 ± 0.07 | 0.91 ± 0.09 | 0.99 | 3.58 ± 0.54 | 3.58 ± 0.56 | 0.97 |
| **Lactating sows' feces** | | | | | | | | | |
| Phylum | 8.18 ± 0.40 | 8.06 ± 0.35 | 0.46 | 0.59 ± 0.04 | 0.54 ± 0.07 | 0.064 | 1.06 ± 0.10 | 0.97 ± 0.15 | 0.091 |
| Family | 21.9 ± 0.76 | 21.9 ± 1.16 | 0.97 | 0.75 ± 0.04 | 0.77 ± 0.03 | 0.51 | 1.85 ± 0.13 | 1.84 ± 0.13 | 0.80 |
| Genera | 48.0 ± 1.77 | 47.6 ± 2.42 | 0.61 | 0.77 ± 0.05 | 0.78 ± 0.04 | 0.42 | 2.10 ± 0.19 | 2.10 ± 0.13 | 0.96 |
| Species | 76. 4 ± 2.86 | 77.9 ± 4.23 | 0.36 | 0.78 ± 0.05 | 0.80 ± 0.05 | 0.34 | 2.36 ± 0.23 | 2.38 ± 0.14 | 0.77 |
| **Milk** | | | | | | | | | |
| Phylum | 8.00 ± < 0.01 | 7.82 ± 0.60 | 0.33 | 0.56 ± 0.09 | 0.58 ± 0.11 | 0.58 | 1.12 ± 0.17 | 1.17 ± 0.21 | 0.57 |
| Family | 45.6 ± 3.45 | 46.6 ± 1.81 | 0.49 | 0.86 ± 0.05 | 0.88 ± 0.04 | 0.34 | 2.49 ± 0.16 | 2.62 ± 0.21 | 0.11 |
| Genera | 97.1 ± 7.81 | 97.1 ± 7.88 | 0.99 | 0.87 ± 0.05 | 0.89 ± 0.04 | 0.36 | 2.80 ± 0.20 | 2.93 ± 0.24 | 0.16 |
| Species | 208 ± 23.6 | 207 ± 25.0 | 0.92 | 0.90 ± 0.06 | 0.92 ± 0.04 | 0.34 | 3.43 ± 0.29 | 3.63 ± 0.30 | 0.14 |
| **Suckling piglet's feces** | | | | | | | | | |
| Phylum | 9.83 ± 0.89 | 9.50 ± 0.74 | 0.064 | 0.68 ± 0.06 | 0.71 ± 0.07 | 0.036 | 1.36 ± 0.18 | 1.46 ± 0.21 | 0.026 |
| Family | 35.8 ± 2.76 | 35.3 ± 2.77 | 0.45 | 0.78 ± 0.07 | 0.83 ± 0.07 | 0.006 | 2.09 ± 0.21 | 2.28 ± 0.29 | 0.001 |
| Genera | 72.7 ± 5.87 | 70.0 ± 7.18 | 0.065 | 0.81 ± 0.07 | 0.84 ± 0.06 | 0.021 | 2.45 ± 0.23 | 2.56 ± 0.25 | 0.054 |
| Species | 118 ± 10.5 | 117 ± 12.9 | 0.62 | 0.82 ± 0.07 | 0.86 ± 0.07 | 0.009 | 2.69 ± 0.28 | 2.89 ± 0.34 | 0.005 |

LCFA, long-chain fatty acid. [1]Values are means ± SD of 11 control and 10 n-3 LCFA samples from sows (gestating and lactating sows' feces, colostrum, and milk) or 44 control and 40 n-3 LCFA samples from piglets (suckling piglets' feces).

*Staphylococcaceae*, and *Lachnospiraceae,* and *Lactobacillus*, *Streptococcus*, *Staphylococcus*, and *Clostridium*, respectively (Figure 2). Differences between the control and the n-3 LCFA treatments were observed for specific groups (Figure 3). Concretely, Phylum Proteobacteria ($P = 0.017$) was increased by dietary n-3 LCFA due to the increased families *Pectobacteriaceae* ($P = 0.020$) and *Enterobacteriaceae* ($P = 0.020$) and the main genera belonging to these families, *Brenneria* ($P = 0.026$) and *Escherichia* ($P = 0.026$), respectively. In addition, other changes by the inclusion of dietary fish oil were detected such as decreases in the family *Ruminococcaceae* ($P = 0.023$) and genera *Ruminococcus* ($P = 0.026$), increases in family *Propionibacteriaceae* ($P = 0.020$) and genera *Cutibacterium* ($P = 0.026$), and decreases in family *Spirochaetaceae* ($P = 0.023$) and genera *Treponema* ($P = 0.028$).

Microbiota composition in the feces of suckling piglets was dominated by phyla Firmicutes, Bacteroidetes, Proteobacteria, Euryarchaeota, and Actinobacteria. *Lachnospiraceae*, *Bacteroidaceae*, *Lactobacillaceae*, *Ruminococcaceae*, and *Clostridiaceae* were the most abundant families, and *Bacteroides*, *Lactobacillus*, *Gemmiger*, *Clostridium*, and *Methanobrevibacter* the most abundant genera (Figure 2). Piglet feces were the type of sample that presented a larger number of differences in microbial communities between treatments. Concretely, one phylum, 15 families, and 27 genera differed between treatments (Figure 3). Phylum Verrucomicrobia ($P = 0.045$) and genera *Akkermansia* ($P = 0.041$) were increased in the feces of piglets from n-3 LCFA-fed sows. In addition, and within Firmicutes, dietary n-3 LCFA reduced the family *Ruminococcaceae* ($P < 0.001$) due to reductions in the genera *Gemmiger* ($P = 0.032$) and *Ruminococcus* ($P = 0.036$). Within the same phylum, the family *Clostridiaceae* ($P = 0.002$) and genera *Clostridium* ($P = 0.001$) were increased by dietary n-3 LCFA. Family *Enterococcaceae* ($P = 0.010$) and genera *Enterococcus* ($P = 0.011$) were also increased by fish oil. It should also be pointed out that the feces from piglets in

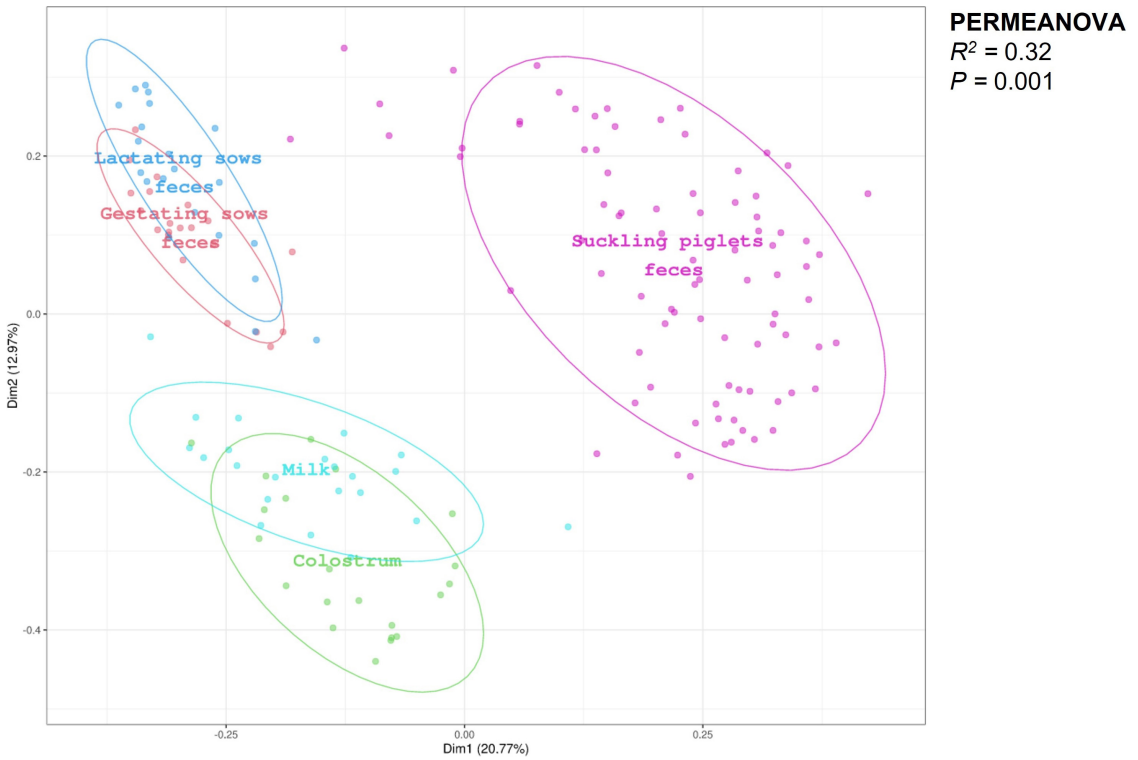


FIGURE 1
Principal coordinates analysis (PCoA) plot of gestating and lactating sows' feces ($n$ = 21, respectively), colostrum ($n$ = 21), milk ($n$ = 21), and suckling piglets' feces ($n$ = 84), regardless of experimental dietary treatments, revealed distinct clusters for each sample type ($R^2$ = 0.32; $P$ = 0.001). PERMANOVA test performed using Bray–Curtis distances to analyze beta-dispersion showed differences between all the different types of samples analyzed (all $P \leq 0.037$) except among gestating and lactating sows' feces ($P$ = 0.235).

the n-3 LCFA group had a reduced abundance of genus *Holdemanella* ($P$ = 0.032), *Flintibacter* ($P$ = 0.024), and *Anaerotaenia* ($P$ = 0.009), and an increased abundance of genus *Streptococcus* ($P$ = 0.023) and *Erysipelatoclostridium* ($P < 0.001$). Regarding phylum Bacteroidetes, dietary fish oil increased in piglets' feces in the families *Tannerellaceae* ($P < 0.001$) and *Odoribacteraceae* ($P$ = 0.042) due to the increase in their respective genera *Parabacteroides* ($P < 0.001$) and *Butyricimonas* ($P$ = 0.002). Within the same phylum also stands out the increased abundance of genera *Bacteroides* ($P$ = 0.009) by n-3 LCFA. In terms of phylum Proteobacteria, an increase of families *Pectobacteriaceae* ($P$ = 0.047) and *Enterobacteriaceae* ($P$ = 0.023), due to the increase in their respective genera *Brenneria* ($P < 0.001$) and *Escherichia* ($P < 0.001$), and a reduction of family *Desulfovibrionaceae* ($P$ = 0.006) were observed in piglets from sows fed the n-3 LCFA. In the phylum of Actinobacteria, n-3 LCFA reduced family *Coriobacteriaceae* ($P < 0.001$) and genera *Collinsella* ($P < 0.001$), and increased family *Actinomycetaceae* ($P < 0.001$) and genera *Schaalia* ($P$ = 0.007), and family *Atopobiaceae* ($P < 0.001$), including genus *Olsenella* ($P < 0.001$) and *Atopobium* ($P < 0.001$). In addition, n-3 LCFA also reduced genera *Synergistes* ($P$ = 0.002) but not modifying phylum *Synergistetes* or family *Synergistaceae*.

Finally, the ratios Firmicutes/Bacteroidetes and *Lactobacillus*/Proteobacteria were also compared between treatments for all types of samples, and this is reported in Figure 4. In the feces of lactating sows, an increase in the ratio Firmicutes/Bacteroidetes ($P$ = 0.040) and a tendency to decrease the ratio *Lactobacillus*/Proteobacteria were observed ($P$ = 0.072) with the n-3 LCFA inclusion, while a tendency to reduce the ratio Firmicutes/Bacteroidetes ($P$ = 0.070) was observed in feces of suckling piglets of n-3 LCFA group. For feces of gestating sows, colostrum, and milk, no differences between treatments were observed for either, Firmicutes/Bacteroidetes ratio (all $P \geq 0.22$) or *Lactobacillus*/Proteobacteria ratio (all $P \geq 0.19$).

## The concentration of lipopolysaccharides in the plasma of suckling piglets

As shown in Figure 5, no differences in plasma LPS concentration were observed between samples from piglets of

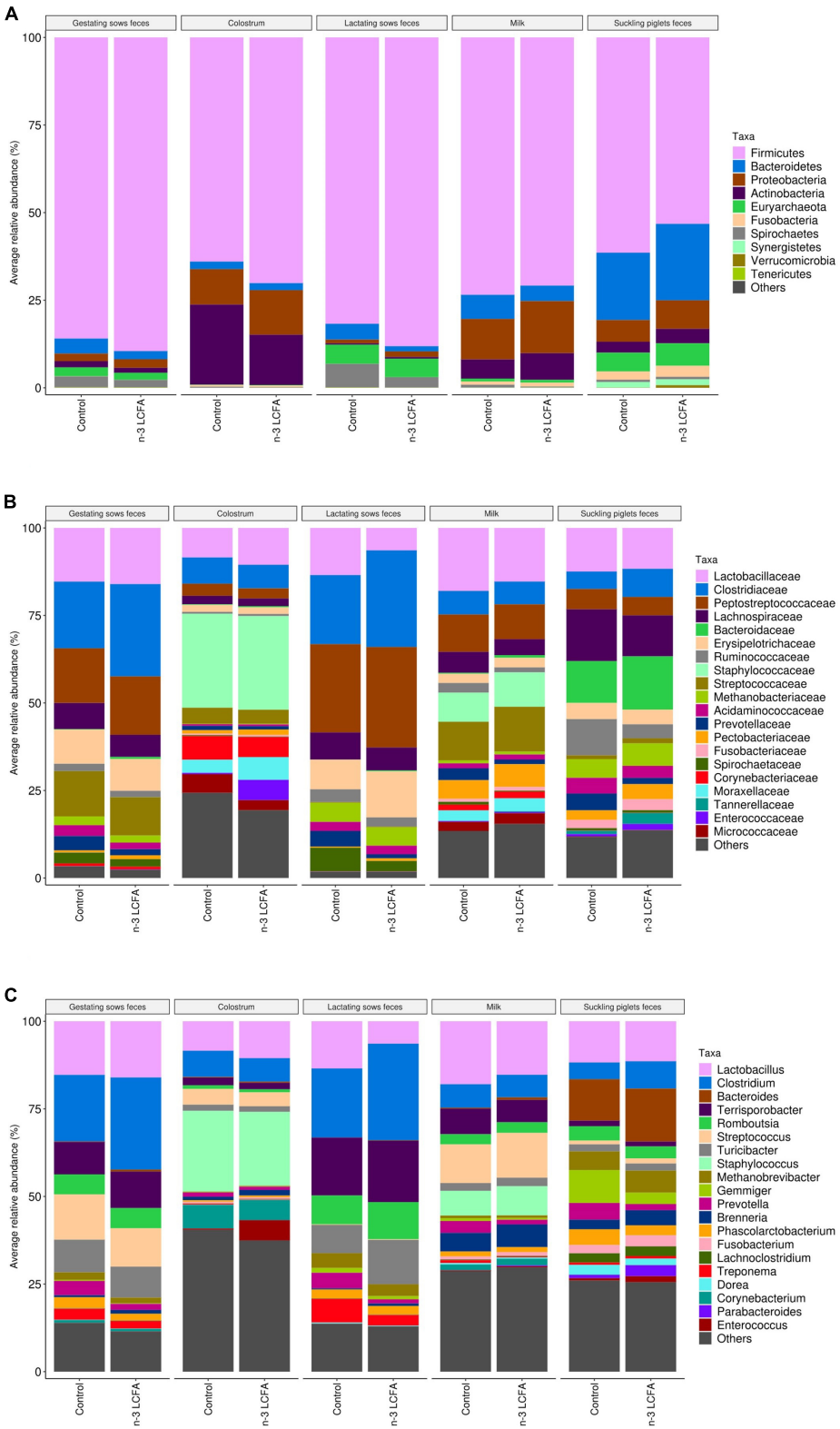


FIGURE 2
Microbiota composition by the relative abundance of the top 10 phyla **(A)**, top 20 families **(B)**, and top 20 genera **(C)** of feces of gestating and lactating sows ($n$ = 21), colostrum ($n$ = 21), milk ($n$ = 21), and feces of suckling piglets ($n$ = 84) according to dietary treatment (control vs. n-3 LCFA). LCFA, long-chain fatty acids.

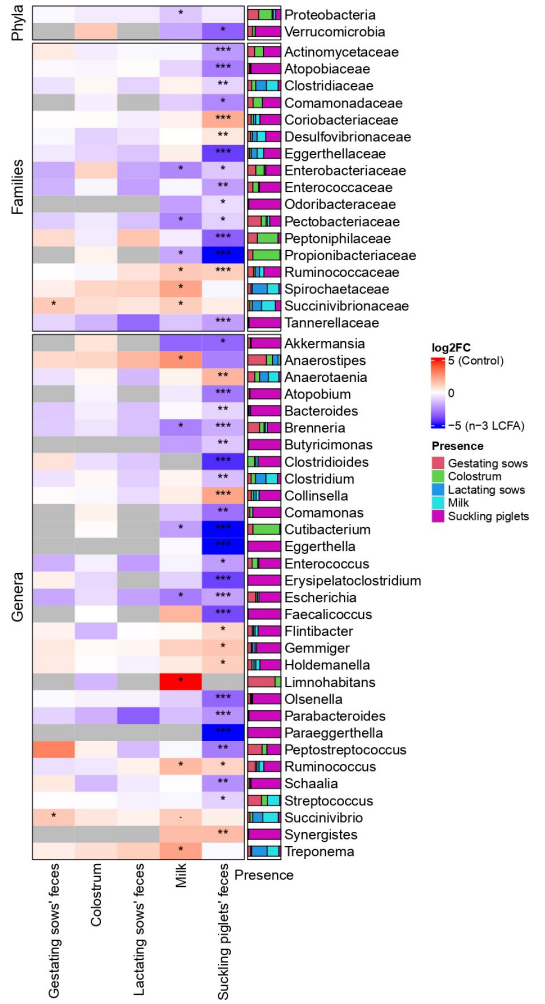


FIGURE 3
Heatmap representing the differentially abundant phyla, families, and genera between control and n-3 LCFA dietary treatments in feces of gestating and lactating sows, colostrum, milk, and feces of suckling piglets. For feces of gestating and lactating sows, colostrum, and milk; $n$ = 11 for control and $n$ = 10 for n-3 LCFA. For feces of suckling piglets; n = 44 for control and n = 40 for n-3 LCFA. Significant differences between treatments were set at $P < 0.05$ (*), $P < 0.01$ (**), and $P < 0.001$ (***). LCFA, long-chain fatty acids.

the control group and piglets of the n-3 LCFA group (181 ± 98.4 and 162 ± 77.8 pg/ml, respectively; $P = 0.700$).

## Correlations of differential microbial populations between sample types

The comparison of feces from lactating sows and their milk rendered positive correlations at the family level, *Ruminococcaceae* in feces with *Ruminococcaceae* and *Spirochaetaceae* in milk, and four positive correlations at the genus level, *Anaerostipes* in feces with *Anaerostipes* in milk, and *Ruminococcus* in feces with *Anaerostipes*, *Ruminococcus,* and *Treponema* in milk (**Figure 6**).

Between milk and feces of suckling piglets, two positive correlations were observed at the family level and five positive correlations at the genus level (**Figure 6**). Concretely, at the family level, *Odoribacteraceae* and *Tannerellaceae* in milk were positively correlated with *Tannerellaceae* in piglets' feces. At the genus level, *Akkermansia*, *Bacteroides*, *Flintibacter*, and *Parabacteroides* in milk were also positively correlated with *Parabacteroides* in feces and *Erysipelatoclostridium* in milk with *Olsenella* in feces.

One positive correlation at family and genus levels was observed between feces from lactating sows and feces from suckling piglets (**Figure 6**). Specifically, the correlations were between the family *Atopobiaceae* and genus *Atopobium* in lactating sows' feces with the family *Peptoniphilaceae* and genus *Akkermansia* in suckling piglets' feces, respectively.

Finally, no significant correlations between microbial populations were observed at any taxonomical level between gestating sow's feces and colostrum, and between bacterial populations in suckling piglets' feces and the growth measurements of suckling piglets during lactation.

# Discussion

The microbiota plays an integral role in influencing host metabolism, its immune system, and the development of a healthy gastrointestinal tract. In addition, the health of offspring is strongly linked to microbial exposure throughout life (Nowland et al., 2019). Considering that weaning is a critical part of pig life characterized by being a complex and stressful event due to the dietary, social and environmental changes (Gresse et al., 2017), the acquisition of an optimal microbiota during the suckling period becomes crucial. Few studies on mice and humans have studied the effect of n-3 fatty acids on the gut microbiota; however, it is described as a poorly understood topic (Costantini et al., 2017). To our knowledge, no prior studies have evaluated the impact of n-3 LCFA on the microbial diversity and composition of sows' feces, colostrum, and milk and the feces of their suckling piglets, and therefore research on the impact of n-3 LCFA on swine microbiota deserves further attention.

The effects of the inclusion of n-3 LCFA in sows' diets on sow's weight, litter characteristics, and growth performance of piglets during lactation have been previously reported (Tanghe and De Smet, 2013). The animals selected for the current study are part of a larger trial studying the impact of n-3 LCFA inclusion in the diets of gestating and lactating diets of sows on different parameters including performance (Llauradó-Calero et al., 2021), and a trend to increase average piglet body weight at weaning is reported, which was in the line with the results of Rooke et al. (2001). However, with the group of sows selected for the current study, no differences in performance between

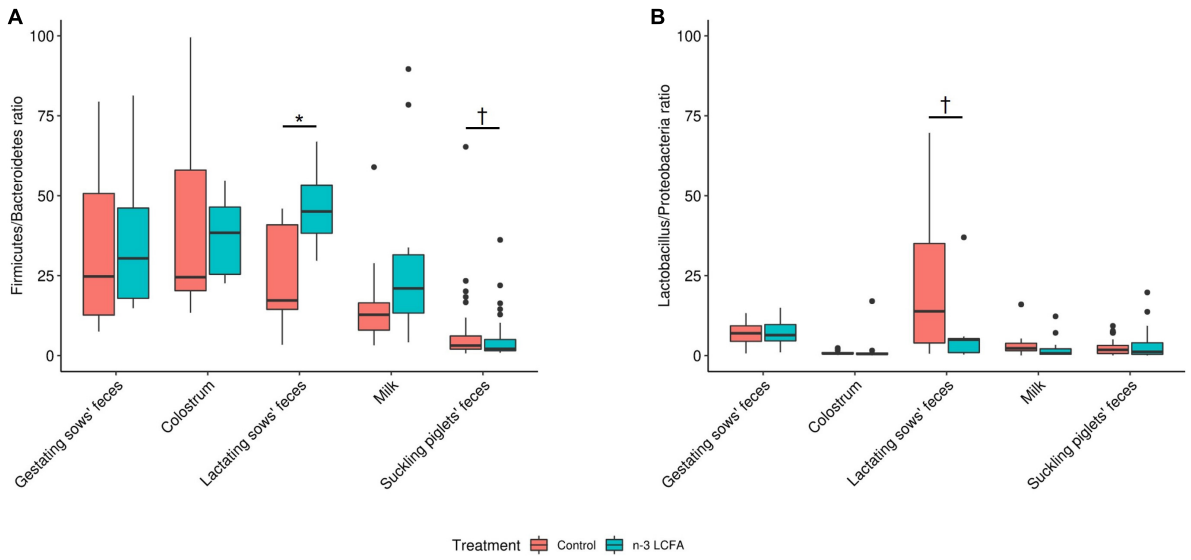


FIGURE 4
Firmicutes/Bacteroidetes **(A)** and *Lactobacillus*/Proteobacteria **(B)** ratios calculated between treatments in all sample types. For feces of gestating and lactating sows, colostrum, and milk; $n$ = 11 for control and $n$ = 10 for n-3 LCFA. For feces of suckling piglets; $n$ = 44 for control and $n$ = 40 for n-3 LCFA. Significant differences between treatments were set at $P < 0.05$ (*), while tendencies were set at $P < 0.10$ (†). Ratios were adjusted at a maximum value of 100 and higher values were removed by being considered outliers. LCFA, long-chain fatty acids.

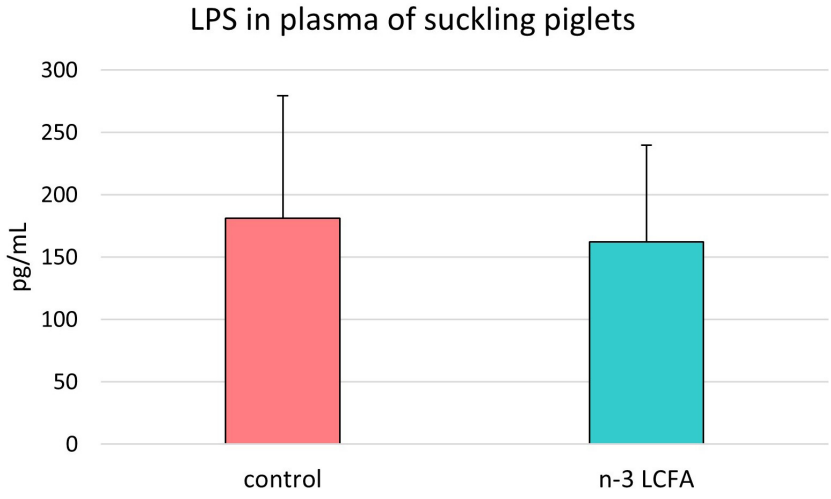


FIGURE 5
Lipopolysaccharide **(LPS)** concentration in plasma of suckling piglets from control and n-3 LCFA-fed sows. $n$ = 44 for control and $n$ = 40 for n-3 LCFA. LCFA, long-chain fatty acids.

treatments were observed, which would also be consistent with the results of Lauridsen and Danielsen (2004) and Leonard et al. (2010).

Despite diet being one of the main factors affecting intestinal microbiota (Duan et al., 2019), in the current study, the inclusion of fish oil as a source of n-3 LCFA in sow diets cause minor changes in microbial populations in the feces of the sows. This may be due to the fact that sows, as adult animals, are characterized by presenting a stable and well-developed microbiota (Simpson et al., 2000; Niu et al., 2019). However, in gestating sows, microbial diversity increased when they were fed with the n-3 LCFA diet. In addition, the decrease of family *Succinivibrionaceae* due to the decrease of genus *Succinivibrio* stands out, which is considered a core microbiome of the proximal colon or cecum of swine and is related to propionate formation and decarboxylation process (Bergamaschi et al., 2020). However, in lactating sows, no differences in diversity or populations at family and genus levels were found between

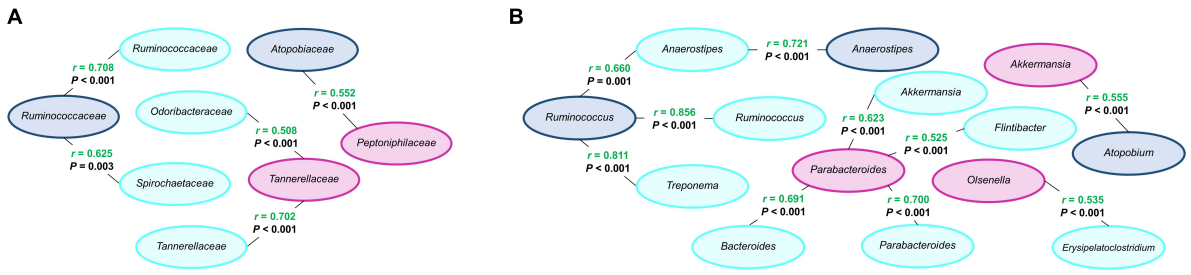


FIGURE 6
Correlations between modified microbial families **(A)** and genera **(B)** from feces of lactating sows (navy blue, $n$ = 21), milk (light blue, $n$ = 21), and feces of suckling piglets (purple, $n$ = 84). Pearson correlation coefficient ($r$) in green indicates a positive correlation. A significant correlation level was set at $r > 0.5$ and $P < 0.05$.

control and n-3 LCFA-fed animals. Even so, in lactating sows, an increase in the ratio of Firmicutes/Bacteroidetes was observed for the n-3 LCFA group. More Firmicutes and fewer Bacteroidetes have been suggested as a characteristic of fat pigs and have been related to fat deposition (Zhao et al., 2015), although we found no differences between treatments for the weight of the sows or their backfat thickness on P2 position at weaning.

Both colostrum and milk play a critical role in piglets' development since they are the first sources of nutrients for newborn piglets which are characterized as having low energy reserves and being immunologically naive. In addition, colostrum and milk not only provide nutrients, energy, and immunity, but they also enable the establishment of commensal microbes (Nowland et al., 2019). Given that optimal colostrum and milk supply are crucial for intestinal microbiota colonization and development (Nowland et al., 2021), the impact of n-3 LCFA on colostrum and milk microbiota composition becomes very relevant. In the current study, the bacterial compositions of colostrum and milk were shown to be different, regardless of dietary treatment. On the one hand, the microbiota of colostrum was dominated mainly by phyla Firmicutes, Actinobacteria, and Proteobacteria and families *Staphylococcaceae*, *Lactobacillaceae*, *Clostridiaceae*, and *Moraxellaceae*. On the other hand, the microbiota of milk was dominated by phyla Firmicutes, Proteobacteria, and Actinobacteria and *Lactobacillaceae*, *Streptococcaceae*, *Peptostreptococcaceae*, *Staphylococcaceae*, and *Lachnospiraceae* at the family level. Interestingly, dietary n-3 LCFA impacted differently on the microbial populations from colostrum and milk. In colostrum, no changes were observed in microbial diversity nor differential populations due to n-3 LCFA supplementation. In milk, however, one phylum, six families, and seven genera were modified by the n-3 LCFA diet, although no changes in microbial diversity were observed. Among these changes, the increase in Proteobacteria should be highlighted since it is one of the most dominant phyla in milk microbiota. This increase was mainly due to the higher abundance of families *Pectobacteriaceae* and *Enterobacteriaceae* and the increase in genera *Brenneria* and *Escherichia*, respectively. While no effects of *Brenneria* have been described on animal microbiota, *Escherichia* is a common inhabitant in swine gut microbiota although some of its species could be pathogenic (Schierack et al., 2007). In addition, the decrease of the family *Ruminococcaceae* due to the decrease of the genus *Ruminococcus* stands out. *Ruminococcaceae* has been reported to play a role in the degradation of complex carbohydrates (Crost et al., 2018) and the production of butyrate, and it is associated with anti-inflammatory effects (Liu et al., 2019). Particularly, *Ruminococcus* OTU was identified as *R. flavefaciens,* which plays an important role in the digestion of hemicellulose and cellulose plant cell walls (Fontes and Gilbert, 2010), and their degradation-derived products may act as prebiotics to gut microbiota (Rajan et al., 2021).

Compared to sows, suckling piglets experienced larger modifications in the diversity and bacterial populations of their fecal microbiota due to n-3 LCFA. However, we cannot be sure whether the higher diversity in the fecal samples from n-3 LCFA piglets is due to the direct effect of dietary n-3 LCFA in creep feed, vertical transmission from the sow at birth, or a combination of both. In addition, no effects of birth weight category (low vs. high birth weight piglets) or interactions between dietary treatment and piglets' birth weight were observed for either diversity or bacterial populations. This contrasts with the results reported by Li et al. (2018), Li N. et al. (2019) who described differences between LBW and normal birth weight piglets during the suckling period for the microbiota of feces and the microbiota of digesta in the ileum and colon. Regarding dietary treatment, bacterial alpha diversity according to Simpson and Shannon indices was increased in suckling piglets on the n-3 LCFA diet. In a previous study, Djuric et al. (2019) described an increase of colonic bacterial diversity in healthy human adults following dietary fish oil supplementation which they related to the anti-inflammatory effects of n-3 LCFA, agreeing with Calder (2019), who suggested a new mechanism by which n-3 LCFA dampen intestinal

inflammation. In terms of differential microbial populations, n-3 LCFA modified one phylum, 15 families, and 27 genera in feces from suckling piglets.

Among the observed changes in the fecal microbiota of suckling piglets, the phylum Verrucomicrobia increased due to the increase in the *Akkermansia* genus with its tentative species *A. muciniphila*. A previous study with mice also reported an increase in *A. muciniphila* after fish oil supplementation (Caesar et al., 2015). Moreover, another study in healthy humans described an increase in the family *Akkermansiaceae* in an n-3 LCFA-treated group (Watson et al., 2018). *A. muciniphila* is the most common species of Verrucomicrobia and colonizes the mucus layer acting as a mucin degrader (Ottman et al., 2017). It is well established that it plays a crucial role in supplying mucin-derived nutrients to other members of the gut microbiota that are unable to degrade the mucin layer by themselves (Tailford et al., 2015). This mucin-degrading capacity makes *A. muciniphila* a modulator for gut homeostasis improving and regulating the gut barrier function (Guo et al., 2017). In addition, its presence in the feces of highly feed-efficient animals (Gardiner et al., 2020) and its possible role as the host immune system modulator (Crespo-Piazuelo et al., 2019) have also been previously described. Other studies propose that excessive mucin degradation may facilitate the access of pathogens to the mucosa (Ganesh et al., 2013). However, in the current study, no differences in LPS concentration in the plasma of suckling piglets at weaning, as a gut barrier integrity marker, were observed between n-3 LCFA and control diets.

Within the important bacterial populations of the Firmicutes phylum that were modified by n-3 LCFA in the feces of suckling piglets, we observed a decrease in the family *Ruminococcaceae* and the increase of family *Clostridiaceae*, which are two of the core families in the swine gastrointestinal tract (Holman et al., 2017). Decreased *Ruminococcaceae* was due to the decrease of genera *Ruminococcus* (tentatively identified as *R. gnavus*) and *Gemmiger*, and increased *Clostridiaceae* due to the increase of the genus *Clostridium* and, specifically, the increases of OTUs identified as *C. innocuum*, *C. cadaveris*, and *C. perfringens*. On the one hand, as already described, *Ruminococcaceae* is associated with anti-inflammatory effects (Liu et al., 2019). Conversely, *R. gnavus* has been associated with a pro-inflammatory role (Hall et al., 2017; Henke et al., 2019). On the other hand, members of the family *Clostridiaceae* are also associated with the production of butyrate, so they can also contribute to decreasing inflammation in the gut of the host (Holman et al., 2017). However, species *C. innocuum*, *C. cadaveris*, and *C. perfringens* can become pathogenic causing infections (Crum-Cianflone, 2009; Gupta et al., 2020; Posthaus et al., 2020). Concretely, *C. perfringens* can cause severe, acute, and necrotic enteritis in humans and livestock, particularly in neonatal pigs (Posthaus et al., 2020). The pathogenicity of *C. perfringens* is given by toxin-α and toxin-β, which are generated by *C. perfringens* type A and C (Baker et al., 2010; Posthaus et al., 2020). Although *C. perfringens* infection causes diarrhea, low weaning weights, and pre-weaning mortality, no differences in body weight nor mortality were observed between treatments for suckling piglets at weaning. Within the same phylum, we also observed an increase in the abundance of *Streptococcus,* which contains some species described as probiotics and has been related to the improvement of colostrum quality, milk quality and quantity, litter size, and piglet vitality, and body weight (Knecht et al., 2020). In addition, *Lactobacillus* species (OTUs tentatively identified as *L. delbrueckii* and *Lactobacillus mucosae*) were also increased in the feces of n-3 LCFA suckling piglets. Previous reports have described an immunomodulatory effect of *L. mucosae* (Ryan et al., 2019), and that the oral administration of *L. delbrueckii* improves intestinal integrity, stimulates the intestinal immune response, and alleviates intestinal oxidative damage in piglets (Li Y. et al., 2019; Chen et al., 2020). Moreover, Yang et al. (2017) described that many beneficial bacteria belonging to the Firmicutes phylum, such as *Enterococcus*, *Streptococcus*, *Lactobacillus*, and *Clostridium,* were reduced in diarrheic piglets. In the current study, these beneficial genera and pertaining species were increased by dietary n-3 LCFA.

Relevant modifications of bacterial populations belonging to phyla Bacteroidetes and Proteobacteria by dietary n-3 LCFA should also be noted in the feces suckling piglets. Regarding Bacteroidetes, n-3 LCFA increased the *Bacteroides* genus, which is one of the core bacterial genera of pigs' microbiota and is reported to be found in more than 90% of healthy pigs of different ages (Luo et al., 2022). Moreover, as described above for *A. muciniphila*, genus *Bacteroides* is also considered to be a mucin glycan degrader (Bell and Juge, 2021) and its low abundance has been associated with post-weaning diarrhea (Ren et al., 2022). It should be mentioned that n-3 LCFA also decreased *Prevotella copri* species, which are present during lactation at low abundances but increase drastically upon weaning (Amat et al., 2020). A high abundance of *P. copri* has recently been related to hosting chronic inflammation responses resulting in excessive fat accumulation in pigs (Chen et al., 2021). In terms of Proteobacteria, n-3 LCFA increased the *Pectobacteriaceae* and *Enterobacteriaceae* families mainly due to the increases of genus *Brenneria* and *Escherichia*, respectively, modifications that match those observed in milk. *Enterobacteriaceae* consists of a set of genera that colonize the intestinal microbiota and includes commensal microbiota as well as pathogens (Schierack et al., 2007). This family contains LPS-producing bacteria and *Escherichia coli* species, which can cause diarrhea and infections in both humans and animals (Schierack et al., 2007; Costantini et al., 2017). However, in the present study, despite *Escherichia* being increased in the feces of n-3 LCFA suckling piglets, no increase in *E. coli* was detected and as already mentioned, there were no differences in piglets' LPS plasma levels.

According to Lauridsen (2020), colostrum, milk and oral-fecal transmission play a crucial role in the acquisition and establishment of the microbiota of newborn piglets. Some correlations were observed between lactating sows' feces and milk, milk and suckling piglets' feces, and lactating sows' feces and suckling piglets' feces showing that the changes observed in the former due to the inclusion of dietary n-3 LCFA may be transferred and have an impact on the latter. First, between lactating sows' feces and milk, the family *Ruminococcaceae* and genus *Ruminococcus* in lactating sows were positively correlated with the same family and genus in milk, but also with the family *Spirochaetaceae*, and genera *Anaerostipes* and *Treponema*. Second, the genera *Akkermansia*, *Bacteroides*, *Flintibacter*, and *Parabacteroides* in milk correlate positively with the genus *Parabacteroides* in suckling piglets' feces. Concretely, different OTUs belonging to these four previous genera in milk were positively correlated with *Parabacteroides distasonis* in suckling piglets' feces, which is described as immunomodulatory health-promoting bacteria (Kverka et al., 2011). Moreover, positive correlations between lactating sows' feces and suckling piglets' feces were also observed. OTUs such as *Olsenella scatoligenes*, *Erysipelatoclostridium ramosum*, *Peptostreptococcus stomatis*, *A. muciniphila*, and *L. delbrueckii* on feces from lactating sows were positively correlated with the *Bacteroides* species *Bacteroides stercoris* and *Bacteroides fluxus* in feces from suckling piglets. Correlations between species are reported in **Supplementary Figure 1**. Therefore, milk appears to be the type of sample with more bacterial populations positively correlated with those in the feces of lactating sows or suckling piglets, suggesting its key role in microbial transfer from sow to piglets. Finally, it is important to mention that the study of correlations was performed using samples that were collected at the same time and although the transference of microbiota is not an immediate process, sampling was carried out when both, sows and piglets, had been eating for several days the same diet and it could be assumed that results are representative of the correlations among the different types of samples. Nevertheless, a future study evaluating microbial transference considering a time window between samplings may be of interest.

## Conclusion

The inclusion of fish oil as a source of n-3 LCFA in sow diets influences the microbiota of the feces of gestating sows, their milk, and the feces of suckling piglets, while no effects were observed in colostrum or the feces of lactating sows. The largest impact of n-3 LCFA supplementation is observed in the feces of suckling piglets, which are young animals that are in the process of acquiring microbiota. Concretely, n-3 LCFA increased piglets' fecal microbial diversity and the relative abundance of beneficial bacteria such as the mucin-degraders genera *Akkermansia* and *Bacteroides*, and different species of *Lactobacillus*, which may contribute to the achievement of a gut anti-inflammatory microbiota. In addition, it can also be concluded that some of these modifications were positively correlated among the feces of lactating sows, their milk, and the feces of the suckling piglets. Milk stands out as the factor with more bacterial populations correlated with both, lactating sows' and suckling piglets' feces.

## 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.ebi.ac.uk/ena, PRJEB53326.

## Ethics statement

This animal study was reviewed and approved by IRTA's Ethical Committee on Animal Experimentation and Generalitat de Catalunya (project no: 10294).

## Author contributions

IB, RL, DT, EE-G, and NT contributed to conception and design of the study. EL-C, ErC, EmC, MB, and NT performed the methodology and statistical analysis. EL-C wrote the first draft of the manuscript. ErC and EmC wrote sections of the manuscript. All authors contributed to manuscript revision, read, 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

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## References


Amat, S., Lantz, H., Munyaka, P. M., and Willing, B. P. (2020). *Prevotella* in pigs: the positive and negative associations with production and health. *Microorganisms* 8:1584. doi: 10.3390/microorganisms8101584

Baker, A. A., Davis, E., Rehberger, T., and Rosener, D. (2010). Prevalence and diversity of toxigenic *Clostridium perfringens* and *Clostridium difficile* among swine herds in the midwest. *Appl. Environ. Microbiol.* 76, 2961–2967. doi: 10.1128/AEM.02459-09

Beaumont, M., Cauquil, L., Bertide, A., Ahn, I., Barilly, C., Gil, L., et al. (2021). Gut microbiota-derived metabolite signature in suckling and weaned piglets. *J. Proteome Res.* 20, 982–994. doi: 10.1021/acs.jproteome.0c00745

Bell, A., and Juge, N. (2021). Mucosal glycan degradation of the host by the gut microbiota. *Glycobiology* 31, 691–696. doi: 10.1093/glycob/cwaa097

Bergamaschi, M., Maltecca, C., Schillebeeckx, C., McNulty, N. P., Schwab, C., Shull, C., et al. (2020). Heritability and genome-wide association of swine gut microbiome features with growth and fatness parameters. *Sci. Rep.* 10:10134. doi: 10.1038/s41598-020-66791-3

Caesar, R., Tremaroli, V., Kovatcheva-Datchary, P., Cani, P. D., and Backhed, F. (2015). Crosstalk between gut microbiota and dietary lipids aggravates WAT inflammation through TLR signaling. *Cell Metab.* 22, 658–668. doi: 10.1016/j.cmet.2015.07.026

Calder, P. C. (2010). Omega-3 fatty acids and inflammatory processes. *Nutrients* 2, 355–374. doi: 10.3390/nu2030355

Calder, P. C. (2019). Is increasing microbiota diversity a novel anti-inflammatory action of marine n-3 fatty acids? *Nutr. J.* 149, 1102–1104. doi: 10.1093/jn/nxz043

Chen, C., Fang, S., Wei, H., He, M., Fu, H., Xiong, X., et al. (2021). *Prevotella* copri increases fat accumulation in pigs fed with formula diets. *Microbiome* 9:175. doi: 10.1186/s40168-021-01110-0

Chen, F., Wang, H., Chen, J., Liu, Y., Wen, W., Li, Y., et al. (2020). *Lactobacillus delbrueckii* ameliorates intestinal integrity and antioxidant ability in weaned piglets after a lipopolysaccharide challenge. *Oxid. Med. Cell. Longev.* 2020:6028606. doi: 10.1155/2020/6028606

Costantini, L., Molinari, R., Farinon, B., and Merendino, N. (2017). Impact of omega-3 fatty acids on the gut microbiota. *Int. J. Mol. Sci.* 18:2645. doi: 10.3390/ijms18122645

Crespo-Piazuelo, D., Migura-Garcia, L., Estellé, J., Criado-Mesas, L., Revilla, M., Castelló, A., et al. (2019). Association between the pig genome and its gut microbiota composition. *Sci. Rep.* 9:8791. doi: 10.1038/s41598-019-45066-6

Crost, E. H., Le Gall, G., Laverde-Gomez, J. A., Mukhopadhya, I., Flint, H. J., and Juge, N. (2018). Mechanistic insights into the cross-feeding of *Ruminococcus gnavus* and *Ruminococcus bromii* on host and dietary carbohydrates. *Front. Microb.* 9:2558. doi: 10.3389/fmicb.2018.02558

Crum-Cianflone, N. (2009). *Clostridium innocuum* bacteremia in a patient with acquired immunodeficiency syndrome. *Am. J. Med. Sci.* 337, 480–482. doi: 10.1097/MAJ.0b013e31819f1e95

de Blas, C., Gasa, J., and Mateos, G. G. (2013). *Necesidades Nutricionales Para Ganado Porcino*. Madrid: Normas FEDNA.

Djuric, Z., Bassis, C. M., Plegue, M., Sen, A., Turgeon, D. K., Herman, K., et al. (2019). Increases in colonic bacterial diversity after omega-3 fatty acid supplementation predict decreased colonic prostaglandin E-2 concentrations in healthy adults. *J. Nutr.* 149, 1170–1179. doi: 10.1093/jn/nxy255

Duan, X., Tian, G., Chen, D., Huang, L., Zhang, D., Zheng, P., et al. (2019). Mannan oligosaccharide supplementation in diets of sow and (or) their offspring improved immunity and regulated intestinal bacteria in piglet. *J. Anim. Sci.* 97, 4548–4556. doi: 10.1093/jas/skz318

Edwards, S. A., and Baxter, E. M. (2015). "Piglet mortality: causes and prevention," in *The Gestating and Lactating Sow*, ed. C. Farmer (Wageningen: Wageningen Academic Publishers), 253–278.

Fontes, C. M. G. A., and Gilbert, H. J. (2010). Cellulosomes: highly efficient nanomachines designed to designed to deconstruct plant cell wall complex carbohydrates. *Annu. Rev. Biochem.* 79, 655–681. doi: 10.1146/annurev-biochem-091208-085603

Ganesh, B. P., Klopfleisch, R., Loh, G., and Blaut, M. (2013). Commensal *Akkermansia muciniphila* exacerbates gut inflammation in *Salmonella* typhimurium-infected gnotobiotic mice. *PLoS One* 8:e74963.

Gardiner, G. E., Metzler-Zebeli, B. U., and Lawlor, P. G. (2020). Impact of intestinal microbiota on growth and feed efficiency in pigs: a review. *Microorganisms* 8:1886. doi: 10.3390/microorganisms8121886

Gresse, R., Chaucheyras-Durand, F., Fleury, M. A., Van de Wiele, T., Forano, E., and Blanquet-Diot, S. (2017). Gut microbiota dysbiosis in postweaning piglets: understanding the keys to health. *Trends Microbiol.* 25, 851–873. doi: 10.1016/j.tim.2017.05.004

Guo, X., Li, S., Zhang, J., Wu, F., Li, X., Wu, D., et al. (2017). Genome sequencing of 39 *Akkermansia muciniphila* isolates reveals its population structure, genomic and functional diverisity, and global distribution in mammalian gut microbiotas. *BMC Genomics* 18:800. doi: 10.1186/s12864-017-4195-3

Gupta, T. B., Jauregui, R., Maclean, P., Nawarathana, A. S., and Brightwell, G. (2020). Draft genome sequence of *Clostridium cadaveris* strain AGRFS2.2, isolated from a bovine dairy farm in New Zealand. *Microbiol. Resour. Announc.* 9, e787–e820. doi: 10.1128/MRA.00787-20

Hall, A. B., Yassour, M., Sauk, J., Garner, A., Jiang, X., Arthur, T., et al. (2017). A novel *Ruminococcus gnavus* clade enriched in inflammatory bowel disease patients. *Genome Med.* 9:103. doi: 10.1186/s13073-017-0490-5

Henke, M. T., Kenny, D. J., Cassilly, C. D., Vlamakis, H., Xavier, R. J., and Clardy, J. (2019). *Ruminococcus* gnavus, a member of the human gut microbiome associated with Crohn's disease, produces an inflammatory polysaccharide. *Proc. Natl. Acad. Sci. U.S.A.* 116, 12672–12677. doi: 10.1073/pnas.1904099116

Holman, D. B., Brunelle, B. W., Trachsel, J., and Allen, H. K. (2017). Meta-analysis to define a core microbiota in the swine gut. *mSystems* 2, e00004–e00017. doi: 10.1128/mSystems.00004-17

Hooper, L. (2004). Bacterial contributions to mammalian gut development. *Trends Microbiol.* 12, 129–134. doi: 10.1016/j.tim.2004.01.001

Huber, L. A., Hooda, S., Fisher-Heffernan, R. E., Karrow, N. A., and de Lange, C. F. M. (2018). Effect of reducing the ratio of omega-6-to-omega-3 fatty acids in diets of low protein quality on nursery pig growth performance and immune response. *J. Anim. Sci.* 96, 4348–4359. doi: 10.1093/jas/sky296

Klindworth, A., Pruesse, E., Schweer, T., Peplies, J., Quast, C., Horn, M., et al. (2013). Evaluation of general 16S ribosomal RNA gene PCR primers for classical

and next-generation sequencing-based diversity studies. *Nucleic Acids Res.* 41:e1. doi: 10.1093/nar/gks808

Knecht, D., Cholewińska, P., Jankowska-Mąkosa, A., and Czyż, K. (2020). Development of swine's digestive tract microbiota and its relation to production indices-A review. *Animals* 10:527. doi: 10.3390/ani10030527

Kverka, M., Zakostelska, Z., Klimesova, K., Sokol, D., Hudcovic, T., Hrncir, T., et al. (2011). Oral administration of *Parabacteroides* distasonis antigens attenuates experimental murine colitis through modulation of immunity and microbiota composition. *Clin. Exp. Immunol.* 163, 250–259. doi: 10.1111/j.1365-2249.2010.04286.x

Lauridsen, C. (2020). Effects of dietary fatty acids on gut health and function of pigs pre- and post-weaning. *J. Anim. Sci.* 98:skaa086. doi: 10.1093/jas/skaa086

Lauridsen, C., and Danielsen, V. (2004). Lactational dietary fat levels and sources influence milk composition and performance of sows and their progeny. *Livest. Prod. Sci.* 91, 95–105. doi: 10.1016/j.livprodsci.2004.07

Leonard, S. G., Sweeney, T., Bahar, B., Lynch, B. P., and O'Doherty, J. V. (2010). Effect of maternal fish oil and seaweed extract supplementation on colostrum and milk composition, humoral immune response, and performance of suckled piglets. *J. Anim. Sci.* 88, 2988–2997. doi: 10.2527/jas.2009-2764

Li, N., Huang, S., Jiang, L., Dai, Z., Li, T., Han, D., et al. (2019). Characterization of the early life microbiota development and predominant *Lactobacillus* Species at distinct gut segments of low- and normal-birth-weight piglets. *Front. Microbiol.* 10:797. doi: 10.3389/fmicb.2019.00797

Li, N., Huang, S., Jiang, L., Wang, W., Li, T., Zuo, B., et al. (2018). Differences in the gut microbiota establishment and metabolome characteristics between low- and normal-birth-weight piglets during early-life. *Front. Microbiol.* 9:1798. doi: 10.3389/fmicb.2018.01798

Li, W., Fu, L., Niu, B., Wu, S., and Wooley, J. (2012). Ultrafast clustering algorithms for metagenomic sequence analysis. *Brief. Bioinform.* 13, 656–668. doi: 10.1093/bib/bbs035

Li, Y., Hou, S., Peng, W., Lin, Q., Chen, F., Yang, L., et al. (2019). Oral administration of *Lactobacillus delbrueckii* during the suckling phase improves antioxidant activities and immune responses after the weaning event in a piglet model. *Oxid. Med. Cell. Longev.* 2019:6919803. doi: 10.1155/2019/6919803

Liu, H., Chen, X., Hu, X., Niu, H., Tian, R., Wang, H., et al. (2019). Alterations in the gut microbiome and metabolism with coronary artery disease severity. *Microbiome* 7:68. doi: 10.1186/s40168-019-0683-9

Liu, Y. (2015). Fatty acids, inflammation and intestinal health in pigs. *J. Anim. Sci. Biotechnol.* 6:41. doi: 10.1186/s40104-015-0040-1

Liu, Y., Chen, F., Odle, J., Lin, X., Jacobi, S. K., Zhu, H., et al. (2012). Fish oil enhances intestinal integrity and inhibits TLR4 and NOD2 signaling pathways in weaned pigs after LPS challenge. *J. Nutr.* 142, 2017–2024. doi: 10.3945/jn.112.164947

Llauradó-Calero, E., Badiola, I., Delpino-Rius, A., Lizardo, R., Torrallardona, D., Esteve-Garcia, E., et al. (2021). Fish oil rich in eicosapentaenoic acid and docosahexaenoic acid in sow diets modifies oxylipins and immune indicators in colostrum and milk. *Animal* 15:100403. doi: 10.1016/j.animal.2021.100403

Lopez-Verge, S., Gasa, J., Farre, M., Coma, J., Bonet, J., and Sola-Oriol, D. (2018). Potential risk factors related to pig body weight variability from birth to slaughter in commercial conditions. *Transl. Anim. Sci.* 2, 383–395. doi: 10.1093/tas/txy082

Love, M. I., Huber, W., and Anders, S. (2014). Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. *Genome Biol.* 15:550. doi: 10.1186/s13059-014-0550-8

Luo, Y., Ren, W., Smidt, H., Wright, A. G., Yu, B., Schyns, G., et al. (2022). Dynamic distribution of gut microbiota in pigs at different growth stages: composition and contribution. *Microbiol Spectr.* 18:e0068821. doi: 10.1128/spectrum.00688-21

Marcel, M. (2011). Cutadapt removes adapter sequences from high-throughput sequencing reads. *EMBnet J.* 17:1. doi: 10.14806/ej.17.1.200

Niu, Q., Li, P., Hao, S., Kim, S. W., Du, T., Hua, J., et al. (2019). Characteristics of gut microbiota in sows and their relationship with apparent nutrient digestibility. *Int. J. Mol. Sci.* 20:870. doi: 10.3390/ijms20040870

Noblet, J., and Etienne, M. (1987). Body-composition, metabolic-rate and utilization of milk nutrients in suckling piglets. *Reprod. Nutr. Dev.* 27, 829–839. doi: 10.1051/rnd:19870609

Nowland, T. L., Kirkwood, R. N., and Pluske, J. R. (2021). Review: can early-life establishment of the piglet intestinal microbiota influence production outcomes? *Animal* 16:100368. doi: 10.1016/j.animal.2021.100368

Nowland, T. L., Plush, K. J., Barton, M., and Kirkwood, R. N. (2019). Development and function of the intestinal microbiome and potential implications for pig production. *Animals* 9:76. doi: 10.3390/ani9030076

Oksanen, J., Guillaume Blanchet, F., Friendly, M., Kindt, R., Legendre, P., McGlinn, D., et al. (2020). *Vegan: Community Ecology Package.* R package version 2.5-7. Available online at: https://CRAN.R-project.org/package=vegan (accessed June, 2022).

Ottman, N., Geerlings, S. Y., Aalvink, S., de Vos, W. M., and Belzer, C. (2017). Action and function of *Akkermansia muciniphila* in microbiome ecology, health and disease. *Best Pract. Res. Clin. Gastroenterol.* 31, 637–642. doi: 10.1016/j.bpg.2017.10.001

Posthaus, H., Kittl, S., Tarek, B., and Bruggisser, J. (2020). *Clostridium perfringens* type C necrotic enteritis in pigs: diagnosis, pathogenesis, and prevention. *J. Vet. Diagn. Invest.* 32, 203–212. doi: 10.1177/1040638719900180

Quiniou, N., Dagorn, J., and Gaudré, D. (2002). Variation of piglets' birth weight and consequences on subsequent performance. *Livest. Prod. Sci.* 78, 63–70. doi: 10.1016/S0301-6226(02)00181-1

R Core Team (2012). *R: A language and environment for statistical computing.* Vienna: R Foundation for Statistical Computing.

Rajan, K., D'Souza, D. H., Kim, K., Choi, J. M., Elder, T., Carrier, D. J., et al. (2021). Production and characterization of high value prebiotics from biorefinery-relevant feedstocks. *Front. Microbiol.* 12:675314. doi: 10.3389/fmicb.2021.675314

Ren, W., Bing, Y., Jie, Y., Ping, Z., Zhiqing, H., Junqiu, L., et al. (2022). Lower abundance of *Bacteroides* and metabolic dysfunction are highly associated with the post-weaning diarrhea in piglets. *Sci. China Life Sci.* 65, 2062–2075. doi: 10.1007/s11427-021-2068-6

Rooke, J. A., Sinclair, A. G., Edwards, S. A., Cordoba, R., Pkiyach, S., Penny, P. C., et al. (2001). The effect of feeding salmon oil to sows throughout pregnancy on pre-weaning mortality of piglets. *Anim. Sci.* 73, 489–500. doi: 10.1017/S135772980005846X

Ryan, P. M., Stolte, E. H., London, L. E. E., Wells, J. M., Long, S. L., Joyce, S. A., et al. (2019). *Lactobacillus* mucosae DPC 6426 as a bile-modifying and immunomodulatory microbe. *BMC Microbiol.* 19:33. doi: 10.1186/s12866-019-1403-0

Schierack, P., Walk, N., Reiter, K., Weyrauch, K. D., and Wieler, L. H. (2007). Composition of intestinal *Enterobacteriaceae* populations of healthy domestic pigs. *Microbiology* 153, 3830–3837. doi: 10.1099/mic.0.2007/010173-0

Simpson, J. M., McCracken, V. J., Gaskins, H. R., and Mackie, R. I. (2000). Denaturing gradient gel electrophoresis analysis of 16S ribosomal DNA amplicons to monitor changes in fecal bacterial populations of weaning pigs after introduction of *Lactobacillus reuteri* strain MM53. *Appl. Environ. Microbiol.* 66, 4705–4714. doi: 10.1128/AEM.66.11.4705-4714.2000

Tailford, L. E., Crost, E. H., Kavanaugh, D., and Juge, N. (2015). Mucin glycan foraging in the human gut microbiome. *Front. Genet.* 6:81. doi: 10.3389/fgene.2015.00081

Tanghe, S., and De Smet, S. (2013). Does sow reproduction and piglet performance benefit from the addition of n-3 polyunsaturated fatty acids to the maternal diet? *Vet. J.* 197, 560–569. doi: 10.1016/j.tvjl.2013.03.051

Watson, H., Mitra, S., Croden, F. C., Taylor, M., Wood, H. M., Perry, S. L., et al. (2018). A randomised trial of the effect of omega-3 polyunsaturated fatty acid supplements on the human intestinal microbiota. *Gut* 67, 1974–1983. doi: 10.1136/gutjnl-2017-314968

Yang, Q., Huang, X., Zhao, S., Sun, W., Yan, Z., Wang, P., et al. (2017). Structure and function of the fecal microbiota in diarrheic neonatal piglets. *Front. Microbiol.* 8:502. doi: 10.3389/fmicb.2017.00502

Zhang, J., Kobert, K., Flouri, T., and Stamatakis, A. (2014). PEAR: a fast and accurate illumina paired-end reAd mergeR. *Bioinformatics* 30, 614–620. doi: 10.1093/bioinformatics/btt593

Zhao, W., Wang, Y., Liu, S., Huang, J., Zhai, Z., He, C., et al. (2015). The dynamic distribution of porcine microbiota across different ages and gastrointestinal tract segments. *PLoS One* 10:e0117441. doi: 10.1371/journal.pone.0117441

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# Differences in meat quality between Angus cattle and Xinjiang brown cattle in association with gut microbiota and its lipid metabolism

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Gut microbiota plays important roles in mediating fat metabolic events in humans and animals. However, the differences of meat quality traits related to the lipid metabolism (MQT-LM) in association with gut microbiota involving in lipid metabolism have not been well explored between Angus cattle (AG) and Xinjiang brown cattle (BC). Ten heads of 18-month-old uncastrated male AG and BC (5 in each group) raised under the identical conditions were selected to test MQT-LM, i.e., the backfat thickness (BFT), the intramuscular fat (IMF) content, the intramuscular adipocyte areas (IAA), the eye muscle area (EMA), the muscle fiber sectional area (MFSA) and the muscle shear force after sacrifice. The gut microbiota composition and structure with its metabolic function were analyzed by means of metagenomics and metabolomics with rectal feces. The correlation of MQT-LM with the gut microbiota and its metabolites was analyzed. In comparison with AG, BC had significant lower EMA, IMF content and IAA but higher BFT and MFSA. Chao1 and ACE indexes of α-diversity were lower. β-diversity between AG and BC were significantly different. The relative abundance of Bacteroidetes, *Prevotella* and *Blautia* and *Prevotella copri*, *Blautia wexlerae*, and *Ruminococcus gnavus* was lower. The lipid metabolism related metabolites, i.e., succinate, oxoglutaric acid, L-aspartic acid and L-glutamic acid were lower, while GABA, L-asparagine and fumaric acid were higher. IMF was positively correlated with *Prevotella copri*, *Blautia wexlerae* and *Ruminococcus gnavus*, and the metabolites succinate, oxoglutaric acid, L-aspartic acid and L-glutamic acid, while negatively with GABA, L-asparagine and fumaric acid. BFT was negatively correlated with *Blautia wexlerae* and the metabolites succinate, L-aspartic acid and L-glutamic acid, while positively with GABA, L-asparagine and fumaric acid. *Prevotella Copri*, *Blautia wexlerae*, and *Ruminococcus gnavus* was all positively correlated with succinate, oxoglutaric acid, while negatively with L-asparagine and fumaric acid. In conclusion, *Prevotella copri*, *Prevotella intermedia*, *Blautia wexlerae*, and *Ruminococcus gnavus* may serve as the potential differentiated bacterial species in association with MQT-LM *via* their metabolites of oxoglutaric acid,

succinate, fumaric acid, L-aspartic acid, L-asparagine, L-glutamic acid and GABA between BC and AG.

KEYWORDS

**Xinjiang brown cattle, Angus cattle, meat quality traits, lipid metabolism, gut microbiota, metabolomics**

# Introduction

Beef is a main source of animal protein in human food, and consumers are paying an increasing amount of attention to meat quality. Most of meat quality traits that are closely related to lipid metabolism (MQT-LM) include the backfat thickness (BFT), intramuscular fat (IMF) content, the intramuscular adipocyte areas (IAA), the eye muscle area (EMA) and the muscle fiber sectional area (MFSA), marbling and tenderness, they are mainly involved in the fat metabolism/deposition in the beef (Picard et al., 2018; Li et al., 2020). Although a large number of studies on MQT-LM have been performed in Angus cattle (AG), few studies have systematically compared MQT-LM in AG with Xinjiang brown cattle (BC), an indigenous dual- purpose of meat and milk cattle breed with excellent adaptability, strong disease resistance, good grazing and tolerance to extreme weather in China (Li et al., 2020). Therefore, the comparison on the difference of meat quality between AG and BC is pivotal for assisting the improvement of meat quality of Xinjiang brown cattle.

The gut microbiota is a highly complex and diverse microecosystem which is strongly associated with various host physiological functions, e.g., metabolism, immune regulation, growth and development and production performance, and to overall host health and disease in human and animals (Turnbaugh and Gordon, 2009; Petri et al., 2013; Lee and Hase, 2014; Myer et al., 2015; Noel et al., 2019). Large body of studies have figured out the contribution of microbiota on host health and indicated marked shifts in the relative abundance of various phyla or species in various physiological statuses (Li et al., 2017; Franzosa et al., 2019; Wu et al., 2020). Regarding the gut microbiota in the interaction with host lipid metabolism and body fat deposition, most studies are human or experimental rodents orientated for human health. For example, the comparisons of the gut microbiota of lean and obese people indicated that the relative abundance of Bacteroidetes and Firmicutes are associated with obesity (Ley et al., 2005, 2006). Additionally, gut microbiota may directly regulate the expression of genes related to fatty acid synthesis and triglyceride storage in animals, thus altering energy balance to determine adipose tissue expansion, or fat deposition, in different parts of the body (Hooper et al., 2001). When gut microbiota obtained from obese mice are transplanted into non-obese, aseptic mice, the previously non-obese mice exhibit greater efficiency in energy absorption in the gut and significant increases in total body fat (Turnbaugh et al., 2006). Bäckhed et al. (2004) experiment showed that the gut microbiota acts through angiopoietin-like protein 4 as to coordinate increased hepatic lipogenesis with increased lipoprotein lipase activity in adipocytes, thereby promoting storage of calories harvested from the diet into fat. In terms of gut microbiota and its lipid metabolic regulating function on MQT-LM / fat deposition in food animals, there are already some studies in pigs (Khanal et al., 2020; Wu et al., 2021; Ma et al., 2022), chickens or broilers (Wen et al., 2019; Yang et al., 2021), and ducks (Lyu et al., 2021). However, the association of MQT-LM with the gut microbiota and its lipid metabolic functions on beef cattle has been seldom elucidated. Zhang and colleagues did the comparison of the gut microbiota in Angus beef cattle reared under the grazing and feedlot conditions and then speculated that the significant differences in gut microbiota composition may have an impact on the meat quality (Zhang et al., 2021). Zheng et al. (2022) studied the association of gut microbiota with host intramuscular differentially expressed genes and metabolites in Angus and Chinese Simmental cattle, unveiled the different associations of gut microbiota and the meat quality between these two breeds. Therefore, we hypothesized that differentials MQT-LM between different AG and BC could be associated with the specific composition and structure in gut microbiota and its metabolic function. MQT-LM namely, the BFT, IMF, IAA, EMA, and MFSA were assessed using 18-month-old BC and AG. The structure, function and metabolic pathways of the gut microbiota were determined by metagenomics and metabolomics and their associations with meat quality traits were determined by correlation analysis so as to provide a potential novel biomarker for improving the meat quality of BC.

# Materials and methods

## Animals, housing, and feeding

Eighteen-month-old uncastrated male AG ($n = 5$) and BC ($n = 5$), which were collected at age of about 5-month-old with initial body weight between 170 and 180 kg from a beef cattle breeding farm in Xinjiang, raised under the identical feeding regime, management and condition (Table 1) were used for the study. The study protocol was approved by the Animal Ethics Committee of Xinjiang Agricultural University (2017015).

TABLE 1 Diet composition and nutrient levels (based on dry matter).

| Feed ingredient | Weight (kg) | Content (%) | Nutritional ingredient | Content |
|---|---|---|---|---|
| Straws | 1.3 | 5.78 | Metabolic energy (MJ/kg) | 5.82 |
| Alfalfas | 0.5 | 2.22 | Crude protein (%) | 16.2 |
| Cossettes | 2 | 8.89 | Crude fat (%) | 2.32 |
| Wheat straws | 1.5 | 6.67 | Calcium (%) | 0.54 |
| Ensilings | 10 | 44.46 | Phosphorus (%) | 0.49 |
| Molasses | 0.7 | 3.11 | Acid detergent fiber (%) | 5.9 |
| Concentrates* | 6.49 | 28.86 | Neutral detergent fiber (%) | 12.98 |
| Total | 22.49 | 100 | Total digestible nutrient (%) | 61.57 |

*Concentrate: corn (53.16%), cotton meal (11.56%), bran (7.40%), magnesium oxide (3.08%), protein feed (23.42) and premix (1.38%) containing (vitamin A, vitamin B1, vitamin B2, vitamin B6, vitamin D, vitamin E, pantothenic acid, nicotinamide, Cu, Fe, Mn, Zn, Se, and Co).

## Sample collection

Bulls were fasted for 24 h, but provided water *ad libitum* up 12 h prior to sacrifice. Following the 24 h fast, cattle were weighed. About 20 g of feces was collected from the rectum of cattle by samplers equipped with aseptic gloves 6 h prior to slaughter, and then placed in a sterile, frozen tube and quickly stored in liquid nitrogen for subsequent testing. All the bulls were slaughtered in the automatic cattle slaughter line of a slaughterhouse head by head in the same day. The bulls' carcass was weighed after sacrifice, and the *longissimus dorsi* muscle was dissected, about 1 $cm^3$ sample of it was collected and fixed in picric acid solution, the remaining muscle was stored at 4°C for shear force examination.

## Meat quality traits detection

The BFT and the EMA were measured *in vivo* by veterinary B-ultrasound (Pyle Co. LTD, Aquila Vet, Netherlands) *via* detector located on the back of *longissimus dorsi* muscle between intercostal space of 12th and 13th ribs. The muscle shear force was measured using a computer-coupled muscle tenderness instrument (Brad Technology Development Co. Ltd., c-lm4, Beijing, China) as described elsewhere (Wen et al., 2020; Bai et al., 2022). The muscle moisture content was measured by the oven drying method, the samples were dried at 101–105°C for 24 h under the constant temperature and pressure condition. The moisture (%) was calculated by calculation formula: (sample weight before drying – weight after drying) / weight before drying × 100 (Mamani-Linares and Gallo, 2013; Wen et al., 2020). The ash content was measured by high temperature burning method, the samples were burned in furnace at ca 500°C. The ash (%) was calculated by calculation formula: (sample weight before burning – weight after burning) / weight before burning × 100 (Latimer, 2012; Luo et al., 2019). IMF was determined by Soxhlet extraction, in which 2.5 g of freeze-dried *longissimus dorsi* muscle was extracted in 85 ml of hexane for 60 min and then placed in a forced draft oven for 30 min at 105°C. The variation in sample weight before and after extraction was used to calculate fat content (Holman et al., 2019; Chen G. et al., 2021). Fixed muscle tissue was dehydrated and paraffin-embedded, paraffin sections were prepared, stained with oil red O, and observed under an optical microscope (Nikon Instruments Co. Ltd., 55I-1,000, Shanghai, China). Adipocyte diameter was measured using a microscopic imaging system (Motic Advanced 3.5). Three non-consecutive muscle sections from each muscle sample were used to measure adipocyte diameter. Within each section, adipocyte diameter was measured in three separate field of vision. All measurements for each bull were averaged and used for statistical analyses. The IAA and the MFSA were measured and calculated (Pertl et al., 2013; Ding et al., 2021).

## Metagenomics of gut microbiota

Total DNA was extracted from cattles' feces with OMEGA Soil DNA Kit (D5625-01), as previously described by Huang et al. (2019). DNA concentration and quality were determined by NanoDropND-1,000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, United States) and agarose gel electrophoresis, respectively. The extracted microbial DNA was processed to construct metagenome shotgun sequencing libraries with insert sizes of 400 bp by using the Illumina TruSeq Nano DNA LT Library Preparation Kit. Each library was sequenced by the Illumina HiSeq X-ten platform (Illumina, United States) with PE150 strategy.

Raw sequencing reads were processed to obtain quality-filtered reads for further analysis. Adapter sequences were removed by Cutadapt (v1.2.1; Marcel, 2011). The sequencing reads were aligned to the host genome using BWA to remove host contamination. Quality-filtered reads were *de novo* assembled to construct the metagenome for each sample by IDBA-UD (Iterative De Bruijn graph Assembler for sequencing data with highly Uneven Depth). The coding sequences (CDS, > 300 bp) were predicted by MetaGeneMark (v3.25; Zhu et al., 2010). CDSs were clustered by CD-HIT (v4.8.1) at 90% amino acid sequence identity to obtain a non-redundant gene catalogue. The alpha diversity index (Chao1 index, Ace index, Simpson index, and Shannon index) was based on Mothur (version 1.30.1). The beta-diversity of both bacterial and fungal communities was assessed by computing weighted UniFrac distance matrices and then ordinated using non-metric multi-dimensional scaling (NMDS). The relative contribution of different biotic and abiotic factors on community dissimilarity was tested with PERMANOVA using the Adonis function. Linear discriminant analysis Effect Size

(LEfSe) was measured the consistency of differences in relative abundance between taxa in the groups analyzed (BC vs. AG), taxa with LDA score > 2 and $p$ value <0.05 set as the significant level. Gene abundance in each sample was estimated by SOAPdenovo2 (v1.0). The taxonomy was annotated by searching against the NCBI-NT database by BLASTN ($e$ value <0.001) and annotated by MEGAN with the lowest common ancestor approach. The functional gene was annotated by searching the sequence of the non-redundant genes against the KEGG databases (release 90.0) by DIAMOND protein aligner (v2.0.4; Cantalapiedra et al., 2021). Gene abundances were derived from mapping the all reads back to the predicted ORF *via* bowtie2 (v2.2.6) and calculated transcripts per kilobase million (TPM) *via* SamTools (v1.5; Rausch et al., 2019). Metagenomic sequencing were analyzed at Personal Biotechnology Co., Ltd. (Shanghai, China).

## Metabolomics analysis

LC–MS was performed as previously described (Fan et al., 2021). For each fecal sample, 100 mg was transferred into a 2 ml centrifuge tube and 500 µl of ddH2O at 4°C added. The supernatant was collected by centrifugation. Chromatographic separation was accomplished in an Thermo Ultimate 3,000 system equipped with an ACQUITY UPLC® HSS T3 (150 × 2.1 mm, 1.8 µm, Waters) 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 done after equilibration. Analytes from fecal samples were obtained *via* chromatographic separation and subjected to electrospray ionization multistage mass spectrometry (ESI-MSn) on the Thermo Q Exactive mass spectrometer. LC–MS was performed by Personal Biotechnology Co., Ltd. (Shanghai, China).

## Statistical analysis

Inter-group variables were analyzed by Mann–Whitney test using GraphPad Prism 9.0.0 (GraphPad Software, San Diego, CA, United States). Data are reported as mean ± SEM. * denotes $p < 0.05$, meaning significant difference, ** denotes $p < 0.01$, meaning extremely significant difference. The differential species in the gut microbiota between BC and AG screened by metagenomics analysis were correlated with the differential MQT-LM and metabolites, separately, and then the correlation of MQT-LM with metabolites was analyzed by Spearman correlation method. Based on R (Version 3.6.2), ggplot2 and corrplot packages were used for correlation analysis and mapping. Spearman correlation coefficient was calculated, and significance test was conducted.

# Results

## Meat quality detection

The body and carcass weight of BC was extremely lower than that of AG ($p < 0.01$). The EMA, IMF content, muscle ash content and IAA were significantly lower in BC ($p < 0.05$), however, the BFT and MFSA were significantly higher in BC ($p < 0.05$; Figure 1; Table 2).

## Metagenomics of gut microbiota

### Composition analysis of gut microbiota

The α-diversity indices of gut microbiota (Simpson, Chao1, ACE, Shannon) and β-diversity were compared in BC and AG shown in Figures 2, 3, respectively. Chao1 and ACE in BC were significantly lower than those in AG ($p < 0.05$). The β-diversity of gut microbiota showed extremely significant independent distribution between AG and BC (Anosim $p < 0.01$).

Results of linear discriminant analysis effect size (LEfSe) showed the gut microbiota was significantly different between AG and BC (Figure 4). At the class level, the Coriobacteriia was enriched in BC and Negativicutes enriched in AG. At the order level, the Coriobacteriales was enriched in BC and Acidaminococcales enriched in AG. At the family level, there were 7 bacteria in gut microbiota significantly different between BC and AG, in which 2 families were enriched in BC and 5 families enriched in AG. At the genus level, there were 21 bacteria significantly different in gut microbiota between BC and AG, in which 5 genera were enriched in BC and 16 genera enriched in AG. At the species level, there were 68 bacteria significantly different in gut microbiota between BC and AG, in which 13 species were enriched in BC and 55 species enriched in AG (Figure 4). As shown in Figure 5A, in both BC and AG gut microbiota were mainly comprised of bacteria within phyla of Firmicutes, Bacteroidetes, Actinobacteria, and Spirochaetes with the relative abundance of Firmicutes and Bacteroidetes being highest. The relative abundance of Bacteroidetes in BC was significantly lower than that in AG ($p < 0.05$; Figure 5B) thus resulting in the ratio of Firmicutes/Bacteroidetes in the relative abundance had a tendency of increase in BC ($p = 0.08$; Figure 5C). *Bifidobacterium*, *Prevotella*, *Bacteroides* and *Eubacterium* were the most abundant genera in both BC and AG (Figure 6A). The relative abundance of *Prevotella* and *Blautia* were significantly lower in BC if compared to AG ($p < 0.05$; Figures 6B,C). The top 20 species in the gut microbiota between breeds were listed in Figure 7A with the highest relative abundance of *Prevotella copri* in AG and *Bifidobacterium pseudolongum* in BC (Figure 7A). The relative abundance of *Prevotella copri* and *Blautia wexlerae* in BC were significantly lower than that in AG ($p < 0.05$; Figures 7B,C). The relative abundance of *Ruminococcus gnavus* was extremely lower in BC than that in AG ($p < 0.01$; Figures 7B,C).

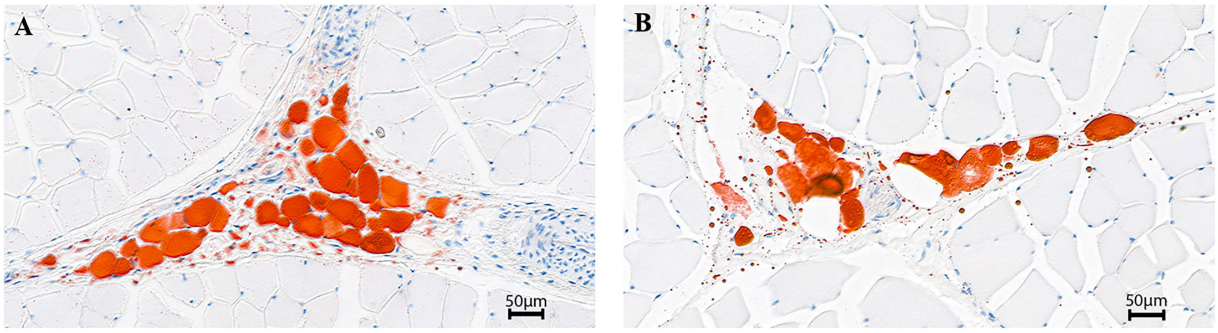


FIGURE 1
Intramuscular fat in longissimus dorsi sections (200×). Angus cattle **(A)**; Xinjiang brown cattle **(B)**.

TABLE 2 Comparison of beef quality traits.

| Characteristics | AG | BC | *p* value |
|---|---|---|---|
| Weight, kg | 625.39 ± 21.51 | 482.65 ± 19.16 | 0.001 |
| Carcass weight, kg | 379.92 ± 17.74 | 278.73 ± 6.88 | 0.001 |
| Backfat thickness (BFT), mm | 5.76 ± 0.27 | 6.62 ± 0.21 | 0.023 |
| Eye muscle area (EMA), $cm^2$ | 80.23 ± 2.78 | 71.05 ± 2.91 | 0.035 |
| Intramuscular fat (IMF), % | 11.09 ± 0.39 | 9.74 ± 0.43 | 0.032 |
| Muscle shear force (MSF), N | 46.35 ± 6.33 | 46.07 ± 6.19 | 0.972 |
| Moisture content (MC), % | 71.28 ± 0.78 | 72.71 ± 1.27 | 0.348 |
| Ash content (AC), % | 7.56 ± 0.31 | 6.58 ± 0.29 | 0.032 |
| Muscle fiber sectional area (MFSA), $\mu m^2$ | 1248.39 ± 154.58 | 1662.83 ± 65.26 | 0.039 |
| Intramuscular adipocyte areas (IAA), $\mu m^2$ | 48310.08 ± 9752.09 | 18312.65 ± 2721.01 | 0.018 |

## Functional analysis of gut microbiota

Protein sequences were annotated using the KEGG database, and the number of KEGG metabolic pathways annotated to different grades and categories was determined according to their KEGG Orthology (KO) classification. As shown in Supplementary Figure 1, enriched KEGG pathways within metabolism function included: carbohydrate metabolism, amino acid metabolism, nucleotide metabolism and lipid metabolism. Protein sequences and KO data were obtained from the gene coding regions of gut microbiota (Table 3). Sixteen functional genes related to lipid metabolism were identified within the KEGG differential lipid metabolism pathway (Pathway ID: ko00564) and enriched in 10 bacterial species: *Methanobrevibacter millerae*, *Methanosphaera stadtmanae*, *Fermentimonas caenicola*, *Prevotella intermedia*, *Paenibacillus terrae*, *Clostridium bornimense*, *Butyrivibrio fibrisolvens*, *Flavonifractor plautii*, *Escherichia coli*, and *Spirochaeta africana*. TPM values of lipid metabolism related functional gene pgpB derived from *Escherichia coli* and GPCPD1 from *Prevotella intermedia* were significantly higher in the gut microbiota of BC than those in the gut microbiota of AG ($p < 0.05$; Figure 8; Table 3).

## Metabolomics of gut microbiota

Metabolomic analysis identified 450 microbiota-derived metabolites that differed between Xinjiang brown and AG (Supplementary Figure 2). These included mainly carboxylic acids and derivatives (15.66%), fatty acyls (14.46%), benzene and substituted derivatives (11.65%), organooxygen compounds (6.43%) and steroids and steroid derivatives (5.02%; Supplementary Figure 2). Two metabolic pathways related to lipid metabolism, i.e., D-glutamine and D-glutamate metabolism and Alanine, aspartate and glutamate metabolism, were used in subsequent analyses based on pathway impact analysis (Supplementary Figure 3). Within the enriched pathways, 7 microbiota-related metabolites were identified as being significantly different between BC and AG. These included oxoglutaric acid, succinate, fumaric acid, L-aspartic acid, L-asparagine, L-glutamic acid and GABA (Table 4). As shown in Figure 9, L-asparagine, fumaric acid ($p < 0.01$) and GABA ($p < 0.05$) were significantly higher, whereas succinate, oxoglutaric acid, L-aspartic acid and L-glutamic acid were significantly lower in BC ($p < 0.01$).

## Correlation analysis

In total there were 76 species with the relative abundance above 0.1% (RA abv. 0.1%) in metagenomic analysis. They were all analyzed in association with 7 metabolites related to lipid metabolism and 10 MQT-LM by Spearman correlation. Among them, 8 species have different correlations with meat quality traits (Supplementary Figure 4A), 7 metabolites have different correlation with meat quality traits (Supplementary Figure 4B)

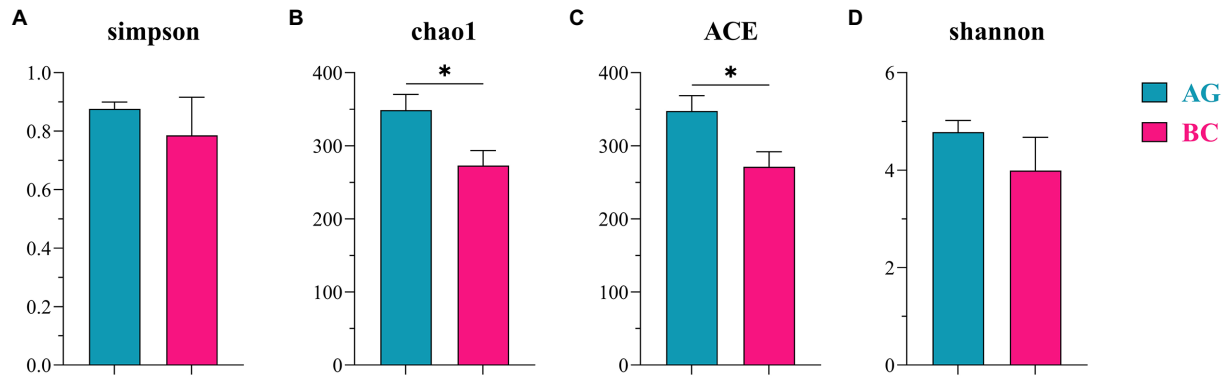


FIGURE 2
Comparison of α-diversity in the gut microbiota between Xinjiang brown cattle (BC) and Angus cattle (AG). ($n$=5 in each breed). Simpson diversity index **(A)**; The Chao1 estimator **(B)**; The ACE estimator **(C)**; Shannon diversity index **(D)**. Values were expressed as means±SEM. * denotes $P$<0.05 indicating significant difference.

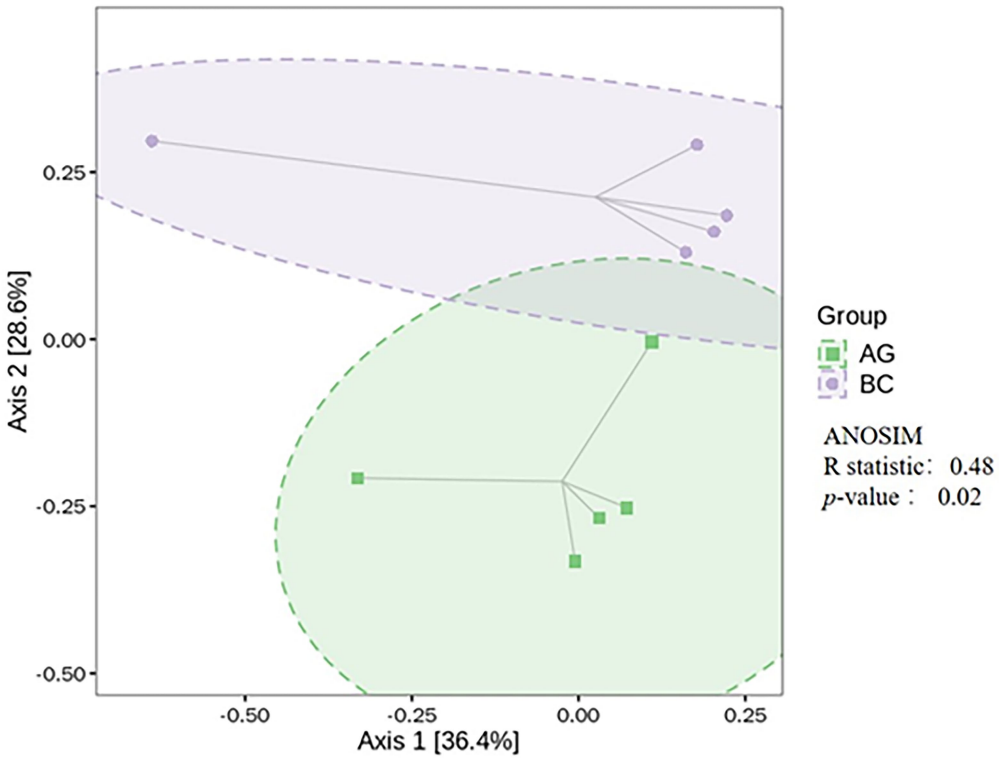


FIGURE 3
Comparison of β-diversity in the gut microbiota between Xinjiang brown cattle (BC) and Angus cattle (AG). ($n$=5 in each breed). ANOSIM, analysis of similarities.

and 12 species have different correlation with 7 metabolites related to lipid metabolism (Supplementary Figure 4C). Based on this result, the differential genus and species, the differential MQT-LM and the differential metabolites related to fat metabolism between AG and BC were further analyzed by Spearman correlation. IMF was positively correlated with *Prevotella copri* ($p$ < 0.01, $r$ = 0.84), *Blautia wexlerae* ($p$ = 0.01, r = 0.78) and *Ruminococcus gnavus* ($p$ < 0.01, $r$ = 0.93; Figure 10A). BFT was negatively correlated with *Blautia wexlerae* ($p$ < 0.01, $r$ = −0.83; Figure 10A). The Spearman correlation analysis of MQT-LM and differential microbiota-related metabolites related to lipid metabolism showed that IMF was positively correlated with levels of succinate ($p$ = 0.01, $r$ = 0.81), oxoglutaric acid ($p$ < 0.01, $r$ = 0.83) and L-aspartic acid ($p$ = 0.03, $r$ = 0.71), and negatively correlated with levels of GABA ($p$ = 0.03,

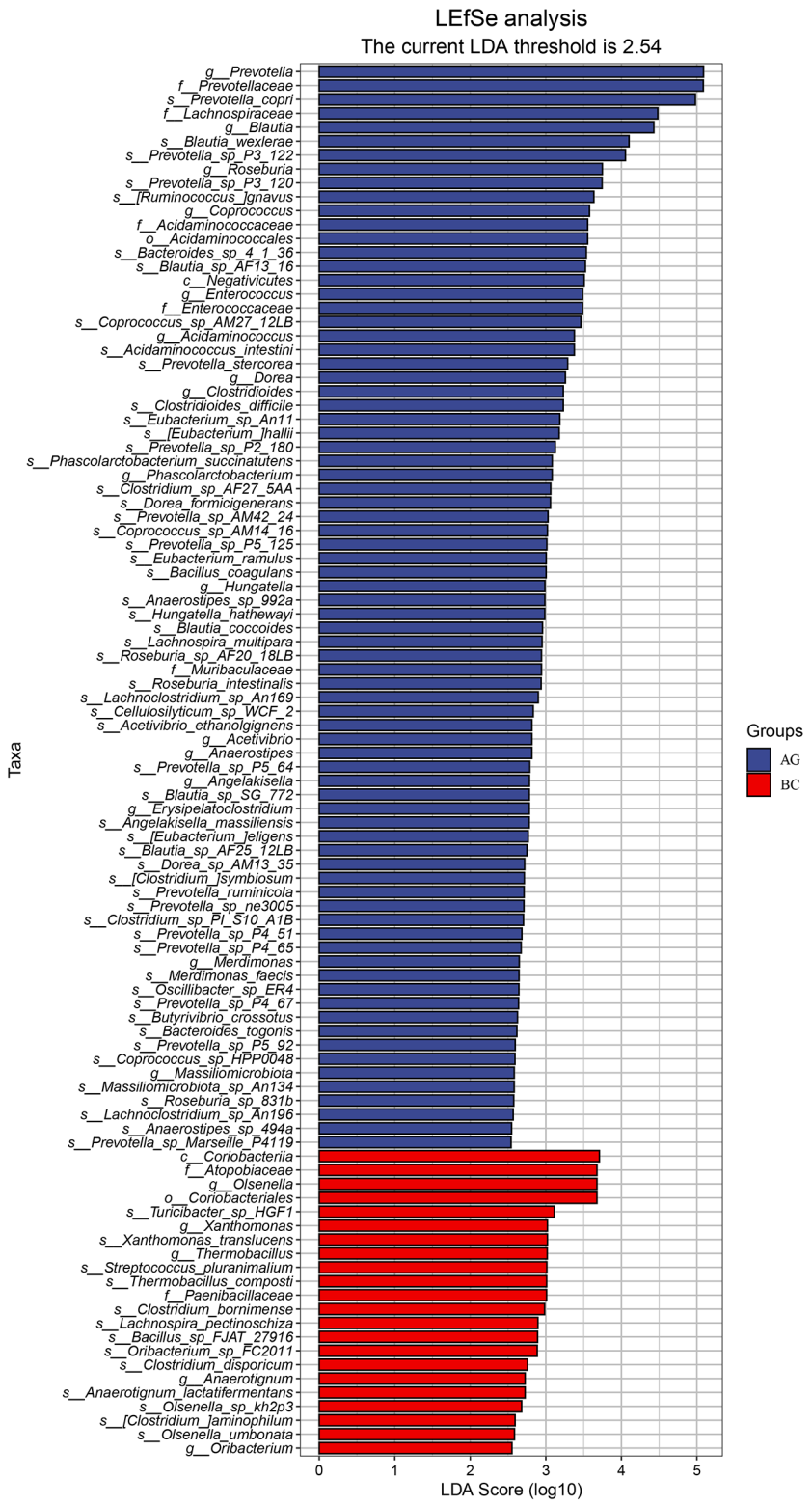


FIGURE 4
LEfSe analysis in the gut microbiota between Xinjiang brown cattle (BC) and Angus cattle (AG). (*n*=5 in each breed). LEfSe, Linear discriminant analysis Effect Size.

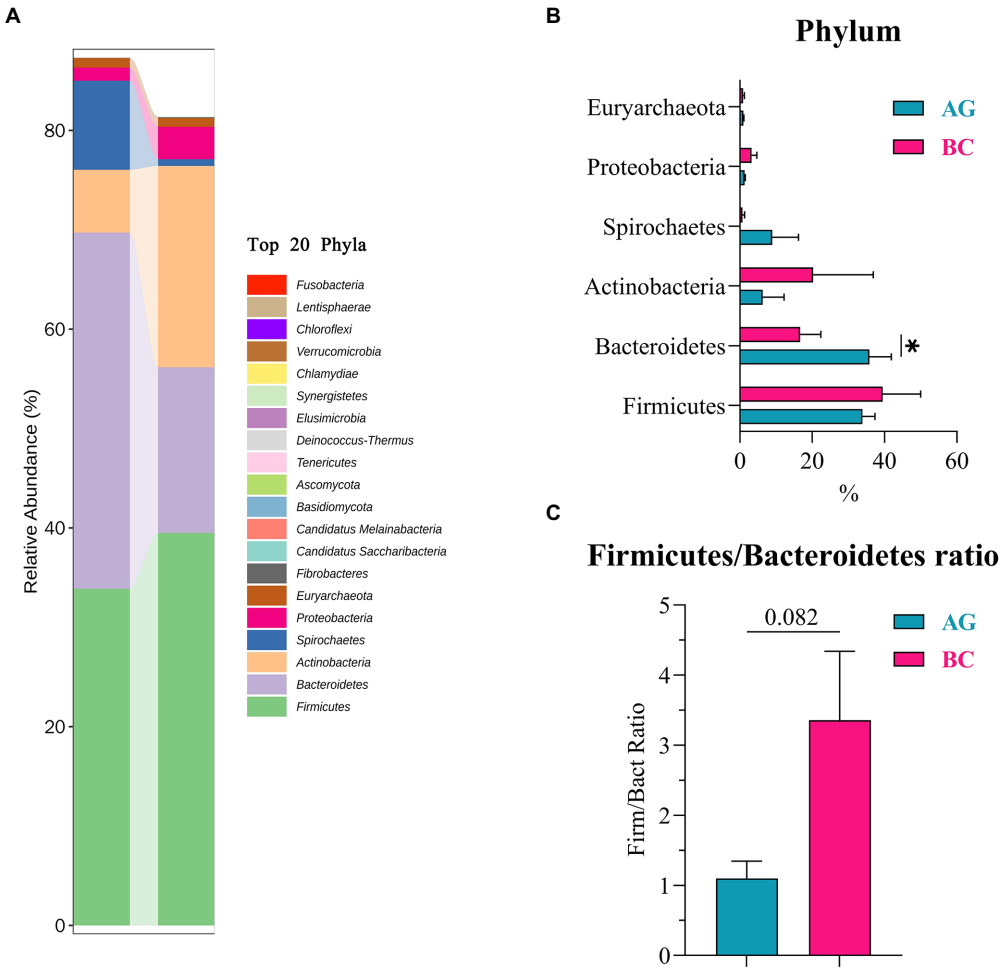


FIGURE 5
The phyla composition and the differentials in the gut microbiota between Xinjiang brown cattle (BC) and Angus cattle (AG; top 20 Phyla). ($n$=5 in each breed). The relative abundance of phyla composition **(A)**; The differential phyla in the different relative abundance **(B)**; Firmicutes/Bacteroidetes Ratio **(C)**. Values were expressed as means±SEM. * denotes $p$<0.05 indicating significant difference.

$r=-0.70$), L-asparagine ($p<0.01$, $r=-0.85$) and fumaric acid ($p<0.01$, $r=-0.87$; Figure 10B). BFT was positively correlated with GABA ($p=0.01$, $r=0.79$), L-asparagine ($p=0.01$, $r=0.80$) and fumaric acid ($p=0.04$, $r=0.66$), and negatively correlated with succinate ($p=0.01$, $r=-0.79$), L-aspartic acid ($p=0.01$, $r=-0.78$) and L-glutamic acid ($p=0.01$, $r=-0.78$; Figure 10B).

Spearman correlation analysis was also performed on the differential metabolites related to fat metabolism with the differential gut microbiota in the BC and AG. Succinate were positively correlated with *Prevotella copri* ($p=0.03$, $r=0.70$), *Blautia wexlerae* ($p=0.02$, $r=0.72$) and *Ruminococcus gnavus* ($p=0.04$, $r=0.67$; Figure 10C). GABA were negatively correlated with *Prevotella copri* ($p<0.01$, $r=-0.84$) and *Blautia wexlerae* ($p<0.01$, $r=-0.83$; Figure 10C). Oxoglutaric acid were positively correlated with *Prevotella copri* ($p<0.01$, $r=0.86$), *Blautia wexlerae* ($p=0.02$, $r=0.73$) and *Ruminococcus gnavus* ($p=0.02$, $r=0.75$; Figure 10C). L-aspartic acid levels were positively correlated with *Prevotella copri* ($p=0.03$, $r=0.70$) and *Blautia wexlerae* ($p=0.03$, $r=0.71$; Figure 10C). L-asparagine levels were negatively correlated with *Prevotella copri* ($p<0.01$, $r=-0.84$), *Blautia wexlerae* ($p=0.02$, $r=-0.73$) and *Ruminococcus gnavus* ($p=0.03$, $r=-0.70$; Figure 10C). L-glutamic acid levels were positively correlated with *Blautia wexlerae* ($p=0.01$, $r=0.82$) levels (Figure 10C). Fumaric acid were negatively correlated with *Prevotella copri* ($p=0.03$, $r=-0.71$), *Blautia wexlerae* ($p<0.01$, $r=-0.83$) and *Ruminococcus gnavus* ($p=0.01$, $r=-0.81$; Figure 10C).

## Discussion

Our present study showed that the body weight, the carcass weight and the EMA, IMF and the IAA of Angus cattle were significantly higher than those of Xinjiang brown cattle, whereas the BFT and the MFSA of Xinjiang brown cattle were higher. The carcass weight, EMA, and the MFSA were indicators of carcass muscle strength and meat yield (Choi and Kim, 2009; Lang et al.,

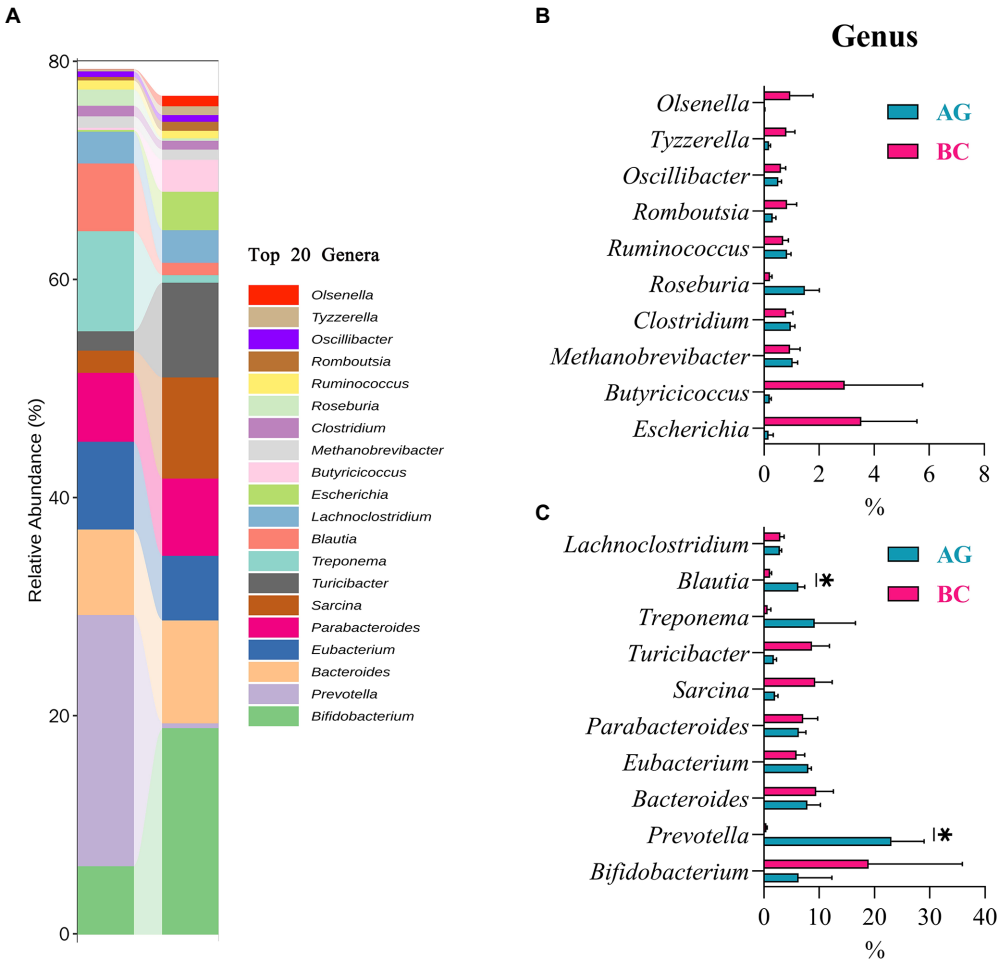


FIGURE 6
The genera composition and the differentials in the gut microbiota between Xinjiang brown cattle (BC) and Angus cattle (AG; top 20 genera). ($n$=5 in each breed). The relative abundance of genera composition **(A)**; The differential genera in the different relative abundance **(B,C)**. Values were expressed as means±SEM. * denotes $p$<0.05 indicating significant difference.

2016). The subcutaneous fat deposition is a necessary stage in the beef cattle fattening process. The intramuscular adipocytes are mainly derived from fibroblasts that reside in the connective tissue surrounding and within skeletal muscle (Lowe et al., 2012). The proportion of IMF content to total fat content is affected by variety, age, nutrition, and other factors (Bong et al., 2012; Gotoh and Joo, 2016). Tenderness, juiciness, flavor, and marbling all increase as IMF content increases (Farrow et al., 2009). Beef tenderness is improved by intramuscular adipose tissue infiltration, which destroys the collagen cross-linking that determines meat toughness (McEvers et al., 2012). Because adipose tissue is denser than muscle tissue, increased amounts of IMF also make lean meat taste more delicate and may help muscles retain more moisture (Campos et al., 2016). The results herein showed that the meat quality of Angus cattle is preferable than that of Xinjiang brown cattle.

Studies have shown that the gut microbiome is closely related to host macronutrient and lipid metabolism, thus affecting the meat quality traits of IMF content, BFT and tenderness. The gut microbiome may play a part in determining meat quality in cattle (Nicholson et al., 2012; Zhang et al., 2017). By analysis of the host-microbiota-metabolic axis revealed that gut microbiota is closely related to fat, sugar, and protein metabolism. Animal lipid metabolism-related meat quality traits are closely related to the composition of gut microbiota involved in lipid metabolism (Nicholson et al., 2012; Sun et al., 2016; Zhang et al., 2017). Zierer et al. (2018) analyzed the relationship between gut microbiota, host phenotype, and complex genetic traits in human twins and found that the microbiota structure is closely related to fat deposition. Gut microbiota imbalances or alterations in microbiota structure have also been shown to cause alterations in whole blood glucose and triglyceride levels, suggesting gut microbiota may regulate carbohydrate and lipid metabolism in the host body (Kuno et al., 2018). In our present study, the results of the α-diversity analysis showed that the richness and diversity of gut microbiota in Angus cattle were greater than those in Xinjiang brown cattle. Furthermore, results of LEfSe analysis showed Angus cattle possess a greater variety of microbiota species in their gut microbiota

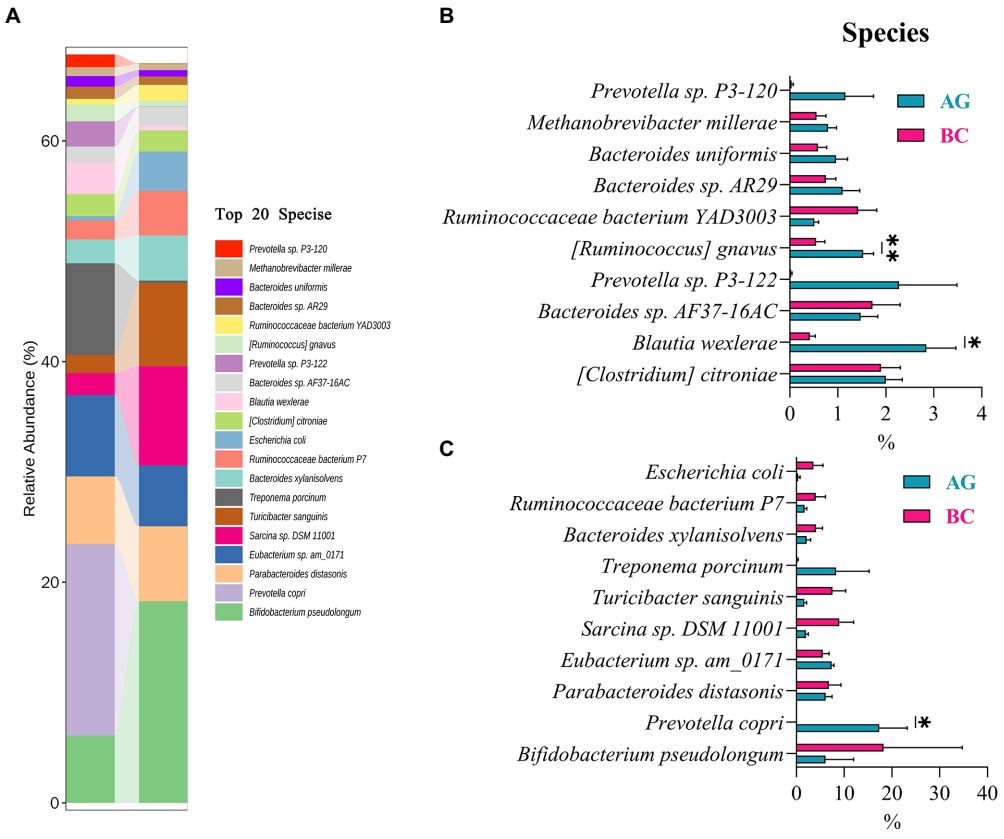


FIGURE 7
The species composition and the differentials in the gut microbiota between Xinjiang brown cattle (BC) and Angus cattle (AG; top 20 species). ($n$=5 in each breed). The relative abundance of species composition **(A)**; The differential species in the different relative abundance **(B,C)**. Values were expressed as means±SEM. * denotes $p$<0.05 indicating significant difference, ** denotes $p$<0.01 indicating extremely significant difference.

compared to Xinjiang brown cattle. Significant differences in microbiota composition were also observed following β diversity analysis. Previous studies have shown that obesity is related to the relative abundance of Bacteroidetes and Firmicutes or changes in the ratio of Firmicutes / Bacteroides win the gut microbiota (Turnbaugh et al., 2006; Parnell and Reimer, 2012). The ratio of Firmicutes / Bacteroides is related to the production of short-chain fatty acids. These acids serve as macromolecular substances that maintain host balance and disease and have been used to prevent or treat obesity and type 2 diabetes (Andrade-Oliveira et al., 2015; Bindels et al., 2015; Zhang et al., 2015; Ohira et al., 2017; Thursby and Juge, 2017). For example, An et al. (2018) showed the gut microbiota in high-fat diet-induced obese rats was composed of higher levels of Firmicutes and lower levels of Bacteroidetes. Increase of Bacteroidetes is related to increased production of short-chain fatty acids, which provide cellular energy, maintain the intestinal epithelial barrier, and regulate the immune system (Greenhalgh et al., 2016). Ley et al. (2005) found that obesity was related to an increase in the number of Firmicutes, a decrease in the number of Bacteroides, or an increase in the ratio of Firmicutes / Bacteroides in the gut. Bacteroidetes abundance is altered by environmental factors, such as dietary protein and fat content, and is also positively correlated with the deposition of animal fat (David et al., 2014; Johnson et al., 2017; Rampelli et al., 2018). Genus *Prevotella* is among the most abundant bacteria found in the rumen, they break down cellulose and use products of cellulose degradation as an energy source in sheep and cattle (Ellison et al., 2017; Delgado et al., 2019), *Prevotella copri* is ubiquitous in the intestine and considered a potential marker for distinguishing high feed efficiency in beef cattle during life span and production cycles (Brooke et al., 2019). A greater abundance of *Prevotella copri* in the gut microbiota is associated with abnormal carbohydrate metabolism during obesity (Duan et al., 2021), along with fat accumulation (Chen C. et al., 2021). Zheng et al. (2022) compared the gut microbiota and intramuscular differentially expressed genes in the Angus and Chinese Simmental cattle. The results show that the relative abundance of *Prevotella copri* was significantly higher in the Simmental cattle. *Blautia wexlerae* is yet another obesity-related bacterium. In contrast to *Prevotella copri*, *Blautia wexlerae* abundance is decreased during obesity (Benítez-Páez et al., 2020). An important function of *Blautia wexlerae* is to produce acetic acid and butyric acid (Jang et al., 2019). A decreased abundance of *Blautia wexlerae* leads to decrease in acetic acid and butyric acid levels (Vital et al., 2018). Our findings to some extents

TABLE 3 Genes of the glycerophospholipid metabolism pathway.

| Protein ID | KO | Gene name | Pathway ID | Taxonomy |
|---|---|---|---|---|
| gene_9708818 | K19664 | carS | ko00564 | *Methanobrevibacter millerae* |
| gene_9222867 | K17830 | GGR | ko00564 | *Methanosphaera stadtmanae* |
| gene_9997979 | K00111 | glpA, glpD | ko00564 | *Fermentimonas caenicola* |
| gene_8259284 | K18695 | GPCPD1 | ko00564 | *Prevotella intermedia* |
| gene_9671445 | K00631 | plsB | ko00564 | *Paenibacillus terrae* |
| gene_9906485 | K00980 | tagD | ko00564 | *Clostridium bornimense* |
| gene_9981399 | K06131 | ClsA/B | ko00564 | *Clostridium bornimense* |
| gene_7283784 | K06130 | LYPLA2 | ko00564 | *Butyrivibrio fibrisolvens* |
| gene_9986649 | K04019 | eutA | ko00564 | *Flavonifractor plautii* |
| gene_9986650 | K03735 | eutB | ko00564 | *Flavonifractor plautii* |
| gene_9986651 | K03736 | eutC | ko00564 | *Flavonifractor plautii* |
| gene_9947823 | K00113 | glpC | ko00564 | *Escherichia coli* |
| gene_12284373 | K01521 | cdh | ko00564 | *Escherichia coli* |
| gene_8324574 | K01096 | pgpB | ko00564 | *Escherichia coli* |
| gene_9999662 | K00995 | pgsA, PGS1 | ko00564 | *Spirochaeta africana* |

were supported by some recent publications. For example, Cao et al. (2021) fund that *Butyrivibrio*, *CF231* and *Dorea* were negative correlated with water loss of sheep meat quality traits significantly. Lei et al. (2021) showed that the higher relative abundances of the genera *Ruminococcaceae_NK4A214_group*, *Parabacteroides*, *Christensenellaaceae_R-7_group*, and *Ruminiclostridium* might corelate with higher intramuscular fat (IMF) content. Zheng et al. (2022) found that the relative abundance of *Roseburia*, *Prevotella*, and *Coprococcus* were positive correlated with IMF of beef quality traits significantly. Animal experiments with Khan and Jena (2016) showed that butyric acid may improve insulin sensitivity and reduce insulin resistance. Recent studies have found that the abundance of *Ruminococcus gnavus* in the intestine of ruminants was increased in obese animals, decreased with malnourishment, and positively correlates with body mass index (Blanton et al., 2016; Jang et al., 2019). In the present studies, the abundance of Bacteroidetes in the gut microbiota of Xinjiang brown cattle, i.e., *Prevotella*, *Blautia* at the genus level, *Prevotella copri*, *Blautia wexlerae*, and *Ruminococcus gnavus* at the species level were significantly lower than that of Angus cattle. Microbiota structure was related to differences in lipid metabolism, fat deposition, and obesity. Correlation analysis between *Prevotella, Blautia*, *Prevotella copri*, *Blautia wexlerae*, *Ruminococcus gnavus*, and meat quality indices related to lipid metabolism showed positive correlations with IMF amount and negative correlations with the BFT. These correlations suggest that *Prevotella*, *Blautia*, *Prevotella copri*, *Blautia wexlerae*, and *Ruminococcus gnavus* play an important role in regulating meat quality in Xinjiang brown cattle. Hosomi et al. (2022) performed a cross-sectional study of Japanese adults and identified that the *Blautia* genus, especially *Blautia wexlerae*, as a commensal bacterium inversely correlated with obesity and type 2 diabetes mellitus, furthermore, they administrated *Blautia wexlerae* orally to the high fat diet (HFD) fed mice, the body weight and the weight of epididymal adipose tissue were significantly decreased than those HFD mice without *Blautia wexlerae* administration demonstrating *Blautia wexlerae* has potential to contribute to the prevention of obesity. This research was partly supported our result that *Blautia wexlerae* was negative correlated with IMF. The correlation of MQT-LM and certain bacterial species showed that these species were mostly related to IMF, the BFT, the IAA, body weight, and carcass weight. Among them, *Prevotella* sp. *P3-122*, *Prevotella sp. P3-120*, *Prevotella sp. P2-180*, and *Prevotella sp. AM42-24* in the genus *Prevotella* play an important role in polysaccharide degradation and fermentation in the rumen (Shinkai et al., 2022). Zeybel et al. (2022) found that the abundances of *Prevotella* sp. *AM42 24* were significantly reduced in subjects with mild hepatic steatosis versus no hepatic steatosis. Eubacterium hallii affects the metabolic balance of the host by forming different short-chain fatty acids in the intestinal tract (Engels et al., 2016), and it was increased in small intestinal biopsies of obese and insulin-resistant subjects upon lean donor fecal transplantation associated with improved insulin sensitivity (Wan et al., 2019) suggesting it may be associated with MQT-LM. However, there is a lack of study on *Blautia sp. AF13-16* in association with lipid metabolism. These data were further supported by the KEGG and KO analyses which showed that lipid metabolism was different in *Prevotella intermedia* between Xinjiang brown and Angus cattle.

Gut microbiota interacts with its host, receiving energy from and providing energy to the host. This constant exchange of energy between microbiota and host is accomplished through the release of enzymes and metabolites such as short-chain fatty acids, amino acids, bile acids, casein B, and lipopolysaccharide (Fetissov, 2017). Importantly, *Escherichia coli* secretes casein B and casein B may enhance the peptide YY (PYY) and glucagon-like peptide (GLP) secretion (Breton et al., 2016). PPY and GLP decrease appetite, leading to decreased caloric intake and fat deposition (Murphy and Bloom, 2006; Berthoud, 2011; Zhang and van den Pol, 2017; Leidmaa et al., 2020). Fatty acids in food are absorbed by intestinal epithelial cells, where they are oxidized and degraded or re-esterified into triglycerides. Newly synthesized triglycerides are incorporated into cytoplasmic lipid droplets or chylous particles that are secreted in the lymphatic system (Iqbal and Hussain, 2009; Nakajima et al., 2014). The digestion of polysaccharides provides monosaccharides to intestinal epithelial cells and reduces the absorption of fatty acids, and promotes storage of newly synthesized triglycerides in cytoplasmic lipid droplets. *Escherichia coli* acts in conjunction with epithelial cells to form mono- and disaccharides that increase fatty acid absorption and oxidation. Increased fatty acid oxidation results in decreased triglyceride synthesis, chylous particle size, and chylous granule secretion, leading to the depletion of cytoplasmic lipid

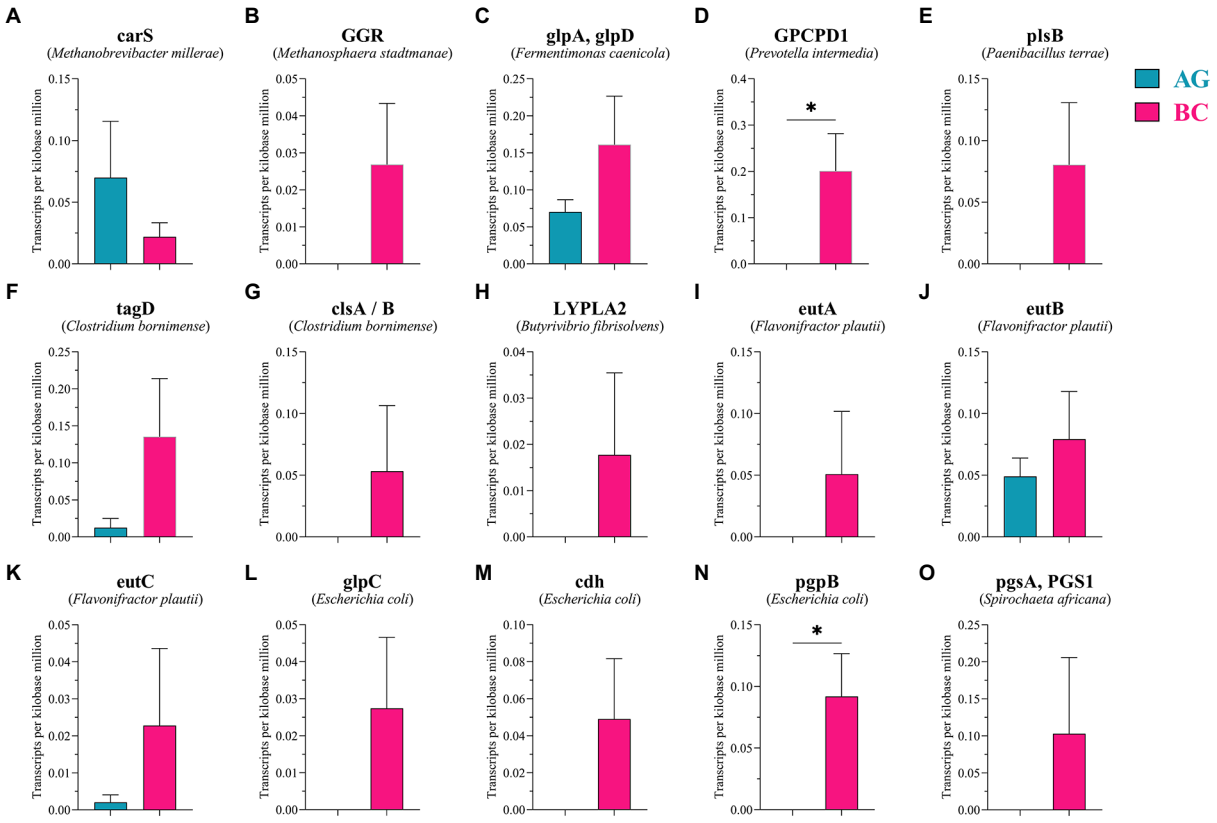


FIGURE 8
The relative abundance of genes related to lipid metabolism in lipid metabolism pathways between Xinjiang brown cattle (BC) and Angus cattle (AG). ($n$=5 in each breed). The expression of genes related to lipid metabolism **(A–O)**. Values were expressed as means±SEM. * denotes $p$<0.05 indicating significant difference.

TABLE 4 Pathways enriched in microbiota-related metabolites.

| Pathway | Compounds |
|---|---|
| Alanine, aspartate and glutamate metabolism (Total: 23; Hits: 7; Raw p: 0.01; –log(p): 4.52; Holm adjust: 0.88; FDR: 0.88; Impact: 0.63) | Fumarate acid |
| | Succinate acid |
| | Oxoglutaric acid |
| | L-asparagine |
| | L-Aspartic acid |
| | L-glutamate acid |
| | GABA |
| D-Glutamine and D-glutamate metabolism (Total: 5; Hits: 2; Raw p: 0.15; –log(p): 1.89; Holm adjust: 1.00; FDR: 1.00; Impact: 1.00) | L-glutamate acid |
| | Oxoglutaric acid |

droplets (Poquet and Wooster, 2016; Tazi et al., 2018). Although it is clear that *Escherichia coli* plays a role in regulating lipid metabolism, but there is no research on the relationship between *Prevotella intermedia* and lipid metabolism. Functional level analysis of bovine gut microbiota in our present study showed that there were differences in the expression of pgpB and GPCPD1 genes within the "Glycerophospholipid metabolism" pathway in *Escherichia coli* and *Prevotella intermedia* suggesting that *Escherichia coli* and *Prevotella* are key bacteria that affect the MQT-LM in Xinjiang brown cattle.

Gut microbiota plays a role in maintaining host homeostasis and health *via* the production of short-chain fatty acids and neuroactive substances (Wang et al., 2020; Han et al., 2021). The neuroactive substance GABA is a major inhibitory neurotransmitter in the central nervous system (Cuevas-Sierra et al., 2021). GABA is also produced by intestinal microbiota to exhibit the largest change of metabolites in the intestine of obese patients in response to fecal transplant using samples from lean patients. This change of GABA was also related to the improvement in insulin sensitivity (Kootte et al., 2017). GABA may also play a role in the treatment of lipid metabolism disorders caused by type 1 and 2 diabetes by inducing pancreatic β-cells and stimulating insulin secretion (Soltani et al., 2011; Tian et al., 2011, 2013). Treatment of obese mice with metabolic dysfunction mice with GABA-producing bacteria attenuated the metabolic dysfunction and reduced mesenteric adipose tissue accumulation (Patterson et al., 2019). GABA can be metabolized to succinate, a metabolic by-product of anaerobic fermentation performed in Bacteroides (Zhang et al., 2008; Feehily and Karatzas, 2013; De Vadder et al., 2016; Louis and Flint, 2017). Interestingly, succinate inhibits fatty acid release from adipocytes. Patients with higher circulating succinate levels have higher blood

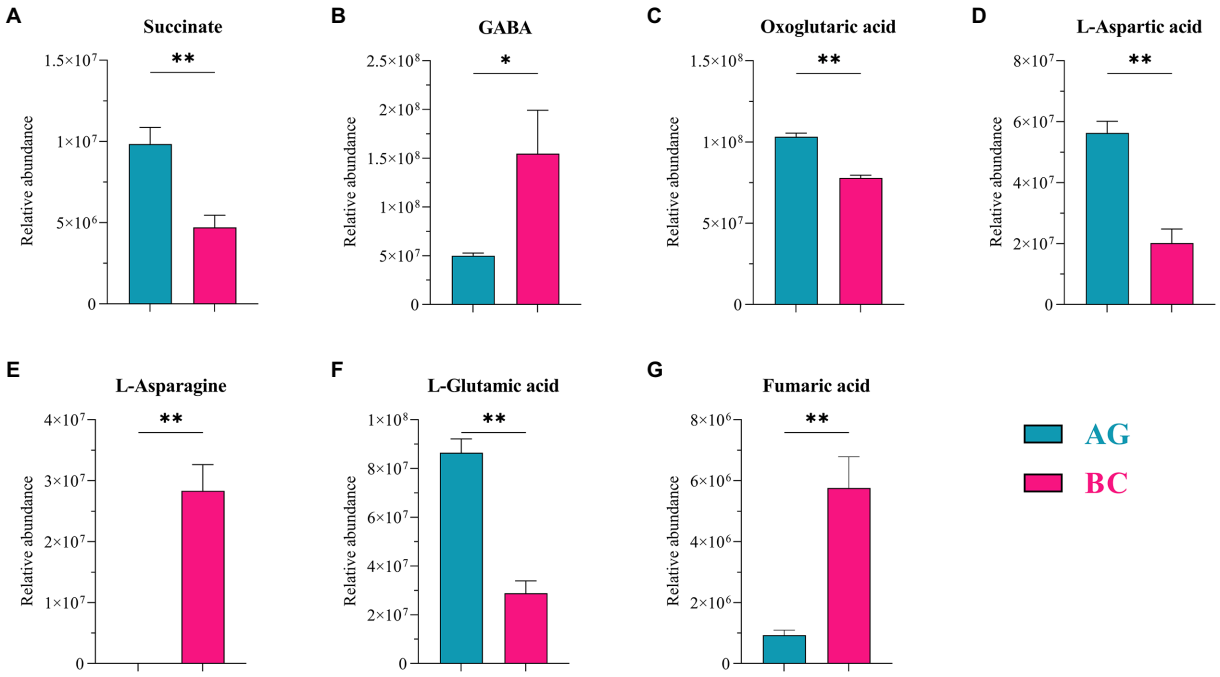


FIGURE 9
Metabolites in gut microbiota related to lipid metabolism between Xinjiang brown cattle (BC) and Angus cattle (AG). ($n$=5 in each breed). The bacterial metabolites related to lipid metabolism **(A–G)** . Values were expressed as means±SEM. * denotes $p$<0.05 indicating significant difference, ** denotes $p$<0.01 indicating extremely significant difference.

glucose levels that may be related to changes in gut microbiota and increased barrier permeability. Thus, microbial-derived succinate may play an important role in obesity and metabolism-related cardiovascular disease (Serena et al., 2018). The oxoglutaric acid, succinate and fumaric acid are metabolites of the citric acid cycle pathway that link metabolic pathways and lead to the formation of citric acid (Akram, 2014). L-glutamic acid is an amino acid that can be utilized for carbohydrate production and is the precursor of glutathione. Meanwhile, L-glutamic acid is the first line of defense against free radicals in the liver and plays an essential role in the pathogenesis of metabolic diseases (Melis et al., 2004; Zhou et al., 2019). Glutamine was previously thought to be associated with obesity (Curtasu et al., 2019) and diabetes (Newgard et al., 2009), as higher levels of glutamine and related metabolites are part of the systematic response to higher blood glucose levels and act to stimulate insulin secretion to lower blood glucose levels (Newsholme et al., 2005). In addition, other studies have shown that plasma L-asparagine levels are negatively correlated with blood lipid levels and positively correlated with type 2 diabetes risk (Ottosson et al., 2018), and exogenous L-aspartic acid feeding limits fatty liver progression (Yanni et al., 2010). The results of the metabolomic analysis in the present study showed that oxoglutaric acid, succinate, fumaric acid, L-aspartic acid, L-asparagine, L-glutamic acid, and GABA levels differed between the two breeds of cattle, and differential metabolites were associated with MQT-LM. Specifically, IMF was positively correlated with succinate, oxoglutaric acid, and L-aspartic acid, and negatively correlated with GABA, L-asparagine, and fumaric acid. The BFT was positively correlated with GABA, L-asparagine, and fumaric acid, and negatively correlated with succinate, L-aspartic acid, and L-glutamic acid, suggesting that these metabolites may reflect alterations in lipid metabolism that determine meat quality. Associations between metabolomes, microbiota, and lipid metabolism functional expressions of the microbiota that differed between Xinjiang brown and Angus cattle showed that succinate, oxoglutaric acid, L-aspartate, and L-glutamic acid were positively correlated with the most abundant microbiota and that differed between breeds. Levels of GABA, L-asparagine, and fumaric acid were negatively correlated with the most abundant microbiota that was different between breeds, suggesting that MQT-LM may be regulated by changing the expression of lipid metabolism-related metabolites in gut microbiota. The correlation of microbiota and species (RA abv.0.1%) showed that except for the species correlation beef quality traits, there are four species of *Prevotella stercorea*, *Ruminococcaceae bacterium YAD3003*, *Clostridium* sp. *af27-5AA*, and *Acetivibrio ethanolgignens* related to lipid metabolites. Pisanu et al. (2020) evaluate the impact of nutritional intervention on the gut microbiota of obese and overweight patients, then patients presented a statistically significant reduction in body weight and fat mass and an increase in the abundance of *Prevotella stercorea*. Yuan et al. (2020) found that alteration of the gut microbiota *Acetivibrio ethanolgignens* under tributyltin exposure was involved in further mediating liver inflammation, causing lipid metabolism abnormalities with the energy supply process. Therefore, *Acetivibrio ethanolgignens* may be a candidate for a potential microbial community marker for assistant diagnosis. But more supporting study regarding the

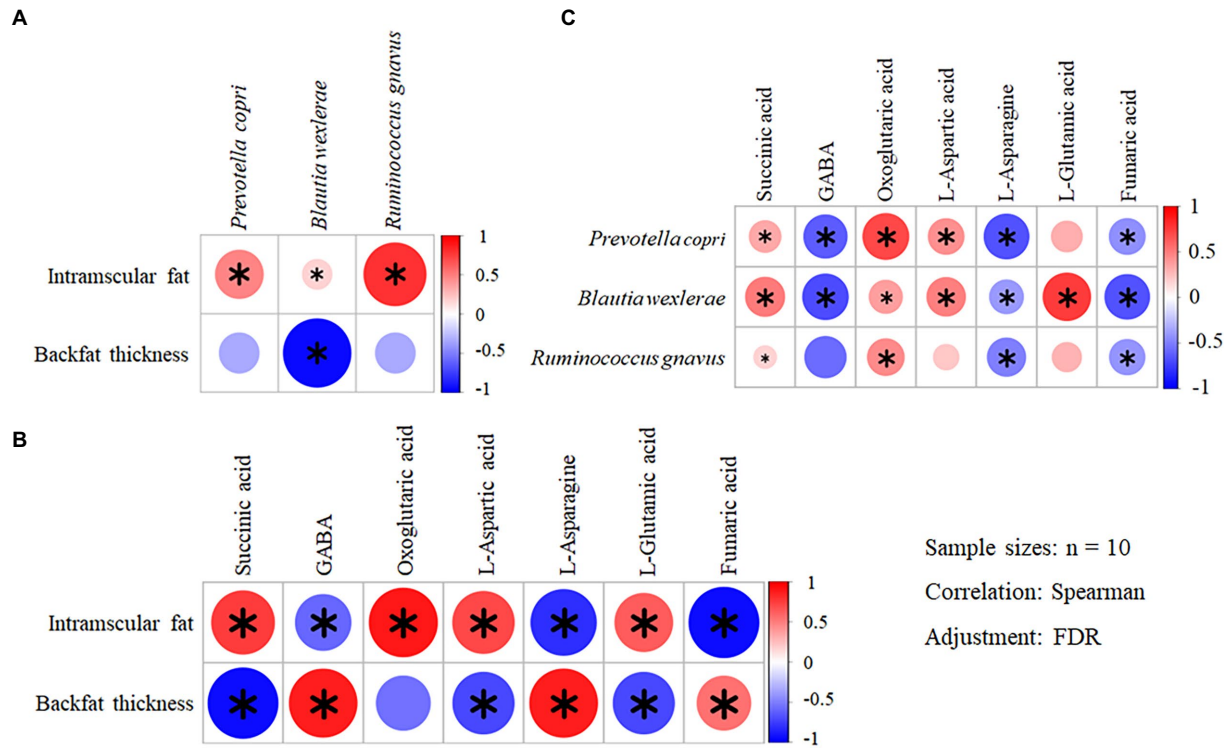


FIGURE 10
Correlation analyses of the differential MQT-LM – species – metabolites in Xinjiang brown cattle (BC) and Angus cattle (AG). ($n$=10). Correlation of the differential MQT-LM and species **(A)**; Correlation of the differential MQT-LM and metabolites **(B)**; Correlation of the differential species and metabolites **(C)**. * denotes $p$<0.05 indicating significant difference.

potentially useful markers of specific gut microbiota from meat animal origin are expected.

## Conclusion

There are significant differences in meat quality traits in association with gut microbiota and its lipid metabolism related metabolites between Xinjiang brown cattle and Angus cattle. The intramuscular fat content was positively correlated with Bacteria species of *Prevotella copri*, *Blautia wexlerae*, and *Ruminococcus gnavus*, and the metabolites succinate, oxoglutaric acid, L-aspartic acid and L-glutamic acid, while negatively with GABA, L-asparagine and fumaric acid. The backfat thickness was negatively correlated with *Blautia wexlerae* and the metabolites succinate, L-aspartic acid and L-glutamic acid, while positively with GABA, L-asparagine and fumaric acid. Furthermore *Prevotella Copri*, *Blautia wexlerae*, and *Ruminococcus gnavus* was all positively correlated with succinate, oxoglutaric acid, while negatively with L-asparagine and fumaric acid. Lipid metabolism genes within the glycerophospholipid metabolism pathway is enriched in *Prevotella intermedia* and *Escherichia coli*. Our data suggest that *Prevotella copri*, *Prevotella intermedia*, *Blautia wexlerae*, and *Ruminococcus gnavus* may serve as the potential differentiated bacterial species in association with meat quality traits related to the lipid metabolism *via* their metabolites of oxoglutaric acid, succinate, fumaric acid, L-aspartic acid, L-asparagine, L-glutamic acid and GABA may serve as potential biomarkers to assistant for improving the meat quality in Xinjiang brown cattle.

## 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/, PRJNA813004.

## Ethics statement

The animal study was reviewed and approved by the Animal Ethics Committee of Xinjiang Agricultural University (2017015).

## Author contributions

ZC and GY designed the study and wrote the manuscript. ZC, HL, and XY are responsible for field survey and experimental determination. ZC, JW, YZ, and LX are responsible for analyzed the data. ZC drafted the manuscript. GY revised the manuscript

and approved final version of 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/fmicb.2022.988984/full#supplementary-material

SUPPLEMENTARY FIGURE S1
KEGG pathways enriched in Xinjiang Brown and Angus cattle microbiomes.

SUPPLEMENTARY FIGURE S2
Classification of differential metabolites.

SUPPLEMENTARY FIGURE S3
Metabolic pathway impact analysis. Alanine, aspartate and glutamate metabolism (-log(p) 4.52; Impact 0.63) (A); D-Glutamine and D-glutamate metabolism (-log(p) 1.89; Impact 1.00).

SUPPLEMENTARY FIGURE S4
Correlations of the species (RA abv 0.1%) - MQT-LMs - metabolites.

## References

Akram, M. (2014). Citric acid cycle and role of its intermediates in metabolism. *Cell Biochem. Biophys.* 68, 475–478. doi: 10.1007/s12013-013-9750-1

An, Y., Li, Y., Wang, X., Chen, Z., Xu, H., Wu, L., et al. (2018). Cordycepin reduces weight through regulating gut microbiota in high-fat diet-induced obese rats. *Lipids Health Dis.* 17:276. doi: 10.1186/s12944-018-0910-6

Andrade-Oliveira, V., Amano, M. T., Correa-Costa, M., Castoldi, A., Felizardo, R. J., de Almeida, D. C., et al. (2015). Gut bacteria products prevent AKI induced by ischemia-reperfusion. *J. Am. Soc. Nephrol.* 26, 1877–1888. doi: 10.1681/ASN.2014030288

Bäckhed, F., Ding, H., Wang, T., Hooper, L. V., Koh, G. Y., Nagy, A., et al. (2004). The gut microbiota as an environmental factor that regulates fat storage. *Proc. Nat. Acad. Sci. U. S. A.* 101, 15718–15723. doi: 10.1073/pnas.0407076101

Bai, H., Yang, B., Dong, Z., Li, X., Song, Q., Jiang, Y., et al. (2022). Research note: effects of cage and floor rearing systems on growth performance, carcass traits, and meat quality in small-sized meat ducks. *Poult. Sci.* 101:101520. doi: 10.1016/j.psj.2021.101520

Benítez-Páez, A., Gómez Del Pugar, E. M., López-Almela, I., Moya-Pérez, Á., Codoñer-Franch, P., and Sanz, Y. (2020). Depletion of Blautia species in the microbiota of obese children relates to intestinal inflammation and metabolic phenotype worsening. *mSystems* 5:e00857-19. doi: 10.1128/mSystems.00857-19

Berthoud, H. R. (2011). Metabolic and hedonic drives in the neural control of appetite: who is the boss? *Curr. Opin. Neurobiol.* 21, 888–896. doi: 10.1016/j.conb.2011.09.004

Bindels, L. B., Delzenne, N. M., Cani, P. D., and Walter, J. (2015). Towards a more comprehensive concept for prebiotics. *Nat. Rev. Gastroenterol. Hepatol.* 12, 303–310. doi: 10.1038/nrgastro.2015.47

Blanton, L. V., Charbonneau, M. R., Salih, T., Barratt, M. J., Venkatesh, S., Ilkaveya, O., et al. (2016). Gut bacteria that prevent growth impairments transmitted by microbiota from malnourished children. *Science* 351:aad3311. doi: 10.1126/science.aad3311

Bong, J. J., Jeong, J. Y., Rajasekar, P., Cho, Y. M., Kwon, E. G., Kim, H. C., et al. (2012). Differential expression of genes associated with lipid metabolism in longissimus dorsi of Korean bulls and steers. *Meat Sci.* 91, 284–293. doi: 10.1016/j.meatsci.2012.02.004

Breton, J., Tennoune, N., Lucas, N., Francois, M., Legrand, R., Jacquemot, J., et al. (2016). Gut commensal *E. coli*. proteins activate host satiety pathways following nutrient-induced bacterial growth. *Cell Metab.* 23, 324–334. doi: 10.1016/j.cmet.2015.10.017

Brooke, C. G., Najafi, N., Dykier, K. C., and Hess, M. (2019). *Prevotella copri*, a potential indicator for high feed efficiency in western steers. *Anim. Sci. J.* 90, 696–701. doi: 10.1111/asj.13197

Campos, C. F., Duarte, M. S., Guimarães, S. E., Verardo, L. L., Wei, S., Du, M., et al. (2016). Review: animal model and the current understanding of molecule dynamics of adipogenesis. *Anim. Int. J. Anim. Biosci.* 10, 927–932. doi: 10.1017/S1751731115002992

Cantalapiedra, C. P., Hernández-Plaza, A., Letunic, I., Bork, P., and Huerta-Cepas, J. (2021). EggNOG-mapper v2: functional annotation, orthology assignments, and domain prediction at the metagenomic scale. *Mol. Biol. Evol* 38, 5825–5829. doi: 10.1093/molbev/msab293

Cao, Q. Q., Lin, L. X., Xu, T. T., Lu, Y., Zhang, C. D., Yue, K., et al. (2021). Aflatoxin B1 alters meat quality associated with oxidative stress, inflammation, and gut-microbiota in sheep. *Ecotoxicol. Environ. Saf.* 225:112754. doi: 10.1016/j.ecoenv.2021.112754

Chen, G., Cai, Y., Su, Y., Wang, D., Pan, X., and Zhi, X. (2021). Study of meat quality and flavour in different cuts of Duroc-Bamei binary hybrid pigs. *Vet. Med. Sci.* 7, 724–734. doi: 10.1002/vms3.409

Chen, C., Fang, S., Wei, H., He, M., Fu, H., Xiong, X., et al. (2021). *Prevotella copri* increases fat accumulation in pigs fed with formula diets. *Microbiome* 9:175. doi: 10.1186/s40168-021-01110-0

Choi, Y. M., and Kim, B. C. (2009). Muscle fiber characteristics, myofibrillar protein isoforms, and meat quality. *Livest. Sci.* 122, 105–118. doi: 10.1016/j.livsci.2008.08.015

Cuevas-Sierra, A., Romo-Hualde, A., Aranaz, P., Goni, L., Cuervo, M., Martínez, J. A., et al. (2021). Diet- and sex-related changes of gut microbiota composition and functional profiles after 4 months of weight loss intervention. *Eur. J. Nutr.* 60, 3279–3301. doi: 10.1007/s00394-021-02508-0

Curtasu, M. V., Knudsen, K., Callesen, H., Purup, S., Stagsted, J., and Hedemann, M. S. (2019). Obesity development in a miniature Yucatan pig model: a multi-compartmental metabolomics study on cloned and normal pigs fed restricted or ad libitum high-energy diets. *J. Proteome Res.* 18, 30–47. doi: 10.1021/acs.jproteome.8b00264

David, L. A., Maurice, C. F., Carmody, R. N., Gootenberg, D. B., Button, J. E., Wolfe, B. E., et al. (2014). Diet rapidly and reproducibly alters the human gut microbiome. *Nature* 505, 559–563. doi: 10.1038/nature12820

De Vadder, F., Kovatcheva-Datchary, P., Zitoun, C., Duchampt, A., Bäckhed, F., and Mithieux, G. (2016). Microbiota-produced succinate improves glucose homeostasis via intestinal gluconeogenesis. *Cell Metab.* 24, 151–157. doi: 10.1016/j.cmet.2016.06.013

Delgado, B., Bach, A., Guasch, I., González, C., Elcoso, G., Pryce, J. E., et al. (2019). Whole rumen metagenome sequencing allows classifying and predicting feed efficiency and intake levels in cattle. *Sci. Rep.* 9:11. doi: 10.1038/s41598-018-36673-w

Ding, S. R., Li, G. S., Chen, S. R., Zhu, F., Hao, J. P., Yang, F. X., et al. (2021). Comparison of carcass and meat quality traits between lean and fat Pekin ducks. *Anim. Biosci.* 34, 1193–1201. doi: 10.5713/ajas.19.0612

Duan, M., Wang, Y., Zhang, Q., Zou, R., Guo, M., and Zheng, H. (2021). Characteristics of gut microbiota in people with obesity. *PLoS One* 16:e0255446. doi: 10.1371/journal.pone.0255446

Ellison, M., Conant, G., Lamberson, W. R., Cockrum, R., Austin, K., Rule, D. C., et al. (2017). Diet and feed efficiency status affect rumen microbial profiles of sheep. *Small Rumin. Res.* 156, 12–19. doi: 10.1016/j.smallrumres.2017.08.009

Engels, C., Ruscheweyh, H. J., Beerenwinkel, N., Lacroix, C., and Schwab, C. (2016). The common gut microbe Eubacterium hallii also contributes to intestinal propionate formation. *Front. Microbiol.* 7:713. doi: 10.3389/fmicb.2016.00713

Fan, S., Zhang, K., Lv, A., Ma, Y., Fang, X., and Zhang, J. (2021). Characteristics of the intestinal microbiota and metabolism in infants with extrauterine growth restriction. *Transl. Pediatr.* 10, 1259–1270. doi: 10.21037/tp-20-431

Farrow, R. L., Loneragan, G. H., Pauli, J. W., and Lawrence, T. E. (2009). An exploratory observational study to develop an improved method for quantifying beef carcass salable meat yield. *Meat Sci.* 82, 143–150. doi: 10.1016/j.meatsci.2008.12.014

Feehily, C., and Karatzas, K. A. (2013). Role of glutamate metabolism in bacterial responses towards acid and other stresses. *J. Appl. Microbiol.* 114, 11–24. doi: 10.1111/j.1365-2672.2012.05434.x

Fetissov, S. O. (2017). Role of the gut microbiota in host appetite control: bacterial growth to animal feeding behaviour. *Nat. Rev. Endocrinol.* 13, 11–25. doi: 10.1038/nrendo.2016.150

Franzosa, E. A., Sirota-Madi, A., Avila-Pacheco, J., Fornelos, N., Haiser, H. J., Reinker, S., et al (2019). Gut microbiome structure and metabolic activity in inflammatory bowel disease. *Nature Microbiology* 4, 293–305. doi: 10.1038/s41564-018-0306-4

Gotoh, T., and Joo, S. T. (2016). Characteristics and health benefit of highly marbled wagyu and Hanwoo beef. *Korean J. Food Sci. Anim. Resour.* 36, 709–718. doi: 10.5851/kosfa.2016.36.6.709

Greenhalgh, K., Meyer, K. M., Aagaard, K. M., and Wilmes, P. (2016). The human gut microbiome in health: establishment and resilience of microbiota over a lifetime. *Environ. Microbiol.* 18, 2103–2116. doi: 10.1111/1462-2920.13318

Han, H., Yi, B., Zhong, R., Wang, M., Zhang, S., Ma, J., et al. (2021). From gut microbiota to host appetite: gut microbiota-derived metabolites as key regulators. *Microbiome* 9:162. doi: 10.1186/s40168-021-01093-y

Holman, B., Bailes, K. L., Meyer, R. G., and Hopkins, D. L. (2019). Effect of modified Soxhlet (Soxtec) and Folch extraction method selection on the total lipid determination of aged beef. *J. Food Sci. Technol.* 56, 3957–3961. doi: 10.1007/s13197-019-03878-4

Hooper, L. V., Wong, M. H., Thelin, A., Hansson, L., Falk, P. G., and Gordon, J. I. (2001). Molecular analysis of commensal host-microbial relationships in the intestine. *Science* 291, 881–884. doi: 10.1126/science.291.5505.881

Hosomi, K., Saito, M., Park, J., Murakami, H., Shibata, N., Ando, M., et al. (2022). Oral administration of *Blautia wexlerae* ameliorates obesity and type 2 diabetes via metabolic remodeling of the gut microbiota. *Nat. Commun.* 13:4477. doi: 10.1038/s41467-022-32015-7

Huang, F., Zheng, X., Ma, X., Jiang, R., Zhou, W., Zhou, S., et al. (2019). Theabrownin from Pu-erh tea attenuates hypercholesterolemia via modulation of gut microbiota and bile acid metabolism. *Nat. Commun.* 10:4971. doi: 10.1038/s41467-019-12896-x

Iqbal, J., and Hussain, M. M. (2009). Intestinal lipid absorption. *Am. J. Physiol. Endocrinol. Metab.* 296, E1183–E1194. doi: 10.1152/ajpendo.90899.2008

Jang, L. G., Choi, G., Kim, S. W., Kim, B. Y., Lee, S., and Park, H. (2019). The combination of sport and sport-specific diet is associated with characteristics of gut microbiota: an observational study. *J. Int. Soc. Sports Nutr.* 16:21. doi: 10.1186/s12970-019-0290-y

Johnson, E. L., Heaver, S. L., Walters, W. A., and Ley, R. E. (2017). Microbiome and metabolic disease: revisiting the bacterial phylum Bacteroidetes. *J. Mol. Med.* 95, 1–8. doi: 10.1007/s00109-016-1492-2

Khan, S., and Jena, G. (2016). Sodium butyrate reduces insulin-resistance, fat accumulation and dyslipidemia in type-2 diabetic rat: a comparative study with metformin. *Chem. Biol. Interact.* 254, 124–134. doi: 10.1016/j.cbi.2016.06.007

Khanal, P., Maltecca, C., Schwab, C., Fix, J., Bergamaschi, M., and Tiezzi, F. (2020). Modeling host-microbiome interactions for the prediction of meat quality and carcass composition traits in swine. *Genet. Select. Evol.* 52:41. doi: 10.1186/s12711-020-00561-7

Kootte, R. S., Levin, E., Salojärvi, J., Smits, L. P., Hartstra, A. V., Udayappan, S. D., et al. (2017). Improvement of insulin sensitivity after lean donor feces in metabolic syndrome is driven by baseline intestinal microbiota composition. *Cell Metab.* 26, 611–619.e6. doi: 10.1016/j.cmet.2017.09.008

Kuno, T., Hirayama-Kurogi, M., Ito, S., and Ohtsuki, S. (2018). Reduction in hepatic secondary bile acids caused by short-term antibiotic-induced dysbiosis decreases mouse serum glucose and triglyceride levels. *Sci. Rep.* 8:1253. doi: 10.1038/s41598-018-19545-1

Lang, Y., Wang, Y., Sun, B., Zhang, S., Xie, P., Sha, K., et al. (2016). Myofiber characteristics and eating quality of three major muscles from Chinese Simmental cattle. *Can. J. Anim. Sci.* 97, 101–108. doi: 10.1139/cjas-2015-0182

Latimer, G., Jr., Ed. (2012). Official Methods of Analysis of A.O.A.C. International; Agriculture, Chemical, Contaminant, Drugs. *19th Edn. Maryland*.

Lee, W. J., and Hase, K. (2014). Gut microbiota-generated metabolites in animal health and disease. *Nat. Chem. Biol.* 10, 416–424. doi: 10.1038/nchembio.1535

Lei, L., Wang, Z., Li, J., Yang, H., Yin, Y., Tan, B., et al. (2021). Comparative microbial profiles of colonic digesta between Ningxiang pig and large white pig. *Animals* 11:1862. doi: 10.3390/ani11071862

Leidmaa, E., Gazea, M., Patchev, A. V., Pissioti, A., Christian Gassen, N., Kimura, M., et al. (2020). Blunted leptin sensitivity during hedonic overeating can be reinstated by activating galanin 2 receptors (Gal2R) in the lateral hypothalamus. *Acta Physiol.* 228:e13345. doi: 10.1111/apha.13345

Ley, R. E., Bäckhed, F., Turnbaugh, P., Lozupone, C. A., Knight, R. D., and Gordon, J. I. (2005). Obesity alters gut microbial ecology. *Proc. Nat. Acad. Sci. U. S. A.* 102, 11070–11075. doi: 10.1073/pnas.0504978102

Ley, R. E., Peterson, D. A., and Gordon, J. I. (2006). Ecological and evolutionary forces shaping microbial diversity in the human intestine. *Cells* 124, 837–848. doi: 10.1016/j.cell.2006.02.017

Li,, Q., Han, Y., Dy, A. B. C., Hagerman, R. J. (2017). The Gut Microbiota and Autism Spectrum Disorders. *Frontiers in Cellular Neuroscience* 11, 120. doi: 10.3389/fncel.2017.00120

Li, N., Yu, Q. L., Yan, X. M., Li, H. B., and Zhang, Y. (2020). Sequencing and characterization of miRNAs and mRNAs from the longissimus dorsi of Xinjiang brown cattle and Kazakh cattle. *Gene* 741:144537. doi: 10.1016/j.gene.2020.144537

Louis, P., and Flint, H. J. (2017). Formation of propionate and butyrate by the human colonic microbiota. *Environ. Microbiol.* 19, 29–41. doi: 10.1111/1462-2920.13589

Lowe, B. K., McKeith, R. O., Segers, J., Safko, J. A., Froetschel, M. A., Stewart, L., et al. (2012). The effects of zilpaterol hydrochloride supplementation on market dairy cow performance, carcass characteristics, and cutability. *Prof. Anim. Sci.* 28, 150–157. doi: 10.15232/S1080-7446(15)30335-1

Luo, Y., Wang, B., Liu, C., Su, R., Hou, Y., Yao, D., et al. (2019). Meat quality, fatty acids, volatile compounds, and antioxidant properties of lambs fed pasture versus mixed diet. *Food Sci. Nutr.* 7, 2796–2805. doi: 10.1002/fsn3.1039

Lyu, W., Liu, X., Lu, L., Dai, B., Wang, W., Yang, H., et al. (2021). Cecal microbiota modulates fat deposition in Muscovy ducks. *Front. Vet. Sci.* 8:609348. doi: 10.3389/fvets.2021.609348

Ma, J., Duan, Y., Li, R., Liang, X., Li, T., Huang, X., et al. (2022). Gut microbial profiles and the role in lipid metabolism in Shaziling pigs. *Anim. Nutr.* 9, 345–356. doi: 10.1016/j.aninu.2021.10.012

Mamani-Linares, L. W., and Gallo, C. B. (2013). Meat quality attributes of the Longissimus lumborum muscle of the Kh'ara genotype of llama (Lama glama) reared extensively in northern Chile. *Meat Sci.* 94, 89–94. doi: 10.1016/j.meatsci.2012.12.013

Marcel, M. (2011). Cutadapt removes adapter sequences from high-throughput sequencing reads. *EMBnet.* 17, 10–12. doi: 10.14806/ej.17.1.200

McEvers, T. J., Hutcheson, J. P., and Lawrence, T. E. (2012). Quantification of saleable meat yield using objective measurements captured by video image analysis technology. *J. Anim. Sci.* 90, 3294–3300. doi: 10.2527/jas.2011-4223

Melis, G. C., ter Wengel, N., Boelens, P. G., and van Leeuwen, P. A. (2004). Glutamine: recent developments in research on the clinical significance of glutamine. *Curr. Opin. Clin. Nutr. Metab. Care* 7, 59–70. doi: 10.1097/00075197-200401000-00011

Murphy, K. G., and Bloom, S. R. (2006). Gut hormones and the regulation of energy homeostasis. *Nature* 444, 854–859. doi: 10.1038/nature05484

Myer, P. R., Smith, T. P., Wells, J. E., Kuehn, L. A., and Freetly, H. C. (2015). Rumen microbiome from steers differing in feed efficiency. *PLoS One* 10:e0129174. doi: 10.1371/journal.pone.0129174

Nakajima, K., Nagamine, T., Fujita, M. Q., Ai, M., Tanaka, A., and Schaefer, E. (2014). Apolipoprotein B-48: a unique marker of chylomicron metabolism. *Adv. Clin. Chem.* 64, 117–177. doi: 10.1016/B978-0-12-800263-6.00003-3

Newgard, C. B., An, J., Bain, J. R., Muehlbauer, M. J., Stevens, R. D., Lien, L. F., et al. (2009). A branched-chain amino acid-related metabolic signature that differentiates obese and lean humans and contributes to insulin resistance. *Cell Metab.* 9, 311–326. doi: 10.1016/j.cmet.2009.02.002

Newsholme, P., Brennan, L., Rubi, B., and Maechler, P. (2005). New insights into amino acid metabolism, beta-cell function and diabetes. *Clin. Sci.* 108, 185–194. doi: 10.1042/CS20040290

Nicholson, J. K., Holmes, E., Kinross, J., Burcelin, R., Gibson, G., Jia, W., et al. (2012). Host-gut microbiota metabolic interactions. *Science* 336, 1262–1267. doi: 10.1126/science.1223813

Noel, S. J., Olijhoek, D. W., Mclean, F., Løvendahl, P., Lund, P., and Højberg, O. (2019). Rumen and fecal microbial community structure of Holstein and Jersey dairy cows as affected by breed, diet, and residual feed intake. *Animals* 9:498. doi: 10.3390/ani9080498

Ohira, H., Tsutsui, W., and Fujioka, Y. (2017). Are short chain fatty acids in gut microbiota defensive players for inflammation and atherosclerosis? *J. Atheroscler. Thromb.* 24, 660–672. doi: 10.5551/jat.RV17006

Ottosson, F., Smith, E., Melander, O., and Fernandez, C. (2018). Altered asparagine and glutamate homeostasis precede coronary artery disease and type 2 diabetes. *J. Clin. Endocrinol. Metab.* 103, 3060–3069. doi: 10.1210/jc.2018-00546

Parnell, J. A., and Reimer, R. A. (2012). Prebiotic fiber modulation of the gut microbiota improves risk factors for obesity and the metabolic syndrome. *Gut Microbes* 3, 29–34. doi: 10.4161/gmic.19246

Patterson, E., Ryan, P. M., Wiley, N., Carafa, I., Sherwin, E., Moloney, G., et al. (2019). Gamma-aminobutyric acid-producing lactobacilli positively affect metabolism and depressive-like behaviour in a mouse model of metabolic syndrome. *Sci. Rep.* 9:16323. doi: 10.1038/s41598-019-51781-x

Pertl, C., Eblenkamp, M., Pertl, A., Pfeifer, S., Wintermantel, E., Lochmüller, H., et al. (2013). A new web-based method for automated analysis of muscle histology. *BMC Musculoskelet. Disord.* 14:26. doi: 10.1186/1471-2474-14-26

Petri, R. M., Schwaiger, T., Penner, G. B., Beauchemin, K. A., Forster, R. J., McKinnon, J. J., et al. (2013). Characterization of the core rumen microbiome in cattle during transition from forage to concentrate as well as during and after an acidotic challenge. *PLoS One* 8:e83424. doi: 10.1371/journal.pone.0083424

Picard, B., Gagaoua, M., Al-Jammas, M., De Koning, L., Valais, A., and Bonnet, M. (2018). Beef tenderness and intramuscular fat proteomic biomarkers: muscle type effect. *PeerJ.* 6:e4891. doi: 10.7717/peerj.4891

Pisanu, S., Palmas, V., Madau, V., Casula, E., Deledda, A., Cusano, R., et al. (2020). Impact of a moderately hypocaloric mediterranean diet on the gut microbiota composition of Italian obese patients. *Nutrients* 12:2707. doi: 10.3390/nu12092707

Poquet, L., and Wooster, T. J. (2016). Infant digestion physiology and the relevance of in vitro biochemical models to test infant formula lipid digestion. *Mol. Nutr. Food Res.* 60, 1876–1895. doi: 10.1002/mnfr.201500883

Rampelli, S., Guenther, K., Turroni, S., Wolters, M., Veidebaum, T., Kourides, Y., et al. (2018). Pre-obese children's dysbiotic gut microbiome and unhealthy diets may predict the development of obesity. *Commun. Biol.* 1:222. doi: 10.1038/s42003-018-0221-5

Rausch, P., Rühlemann, M., Hermes, B. M., Doms, S., Dagan, T., Dierking, K., et al. (2019). Comparative analysis of amplicon and metagenomic sequencing methods reveals key features in the evolution of animal metaorganisms. *Microbiome* 7:133. doi: 10.1186/s40168-019-0743-1

Serena, C., Ceperuelo-Mallafré, V., Keiran, N., Queipo-Ortuño, M. I., Bernal, R., Gomez-Huelgas, R., et al. (2018). Elevated circulating levels of succinate in human obesity are linked to specific gut microbiota. *ISME J.* 12, 1642–1657. doi: 10.1038/s41396-018-0068-2

Shinkai, T., Ikeyama, N., Kumagai, M., Ohmori, H., Sakamoto, M., Ohkuma, M., et al. (2022). *Prevotella lacticifex* sp. nov., isolated from the rumen of cows. *Int. J. Syst. Evol. Microbiol.* 72: 005278. doi: 10.1099/ijsem.0.005278

Soltani, N., Qiu, H., Aleksic, M., Glinka, Y., Zhao, F., Liu, R., et al. (2011). GABA exerts protective and regenerative effects on islet beta cells and reverses diabetes. *Proc. Nat. Acad. Sci. U. S. A.* 108, 11692–11697. doi: 10.1073/pnas.1102715108

Sun, Y., Yu, K., Zhou, L., Fang, L., Su, Y., and Zhu, W. (2016). Metabolomic and transcriptomic responses induced in the livers of pigs by the long-term intake of resistant starch. *J. Anim. Sci.* 94, 1083–1094. doi: 10.2527/jas.2015-9715

Tazi, A., Araujo, J. R., Mulet, C., Arena, E. T., Nigro, G., Pédron, T., et al. (2018). Disentangling host-microbiota regulation of lipid secretion by enterocytes: insights from commensals *Lactobacillus paracasei* and *Escherichia coli*. *mBio* 9:e01493-18. doi: 10.1128/mBio.01493-18

Thursby, E., and Juge, N. (2017). Introduction to the human gut microbiota. *Biochem. J.* 474, 1823–1836. doi: 10.1042/BCJ20160510

Tian, J., Dang, H., Chen, Z., Guan, A., Jin, Y., Atkinson, M. A., et al. (2013). γ-Aminobutyric acid regulates both the survival and replication of human β-cells. *Diabetes* 62, 3760–3765. doi: 10.2337/db13-0931

Tian, J., Dang, H., and Kaufman, D. L. (2011). Combining antigen-based therapy with GABA treatment synergistically prolongs survival of transplanted ß-cells in diabetic NOD mice. *PLoS One* 6:e25337. doi: 10.1371/journal.pone.0025337

Turnbaugh, P. J., and Gordon, J. I. (2009). The core gut microbiome, energy balance and obesity. *J. Physiol.* 587, 4153–4158. doi: 10.1113/jphysiol.2009.174136

Turnbaugh, P. J., Ley, R. E., Mahowald, M. A., Magrini, V., Mardis, E. R., and Gordon, J. I. (2006). An obesity-associated gut microbiome with increased capacity for energy harvest. *Nature* 444, 1027–1031. doi: 10.1038/nature05414

Vital, M., Howe, A., Bergeron, N., Krauss, R. M., Jansson, J. K., and Tiedje, J. M. (2018). Metagenomic insights into the degradation of resistant starch by human gut microbiota. *Appl. Environ. Microbiol.* 84:e01562-18. doi: 10.1128/AEM.01562-18

Wan, Y., Tong, W., Zhou, R., Li, J., Yuan, J., Wang, F., et al. (2019). Habitual animal fat consumption in shaping gut microbiota and microbial metabolites. *Food Funct.* 10, 7973–7982. doi: 10.1039/c9fo01490j

Wang, S. Z., Yu, Y. J., and Adeli, K. (2020). Role of gut microbiota in neuroendocrine regulation of carbohydrate and lipid metabolism via the microbiota-gut-brain-liver axis. *Microorganisms* 8:527. doi: 10.3390/microorganisms8040527

Wen, Y., Liu, H., Liu, K., Cao, H., Mao, H., Dong, X., et al. (2020). Analysis of the physical meat quality in partridge (*Alectoris chukar*) and its relationship with intramuscular fat. *Poult. Sci.* 99, 1225–1231. doi: 10.1016/j.psj.2019.09.009

Wen, C., Yan, W., Sun, C., Ji, C., Zhou, Q., Zhang, D., et al. (2019). The gut microbiota is largely independent of host genetics in regulating fat deposition in chickens. *ISME J.* 13, 1422–1436. doi: 10.1038/s41396-019-0367-2

Wu, C., Lyu, W., Hong, Q., Zhang, X., Yang, H., and Xiao, Y. (2021). Gut microbiota influence lipid metabolism of skeletal muscle in pigs. *Front. Nutr.* 8:675445. doi: 10.3389/fnut.2021.675445

Yang, Y., Gao, H., Li, X., Cao, Z., Li, M., Liu, J., et al. (2021). Correlation analysis of muscle amino acid deposition and gut microbiota profile of broilers reared at different ambient temperatures. *Anim. Biosci.* 34, 93–101. doi: 10.5713/ajas.20.0314

Yanni, A. E., Agrogiannis, G., Nomikos, T., Fragopoulou, E., Pantopoulou, A., Antonopoulou, S., et al. (2010). Oral supplementation with L-aspartate and L-glutamate inhibits atherogenesis and fatty liver disease in cholesterol-fed rabbit. *Amino Acids* 38, 1323–1331. doi: 10.1007/s00726-009-0340-x

Yuan, G. H., Zhang, Z., Gao, X. S., Zhu, J., Guo, W. H., Wang, L., et al. (2020). Gut microbiota-mediated tributyltin-induced metabolic disorder in rats. *RSC Adv.* 10, 43619–43628. doi: 10.1039/d0ra07502g

Zeybel, M., Arif, M., Li, X., Altay, O., Yang, H., Shi, M., et al. (2022). Multiomics analysis reveals the impact of microbiota on host metabolism in hepatic steatosis. *Adv. Sci.* 9:e2104373. doi: 10.1002/advs.202104373

Zhang, Y. J., Li, S., Gan, R. Y., Zhou, T., Xu, D. P., and Li, H. B. (2015). Impacts of gut bacteria on human health and diseases. *Int. J. Mol. Sci.* 16, 7493–7519. doi: 10.3390/ijms16047493

Zhang, S., Nagana Gowda, G. A., Asiago, V., Shanaiah, N., Barbas, C., and Raftery, D. (2008). Correlative and quantitative 1H NMR-based metabolomics reveals specific metabolic pathway disturbances in diabetic rats. *Anal. Biochem.* 383, 76–84. doi: 10.1016/j.ab.2008.07.041

Zhang, X., and van den Pol, A. N. (2017). Rapid binge-like eating and body weight gain driven by zona incerta GABA neuron activation. *Science* 356, 853–859. doi: 10.1126/science.aam7100

Zhang, Z., Yang, L., He, Y., Luo, X., Zhao, S., and Jia, X. (2021). Composition of fecal microbiota in grazing and feedlot Angus beef cattle. *Animals* 11:3167. doi: 10.3390/ani11113167

Zhang, C. L., Zhou, J. J., Jiang, L. L., Wang, X. L., Zhu, J. M., Quan, C. S., et al. (2017). Advances in microbionomics and its applications. *J. Microbiol.* 037, 74–81. doi: 10.3969/j.issn.1005-7021.2017.04.012

Zheng, Y., Chen, J., Wang, X., Han, L., Yang, Y., Wang, Q., et al. (2022). Metagenomic and transcriptomic analyses reveal the differences and associations between the gut microbiome and muscular genes in Angus and Chinese Simmental cattle. *Front. Microbiol.* 13:815915. doi: 10.3389/fmicb.2022.815915

Zhou, P., Yang, X., Yang, Z., Huang, W., Kou, J., and Li, F. (2019). Akebia saponin D regulates the metabolome and intestinal microbiota in high fat diet-induced hyperlipidemic rats. *Molecules* 24:1268. doi: 10.3390/molecules24071268

Zhu, W., Lomsadze, A., and Borodovsky, M. (2010). Ab initio gene identification in metagenomic sequences. *Nucleic Acids Research*. 38:e132. doi: 10.1093/nar/gkq275

Zierer, J., Jackson, M. A., Kastenmüller, G., Mangino, M., Long, T., Telenti, A., et al. (2018). The fecal metabolome as a functional readout of the gut microbiome. *Nat. Genet.* 50, 790–795. doi: 10.1038/s41588-018-0135-7

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# Effects of whole maize high-grain diet feeding on colonic fermentation and bacterial community in weaned lambs

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High-grain diet is commonly used in intensive production to boost yield in short term, which may cause adverse effects such as rumen and colonic acidosis in ruminants. Maize is one of the key components of high-grain diet, and different processing methods of maize affect the digestive absorption and gastrointestinal development of ruminants. To investigate the effects of maize form in high-grain diets on colonic fermentation and bacterial community of weaned lambs, twenty-two 2.5-month-old healthy Hu lambs were fed separately a maize meal low-grain diet (19.2% grain; CON), a maize meal high-grain diet (50.4% grain; CM), and a whole maize high-grain diet (50.4% grain; CG). After 7 weeks of feeding, the total volatile fatty acid concentration ($P$ = 0.035) were significantly higher in lambs from CM than that from CON. The sequencing results of colonic content microbial composition revealed that the relative abundance of genera *Parasutterella* ($P$ = 0.028), *Comamonas* ($P$ = 0.031), *Butyricicoccus* ($P$ = 0.049), and *Olsenella* ($P$ = 0.010) were higher in CM than those in CON; compared with CM, the CG diet had the higher relative abundance of genera *Bacteroides* ($P$ = 0.024) and *Angelakisella* ($P$ = 0.020), while the lower relative abundance of genera *Olsenella* ($P$ = 0.031) and *Paraprevotella* ($P$ = 0.006). For colonic mucosal microbiota, the relative abundance of genera *Duncaniella* ($P$ = 0.024), *Succiniclasticum* ($P$ = 0.044), and *Comamonas* ($P$ = 0.012) were significantly higher in CM than those in CON. In comparison, the relative abundance of genera *Alistipes* ($P$ = 0.020) and *Campylobacter* ($P$ = 0.017) were significantly lower. And the relative abundance of genera *Colidextribacter* ($P$ = 0.005), *Duncaniella* ($P$ = 0.032), *Christensenella* ($P$ = 0.042), and *Lawsonibacter* ($P$ = 0.018) were increased in the CG than those in the CM. Furthermore, the CG downregulated the relative abundance of genes encoding infectious-disease-parasitic ($P$ = 0.049), cancer-specific-types ($P$ = 0.049), and neurodegenerative-disease ($P$ = 0.037) in colonic microbiota than those in the CM. Overall, these results indicated that maize with different grain sizes might influence the colonic health

of weaned lambs by altering the composition of the colonic bacterial community.

KEYWORDS

lambs, maize, colonic content, colonic mucosa, bacterial community

# Introduction

In the current intensive farming system, feeding ruminants a high-grain diet is extensively used to boost yield and earn immediate financial gains (Plaizier et al., 2008). However, the high proportion of starch and low level of fiber in high-grain diets can lead to more volatile fatty acids (VFAs) and lipopolysaccharide in the colon due to increased carbohydrate fermentation in the post-rumen. Then it was followed by a decrease in colonic pH and further changes of microbiota in the colon and injuries to the colonic epithelium (Metzler-Zebeli et al., 2013; Wang et al., 2017).

The ruminant colonic epithelium exists as a physical and immunological barrier to protect animal health (Kostic et al., 2013; Steele et al., 2016). Meanwhile, the microbial communities associated with colonic content play an important role in nutrient digestion of ruminants. It was reported that the nitrogen digestion and VFAs production by microbiota in the large intestine of ruminant can account for up to 7 and 17% of the total intestine (Hoover, 1978; Vanhatalo and Ketoja, 1995). Furthermore, there are remarkable differences between the microbiota of colonic contents and mucosa in pre-weaned calves, suggesting that they may serve different functions (Malmuthuge et al., 2014), and the gastrointestinal mucosa-associated microbiota is believed to be vital for regulating barrier function and the immune system (Hooper et al., 2012). Colonic fermentation and immunity are crucial for the growth and health of ruminants, so it is essential to prevent or mitigate the adverse effects of high-grain diets on the colonic content or mucosal microbiota through nutritional strategies.

Maize, the main grain component of feed (Humer and Zebeli, 2017), can be processed by physical, chemical, and biological methods. Callison et al. (2001) demonstrated with primiparous Holstein cows that the digestibility of nonstructural carbohydrates in the rumen increased when the size of the corn crush was decreased. Chester-Jones et al. (1991) found that feeding whole corn to weaned calves tended to increase the average daily gain (ADG) and feed conversion ratio (FCR). Lopez et al. (2014) demonstrated that whole maize high-grain diets had beneficial effects on the metabolic energy of goats. These studies have shown that feeding larger particle sizes of grains facilitates the promotion of nutrient digestion and ruminant performance, which might be related to the beneficial fermentation of the microbial community under these conditions in the digestive tract. Unfortunately, limited studies have focused on the effects of maize forms on the colonic microbial community and mucosal morphology structure in fattening lambs.

Studies have shown that the entire digestive tract is not fully developed in weaned lambs (Kelly and Coutts, 2000). Early nutritional intervention can substantially impact their intestinal mucosal morphology and microbial composition (Abecia et al., 2013; Yanez-Ruiz et al., 2015), which will further influence their lifelong health and performance (Jiao et al., 2016). Therefore, we hypothesized that replacing crushed maize with whole maize could alleviate the deleterious effects of high-grain diets on developing ruminant colon in this study. To test the hypothesis, the colonic epithelial morphology and microbial community were examined in weaned lambs reared in a maize meal low-grain diet, maize meal high-grain diet, and whole maize high-grain diet.

# Materials and methods

## Experimental animals and feeding management

The animal experiment was approved by the Animal Protection and Use Committee of Nanjing Agricultural University [Authorisation SYXK(Su)2019-0074] and was performed following the Regulations for the Administration of Affairs Concerning Experimental Animals (the State Science and Technology Commission of P. R. China, 1988). The study was conducted at the experimental station located at Nanjing Agricultural University, Jiangsu Province, China, with twenty-two healthy 2.5-month-old male lambs being used and randomly allocated to three groups: one group was fed a maize meal low-grain diet (19.2% grain; CON, $n$ = 7), another group was fed a maize meal high-grain diet (50.4% grain; CM, $n$ = 8), and the other group was fed a whole maize high-grain diet (50.4% grain; CG, $n$ = 7). The formulas of lambs were designed according to the feeding standard of NY/T 816-2004 fattening lambs (MOA, 2004), and the crude protein, starch, neutral detergent fiber, acid detergent fiber, and crude ash were measured using standard methods of AOAC (2007). Components and nutrient compositions of the diets can be found in **Supplementary Table 1**. The maize meal was directly crushed by hammer mill without sieve, with an average grain size of 2.57 mm; whole maize was fed directly without processing. The total experimentation period lasted 49 days

after a 7-day acclimatization phase. At 8:00 and 17:00 each day for the course of the trial, all lambs were provided water and food. Food intake was recorded daily, and weight changes were monitored weekly.

## Sample collection

On day 50, all lambs were slaughtered 4 h after the morning feeding. Colon samples were separated with a blunt instrument immediately after evisceration. The pH of the representative colonic fluid was measured using a portable pH meter (HI 9024C; HANNA Instruments, Woonsocket, RI, USA). Ten g samples of the content were mixed with a double amount of distilled water and immediately centrifuged at 2,000 x *g* for 10 min and stored at −20°C for analysis of VFAs (GC-14B, Shimadzu, Japan). The colon content was collected and stored in liquid nitrogen for subsequent microbial DNA extraction. Meanwhile, a section of colonic tissue was collected and washed three times in ice-cold phosphate-buffered saline solution immediately (within 5 min) after slaughter. Then, the washed colon tissue was divided into two parts. The first part of the sample was cut into approximately 2 × 2 cm and the mucosal tissue scraped with sterile sections before being stored in liquid nitrogen until microbial DNA extraction. And samples from the second part were immediately fixed in 4% paraformaldehyde (Sigma, St. Louis, MO, USA) for histomorphometric microscopic analysis.

## Determination of fermentation parameters

The concentration of VFAs in colonic content was measured by capillary column gas chromatography (GC-14B; Shimadzu company; Japan) (Qin, 1983). One gram of sample was taken to the centrifuge tube, and 5–10 times of double-distilled water was added and mixed thoroughly. Then 0.2 ml of 25% (w/v) metaphosphoric acid was added to 1 mL of supernatant and stored overnight at −20°C. After thawing, we centrifuged the sample at 12,000 rpm for 10 min and retained the supernatant, which was then centrifuged at 12,000 rpm for another 10 min before measurement. The relative correction factors of organic acids could be calculated from the respective peak area of standard samples and internal standard crotonic acid. The concentrations andproportions of VFAs in each sample could be calculated according to the weight (or concentration) of VFAs in proportion to their peak area.

## Histological measurements

The colonic tissues were embedded with paraffin and then sliced (6 μm) and stained with hematoxylin and eosin. The microscopist was blinded to the treatment conditions during histomorphometric analysis. Three slides were prepared for each lamb, and two images were taken for each slide. The tissues were fixed with 2.5% glutaraldehyde for at least 24 h, post-fixed in 1% osmium, and embedded in Epon Araldite. Semi-thin sections (0.25–0.5 μm) and ultrathin sections (70–90 nm) were cut using a glass cutter. Then the semithin sections were stained with 1% toluidine blue and 1% sodium borate before the ultrathin sections were stained with uranyl acetate and lead citrate. We used a transmission electron microscope (H-7650; Hitachi Technologies, Tokyo, Japan) to determine the ultrastructures of the colonic epithelial cells.

## Microbial DNA extraction

The colonic content and mucosa tissue samples were processed separately, and the microbial DNA was extracted using the DNA Extraction Kit (Mobio laboratory, Carlsbad, CA, USA). The concentration and purity of the extracted DNA samples were measured by the Nanodrop 2000 spectrophotometer (Thermo Fisher Scientific, USA), ensuring that the OD260/280 value was between 1.80 and 2.00. Then the DNA samples were stored at −80°C for subsequent sequencing analysis.

## Sequencing data processing of bacterial 16S rRNA gene

Bacterial 16S rRNA gene sequences were amplified by forwarding primer (5'-CCA TCT CAT CCC TGC GTG TCT CCG ACT CAG-3') and reverse primer (5'-CCT ATC CCC TGT GTG CCT TGG CAG TCTCAG-3'). Sample identification in the forwarding primer was a unique 12-base barcode. The PCR reaction was performed in a 50 μL C1000 thermal cycler (Bio-Rad, USA) with 10 μL 5x Fast Pfu buffer, 50 ng of DNA, 0.4 μM of each primer, 0.5U FastPfu polymerase, and 2.5 mM dNTPs. The PCR amplification process was as follows: preliminary denaturation at 95°C for 2 min; cycles (30 times for bacteria/archaea/protozoa; 35 times for fungi) of denaturation (95°C, 20 s); annealing (60°C for bacteria/58°C for archaea/54°C for protozoa/50°C for fungi, 20 s) and elongation (65°C, 1 min); and a final extension at 65°C for 7 min (Mao et al., 2012; Kittelmann et al., 2013). The bacterial amplicon sequence was then sequenced by the Illumina MiSeq platform (Illumina, San Diego, CA, USA). The sequenced data processing was operated using the Divisive Amplicon Denoising Algorithm 2 (DADA2) R package (Callahan et al., 2016). The representative amplicon sequence variant (ASV) sequences were identified by the SILVA (v183) and classified into specific bacterial taxonomy. Single sample diversity analysis was accomplished by Alpha diversity index using QIIME: ACE, Chao1, Shannon, and Simpson.

## Functional gene prediction

Phylogenetic investigation of communities by reconstruction of unobserved states (PICRUSt), a bioinformatics tool to predict the metagenome gene's functional content using 16S rRNA genes, was applied to obtain an overview of the metagenomic contribution of the colonic content and mucosal bacterial community. Predictions were made for genes in databases, including the metagenome inference of Kyoto Encyclopedia of Genes and Genomes (KEGG).

## Statistical analyses

The independent-sample *t*-test was employed to calculate the differences in fermentation parameters between groups. The two-way repeated analysis of variance (ANOVA) evaluation was used to calculate the difference in diversity index between groups. Analysis of similarities (ANOSIM) of bacterial community based in unweighted unifrac distance among the three groups was performed by a principal coordinate analysis (PCoA) using the SIMCA-P (version 14.0) software package (Umetrics, Umea, Sweden). The taxonomy data from the experiment were initially organized in Excel and then statistically calculated by the SPSS software package v.25 (SPSS Inc., Chicago, IL, USA). The normality of the distribution of variables was tested using the Shapiro Wilk. When the variable distributions were assumed normal, the independent samples *t*-test procedure was used; otherwise, the Kruskal-Wallis procedure was used. $P < 0.05$ indicates a significant difference, $P < 0.01$ indicates a significantly difference.

# Results

## Colonic pH, concentrations of volatile fatty acids, and epithelium morphological structure in weaned lambs

As shown in **Table 1**, compared with CON, colonic pH in CM showed a decreasing trend ($P = 0.069$), and a significant increase in the concentration of total VFA ($P = 0.035$). Between the CM and the CG groups, there was no significant difference in colonic pH and the proportion of VFAs.

Compared with CON group, the intestinal villus and colonic epithelial mitochondria were damaged and vacuoles appeared in the colonic epithelial cell in CM group (**Figure 1**). Although the same high-gain level diet was used, the morphology of the colonic epithelium was significantly improved in CG compared to CM, suggesting that the whole maize form could protect the colonic environment in a high-gain diet feeding.

## Colonic content microbiota in weaned lambs

### Diversity of the colonic content microbiota

The bacterial rarefaction profile of colon content flattened, indicating that the sequencing depth and data volume were sufficient to cover most microbes (**Figure 2A**). The microbial composition was significantly distinct in CON and CM (ANOSIM, $R = 0.4096$, $P = 0.001$) but not in CM and CG (ANOSIM, $R = 0.0146$, $P = 0.342$) (**Figures 2B,C**).

As shown in **Figures 2D,E**, there was a decreasing trend of Chao 1 ($P = 0.092$) and Shannon's estimate ($P = 0.099$) for colonic content in CM compared to CON. The diversity index of the content microbiota did not significantly differ between the CM and the CG ($P > 0.05$), despite the CG having a higher number of Chao 1, Shannon indexes, and lower Simpson index (**Figures 2D–F**). These findings indicated that the colonic content microbial diversity within a high-gain diet could not be considerably improved by whole maize form.

### Composition of the colonic content microbiota

There were nine common phyla detected in the colonic content samples among the three groups: Firmicutes, Proteobacteria, Bacteroidetes, Spirochaetae, Fibrobacteres, Unclassified Bacteria, Actinobacteria, Verrucomicrobia, and Euryarchaeota (**Figure 3A**). The relative abundance of Candidatus, Saccharibacteria, and Unclassified Bacteria in the CM were substantially lower than those in the CON ($P < 0.05$).

The relative abundance of 183 genera was more than 0.1 %. In **Figures 4A,B**, some of the genera with notable differences are depicted. Compared with the CON, the relative abundance of *Vibrio*, *Kineothrix*, *Alistipes*, *Lacrimispora*, *Paramuribaculum*, *Flavonifractor,* and *Anaerobutyricum* in colonic content of the CM were highly significantly decreased ($P < 0.01$); the relative abundance of *Comamonas*, *Olsenella*, *Sporobacter*, *Fluviicola*, *Parasutterella,* and *Butyricicoccus* were significantly increased ($P < 0.05$). Compared with the CM, the CG decreased the relative abundance of *Paraprevotella* ($P < 0.01$) and *Olsenella* ($P < 0.05$) while increased the relative abundance of Unclassified Oscillospiraceae, *Bacteroides*, *Pseudoflavonifractor*, *Angelakisella,* and Unclassified Enterobacteriaceae significantly ($P < 0.05$). These findings suggested that the whole maize form throughout a long-term high-gain diet restored the alterations in the content microbial community.

### Functional prediction of colonic content microbiota

The functional prediction of colonic content microbiota was shown in **Figures 5A,B**. Compared with the CON, the

TABLE 1 The effect of CON, CM, and CG on colonic fermentation parameters of weaned lambs[1].

| Item | CON[3] | CM[3] | CG[3] | *P*-value | |
|---|---|---|---|---|---|
| | | | | CON vs. CM | CM vs. CG |
| Colonic pH | 6.80 ± 0.05 | 6.45 ± 0.17 | 6.59 ± 0.07 | 0.069 | 0.418 |
| Total VFA[2], mM | 73.56 ± 6.74 | 102.69 ± 9.88 | 89.97 ± 3.80 | 0.035 | 0.228 |
| Acetate, mol/100 mol of VFA | 74.11 ± 1.10 | 69.25 ± 2.96 | 71.13 ± 1.49 | 0.172 | 0.588 |
| Propionate, mol/100 mol of VFA | 16.07 ± 0.99 | 16.89 ± 0.80 | 15.99 ± 0.86 | 0.535 | 0.460 |
| Isobutyrate, mol/100 mol of VFA | 1.11 ± 1.52 | 0.81 ± 0.17 | 0.81 ± 0.13 | 0.224 | 0.980 |
| Butyrate, mol/100 mol of VFA | 6.48 ± 0.62 | 11.26 ± 2.92 | 10.00 ± 1.03 | 0.165 | 0.698 |
| Isovalerate, mol/100 mol of VFA | 1.00 ± 1.74 | 0.75 ± 0.18 | 0.78 ± 0.14 | 0.331 | 0.881 |
| Valerate, mol/100 mol of VFA | 1.23 ± 0.13 | 1.03 ± 0.12 | 1.29 ± 0.18 | 0.285 | 0.258 |

[1]The values shown are means ± SEM (standard error of the mean); $P < 0.05$ indicated that mean values were significantly difference.
[2]VFA, volatile fatty acids.
[3]CON = maize meal low-grain diet, CM = maize meal high-grain diet, CG = whole maize high-grain diet.

relative abundance of genes encoding metabolism of cofactors and vitamins ($P = 0.004$), folding, sorting and degradation ($P = 0.001$), translation, replication, and repair ($P = 0.037$) in colonic microbiota of the CM were significantly decreased. In contrast, the relative abundance of genes encoding metabolism of other amino acids ($P = 0.037$), xenobiotics biodegradation and metabolism ($P = 0.015$) were significantly increased. Additionally, the colonic microbiota of the CG showed substantially lower relative abundance of genes encoding infectious disease_ parasitic ($P = 0.049$), cancer_ specific kinds ($P = 0.049$), and neurodegenerative disease ($P = 0.037$) compared to the CM, demonstrating how the whole maize form improved colonic microbiota's capability to combat infection under high-gain diet feeding.

## Colonic mucosal microbiome of weaned lambs

### Diversity of colonic mucosal microbiota

The rarefaction curves of colonic mucosal microbiota tended to be flat, indicating reasonable sampling and sufficient sequencing depth to reflect the bacterial community in the samples (Figure 6A). Figures 6B,C show the bacterial community profiles in the colonic mucosa of the CON, the CM, and the CM and the CG. The results of the Bray-Curtis metric revealed clear dissimilarities between CON and CM (ANOSIM, $R = 0.4519$, $P = 0.001$), while there was no significant segregation between CM and CG (ANOSIM, $R = 0.0029$, $P = 0.432$).

As shown in Figures 6D–F, no significant difference was observed in the colonic mucosal bacterial community diversity index between CON and CM ($P > 0.05$). And compared with the CON and the CM, the Shannon index of colonic mucosa in the CG was significantly increased ($P < 0.05$), indicating a higher microbial diversity in the whole maize from diet feeding.

### Composition of the colonic mucosal microbiota

The common phyla detected in the colonic mucosa samples of the three groups were: Proteobacteria, Firmicutes, Bacteroidetes, Spirochaetes, Verrucomicrobia, Unclassified Bactiera, Fibrobacteres, Actinobacteria, and Elusimicrobia (Figure 3B). Compared with the CM, the relative abundance of Proteobacteria in the CG was significantly decreased ($P < 0.05$), and the relative abundance of Firmicutes, Fibrobacteres, and Unclassified Bactiera were significantly increased ($P < 0.05$).

At least one set of relative abundance greater than 0.1 % was found in 159 genera. It can be seen from Figure 4C that compared with the CON, the relative abundance of *Paramuribaculum* in the colonic mucosa of the CM was highly significantly decreased ($P < 0.01$); the relative abundance of *Alistipes* and *Campylobacter* decreased significantly ($P < 0.05$); and the relative abundance of *Comamonas*, *Duncaniella*, *Succiniclasticum*, *Eisenbergiella*, *Blautia,* and *Lachnoclostridium* were dramatically increased ($P < 0.05$). And as shown in Figure 4D, compared with the CM, the relative abundance of *Colidextribacter* in the colonic mucosa of the CG was highly considerably increased ($P < 0.01$); the relative abundance of *Duncaniella*, Unclassified Gammaproteobacteria, Unclassified Oscillospiraceae, Unclassified Bacyeria, *Fibrobacter*, *Lawsonibacter*, *Faecalimonas*, *Stenotrophomonas,* and *Christensenella* were pronouncedly increased ($P < 0.05$). These findings demonstrated that the maize particle size significantly influenced the mucosal microbial population under a prolonged high-gain diet.

### Functional prediction of colonic mucosal microbiota

Figures 5C,D show the functional prediction of colonic mucosal microbiota. Compared with the CON, the relative abundance of genes encoding nucleotide metabolism

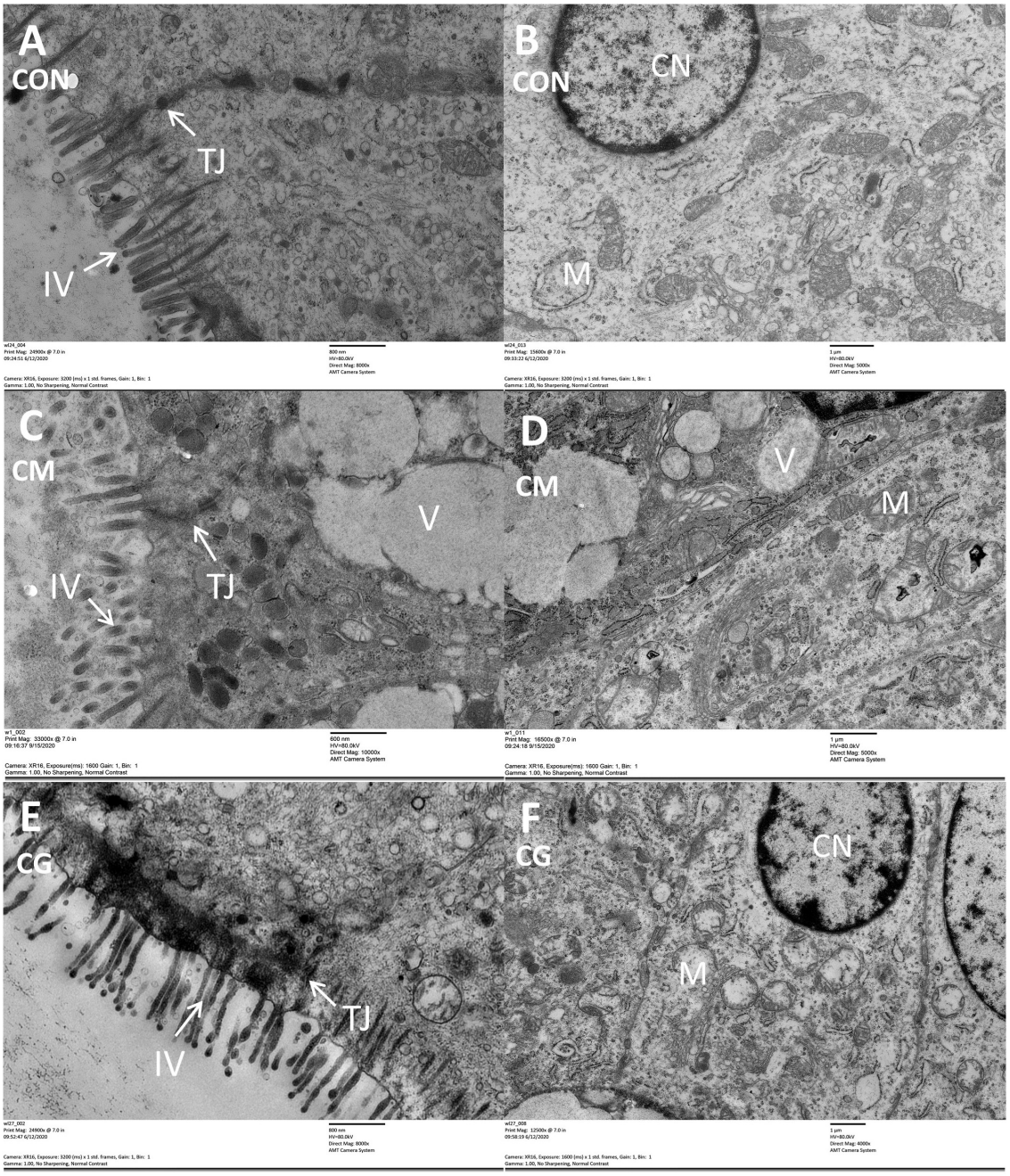


FIGURE 1
The histology of colon tissue among low-grain fed (CON), maize meal high-grain fed (CM), and whole maize high-grain fed (CG) lambs. Comparison of colonic epithelial ultrastructure of junctional complexes among the CON [**(A)** scale bar = 800 nm], CM [**(C)** scale bar = 600 nm], and CG [**(E)** scale bar = 800 nm]. The colonic epithelial ultrastructure in representative CON [**(B)** scale bar = 1 μm], CM [**(D)** scale bar = 1 μm], and CG [**(F)** scale bar = 1 μm]. TJ, tight junction; IV, intestinal villus; CN, cell nucleus; M, mitochondria; V, vacuole.

($P$ = 0.021), glycan biosynthesis and metabolism ($P$ = 0.015), translation ($P$ = 0.028), replication and repair ($P$ = 0.021), cell motility ($P$ = 0.049) in the CM were significantly increased, while the relative abundance of genes encoding xenobiotics biodegradation and metabolism ($P$ = 0.049), signal transduction ($P$ = 0.021) were significantly decreased. And compared with the CM, the CG significantly increased the relative abundance of genes encoding transcription ($P$ = 0.028) and cardiovascular disease ($P$ = 0.037). In contrast, the relative abundance of genes encoding cellular community_eukaryotes ($P$ = 0.021), cellular community_ prokaryotes ($P$ = 0.049), and endocrine system ($P$ = 0.037) were significantly decreased.

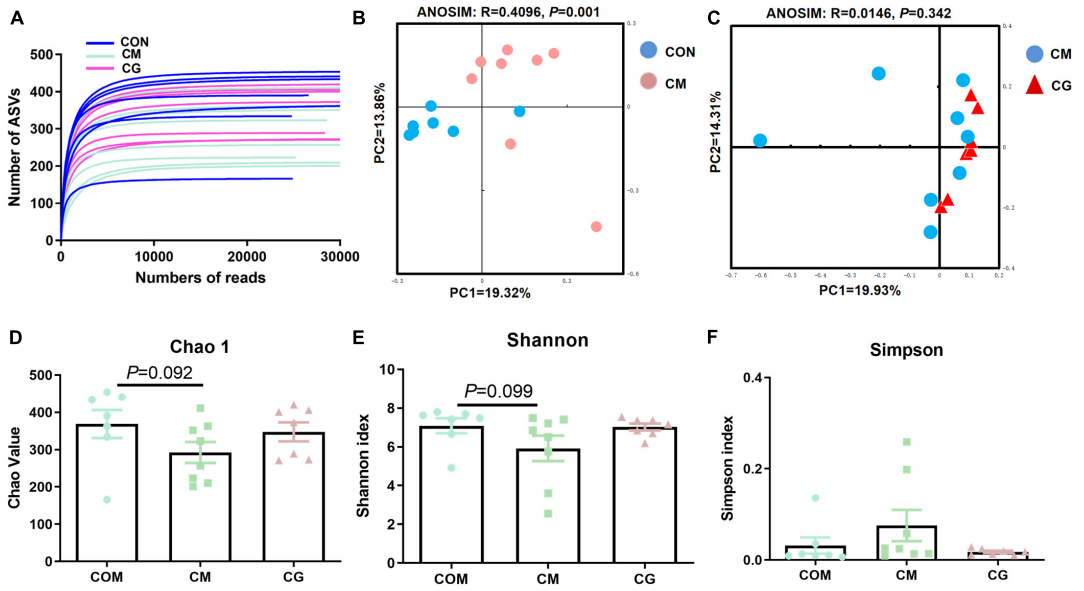


FIGURE 2
Effects of low-grain fed (CON), maize meal high-grain fed (CM), and whole maize high-grain fed (CG) on the diversity of colonic content bacterial structures. **(A)** Bacterial dilution curve of colonic content bacterial community. Horizontal coordinate: the amount of randomly selected sequencing data; vertical coordinate: the number of observed amplicon sequence variants (ASVs). **(B)** Unweighted UniFrac principal coordinate analysis (PCoA) of bacterial communities in the colonic content between the CON and CM. **(C)** Unweighted UniFrac principal coordinate analysis (PCoA) of bacterial communities between the CM and CG. Effects of the CON, CM, and CG on the diversity of colonic content bacterial community: Chao1 **(D)**, Shannon **(E)**, Simpson **(F)**.

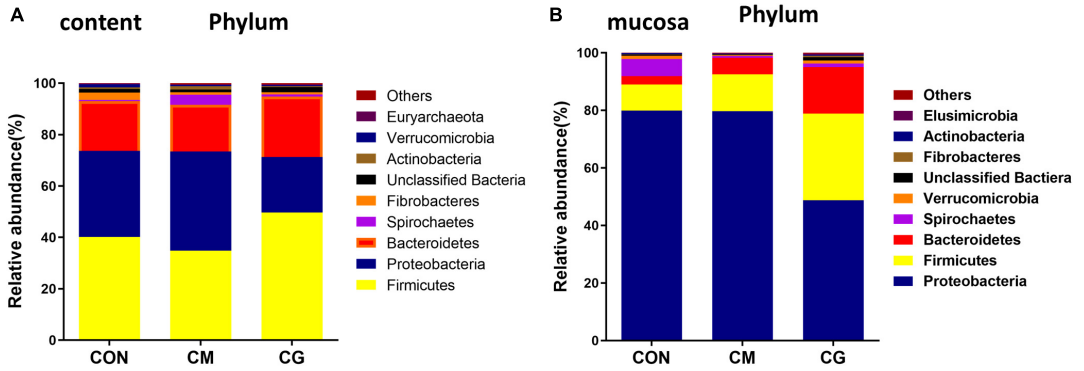


FIGURE 3
Effects of low-grain fed (CON), maize meal high-grain fed (CM), and whole maize high-grain fed (CG) on the relative abundance of phylum level in colonic content and mucosa. **(A)** The distribution of phylum for each sample in colonic content. **(B)** Distribution of the phylum in colonic mucosa among the CON, CM, and CG groups.

# Discussion

## Colonic pH, concentrations of volatile fatty acids, and epithelium morphological structure

In this experiment, the CM tended to have lower colonic pH and greater concentrations of VFAs than the CON, which is similar to the findings of earlier studies on the goat colon (Ye et al., 2016). When a high-grain diet was fed for a long time, excessive carbohydrates could escape from the rumen fermentation and enter the ruminant's intestine (Metzler-Zebeli et al., 2013). Microbes could largely use these fermented carbohydrates in the colon to produce short-chain fatty acids that will likely lower the colonic pH and cause hindgut acidosis as well as inflammation (Tao et al., 2015). The CM and CG did not differ substantially in terms of colonic pH, total VFAs, or any specific short-chain fatty acids.

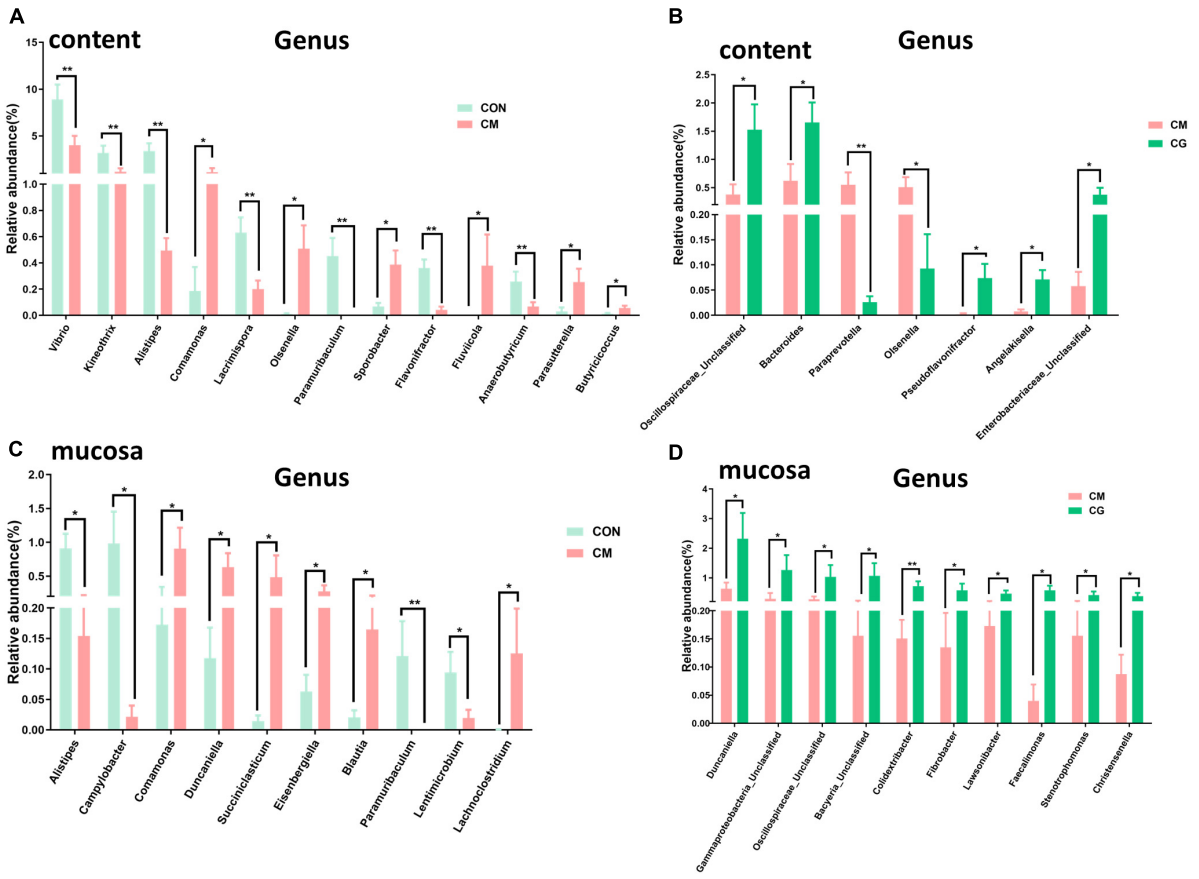


FIGURE 4
Effects of low-grain fed (CON), maize meal high-grain fed (CM), and whole maize high-grain fed (CG) on the genus level bacteria in colonic content and mucosa. **(A)** The comparison of genus level bacterial relative abundance in colonic content between the CON and CM group. **(B)** The differences of bacterial relative abundance in colonic content between the CM and CG group. **(C)** Effects of low-grain fed (CON), and maize meal high-grain fed (CM) on colonic mucosal bacterial relative abundance. **(D)** The colonic mucosal bacteria respond to the maize meal high-grain fed (CM) and whole maize high-grain fed (CG). $*P < 0.05$ indicated that mean values were significantly difference. $**P < 0.01$ indicated that mean values were greatly significantly difference.

The surface layer epithelium, intercellular tight junction, and cell mitochondria of the CM, however, were significantly damaged compared to the CON during microscopic observation of the lamb colonic epithelium, which is in line with the previous investigation in the goat colon (Ye et al., 2016). And the colonic epithelium of the CG was relatively intact. It was speculated that the feeding form of whole maize mitigated the extent of colonic epithelial damage from a high-grain diet and was beneficial for further nutrient absorption and immune function for the lambs.

## Adaptability of the colonic content microbiota in weaned lambs

The gastrointestinal microbiota is vital for ruminants and plays an indispensable role in the digestion and absorption of feed and gastrointestinal development (Yanez-Ruiz et al., 2015). In the present study, we investigated the adaptive response of colonic content microbiota with different particle sizes maize feeding through high-throughput sequencing. The dominant phyla of the three groups were Firmicutes, Bacteroidetes, and Proteobacteria, consistent with the bacterial community previously found in the colonic tissue of male goats (Ye et al., 2016). Several previous studies have shown that phyla Firmicutes and Bacteroidetes function in fermentation, metabolism, and degradation of carbon sources, oligosaccharides (Wetzels et al., 2015), protein and amino acids (Tang et al., 2005).

In comparison to CON, *Parasutterella*, *Comamonas*, and *Butyricicoccus* had considerably greater relative abundance in CM. Although genera *Parasutterella* has been reported in the gastrointestinal tract of cattle, its precise function is yet unclear (Kim and Wells, 2016; Zhang et al., 2017). Previous studies have shown that *Comamonas* and *Butyricicoccus* are important butyrates and hydroxybutyric acid producers (Ys and Yd, 1994; Pryde et al., 2002). And genera *Comamonas* has also been found

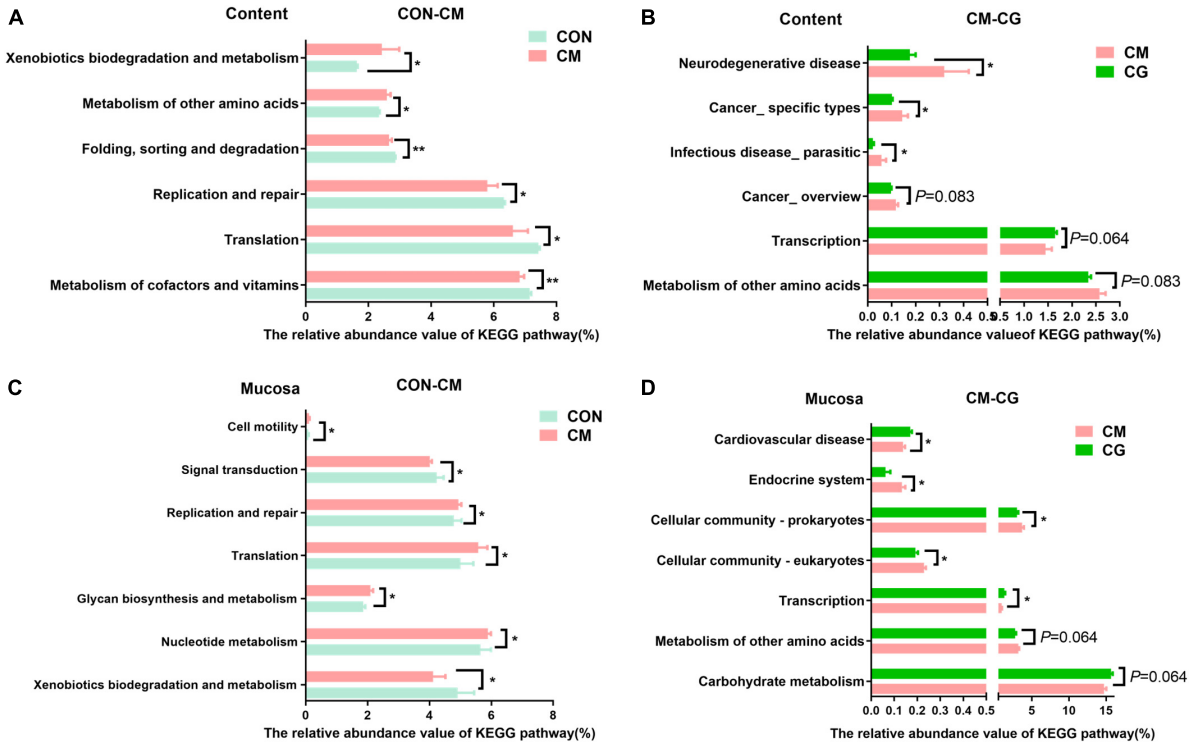


FIGURE 5
Effects of low-grain fed (CON), maize meal high-grain fed (CM) and whole maize high-grain fed (CG) on the function of colonic content and mucosal bacteria. **(A)** Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways of colonic content bacteria in the CON and CM group. **(B)** KECG pathways of colonic content bacteria in the CM and CG group. **(C)** Microbial KEGG pathway of colonic mucosal bacteria in the CON and CM group. **(D)** Microbial KEGG pathway of colonic mucosal bacteria in the CM and CG group. *$P < 0.05$ indicated that mean values were significantly difference. **$P < 0.01$ indicated that mean values were greatly significantly difference.

in the rumen of severe feed restriction and tissue-associated community of heifers (Petri et al., 2013; Fan et al., 2018). High-grain diets allow more soluble carbohydrates to enter the colon as fermentation substrates; therefore, the relative abundance of these butyrate-producing microbiota is elevated.

The relative abundance of the genera *Bacteroides* and *Angelakisella* were substantially greater in the CG compared to the CM. It has been shown that genera *Bacteroides* involve the degradation of starch and fiber (Dias et al., 2018) and can ferment various monosaccharides (Holdeman et al., 1976). Genera *Angelakisella* has been isolated from the human ileum (Mailhe et al., 2017) and is thought to be able to regulate the short-chain fatty acids production (Qiu et al., 2020). The enhancement of these fermentation-associated microbiotas has the opportunity to facilitate energy production and nutrient utilization in the colon.

It was noteworthy that the relative abundance of *Olsenella* was significantly higher in CM than in the CON and CG groups. Petri et al. (2013) reported that *Olsenella* dominates in beef cattle during subacute ruminal acidosis by pyrosequencing techniques. We hypothesize that the feeding strategy of whole maize might lessen the likelihood of acidosis brought on by high-grain diets since the relative quantity of *Olsenella* is lower in CG. However, our measured colonic fermentation profiles did not reveal any discernible differences in VFAs, pH, etc., between the CM and the CG, so follow-up studies are needed to explore the effect of maize particle size on colonic fermentation.

## Adaptability of the colonic mucosal microbiota in weaned lambs

The colonic epithelium, covered with mucosa-associated bacteria, serves as the host's physical and immunological barrier (Kostic et al., 2013). Changes in the relative abundance of these microbiotas may affect the colonic immune functions and even the host's health status (Donaldson et al., 2016). In this experiment, we explored the adaptive response of lamb colonic mucosal microbiota to various feeding regimens. Proteobacteria, Firmicutes, and Bacteroidetes were the most prevalent phyla in all three groups. Phyla Firmicutes and Bacteroidetes were also previously shown to be the dominant phyla in the colonic mucosal flora of goats (Ye et al., 2016) and calves (Malmuthuge et al., 2014). Bacteria belonging to phyla Bacteroidetes and Firmicutes can promote the fermentation of

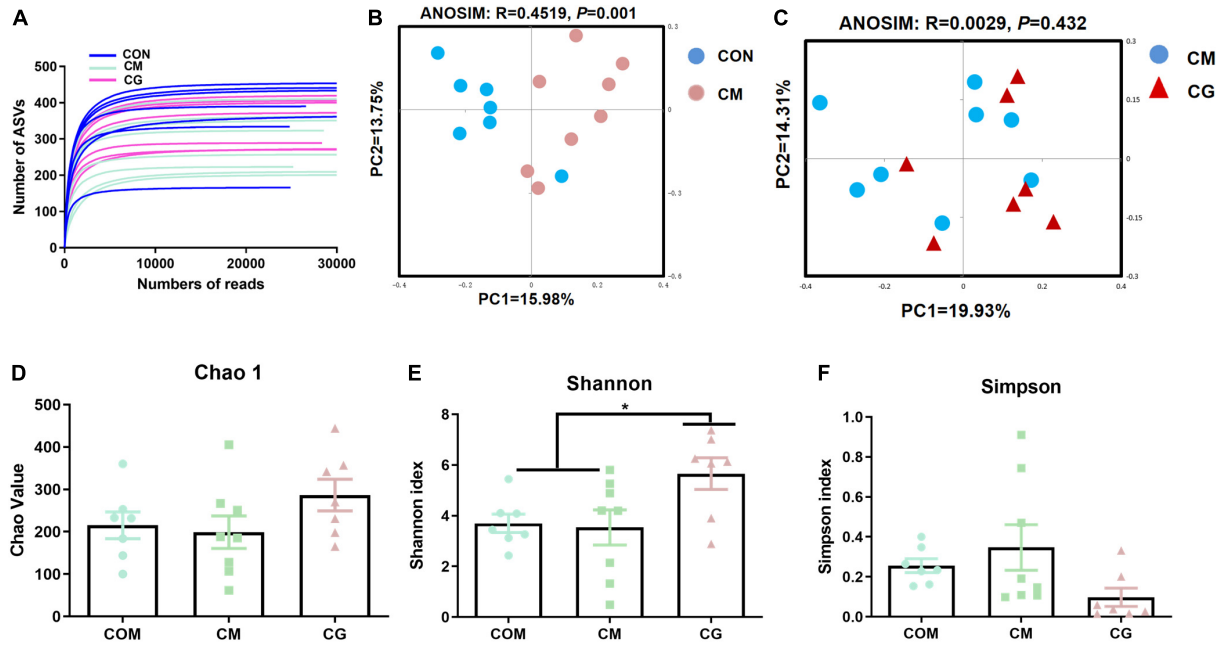


FIGURE 6
Effects of low-grain fed (CON), maize meal high-grain fed (CM), and whole maize high-grain fed (CG) on the diversity of colonic mucosal bacterial structures. **(A)** Bacterial dilution curve of colonic mucosal bacterial community. Horizontal coordinate: the amount of randomly selected sequencing data; vertical coordinate: the number of observed amplicon sequence variants (ASVs). **(B)** Unweighted UniFrac principal coordinate analysis (PCoA) of bacterial communities in the colonic mucosa between the CON and CM. **(C)** Unweighted UniFrac principal coordinate analysis (PCoA) of bacterial communities between the CM and CG. Effects of the CON, CM, and CG on the diversity of colonic mucosal bacterial community: Chao1 **(D)**, Shannon **(E)**, Simpson **(F)**. *$P < 0.05$ indicated that mean values were significantly difference.

plant-based fibrous polysaccharides and regulate the absorption of proteins and other nutrients, which is vital for the host health (Liu et al., 2017).

In line with variations in the colonic content, the relative abundance of the acetate producer *Alistipes* at the genus level was much lower in the CM than it was in the CON (Oliphant and Allen-Vercoe, 2019). As an anaerobic bacterium, *Alistipes* was found mainly in the intestines of healthy animals (Shkoporov et al., 2015). It can regulate intestinal inflammation and relieve disease through short-chain fatty acids (Harrisham et al., 2017). However, some studies showed that *Alistipes* might be associated with colorectal cancer (Moschen et al., 2016), cardiovascular disease (Zuo et al., 2019) and hepatic encephalopathy (Chang et al., 2019). So different species of *Alistipes* may play various roles in host's health (Parker et al., 2020). Thus, further studies are needed to clarify the mechanism. Meanwhile, the relative abundance of genera *Campylobacter* was significantly reduced in the CM than that in the CON. As a common gastrointestinal pathogen, microbes belonging to the genera *Campylobacter* are frequently found in the gastrointestinal tract of ruminants, including cattle and sheep, the relative abundance of which can be as high as 20% in lamb rumen (Diker et al., 1990; Guyard-Nicodeme et al., 2016). Numerous studies have revealed that certain bacteria in this genus are susceptible to low pH levels and that some members of this genus have been linked to localized colonic inflammation in people or animals (Blaser et al., 1980; Grayson et al., 1989; Russell et al., 1993; Chen et al., 2007). In this experiment, there was a decreased trend of pH in the colon of the CM compared to the CON, consistent with the conclusion mentioned above. We also found that the relative abundance of butyrate-producing genera *Succiniclasticum* and *Comamonas* were significantly elevated in the CM compared to the CON (Ys and Yd, 1994; Koike and Kobayashi, 2009). Butyrate has been shown in numerous studies to demonstrate beneficial effects on the repair of the rumen epithelium (Poller et al., 2004; Zhang et al., 2018), the development of the cecum epithelium (Kien et al., 2007; Malhi et al., 2013), and the prevention of the occurrence of colonic inflammation (Inan et al., 2000). The gut mucosa-associated microbiota can change their densities to adapt to the altered host's internal environment (Blaser and Kirschner, 2007). Combined with the results of colon damage observed under transmission electron microscopy, we speculate that the increased relative abundance of genera *Succiniclasticum* and *Comamonas* in the CM may be reactive response of lambs to avoid more severe damage to the colonic epithelium.

Furthermore, the relative abundance of genera *Duncaniella* was significantly increased in the CM relative to the CON, while it further rose considerably in the CG. Although research has shown that the *Duncaniella* genus predominates in the mouse gut, its precise role is yet unknown (Miyake et al., 2020). The

relative abundance of genera *Christensenella*, *Lawsonibacter*, and *Coldextribacter* in the CG were significantly higher than that in the CM. Genera *Christensenella* is a common resident in the rumen and plays an essential role in maintaining the structure and function of the gastrointestinal tract (Jenkins et al., 2015). Genera *Lawsonibacter* can produce butyrate (Sakamoto et al., 2018), which is linked to the prevention of colitis and colorectal cancer (Mcintyre et al., 1993; Archer et al., 1998). Genera *Colidextribacter* has been reported to reduce tissue damage and inflammation through modulating signaling pathways (Mager et al., 2020; Guo et al., 2021). All three genera are associated with body health, and the higher relative abundance shows that the whole maize feeding pattern may have favorable effects on the development and function of the intestinal mucosa. Our microscopic observations of the colonic epithelium also indicated that the colonic epithelium in the CM was severely damaged, whereas the CG was relatively intact and similar to the CON. It was proved again that whole maize could alleviate the damage of colon epithelium caused by high-grain diets and promote the development of lamb colon.

## Functional prediction of colonic content and mucosal microbiome

Intestinal bacterial community plays an important role in the immune system and maintaining the body's health (Hooper et al., 2012). According to earlier research, diet is a key factor in the interaction between the host genome and the microbiota (Holmes et al., 2012). PICRUSt was also used to investigate the metagenomic and potential functions of the colonic content and mucosal microbiota in lambs in this experiment. We found considerably higher relative abundance of genes encoding degradation and metabolism in the colonic content microbiota of the CM compared to the CON, which is likely a result of larger quantity of degradable carbohydrates entering the colon from the high-grain diet. Meanwhile, we discovered a significant decrease in genes encoding various diseases in the colonic microbiota of the CG than that in CM. This alteration in the relative abundance of digestion-related genes hypothesized that feeding the whole maize high-grain diet would reduce the incidence of intracolonic diseases resulting from the high-grain diet.

## Adaptation of differences between colonic content and mucosa microbiome to different grain sizes

In this experiment, 183 genera of content microbiota were detected, including 7 genera with significant difference in the CM and the CG; and 159 genera of mucosal microbiota were detected, including 18 genera with significant difference between the CM and the CG. The results revealed that the impact of various maize forms on microbiota in lamb colonic mucosa was more substantial than that in colonic content, which may be related to the two colonic areas' various activities. The microbiota in the colonic content plays a vital role in the

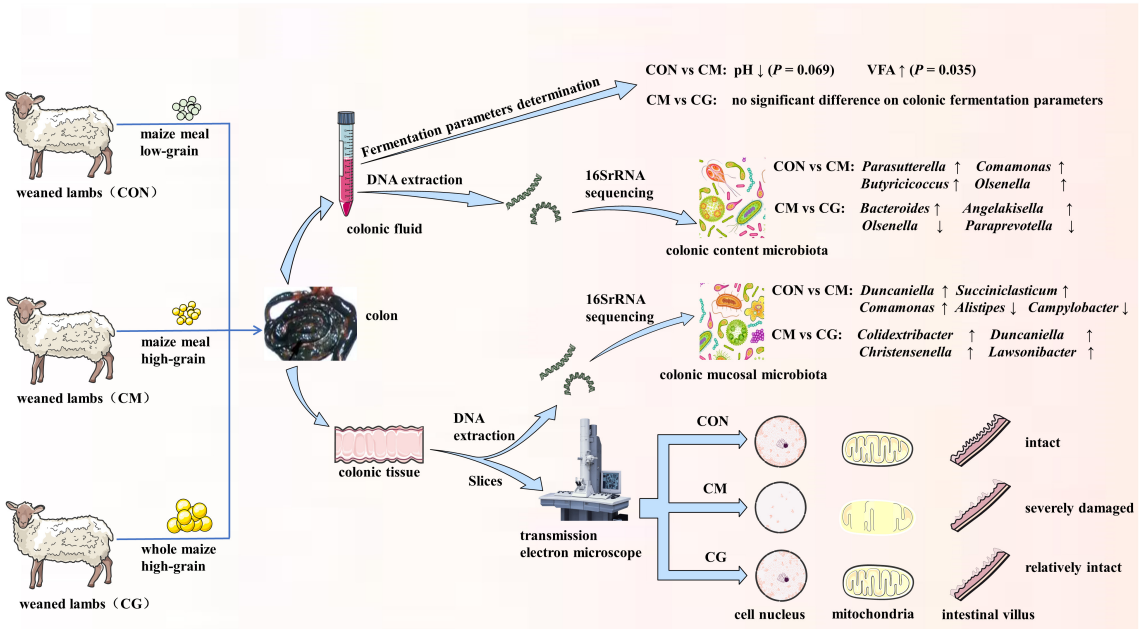


FIGURE 7
The schematic diagram of experimental design and main results in this study.

digestion and metabolism of nutrients. In contrast, the colonic mucosal microbiota, as an important component of the colonic epithelial barrier, is mainly related to the maintenance of the colonic epithelial barrier (Nagy-Szakal et al., 2012; Kostic et al., 2013). Pryde et al. (1999) found that microbiota colonizing in the pig's colonic wall differed strongly from that in the colonic and cecal lumen. Microbiota in the intestinal wall of animals plays a potential function in the interaction with the host (Bengmark, 1996); hence it is hypothesized that the changes made by microbiota in the intestinal wall in response to external stimuli are more pronounced than in the intestinal lumen. Second, the more sensitive response of colonic mucosal microbiota to the maize form may be related to the colonic structure. The surface of colonic mucosa is smooth and is dwelled by numerous microbiota. The microbiota in the goats' hind gut mucosa has previously been demonstrated to be extremely responsive to diet (Liu et al., 2014; Ye et al., 2016). Therefore, the fermentation environment in the colon follows when the maize form changes, which will further lead to the relative abundance changes of the bacterial community in the colonic mucosa to achieve homeostatic reconstruction. Third, having more carbohydrates in the colon leads to an increase in the osmotic pressure of the colon wall, which might impair the structure of the colonic mucosa by increasing the permeability of the colonic epithelium (Gressley et al., 2011), and further leads to the altered mucosal microbial community to a deeper extent than the content microbiota. The increased permeability of the intestinal tract was speculated to cause the absorption of toxins such as histamine in the content (Gressley et al., 2011). We experimentally observed that a prolonged high-grain diet significantly led to damage to the colonic epithelium, and the damage was mitigated in the colonic mucosa of lambs fed a whole-grain diet.

## Conclusion

We found that feeding weaned lambs a whole-maize, high-grain diet may reorganize the colon's bacterial population and restore some of harm brought on by a high-grain diet (**Figure 7**). Furthermore, microbiota associated with the colonic mucosal region responded greater than those in the colonic content to different maize forms. This study provides theoretical guidance for the application of whole maize high-grain diet in practical production.

## Data availability statement

The sequencing data have been deposited in the NCBI Sequence Read Archive (SRA) database under the accession code: SRP387601.

## Ethics statement

The animal experiment was approved by the Animal Protection and Use Committee of Nanjing Agricultural University (Authorisation SYXK(Su)2019-0074) and was performed following the Regulations for the Administration of Affairs Concerning Experimental Animals (The State Science and Technology Commission of P. R. China, 1988).

## Author contributions

CC and YY carried out most of the experiment including animal care, VFA analyses, DNA isolation, and histological measurements. CC and GB were responsible for 16S rRNA high throughput data processing, analyses, interpretation, and manuscript preparation. GB contributed to the conception of the project. 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/fmicb.2022.1018284/full#supplementary-material

# References


Abecia, L., Martín-García, I., Martínez, G., Newbold, C. J., and Yáñez-Ruiz, D. R. (2013). Nutritional intervention in early life to manipulate rumen microbial colonization and methane output by kid goats postweaning. *J. Anim. Sci.* 91, 4832–4840. doi: 10.2527/jas.2012-6142

AOAC, (2007). *Official methods of analysis*, 18th Edn. Gaithersburg, MD: Association of Official Analytical Chemists.

Archer, S. Y., Meng, S., Shei, A., and Hodin, R. A. (1998). WAF1) is required for butyrate-mediated growth inhibition of human colon cancer cells. *Proc. Natl. Acad. Sci. U.S.A.* 95, 6791–6796. doi: 10.1073/pnas.95.12.6791

Bengmark, S. (1996). Econutrition and health maintenance–A new concept to prevent GI inflammation, ulceration and sepsis. *Clin. Nutr.* 15, 1–10. doi: 10.1016/s0261-5614(96)80253-6

Blaser, M. J., and Kirschner, D. (2007). The equilibria that allow bacterial persistence in human hosts. *Nature* 449, 843–849. doi: 10.1038/nature06198

Blaser, M. J., Hardesty, H. L., Powers, B., and Wang, W. L. (1980). Survival of Campylobacter fetus subsp. jejuni in biological milieus. *J. Clin. Microbiol.* 11, 309–313. doi: 10.1128/jcm.11.4.309-313.1980

Callahan, B. J., McMurdie, P. J., Rosen, M. J., Han, A. W., Johnson, A. J., and Holmes, S. P. (2016). DADA2: High-resolution sample inference from Illumina amplicon data. *Nat. Methods* 13, 581–583. doi: 10.1038/nmeth.3869

Callison, S. L., Firkins, J. L., and Eastridge, M. L. (2001). Site of nutrient digestion by dairy cows fed corn of different particle sizes or steam-rolled. *J. Dairy Sci.* 84, 1458–1467. doi: 10.3168/jds.S0022-0302(01)70179-8

Chang, M. S., Lin, Y. F., Chen, K. F., Ke, H. M., and Hsieh, S. Y. (2019). Predicting clinical outcomes of cirrhosis patients with hepatic encephalopathy from the fecal microbiome. *Cell. Mol. Gastroenterol. Hepatol.* 8, 301–318. doi: 10.1016/j.jcmgh.2019.04.008

Chen, M. L., Ge, Z., Fox, J. G., and Schauer, D. B. (2007). Disruption of tight junctions and induction of proinflammatory cytokine responses in colonic epithelial cells by *Campylobacter jejuni*. *Infect. Immun.* 74, 6581–6589. doi: 10.1128/iai.00958-06

Chester-Jones, H., Ziegler, D. M., and Meiske, J. C. (1991). Feeding whole or rolled corn with pelleted supplement to holstein steers from weaning to 190 Kilograms1. *J. Dairy Sci.* 74, 1765–1771. doi: 10.3168/jds.S0022-0302(91)78340-9

Dias, J., Marcondes, M. I., Motta de Souza, S., Cardoso da Mata e Silva, B., Noronha, M. F., Resende, R. T., et al. (2018). Bacterial community dynamics across the gastrointestinal tracts of dairy calves during preweaning development. *Appl. Environ. Microbiol.* 84:e02675-17. doi: 10.1128/AEM.02675-17

Diker, K. S., Diker, S., and Ozlem, M. B. (1990). Bovine diarrhea associated with *Campylobacter hyointestinalis*. *J. Vet. Med.* 37, 158–160. doi: 10.1111/j.1439-0450.1990.tb01040.x

Donaldson, G. P., Lee, S. M., and Mazmanian, S. K. (2016). Gut biogeography of the bacterial microbiota. *Nat. Rev. Microbiol.* 14, 20–32. doi: 10.1038/nrmicro3552

Fan, H., Xue, Y., Guo, C., Liu, J., and Mao, S. (2018). The response of ruminal fermentation, epithelium-associated microbiota, and epithelial barrier function to severe feed restriction in pregnant ewes. *J. Anim. Sci.* 10, 4293–4305. doi: 10.1093/jas/sky306

Grayson, M. L., Eliopoulos, G. M., Ferraro, M. J., and Moellering, R. C. Jr. (1989). Effect of varying pH on the susceptibility of Campylobacter pylori to antimicrobial agents. *Eur. J. Clin. Microbiol. Infect. Disease.* 8, 888–889. doi: 10.1007/BF01963775

Gressley, T. F., Hall, M. B., and Armentano, L. E. (2011). Ruminant nutrition symposium: Productivity, digestion, and health responses to hindgut acidosis in ruminants. *J. Anim. Sci.* 89, 1120–1130. doi: 10.2527/jas.2010-3460

Guo, W., Xiang, Q., Mao, B., Tang, X., and Chen, W. (2021). Protective effects of microbiome-derived inosine on lipopolysaccharide-induced acute liver damage and inflammation in mice via mediating the TLR4/NF-κB Pathway. *J. Agric. Food Chem.* 69, 7619–7628. doi: 10.1021/acs.jafc.1c01781

Guyard-Nicodeme, M., Keita, A., Quesne, S., Amelot, M., Poezevara, T., Le Berre, B., et al. (2016). Efficacy of feed additives against Campylobacter in live broilers during the entire rearing period. *Poultry Sci.* 95, 298–305. doi: 10.3382/ps/pev303

Harrisham, K., Chandrani, D., and Mande, S. S. (2017). In silico analysis of putrefaction pathways in bacteria and its implication in colorectal cancer. *Front. Microbiol.* 8:2166. doi: 10.3389/fmicb.2017.02166

Holdeman, L. V., Good, I. J., and Moore, W. E. (1976). Human fecal flora: Variation in bacterial composition within individuals and a possible effect of emotional stress. *Appl. Environ. Microbiol.* 31, 359–375. doi: 10.1128/aem.31.3.359-375.1976

Holmes, E., Kinross, J., Gibson, G. R., Burcelin, R., Jia, W., Pettersson, S., et al. (2012). Therapeutic modulation of microbiota-host metabolic interactions. *Sci. Transl. Med.* 4, rv136–rv137. doi: 10.1126/scitranslmed.3004244

Hooper, L. V., Littman, D. R., and Macpherson, A. J. (2012). Interactions between the microbiota and the immune system. *Science* 336, 1268–1273. doi: 10.1126/science.1223490

Hoover, W. H. (1978). Digestion and absorption in the hindgut of ruminants. *J. Anim. Sci.* 46, 1789–1799. doi: 10.2527/jas1978.4661789x

Humer, E., and Zebeli, Q. (2017). Grains in ruminant feeding and potentials to enhance their nutritive and health value by chemical processing. *Anim. Feed Sci. Technol.* 226, 133–151. doi: 10.1016/j.anifeedsci.2017.02.005

Inan, M. S., Rasoulpour, R. J., Yin, L., Hubbard, A. K., Rosenberg, D. W., and Giardina, C. (2000). The iuminal short-chain fatty acid butyrate modulates NF-kappaB activity in a human colonic epithelial cell line. *Gastroenterology* 118, 724–734. doi: 10.1016/s0016-5085(00)70142-9

Jenkins, S. N., Waite, I. S., Mansfield, J., Kim, J. C., and Pluske, J. R. (2015). Relationships between diets different in fibre type and content with growth, *Escherichia coli* shedding, and faecal microbial diversity after weaning. *Anim. Prod. Sci.* 55, 1451–1451. doi: 10.1071/ANv55n12Ab125

Jiao, J., Lu, Q., Forster, R. J., Zhou, C., Wang, M., Kang, J., et al. (2016). Age and feeding system (supplemental feeding versus grazing) modulates colonic bacterial succession and host mucosal immune maturation in goats. *J. Anim. Sci.* 94, 2506–2518. doi: 10.2527/jas.2015-0081

Kelly, D., and Coutts, A. G. P. (2000). Development of digestive and immunological function in neonates: Role of early nutrition. *Livestock Prod. Sci.* 66, 161–167. doi: 10.1016/S0301-6226(00)00223-2

Kien, C. L., Blauwiekel, R., Bunn, J. Y., Jetton, T. L., Frankel, W. L., and Holst, J. J. (2007). Cecal infusion of butyrate increases intestinal cell proliferation in piglets. *J. Nutr.* 137, 916–922. doi: 10.1093/jn/137.4.916

Kim, M., and Wells, J. E. (2016). A meta-analysis of bacterial diversity in the feces of cattle. *Curr. Microbiol.* 72, 145–151. doi: 10.1007/s00284-015-0931-6

Kittelmann, S., Seedorf, H., Walters, W. A., Clemente, J. C., Knight, R., Gordon, J. I., et al. (2013). Simultaneous amplicon sequencing to explore co-occurrence patterns of bacterial, archaeal and eukaryotic microorganisms in rumen microbial communities. *PLoS One* 8:e47879. doi: 10.1371/journal.pone.0047879

Koike, S., and Kobayashi, Y. (2009). Fibrolytic rumen bacteria: Their ecology and functions. *Asian Austr. J. Anim. Sci.* 40, 1141–1147. doi: 10.5713/ajas.2009.r.01

Kostic, A. D., Howitt, M. R., and Garrett, W. S. (2013). Exploring host-microbiota interactions in animal models and humans. *Genes Dev.* 27, 701–718. doi: 10.1101/gad.212522.112

Liu, J., Bian, G., Sun, D., Zhu, W., and Mao, S. (2017). Starter feeding altered ruminal epithelial bacterial communities and some key immune-related genes' expression before weaning in lambs. *J. Anim. Sci.* 95, 910–921. doi: 10.2527/jas.2016.0985

Liu, J., Xu, T., Zhu, W., and Mao, S. (2014). High-grain feeding alters caecal bacterial microbiota composition and fermentation and results in caecal mucosal injury in goats. *Br. J. Nutr.* 112, 416–427. doi: 10.1017/S0007114514000993

Lopez, M. C., Estelles, F., Moya, V. J., and Fernandez, C. (2014). Use of dry citrus pulp or soybean hulls as a replacement for corn grain in energy and nitrogen partitioning, methane emissions, and milk performance in lactating Murciano-Granadina goats. *J. Dairy Sci.* 97, 7821–7832. doi: 10.3168/jds.2014-8424

Mager, L., Burkhard, R., Pett, N., Cooke, N., Brown, K., Ramay, H., et al. (2020). Microbiome-derived inosine modulates response to checkpoint inhibitor immunotherapy. *Science* 369, 1481–1489. doi: 10.1126/science.abc3421

Mailhe, M., Ricaboni, D., Vitton, V., Cadoret, F., Fournier, P. E., and Raoult, D. (2017). 'Angelakisella massiliensis' gen. nov., sp. nov., a new bacterial species isolated from human ileum. *New Microb. New Infect.* 16, 51–53. doi: 10.1016/j.nmni.2017.01.003

Malhi, M., Gui, H., Yao, L., Aschenbach, J. R., Gabel, G., and Shen, Z. (2013). Increased papillae growth and enhanced short-chain fatty acid absorption in the rumen of goats are associated with transient increases in cyclin D1 expression after ruminal butyrate infusion. *J. Dairy Sci.* 96, 7603–7616. doi: 10.3168/jds.2013-6700

Malmuthuge, N., Griebel, P. J., and Guan, L. L. (2014). Taxonomic identification of commensal bacteria associated with the mucosa and digesta throughout the gastrointestinal tracts of preweaned calves. *Appl. Environ. Microbiol.* 80, 2021–2028. doi: 10.1128/AEM.03864-13

Mao, S., Zhang, R., Wang, D., and Zhu, W. (2012). The diversity of the fecal bacterial community and its relationship with the concentration of volatile fatty acids in the feces during subacute rumen acidosis in dairy cows. *BMC Vet. Res.* 8:237. doi: 10.1186/1746-6148-8-237

Mcintyre, A., Gibson, P. R., and Young, G. P. (1993). Butyrate production from dietary fibre and protection against large bowel cancer in a rat model. *Gut* 34, 386–391. doi: 10.1136/gut.34.3.386

Metzler-Zebeli, B. U., Schmitz-Esser, S., Klevenhusen, F., Podstatzky-Lichtenstein, L., Wagner, M., and Zebeli, Q. (2013). Grain-rich diets differently alter ruminal and colonic abundance of microbial populations and lipopolysaccharide in goats. *Anaerobe* 20, 65–73. doi: 10.1016/j.anaerobe.2013.02.005

Miyake, S., Ding, Y., Soh, M., Low, A., and Seedorf, H. (2020). Cultivation and description of Duncaniella dubosii sp. nov., *Duncaniella freteri* sp. nov. and emended description of the species Duncaniella muris. *Int. J. Syst. Evol. Microbiol.* 70, 3105–3110. doi: 10.1099/ijsem.0.004137

MOA (2004). *Feeding standard of meat-producing sheep and goats (NY/T 816-2004)*. Beijing: MOA.

Moschen, A., Gerner, R., Wang, J., Klepsch, V., Adolph, T., Reider, S., et al. (2016). Lipocalin 2 protects from inflammation and tumorigenesis associated with gut microbiota alterations. *Cell Host Microbe.* 19, 455–469. doi: 10.1016/j.chom.2016.03.007

Nagy-Szakal, D., Ross, M. C., Dowd, S. E., Mir, S. A., Schaible, T. D., Petrosino, J. F., et al. (2012). Maternal micronutrients can modify colonic mucosal microbiota maturation in murine offspring. *Gut Microbes* 3, 426–433. doi: 10.4161/gmic.20697

Oliphant, K., and Allen-Vercoe, E. (2019). Macronutrient metabolism by the human gut microbiome: Major fermentation by-products and their impact on host health. *Microbiome* 7, 1–15. doi: 10.1186/s40168-019-0704-8

Parker, B. J., Wearsch, P. A., Veloo, A., and Rodriguez-Palacios, A. (2020). The genus alistipes: Gut bacteria with emerging implications to inflammation, cancer, and mental health. *Front. Immunol.* 11:906. doi: 10.3389/fimmu.2020.00906

Petri, R. M., Schwaiger, T., Penner, G. B., Beauchemin, K. A., Forster, R. J., Mckinnon, J. J., et al. (2013). Changes in the rumen epimural bacterial diversity of beef cattle as affected by diet and induced ruminal acidosis. *Appl. Environ. Microbiol.* 79, 3744–3755. doi: 10.1128/AEM.03983-12

Plaizier, J. C., Krause, D. O., Gozho, G. N., and Mcbride, B. W. (2008). Subacute ruminal acidosis in dairy cows: The physiological causes, incidence and consequences. *Vet. J.* 176, 21–31. doi: 10.1016/j.tvjl.2007.12.016

Poller, L., Keown, M., Chauhan, N., van den Besselaar, A. M., Tripodi, A., Shiach, C., et al. (2004). European concerted action on anticoagulation. quality assessment of the coaguchek mini and TAS PT-NC point-of-care whole-blood prothrombin time monitors. *Clin. Chem.* 50, 537–544. doi: 10.1373/clinchem.2003.019653

Pryde, S., Duncan, S., Hold, G., Stewart, C., and Flint, H. (2002). The microbiology of butyrate formation in the human colon. *FEMS Microbiol. Lett.* 217, 133–139. doi: 10.1016/s0378-1097(02)01106-0

Pryde, S. E., Richardson, A. J., Stewart, C. S., and Flint, H. J. (1999). Molecular analysis of the microbial diversity present in the colonic wall, colonic lumen, and cecal lumen of a pig. *Appl. Environ. Microbiol.* 65, 5372–5377. doi: 10.1128/aem.65.12.5372-5377.1999

Qin, W. L. (1983). Determination of rumen volatile fatty acids by means of gas chromatography. *J. Nanjing Agric. Univ.* 4, 110–116. doi: 10.3168/jds.S0022-0302(68)86962-0

Qiu, X., Macchietto, M. G., Liu, X., Lu, Y., Ma, Y., Guo, H., et al. (2020). Identification of gut microbiota and microbial metabolites regulated by an antimicrobial peptide lipocalin 2 in high fat diet-induced obesity. *Int. J. Obesity* 45, 143–154. doi: 10.1038/s41366-020-00712-2

Russell, R. G., O'Donnoghue, M., Blake, D. C. Jr., Zulty, J., and DeTolla, L. J. (1993). Early colonic damage and invasion of Campylobacter jejuni in experimentally challenged infant *Macaca mulatta*. *J. Infect. Disease.* 168, 210–215. doi: 10.1093/infdis/168.1.210

Sakamoto, M., Iino, T., Yuki, M., and Ohkuma, M. (2018). Lawsonibacter asaccharolyticus gen. nov., sp. nov., a butyrate-producing bacterium isolated from human faeces. *Int. J. Syst. Evol. Microbiol.* 68, 2074–2081. doi: 10.1099/ijsem.0.002800

Shkoporov, A. N., Chaplin, A. V., Khokhlova, E. V., Shcherbakova, V. A., Motuzova, O. V., Bozhenko, V. K., et al. (2015). Alistipes inops sp. nov. and Coprobacter secundus sp. nov., isolated from human faeces. *Int. J. Syst. Evol. Microbiol.* 65:4580. doi: 10.1099/ijsem.0.000617

Steele, M. A., Penner, G. B., Chaucheyras-Durand, F., and Guan, L. L. (2016). Development and physiology of the rumen and the lower gut: Targets for improving gut health. *J. Dairy Sci.* 99, 4955–4966. doi: 10.3168/jds.2015-10351

Tang, Y., Shigematsu, T., Morimura, S., and Kida, K. (2005). Microbial community analysis of mesophilic anaerobic protein degradation process using bovine serum albumin (BSA)-fed continuous cultivation. *J. Biosci. Bioeng.* 99, 150–164. doi: 10.1263/jbb.99.150

Tao, S., Tian, J., Cong, R., Sun, L., Duanmu, Y., Dong, H., et al. (2015). Activation of cellular apoptosis in the caecal epithelium is associated with increased oxidative reactions in lactating goats after feeding a high-concentrate diet. *Exp. Physiol.* 100, 278–287. doi: 10.1113/expphysiol.2014.083352

Vanhatalo, A., and Ketoja, E. (1995). The role of the large intestine in post-ruminal digestion of feeds as measured by the mobile-bag method in cattle. *Br. J. Nutr.* 73, 491–505. doi: 10.1079/BJN19950054

Wang, Y., Lei, X., Liu, J., Zhu, W., and Mao, S. (2017). A high grain diet dynamically shifted the composition of mucosa-associated microbiota and induced mucosal injuries in the colon of sheep. *Front. Microbiol.* 8:2080. doi: 10.3389/fmicb.2017.02080

Wetzels, S. U., Mann, E., Metzler-Zebeli, B. U., Wagner, M., Klevenhusen, F., Zebeli, Q., et al. (2015). Pyrosequencing reveals shifts in the bacterial epimural community relative to dietary concentrate amount in goats. *J. Dairy Sci.* 98, 5572–5587. doi: 10.3168/jds.2014-9166

Yanez-Ruiz, D. R., Abecia, L., and Newbold, C. J. (2015). Manipulating rumen microbiome and fermentation through interventions during early life: A review. *Front. Microbiol.* 6:1133. doi: 10.3389/fmicb.2015.01133

Ye, H., Liu, J., Feng, P., Zhu, W., and Mao, S. (2016). Grain-rich diets altered the colonic fermentation and mucosa-associated bacterial communities and induced mucosal injuries in goats. *Sci. Rep.* 6:20329. doi: 10.1038/srep20329

Ys, A., and Yd, B. (1994). Biosynthesis and characterization of poly(3-hydroxybutyrate- co -4-hydroxybutyrate) by *Comamonas acidvorans*. *Stud. Poly. Sci.* 12, 394–399. doi: 10.1016/b978-0-444-81708-2.50042-7

Zhang, J., Xu, C., Huo, D., Hu, Q., and Peng, Q. (2017). Comparative study of the gut microbiome potentially related to milk protein in murrah buffaloes (*Bubalus bubalis*) and Chinese Holstein cattle. *Sci. Rep.* 7:42189. doi: 10.1038/srep42189

Zhang, K., Meng, M., Gao, L., Tu, Y., and Bai, Y. (2018). Sodium butyrate improves high-concentrate-diet-induced impairment of ruminal epithelium barrier function in goats. *J. Agric. Food Chem.* 66, 8729–8736. doi: 10.1021/acs.jafc.8b03108

Zuo, K., Li, J., Li, K., Hu, C., Gao, Y., Chen, M., et al. (2019). Disordered gut microbiota and alterations in metabolic patterns are associated with atrial fibrillation. *GigaScience* 8:058. doi: 10.1093/gigascience/giz058

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# Modulating gut microbiota and metabolites with dietary fiber oat β-glucan interventions to improve growth performance and intestinal function in weaned rabbits

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The effect of oat β-glucan on intestinal function and growth performance of weaned rabbits were explored by multi-omics integrative analyses in the present study. New Zealand White rabbits fed oat β-glucan [200 mg/kg body weight (BW)] for 4 weeks, and serum markers, colon histological alterations, colonic microbiome, colonic metabolome, and serum metabolome were measured. The results revealed that oat β-glucan increased BW, average daily gain (ADG), average daily food intake (ADFI), and decreased serum tumor necrosis factor-α (TNF-α) interleukin-1β (IL-1β), and lipopolysaccharide (LPS) contents, but did not affect colonic microstructure. Microbiota community analysis showed oat β-glucan modulated gut microbial composition and structure, increased the abundances of beneficial bacteria *Lactobacillus*, *Prevotellaceae_UCG-001*, *Pediococcus*, *Bacillus*, etc. Oat β-glucan also increased intestinal propionic acid, valeric acid, and butyric acid concentrations, decreased lysine and aromatic amino acid (AAA) derivative contents. Serum metabolite analysis revealed that oat β-glucan

altered host carbohydrate, lipid, and amino acid metabolism. These results suggested that oat β-glucan could inhibit systemic inflammation and protect intestinal function by regulating gut microbiota and related metabolites, which further helps to improve growth performance in weaned rabbits.

KEYWORDS

**oat β-glucan, gut microbiota, metabolites, intestinal function, growth performance**

## 1 Introduction

Young animals, especially weaned animals are vulnerable to pathogens due to the immaturity of the digestive system and immune system, which always causing intestinal mucosal damage and gut microbial dysbiosis, and further leading to growth retardation or even death of animals (Ren et al., 2018). Prebiotics are considered as substrates that are selectively utilized by host microorganisms, in the process host gut microbiota also be restructured, increasing beneficial bacteria abundances but inhibiting the growth of pathogens (Sanders et al., 2019). In addition, consumption of some prebiotics, like dietary fiber, could produce short-chain fatty acids (SCFAs), which can act to improve barrier function in the gut and modulate immune cell activity (van der Hee and Wells, 2021; Johnson et al., 2022). Based on this, prebiotics can be used as non-antimicrobial alternative feed additives in young animal rearing to promote host health.

Beta-glucan is regarded as a dietary fiber that has prebiotic potential, and cereals, especially oat, are the main source of β-glucan (Bai et al., 2021a). The U.S. Food and Drug Administration, the Joint Health Claims Initiative, and the French Food Safety Agency have approved health claims for oat β-glucan as it presents a broad range of biological activities, such as lowering blood cholesterol, anti-diabetes, immunomodulatory, and anti-inflammatory activities (Jayachandran et al., 2018; Yu et al., 2022). Nowadays, accumulating researches have also suggested that it has potential health promoting properties on intestinal function. For example, supplementation with whole oat meal or β-glucan induced increase in intestinal $Na^+$ $K^+$-ATPase activity, $Ca^{2+}$ $Mg^{2+}$-ATPase activity, and energy charge in rats (Zhang et al., 2012). Several studies have also proposed its role in gut development and microbiota regulation; a previous study suggested that oat β-glucan treatment not only increased duodenum and ileum villus height/crypt depth ratios but also showed higher expression levels of genes associated with intestinal barrier function (*zonula occludens 1* and *claudin 1*) in weaned pigs (Wu et al., 2018). Additionally, dietary oat-derived β-glucan was found to alter the colonic bacterial community of mice, which increasing the phyla Bacteroidetes and Proteobacteria, while decreasing the phyla Firmicutes in BALB/c mice (Luo et al., 2017).

The functionalities of oat β-glucan are inextricably related to gut microbiota and its catabolite. Bai et al. (2021b) found that oat β-glucan ameliorated dextran sulfate sodium (DSS)-induced colitis in mice simultaneously by regulating gut-derived SCFAs and microbial metabolic biomarkers. Another study pointed out that oat β-glucan treatment could modulate intestinal microbiota toward a healthier profile, promote the growth of *Lactobacillus* and the release of butyrate, which could ameliorate inflammation and consequently improve renal function in diabetic nephropathy rats (Wang et al., 2022). However, to our known, only a few studies reported the role of oat β-glucan fermentation in the intestinal function. Shen et al. (2012) found oat β-glucan exerts favorable effects on improving intestinal function and health by promoting intestinal SCFAs production and improving colonic *Bifidobacterium* and *Lactobacillus*, lowering colonic *Enterobacteriaceae* counts, but their study only focused on a few specific bacteria and metabolites due to the limitation of research technology. As intestinal function is highly associated with animal growth (Zou et al., 2022), there is still need a comprehensive study to systemically explore whether oat β-glucan exerts its beneficial effects on intestinal function *via* microbiota and its metabolites, as well as its effect on the overall metabolism of the body.

Rabbit is an animal model that is widely used in clinical trials. It does not secrete cellulolytic enzymes by itself, and mainly depends on bacteria in the hindgut for the utilization and digestion of dietary fiber (Liu et al., 2022). On the other hand, rabbit meat is thought to be a functional food as it rich in highly digestible protein, essential amino acids, unsaturated fatty acid, calcium and phosphorus, while contains less fat, cholesterol and sodium when compared to traditional red meat. These nutrients properties of rabbit meat also meet people's demand of having a healthy lifestyle, thus promoting the consumption of rabbit meat in the worldwide (Nasr et al., 2017; Krunt et al., 2022). The current study therefore used a weaned rabbit model to evaluate the effect of oat β-glucan on growth and intestinal development and to determine whether this effect was related to gut microbiota and related metabolites using integrated omics analyses, including 16S rDNA sequencing analysis and metabolomics analysis. Our

study will provide a better understanding of the role of oat β-glucan in intestinal function, meanwhile, it is no longer limited to local gut microbiota metabolism, but expanded to host metabolism. The study also helps to clarify the effect of oat β-glucan on the growth of weaned rabbits, promote the application of oat β-glucan as a non-antimicrobial alternative feed additive in rabbit or other young animals rearing.

# 2 Materials and methods

## 2.1 Animals, management, and experiment design

This study was conducted in strict accordance with the guidelines for the Care and Use of Laboratory Animals of China and all procedures were approved by the Animal Care and Use Committee of Sichuan Agricultural University (No. DYY-2018203039). Twenty-four 3 week old weaned female New Zealand white rabbits were obtained from Chengdu Dossy Experimental Animals Co., Ltd., (Chengdu, China) and individually housed in cages at a controlled temperature (22 ± 2°C), with a 12 h light/dark period in the Laboratory Animal Center of Sichuan Agriculture University (Yaan, China), rabbits have free access to water and standard diet. During 1 week of acclimatization, the rabbits were fed a basal diet. The rabbits were randomly divided into two groups after acclimation. Rabbits in the control group ($n$ = 12, CT group) and oat β-glucan group ($n$ = 12, BG group) were fed a normal basal diet, and rabbits in the BG group were also administered oat β-glucan (200 mg/kg BW, dissolved in 20 ml drinking water), according to the dosage of Zhang et al. (2012). Rabbits would be supplemented with fresh water after drinking up the water dissolved with oat β-glucan every day. The feeding period lasted for 4 weeks (**Figure 1A**). Oat β-glucan (88.69% purity on an air-dry basis; batch number SF-OGBO-210616) was purchased from Baixing Biological Technology Co., Ltd., Shandong, China.

At the end of the experiment at week four, fasting blood samples (6 h fasted) were collected from the rabbit hearts, followed by centrifuging at 1,500 × g for 10 min at 4°C, and the serum samples were isolated and frozen at −80°C for further analysis. Rabbits were anesthetized with an intravenous injection of 10% urethane (3 ml/kg BW) and euthanized by cervical dislocation. Colonic contents from the gut of each rabbit was collected into germ-free frozen tubes and stored at −80°C for intestinal microbiota analysis and metabolomics analysis. Six rabbits were randomly selected from each group, and distal colon tissues (approximately 3 cm) were collected, immediately flushed with cold saline, and fixed in 4% paraformaldehyde solution for histological analysis.

## 2.2 Food intake and growth

Body weight (BW) and food consumption were recorded weekly throughout the experiment, and the average daily gain (ADG), average daily food intake (ADFI), and the feed to gain ratio (F/G) were calculated for each week and the whole feeding period.

## 2.3 Measurement of serum biochemical indices

The concentrations of tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β), and LPS in the serum of each rabbit were measured using commercially available test kits from Nanjing Jiangcheng Bioengineering Institute, Nanjing, China (#H052-1-1, #H002-1-1, and #H255-1-1, respectively). Serum diamine oxidase (DAO) was measured using a commercial kit from Beijing Solarbio Science and Technology Co., Ltd., Beijing, China (#BC1285).

## 2.4 Histological analysis

After fixation in 4% paraformaldehyde solution for 48 h, the collected colon tissues were embedded in paraffin, sectioned at 5 μm using a rotary microtome (Leica RM2235, Germany), and stained with hematoxylin and eosin and periodic acid–Schiff. All the sections were visualized using a microimaging system (Leica DM2000, Germany). Villous height, crypt depth, muscular layer width, and mucin area proportion were measured using Image-Pro Plus version 6.0 (Image-Pro Plus software, Media Cybernetics, USA), and the villous height to crypt depth ratio was calculated. Ten microscopic fields were randomly selected from each section of the testes.

## 2.5 Intestinal microbiota analysis

The colonic content microbial genome was obtained using a Magnetic Soil and Stool DNA Kit (Tiangen Biotech (Beijing) Co., Ltd., Beijing, China), and the hypervariable V3–V4 region of the bacterial 16S rRNA gene was sequenced using primers (338F:5′-ACTCCTACGGGAGGCAGCA-3′; 806R:5′-GGACTACHVGGGTWTCTAAT-3′) on the Illumina Novaseq 6000 system at Shanghai Biotree Biotech Co., Ltd., Shanghai, China. The 16S rDNA gene sequencing data were merged using FLASH software (version 1.2.7) and filtered using Trimmomatic software (version 0.33), after which Cutadapt software (version 1.9.1) was used to identify and remove the primer pairs to acquire clean reads. UCHIME software (version 4.2) was used to identify and remove chimeric sequences to obtain effective reads. Operational taxonomic units (OTUs) were clustered

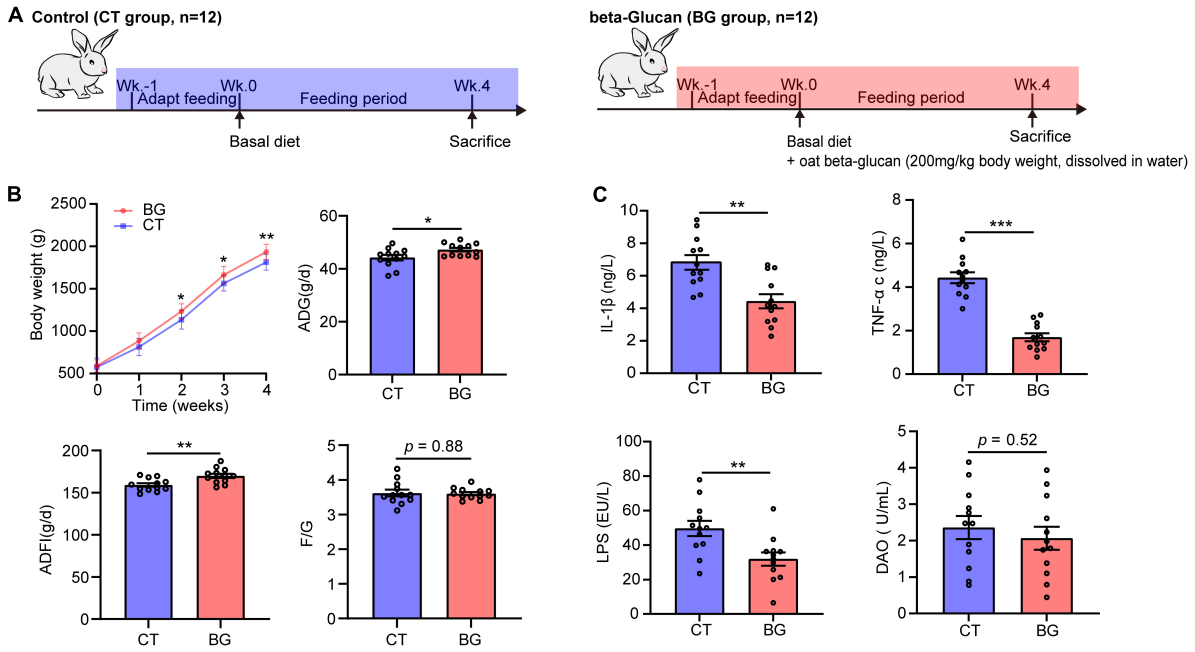


FIGURE 1
**(A)** Experimental setup for control (CT) and oat β-glucan (BG) groups. **(B)** Oat β-glucan improved growth performance. ADG, average daily gain; ADFI, average daily food intake; F/G, feed-to-gain ratio. Body weight (BW) data are presented as means ± SD. **(C)** Oat β-glucan decreases serum TNF-α, IL-1β, LPS, and DAO contents. TNF-α, tumor necrosis factor-α; IL-1β, interleukin-1β; LPS, lipopolysaccharide; DAO, diamine oxidase. $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$.

with an identity threshold of 97% using the Usearch software (version 10.0). A representative sequence for each OTU was selected, and a classify-sklearn naive Bayes taxonomy classifier was employed to annotate the taxonomic information for each representative sequence against the SILVA database (Release 132).[1] Rarefaction curve analysis was performed using Mothur software.[2] QIIME 2 was[3] used to evaluate the alpha diversity index (Chao1 and Shannon) and analyze beta diversity (non-metric multidimensional scaling, NMDS) using unweighted UniFrac methods. Bioinformatics analysis was performed using the online platforms Wekemo Bioincloud[4] and Omicstudio.[5]

## 2.6 Untargeted metabolomics analysis

Liquid chromatography-mass spectrometry based metabolomics was used to obtained serum and colon metabolic profiles. The extraction and measurement procedures were described in detail in the **Supplementary material**. The metabolites of the serum samples were identified according to Luo Z. Z. et al. (2019). Metabolites of colonic content samples were identified based on MS/MS spectra and matched against an in-house MS2 database (BiotreeDB, version 2.1). Principal component analysis (PCA) was conducted to evaluate the differences between samples within and between groups. Variable importance in the projection (VIP) values in the orthogonal partial least squares discrimination analysis (OPLS-DA) model and $p$ values from the Student's $t$-test were implemented to screen the discriminatory components. Metabolites with VIP $> 1$ and $p < 0.05$ were set as significantly differential metabolites, while those with VIP $> 1$, and $0.05 \leq p < 0.1$ were considered as metabolites with different trends. The differential metabolites were visualized, and the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis was performed using MetaboAnalyst 5.0.[6]

## 2.7 Intestinal short chain fatty acids analysis

Six colonic content samples were randomly selected from each group, and concentrations of SCFAs in them were determined using a gas chromatography mass spectrometer (Agilent 7890A/5975C, USA) at Shanghai Applied Protein

1 http://www.arb-silva.de
2 http://www.mothur.org/
3 https://qiime2.org
4 https:/bioincloud.tech/
5 https://www.omicstudio.cn/tool
6 https://www.metaboanalyst.ca/

Technology Co., Ltd., Shanghai, China. Briefly, a total of 30 mg colonic content was thawed, suspended in 900 μL of 0.5% phosphoric acid, and vortexed for 2 min. Each sample was centrifuged at 14,000 × g for 10 min and the phases were separated. The supernatant (800 μL) was then transferred into a 2 ml centrifuge tube and mixed with 800 μL of ethyl acetate to extract SCFAs. After centrifugation at 14,000 × g for 10 min, 600 μL of the upper layer was taken, 25 μL of 4-methylvaleric acid (500 μM) was added as an internal standard, and 1 μL of the mixture was injected into a sample injection bottle for further analysis.

All samples were analyzed with a 10:1 split ratio and separated using a gas chromatograph (Agilent DB-WAX, USA; capillary column: 30 m × 0.25 mm ID × 0.25 μm) at an oven temperature of 90°C, increased to 120°C at a rate of 10°C/min, then increased to 180°C at 5°C/min, finally at a rate of 25°C/min to 250°C, and held for 2 min; the flow rate of hydrogen was 1 ml/min. SCFA concentration was determined using the external standard method with corresponding standards and expressed in mg/g of colonic content. The total SCFA concentration was calculated as the sum of acetic, propionic, butyric, valeric, isovaleric, and isobutyric acids.

## 2.8 Statistical analysis

Differences between groups were evaluated by two-tailed unpaired Student's *t*-tests using the SPSS software (version 17.0, Chicago, USA). Two-way ANOVA analysis and graph drawing were conducted using GraphPad Prism software (version 8.0.2, La Jolla, CA, USA) unless otherwise specified. The significance threshold was set at $p < 0.05$; trends were declared at $0.05 \leq p < 0.10$. Correlation analyses among the screened differential metabolites, SCFAs, biochemical indices, and the relative intestinal microbiota abundance were performed by Spearman's correlation analysis using the R software. Data are presented as mean ± standard error unless otherwise indicated.

# 3 Results

## 3.1 Oat β-glucan improved growth performance in weaned rabbits

The BW, ADG ADFI, and F/G are shown in **Figure 1B**. Rabbits supplemented with oat β-glucan showed an increase in BW, which was significantly increased after feeding for 2 weeks ($p < 0.05$) and 3 weeks ($p < 0.05$), and significantly increased at 4 weeks ($p < 0.01$). Additionally, oat β-glucan accelerated ADG and ADFI ($p < 0.05$), but did not affect F/G ($p > 0.05$). In detail, rabbits in the BG group had a higher ADG ($p < 0.05$) after feeding for 1 week and had much more AFDI ($p < 0.05$) at 3 weeks. Oat β-glucan also significantly reduced the F/G ratio ($p < 0.05$) after feeding for 1 week. Moreover, feeding time significantly affected growth performance traits (except for F/G) ($p < 0.05$), whereas the interaction effect between β-glucan and feeding time was not significant on BW, ADG, ADFI, and F/G ($p > 0.05$, **Supplementary Table 1**).

## 3.2 Oat β-glucan altered serum biochemical indices and intestinal microstructure

Compared to the control group, oat β-glucan markedly reduced serum TNF-α, IL-1β, and LPS concentrations ($p < 0.001$), while the serum DAO concentration did not differ between treatments ($p = 0.52$), but showed a slight decrease in the BG group (**Figure 1C**). Oat β-glucan considerable increased the intestinal muscular layer width, villus height, villous height, crypt depth ratio, and mucin area proportion, and decreased crypt depth ($p > 0.05$, **Supplementary Table 2** and **Supplementary Figure 1**).

## 3.3 Oat β-glucan modulated intestinal microbiota

After removing the chimeric sequences, 1,877,535 effective reads were generated from 24 colonic content samples, with an average of 78,230 effective reads per sample. Venn plot analysis showed that there were 810 OTUs shared between the two groups, while only six and five OTUs were uniquely present in the BG and CT groups, respectively (**Supplementary Figure 2A**). The rarefaction curve reached a plateau at a sequencing depth of 20,000, indicating that the sequencing depth was sufficient to represent most bacterial OTUs (**Supplementary Figure 2B**). The Chao1 and Shannon index values, used to evaluate community richness and diversity in each microbiome sample, were not significantly different between the two groups ($p > 0.05$, **Figure 2A**), whereas the NMDS by unweighted UniFrac distance analysis demonstrated that the BG group was distant from the CT groups (**Figure 2B**), indicating that oat β-glucan did not significantly change the bacterial diversity but altered the overall microbiota structure.

We further analyzed the composition and structure of gut microbial populations at the phylum and genus levels. According to the phylum assignment results, Firmicutes, Bacteroidetes, Verrucomicrobia, Tenericutes, and Proteobacteria were the most dominant bacteria, accounting for over 95% of the taxonomic groups identified. Compared to the CT group, the relative Proteobacteria and Thermotogae abundances were significantly increased (**Figure 2C**, $p < 0.01$). Inter-group comparisons of taxonomic profiles at the genus level showed that the BG group exhibited a higher relative abundance of *Ruminiclostridium_5*,

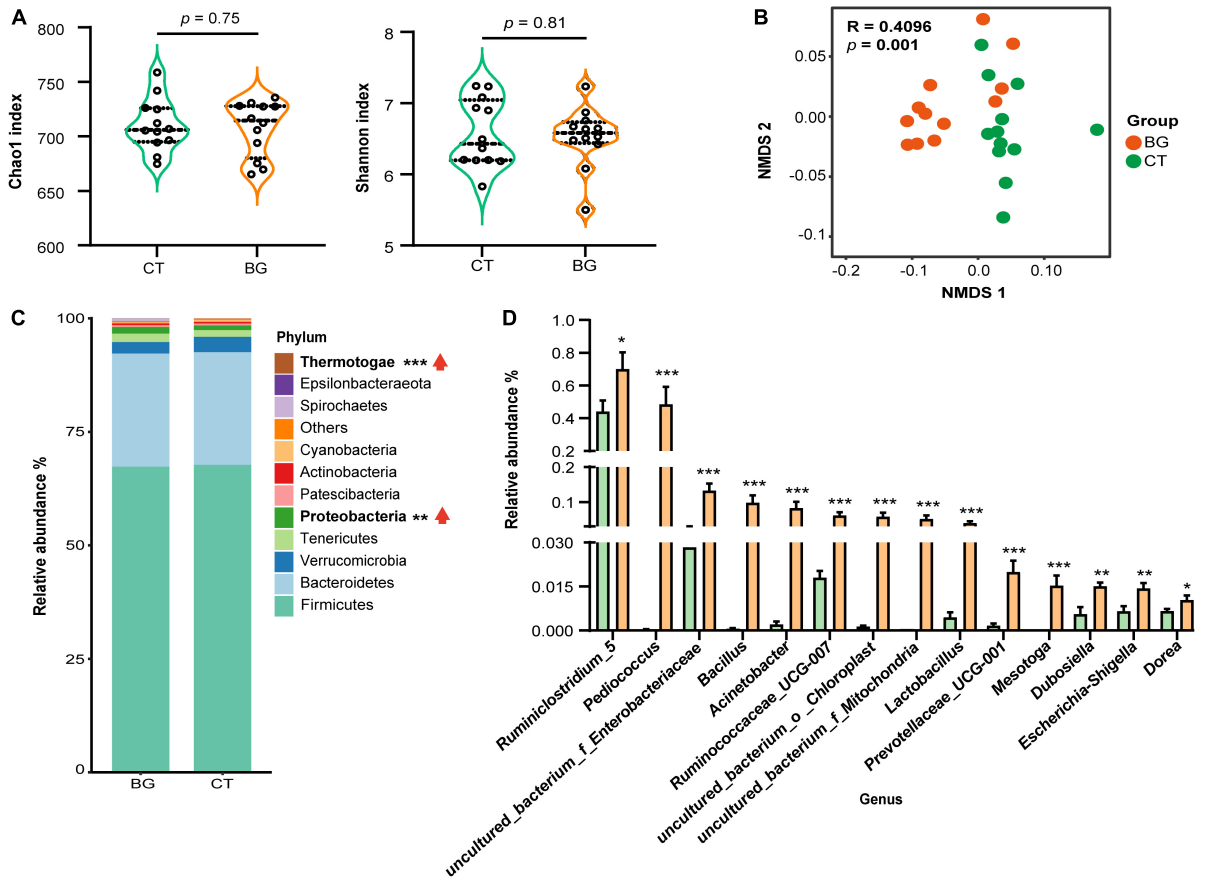


FIGURE 2
Oat β-glucan altered gut microbiota abundance of weaned rabbits. **(A)** Alpha-diversity of bacterial communities in colon. **(B)** Non-metric multidimensional scaling (NMDS) analysis plots of bacteria communities in oat β-glucan (BG) and control (CT) groups. **(C)** The abundance of gut microbiota at the phylum level. **(D)** Differential gut bacteria at the genus level. *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$. The upward red arrow indicates the relative abundance of this phylum was increased after feeding oat β-glucan.

*Pediococcus*, *uncultured_bacterium_f_Enterobacteriaceae*, *Bacillus*, *Acinetobacter*, *Ruminococcaceae_UCG-007*, *uncultured_bacterium_o_Chloroplast*, *uncultured_bacterium_f_Mitochondria*, *Lactobacillus*, *Prevotellaceae_UCG-001*, *Mesotoga*, *Dubosiella*, *Escherichia-Shigella*, and *Dorea* as compared to the CT group (Figure 2D, $p < 0.05$).

## 3.4 Oat β-glucan changed intestinal metabolism

To further explore the effects of oat β-glucan supplementation on intestinal microbiota metabolism, we performed an untargeted metabolome assay. The PCA score plot showed that the BG and CT groups were clearly clustered into two separate groups in both the positive and negative ion modes (Supplementary Figures 3A, B), indicating that oat β-glucan might modulate gut microbiota metabolism. The OPLS-DA score plot exhibited a similar trend of sample distribution, with the permutation tests for the model of each group showing a $Q^2$ intercept of <0.05, indicating that there was no overfitting (Supplementary Figures 3C–F). The results indicated that the established OPLS-DA model was suitable. Then, the VIP value obtained from the OPLS-DA mode and the *p*-value from Student's *t*-test were used to screen potential differential components. In total, 264 metabolites that differed between the BG and CT groups were screened (VIP > 1, $p < 0.1$). We then determined the origin of these differential metabolites using MetOrigin[7], and found 17 bacterial metabolites, 15 bacteria–host metabolites, 2 host-specific metabolite, and 230 others (27 drug-related metabolites, 153 food-related metabolites, 1 environment metabolite, and 49 unknown metabolites), as shown in Supplementary Figure 3G.

Next, we focused on the differential metabolites from the host, microbiota, and shared by both and mapped them in the

7 http://metorigin.met-bioinformatics.cn/

KEGG database[8] for KEGG second-grade pathway analysis. Finally, 23 metabolites were identified (**Supplementary Table 3**), including lysine derivatives (saccharopine, 5-aminopentanamide, and 4-trimethylammoniobutanal), tyrosine derivatives (dopamine, 5, 6-dihydroxyindole, and maleic acid), tryptophan derivatives (5-hydroxy-L-tryptophan, indole, and 3-methyldioxyindole), phenylalanine derivatives (phenylacetaldehyde), and purine metabolites (deoxyadenosine and adenosine). The levels of all these 23 metabolites, which were mainly involved in lysine degradation, purine metabolism, and aromatic amino acid (AAA) metabolism according to KEGG metabolic enrichment pathway analysis (**Supplementary Figure 3H**), were found to be decreased in the BG group (**Figure 3**). Taken together, these data suggest that supplementation with oat β-glucan can alter intestinal metabolic profiles in weaned rabbits.

## 3.5 Oat β-glucan changed serum metabolism

Microbial metabolites can be absorbed across the host gut, are measurable in the host circulation, and serve as signaling molecules and substrates for metabolic reactions that affect host physiology (Krautkramer et al., 2021). Therefore, we analyzed the serum metabolites of rabbits to assess whether local intestinal metabolic alterations would influence the serum metabolism profiles. The OPLS-DA mode exhibited a significant separation of clusters between the BG and CT groups, and the permutation tests for the model of each group showed a Q2 intercept of <0.05 (**Supplementary Figures 4A–D**), suggesting that the OPLS-DA model is suitable for screening differential metabolites. Compared with the serum metabolomics of the controls, we identified 42 differential metabolites (VIP $>$ 1, $p < 0.1$), of which 26 were considered to be significantly expressed (VIP $>$ 1, $p < 0.05$). Among them, two were bacterial metabolites, 19 were bacteria–host metabolites, and 21 belonged to others (**Supplementary Figure 4E**). KEGG metabolic enrichment pathway analysis found these metabolites mainly involved in carbohydrate, lipid, and amino acid metabolism (**Supplementary Figure 4F**). Furthermore, according to the Human Metabolome Database (HMDB),[9] classification, seven belonged to carboxylic acids and derivatives, six metabolites belonged to sphingolipids, five belonged to organooxygen compounds, five belonged to glycerophospholipids, three belonged to quinolines and derivatives, three belonged to steroids and steroid derivatives, three belonged to benzene and substituted derivatives, two belonged to hydroxy acids and derivatives, two belonged to fatty acids, two belonged to prenol lipids, and the rest belonged to others. Heatmap analysis revealed 22 differentially expressed metabolites that were upregulated and 20 that were downregulated (**Figure 3** and **Supplementary Table 4**).

## 3.6 Oat β-glucan promoted intestinal SCFA fermentation

Previous studies found that β-glucan was utilized by gut microbiota to produce SCFAs; therefore, we also measured concentrations of SCFAs in the colonic content. The results showed that after supplementation with oat β-glucan for 4 weeks, the total concentration of SCFAs increased compared to that in the control group, but the difference was not significant ($p = 0.12$, **Figure 4A**). Particularly, propionic and valeric acid concentrations increased significantly ($p < 0.05$) in the BG group, and butyric acid also demonstrated an increasing tendency ($p = 0.06$, **Figure 4B**).

## 3.7 Correlation analyses among phenotype, intestinal microbiota, and related metabolites

Spearman's correlation analysis between the microbiome and clinical phenotype indices (LPS, IL-1β, TNF-α, ADG, and ADFI) revealed that most of these differential gut bacteria at the genus level negatively correlated with LPS, IL-1β, and TNF-α, and positively correlated with ADG and ADFI (**Figure 5**).

Spearman's correlation analysis between the microbiome and related differentially expressed gut metabolites revealed that most of these metabolites negatively correlated with the gut bacteria. Particularly, dopamine and saccharopine showed strong correlations with most of the microbial taxa ($p < 0.05$, **Supplementary Figure 5**). Correlation analysis of the gut bacteria and serum metabolites showed that gut bacteria negatively associated with metabolites that involved in lipid metabolism, and positively correlated with metabolites which participated in carbohydrate and amino acid metabolism (**Supplementary Figure 6**). Then between layers correlation of gut metabolites and serum metabolites calculated, the results showed that the correlation between the two layers was not strong (**Supplementary Figure 7**).

Further correlation analysis of gut metabolites and clinical phenotype indices suggested that most of these metabolites significantly positively correlated with LPS, IL-1β, and TNF-α levels ($p < 0.05$, **Figure 6A**).

Spearman's correlation analysis between serum metabolites and clinical phenotype indices was performed, and significant correlations were present in a heatmap (**Figure 6A**). The results showed that LPS, IL-1β, and TNF-α significantly correlated with most serum metabolites ($p < 0.05$); however, ADG and ADFI showed no significant correlations with most serum metabolites

8 https://www.genome.jp/kegg/

9 https://hmdb.ca/

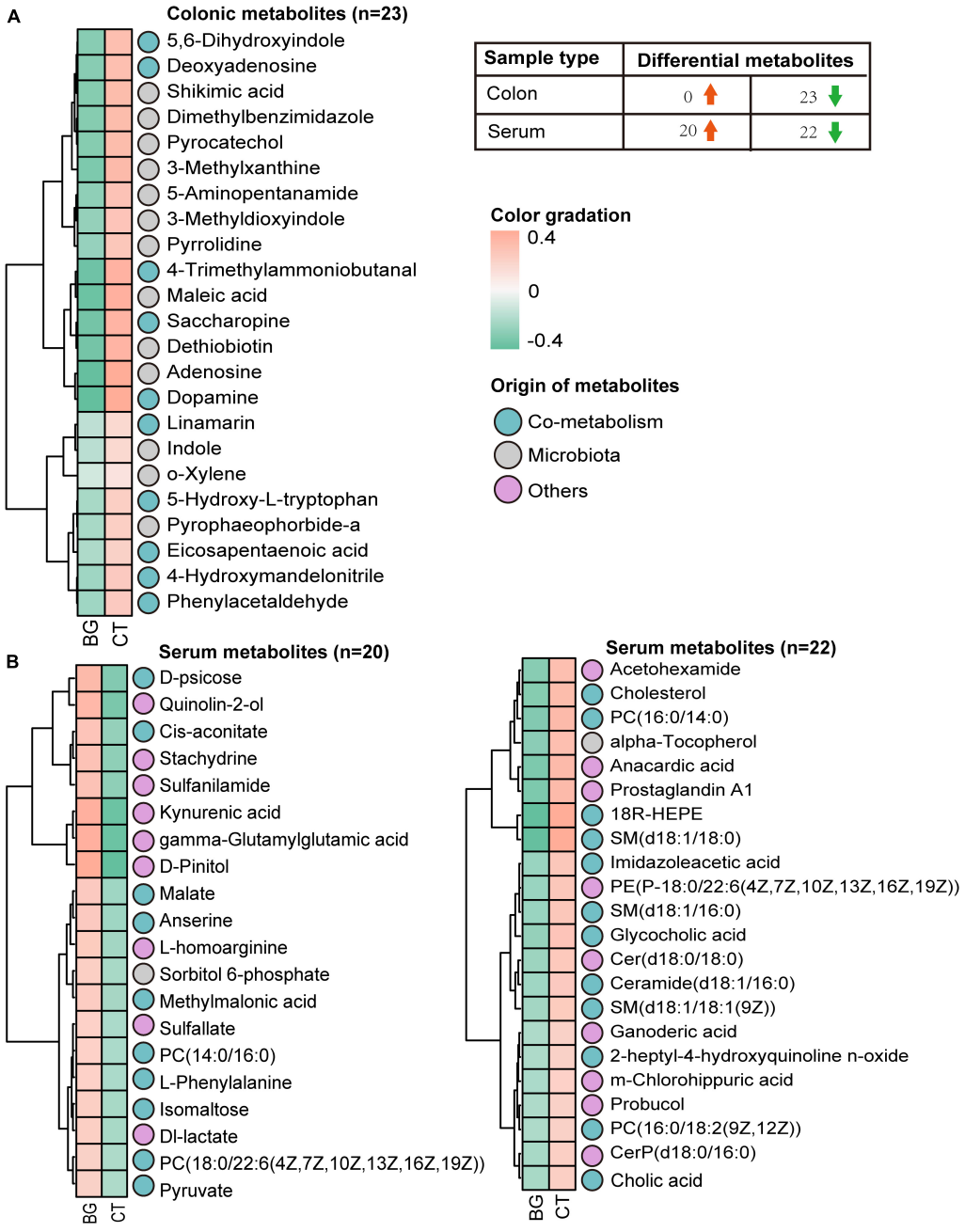


FIGURE 3
The heatmap. **(A)** Heatmap visualization of the colonic metabolites that differed between the oat β-glucan (BG) and control (CT) groups. **(B)** Heatmap visualization of the serum metabolites that differed between the oat BG and CT groups.

($p > 0.05$). Interestingly, inflammation-related indices (LPS, IL-1β, and TNF-α) and growth-related indices (ADG and ADFI) showed opposite relationships with serum metabolites.

Spearman's correlation analysis between SCFAs and the microbiome revealed that colonic propionic acid and valeric acid concentrations exhibited a strong positive correlation with most of these bacteria ($p < 0.05$). Additionally, acetic and butyric acids positively correlated with *Ruminiclostridium_5* and *uncultured_bacterium_o_Chloroplast* ($p < 0.05$), whereas isobutyric acid negatively correlated with *Dubosiella* ($p < 0.05$, Supplementary Figure 8). These results suggest that changes in microbiome may promote the production of SCFAs in weaned rabbit guts.

Short-chain fatty acids (SCFAs) not only play a role in maintaining intestinal homeostasis but also circulate in blood and directly affect metabolism or function of peripheral tissues

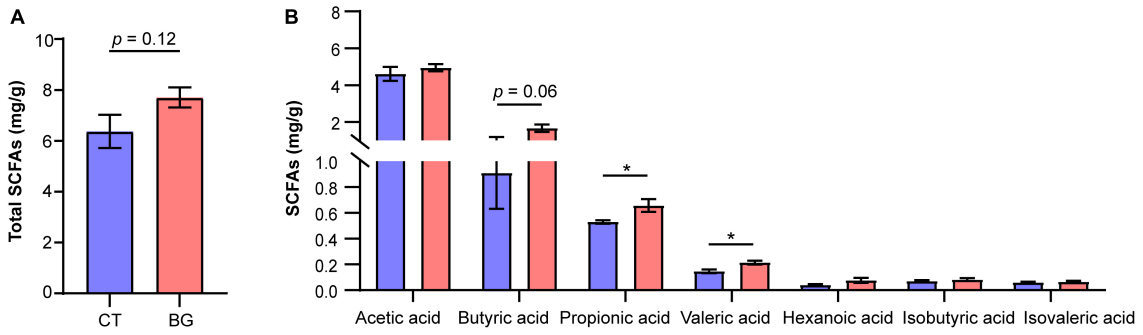


FIGURE 4
**(A)** Total concentration of short-chain fatty acids (SCFAs). **(B)** The concentration of acetic, propionic, butyric, valeric, isovaleric, and isobutyric acids. *$p < 0.05$.

(van der Hee and Wells, 2021); thus, we performed Spearman's correlation analysis to identify the associations between SCFAs and clinical phenotype indices. Consequently, a negative correlation between inflammation-related indices and SCFAs was observed. Particularly, serum TNF-α significantly negatively correlated with acetic, butyric, and valeric acids, and IL-1β significantly negatively correlated with valeric acid ($p < 0.05$). Conversely, ADG and ADFI were positively correlated with SCFAs, of which ADFI significantly positively associated with acetic, butyric, and valeric acids ($p < 0.05$, **Figure 6B**). These results prompted us to identify the links between these inflammatory and growth-related indices and found that they negatively correlated; indeed, TNF-α significantly negatively correlated with ADFI ($p < 0.05$, **Figure 6C**). Taken together, these findings indicate that an altered gut microbiome changes gut metabolism and promotes the production of SCFAs, and further influences serum metabolic profiles, thus regulating the production of inflammatory cytokines and improving growth performance.

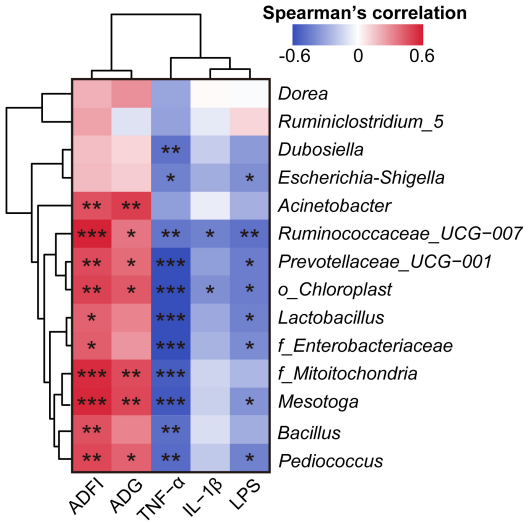


FIGURE 5
Spearman's correlation analysis of clinical phenotype indices and the relative abundance of differential bacteria at the genus level. *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$. The Spearman's correlation coefficient of significant correlations >0.4 (or <−0.4).

## 4 Discussion

In this study, we investigated the effects of oat β-glucan supplementation on weaned rabbits. After 4 weeks of oat β-glucan treatment, a significant increase in BW, daily gain, and food intake were observed. The effects of different β-glucan sources on growth performance have been previously reported. Luo J. et al. (2019) found that a basal diet supplemented with 100 mg/kg bacterial β-glucan improved ADG in weaned piglets. Oral administrated with 75 mg/kg oat β-glucan also increased BW in pre-weaning dairy calves (Luo et al., 2022). Additionally, oral administration of pharmaceutical grade 1, 3 β-glucan at doses of 0.25 and 0.5 ml/L of drinking water significantly accelerated BW gain and reduced the feed conversion ratio in 6-week-old rabbits (Abo Ghanima et al., 2020). Claves supplemented with 1 g/day seaweed β-glucan had lower food intake and ADG, which was probably due to excessive β-glucan addition (McDonnell et al., 2019). However, to the best of our knowledge, limited research has focused on its role in promoting growth, particularly in young animals. Our results suggest that feeding oat β-glucan can improve growth performance; this might not only reflect the feeding amount of oat β-glucan in this work is suitable, but also provide a basis for its application in young animal rearing in the future.

We also observed a decrease in serum LPS levels. LPS is a component of the gram-negative bacterial cell wall and is generally considered as a trigger inducing localized or systemic inflammation by increasing the production of proinflammatory cytokines, such as IL-1β, interleukin-6 (IL-6), and TNF-α, and

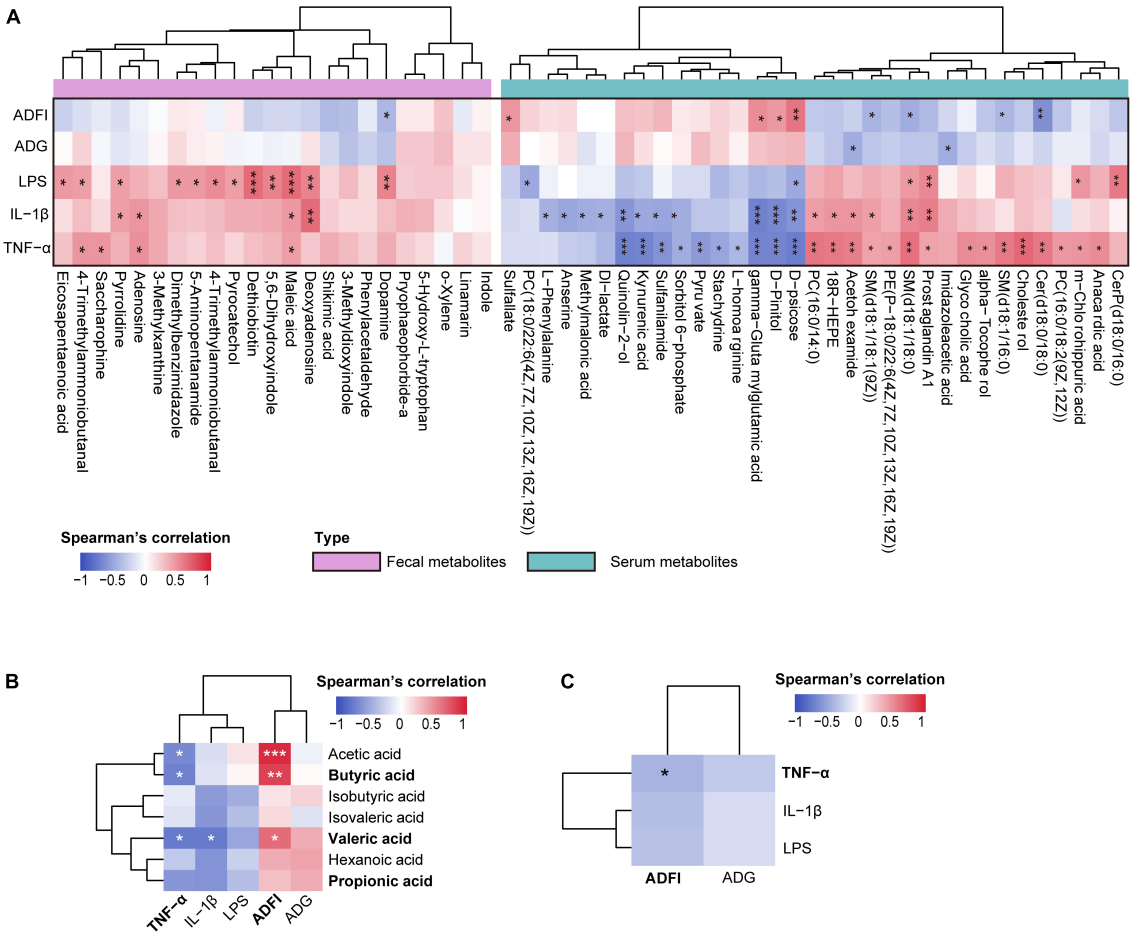


FIGURE 6
**(A)** Spearman's correlation analysis between differential metabolites (colonic contents and serum) and clinical phenotype indices. **(B)** Spearman's correlation analysis between the colonic short-chain fatty acids (SCFAs) and clinical phenotype. **(C)** Spearman's correlation analysis between the inflammatory-related indices (tumor necrosis factor-α [TNF-α], interleukin-1β [IL-1β], and lipopolysaccharide [LPS]) and growth performance-related indices (average daily gain [ADG], average daily food intake [ADFI]). *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$. The spearman's correlation coefficient of significant correlations > 0.4 (or <−0.4).

excess LPS leaked from the gut to the bloodstream usually reflects gut barrier integrity damage (Cox and Blaser, 2013; You et al., 2013; Guo et al., 2015). Therefore, the decrease in serum LPS has a causative role in the decrease of serum IL-1β and TNF-α levels in this study.

Numerous studies have shown a strong correlation between intestinal function and growth performance in animals (Zou et al., 2022). Therefore, we measured the colon microstructure and found that oat β-glucan could promote intestinal development to some extent. Like LPS, DAO is usually located at the upper villi of the small intestinal mucosa and enters the circulation when epithelial cells are damaged (Zhang et al., 2020). Thus, both serum DAO and LPS levels can reflect gut epithelial permeability and integrity. In this study, a decrease in serum DAO and LPS concentrations were found in the BG group, which might indicate that oat β-glucan also improved the intestinal epithelial barrier function. TNF-α is mainly produced by inflammatory cells such as macrophages and activated T-cells, and initiates inflammatory responses *via* the nuclear factor-κB (NF-κB) and mitogen-activated protein kinase signaling pathways (Oceandy et al., 2019). It has also been shown to induce apoptosis and inflammatory responses in intestinal epithelial cells and impair the intestinal barrier through cytoskeletal rearrangement and tight junction protein expression regulation. Previous studies found that IL-1β increases in the intestinal mucosa under inflammatory conditions, and like TNF-α, it impairs the intestinal tight junction barrier through decreases in occludin and cytoskeletal rearrangement (Suzuki, 2013). In this regard, we assumed that oat β-glucan might improve intestinal barrier function by regulating TNF-α and IL-1β expressions. In brief, oat β-glucan may improve the growth performance of weaned rabbits by enhancing gut function, not only promoting intestinal development but also protecting barrier integrity.

Accumulating evidence demonstrates that gut microbiota and its catabolites are closely associated with gut function (Valdes et al., 2018). A previous study found that β-glucan ingestion suppressed inflammation and improved colonic mucosal barrier function in ulcerative colitic mice, which was mediated by gut-derived SCFAs and intestinal microbial metabolic biomarkers (Bai et al., 2021b). In this study, oat β-glucan supplementation significantly altered the gut microbiota structure and composition in weaned rabbits. Particularly, at the genus level, the relative abundance of some beneficial bacteria, such as *Lactobacillus*, *Prevotellaceae_UCG-001*, *Pediococcus*, and *Bacillus*, significantly increased in the BG group. Among these, *Lactobacillus*, *Pediococcus*, and *Bacillus* are the three most common probiotics. *Lactobacillus* is implicated with beneficial effects in preventing and reducing inflammation-related diseases, such as inflammatory bowel disease (IBD) and non-alcoholic fatty acid liver diseases, accompanied by gastrointestinal barrier function enhancement and a decrease in pro-inflammatory cytokine levels (such as IL-6 and IL-1β) (Azad et al., 2018; Aghamohammad et al., 2022). A previous study demonstrated that oat β-glucan increased the population of *Lactobacillus* during *in vitro* fermentation when inoculated with mouse fecal microbiota (Jayachandran et al., 2018). Additionally, dietary supplementation with β-glucan from *Candida glabrata* also increased the relative abundance of *Lactobacillus* in colitic mice (Charlet et al., 2018). Similar to *Lactobacillus*, *Pediococcus* also exhibits beneficial effects in non-alcoholic fatty acid liver diseases by modulating the gut microbiome and inflammatory pathways (Lee et al., 2020). A recent study demonstrated that the increase in *Pediococcus* caused by feeding selenium-enriched *Pediococcus acidilactici* MRS-7 contributed to intestinal barrier function restoration and alleviation of the inflammatory response and oxidative stress in jejunum-injured mice (Chen et al., 2022). *Bacillus* strains have been widely used as potential probiotics to combat pathogen invasion and improve growth performance (Wang et al., 2020; Sam-On et al., 2022). Zou et al. (2022) found that dietary supplementation with *Bacillus subtilis* decreased proinflammatory cytokine expression and improved antioxidative status and intestinal barrier integrity by regulating the intestinal microenvironment and related microbiota composition in laying hens. As one of the most common members of intestinal bacteria, accumulating studies have presented a lower relative abundance of *Prevotellaceae_UCG-001* in the gut of animals with colitis and have rebalanced it after colitis alleviation. A previous report also showed that metabolites of *Prevotellaceae UCG-001* can activate the adenosine monophosphate (AMP)-activated protein kinase signaling pathway to improve gut health (Song et al., 2019). In the BG group, we also observed an increased abundance of *Ruminococcaceae_UCG-007* and *Ruminiclostridium_5*, which have rarely been reported previously. *Ruminococcaceae* is generally considered a beneficial bacterium colonized in the cecum and colon and can degrade various polysaccharides and fibers to produce SCFAs (Huang et al., 2021), it also presents an effect to reduce intestinal inflammation (Hu et al., 2021). Moreover, findings from prior research showed that the increase of *Ruminiclostridium_5* was associated with positive health states, whereas reduced levels are associated with negative health states. For instance, a prebiotic diet produced a rapid and stable increase in the relative abundance of *Ruminiclostridium_5* in mice with the chronic disruption of rhythm (Thompson et al., 2021). *Ruminiclostridium_5* was also abundant owing to the alleviation of DSS-induced colitis (Shao et al., 2020). These results may explain the higher SCFA content in the BG group. These genera are SCFA-producing bacteria, as previously reported. SCFAs can modulate the expression of tight junction proteins to maintain gut epithelial barrier integrity and protect gut epithelium by enhancing mucin 2 expression, modulating oxidative stress, and immune response (Feng et al., 2018). Therefore, SCFAs play a crucial role in the maintenance of intestinal homeostasis. Moreover, *Lactobacillus* and *Ruminiclostridium_5* were reported to produce butyric acid, which can nourish and protect the intestinal epithelium (Fu et al., 2019; Shao et al., 2020).

It is also worth noting that oat β-glucan increased the abundances of phyla Proteobacteria and genus *uncultured_bacterium_f_Enterobacteriaceae*, *uncultured_bacterium_f_Mitochondria*, *Acinetobacter*, and *Escherichia-Shigella* (phylum Proteobacteria), which are often considered as pathogens. This is consistent with a previous report that Proteobacteria were adapted for food rich in non-starch polysaccharides such as xylan and cellulose. They speculated that this might be due to increased fiber intake that decreases fat and protein digestibility, which ultimately increases the amount of fermentation substrates available for various gut microorganisms, such as Proteobacteria (Tian et al., 2017). A previous study also found that dietary supplementation with oat-derived β-glucan increased the relative abundance of Betaproteobacteria, Gammaproteobacteria (including *Enterobacteriaceae*), and Deltaproteobacteria in BALB/c mice (Luo et al., 2017). Taken together, these data indicate that oat β-glucan might enhance intestinal function by increasing the abundance of beneficial gut microbiota communities and their metabolites, SCFAs.

Interestingly, except for SCFAs, correlation analysis also indicated that these beneficial bacteria have profound relationships with other intestinal metabolites. In this study, the levels of saccharopine and the two other metabolites, 5-aminopentanamide and 4-trimethylammoniobutanal were lower in the BG group, and KEGG pathway analysis revealed that they participated in the lysine degradation pathway. Lysine is an essential amino acid, and its degradation has two distinct pathways. In the saccharopine pathway, lysine is converted to saccharopine *via* α-aminoadipic semialdehyde synthase, whereas in the pipecolate pathway, lysine is first converted to

5-aminopentanamide (Crowther et al., 2019). Saccharopine accumulation induces mitochondrial dysfunction in mammals (Leandro and Houten, 2019), and has also been shown to participate in gluconeogenesis through its role in the propionyl-CoA synthesis pathway and tricarboxylic acid cycle *via* the acetyl-CoA production pathway (Safi-Stibler et al., 2020). In this regard, the decrease in saccharopine might partially reflect an alteration in the way gut microbiota acquires energy. Additionally, higher 5-aminopentanamide content in the gut has been strongly associated with IBDs, such as ulcerative colitis and Crohn's disease (Lin et al., 2010; Feng et al., 2020). Besides lysine degradation, we observed alterations in the AAA metabolism. Research has shown that increasing carbohydrate availability in the hindgut leads to microbial AAA metabolism inhibition and an increase in systemic AAA availability in piglets (Gao et al., 2019).

In line with this previous study, oat β-glucan also decreased the intestinal contents of AAA metabolites in weaned rabbits in this study, and the serum contents of l-phenylalanine and kynurenic acid (formed from tryptophan) were higher in the BG group. An explanation for this occurrence is that the fiber supplementation promotes intestinal microbiota to preferentially ferment easily fermented carbohydrates, but not proteins, thus decreasing the production of amino acid (AAA and lysine) metabolites (Gao et al., 2018). Meanwhile, there was no AAA elevation in the colon; therefore, we speculated that the upregulation of serum l-phenylalanine and kynurenic acid might reflect the enhancement of amino acid transport ability from the gut to the blood, as circulating AAA is mainly derived from gut absorption (Fernstrom, 2013). In contrast, the content of AAA biosynthesis precursor shikimic acid decreased in the BG group, which might suggest an increase in gut AAA biosynthesis, but this needs further investigation. Microbial AAA metabolites serve as signaling molecules that affect host physiology, such as by regulating intestinal epithelial cell homeostasis and immune cell response (Liu et al., 2020). In this study, we found that the microbial tryptophan metabolites indole and 3-methyldioxyindole were decreased in the BG group. 3-methyldioxyindole is an oxidation product of 3-methylindole (also called skatole) *in vivo* (Xu et al., 2020). Skatole is most probably a product in the colon and has been found to have a damaging effect on intestinal epithelial tissue activity and function after absorption but before detoxification (Yokoyama and Carlson, 1979). A previous study also suggested that indoles and skatoles exert an inhibitory effect on *CYP11A1* expression, which leads to a decrease in glucocorticoid production and further disturbs intestinal homeostasis (Mosa et al., 2016; Gao et al., 2018). Combined with the above results, we speculate that oat β-glucan supplementation enriched the available nutrient substrates in the intestine, which perhaps promotes the alteration of microbiota nutrition patterns and alters microbiota metabolism, further improving intestinal health by promoting the production of SCFAs and decreasing the levels of lysine and AAA-related metabolites.

Serum metabolic profile analysis revealed that oat β-glucan administration mainly altered host energy metabolism, including carbohydrate, lipid, and amino acid metabolisms. Correlation analysis suggested that altered serum metabolites were highly associated with inflammation-related indices LPS, IL-1β, and TNF-α. Most notably, sphingolipid metabolism changed significantly, which is consistent with a previous report that oat β-glucan treatment alters gut microbial sphingolipid metabolism in colitic mice (Bai et al., 2021b). Moreover, six sphingolipids, including SM (d18:1/16:0), ceramide (d18:1/16:0), SM [d18:1/18:1 (9Z)], Cer (d18:0/18:0), SM (d18:1/18:0), and CerP (d18:0/16:0), which were identified as serum metabolic biomarkers in this study, were all decreased in the BG group. Studies have suggested that TNF-α can initiate an inflammatory response through the acid and neutral sphingomyelinase pathways, which leads to increased ceramide production and eventually activates the pro-inflammatory transcription factor NF-κB (Sedger and McDermott, 2014). Similar to TNF-α, IL-1β exerts an effect on the induction of the ceramide-signaling pathway (Schneider et al., 2005). Ceramide is the center of sphingolipid synthesis and degradation and serves as a precursor for the synthesis of complex sphingolipids (Nixon, 2009). Additionally, TNF-α also activates phospholipase $A_2$ enzymes, resulting in the metabolism of glycerophospholipids to arachidonic acid, which then degrades to prostaglandins *via* the cyclooxygenase pathway (Oceandy et al., 2019; Yuan et al., 2020). Based on these findings, we assumed that there is a close relationship between serum IL-1β and TNF-α concentrations, and serum sphingolipid and glycerophospholipid content; that is, oat β-glucan might inhibit the systemic inflammatory response by regulating serum sphingolipid and glycerophospholipid metabolism.

It is also worth noting that serum metabolites just showed slight correlations with growth performance related indices. However, both ADG and ADFI were negatively associated with LPS, IL-1β, and TNF-α, indicating that oat β-glucan may promote growth by inhibiting systemic inflammation development. Furthermore, SCFAs have been reported to exhibit anti-inflammatory effects by directly activating G-protein-coupled receptors (GPCRSs) and inhibiting nuclear class I histone deacetylases, leading to decreased proinflammatory cytokines, such as IL-6, interleukin-8, and TNF-α (Feng et al., 2018). Besides maintaining intestinal hemostasis and anti-inflammatory effects, gut-derived SCFAs are also involved in appetite control through hormonal and central effects. For instance, SCFAs can activate ghrelin-related signaling and inhibit insulin secretion by activating GPCR 3, which also crosses the blood-brain barrier to directly affect appetite-related neurons in the brain (Han et al., 2021). In this study, acetic, butyric, and valeric acids positively associated with ADFI, indicating that these play a pivotal role in promoting

food intake. Moreover, a study found that high carbohydrate availability in the hindgut also led to an increase in AAA-derived neurotransmitters (serotonin and dopamine) in the hypothalamus of piglets (Gao et al., 2019), both of which are critical mediators of appetite control (Meguid et al., 2000; Han et al., 2021). Therefore, we assumed that, in rabbits, oat β-glucan administration might also stimulate food intake by modulating central serotonin and dopamine signaling, but the mechanisms deserve further investigation.

## 5 Conclusion

This study revealed that oat β-glucan can regulate gut microbiota structure and increase the abundance of potentially beneficial bacteria. Simultaneously, it can increase the concentration of SCFAs in the colon and decrease the concentrations of AAA and lysine-related metabolites. Oat β-glucan can also alter serum metabolic profiles and change the serum contents of 42 metabolites, including sphingolipids and glycerophospholipids. These results demonstrate the positive effect of oat β-glucan on lowering systemic inflammatory levels and promoting intestinal health, which further helps to improve growth performance. This study provides new insights into the prebiotic effects of oat β-glucan in a weaned rabbit model and promotes the application of oat β-glucan in young animals rearing. However, we have not explored the precise mechanism behind these effects. Further research is needed to clarify the interactions between SCFAs, AAA derivatives and intestinal epithelial tissue, and explore their role in the gut–brain axis.

## 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: NCBI-PRJNA901541.

## Ethics statement

This study was conducted in strict accordance with the guidelines for the Care and Use of Laboratory Animals of China and all procedures were approved by the Animal Care and Use Committee of Sichuan Agricultural University (No. DYY-2018203039).

## Author contributions

LM and ZL: conceptualization, methodology, investigation, writing – original draft, writing – reviewing and editing, and visualization. YXH: writing – original draft, writing – reviewing and editing, and visualization. YL and JG: methodology, investigation, writing – original draft, and writing – reviewing and editing. TZ, ZD, KY, XY, LS, and SY: investigation, writing – original draft, and writing – reviewing and editing. ZZ, YCH, and GP: writing – original draft and writing – reviewing and editing. XS: funding acquisition, project administration and supervision, writing – original draft, and writing – reviewing and editing. SC: conceptualization, writing – original draft, writing – reviewing and editing, funding acquisition, project administration, and supervision. 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/fmicb.2022.1074036/full#supplementary-material

# References

Abo Ghanima, M. M., Abd El-Aziz, A. H., Noreldin, A. E., Atta, M. S., Mousa, S. A., and El-Far, A. H. (2020). Beta-glucan administration improves growth performance and gut health in new zealand white and apri rabbits with different breed responses. *PLoS One* 15:e0234076. doi: 10.1371/journal.pone.0234076

Aghamohammad, S., Sepehr, A., Miri, S. T., Najafi, S., Pourshafie, M. R., and Rohani, M. (2022). Anti-inflammatory and immunomodulatory effects of lactobacillus spp. As a preservative and therapeutic agent for ibd control. *Immun. Inflamm. Dis.* 10:e635. doi: 10.1002/iid3.635

Azad, M. A. K., Sarker, M., Li, T., and Yin, J. (2018). Probiotic species in the modulation of gut microbiota: An overview. *Biomed. Res. Int.* 2018:9478630. doi: 10.1155/2018/9478630

Bai, J., Li, T., Zhang, W., Fan, M., Qian, H., Li, Y., et al. (2021a). Systematic assessment of oat beta-glucan catabolism during in vitro digestion and fermentation. *Food Chem.* 348, 129116. doi: 10.1016/j.foodchem.2021.129116

Bai, J., Zhao, J., Al-Ansi, W., Wang, J., Xue, L., Liu, J., et al. (2021b). Oat beta-glucan alleviates dss-induced colitis via regulating gut microbiota metabolism in mice. *Food Funct.* 12, 8976–8993. doi: 10.1039/d1fo01446c

Charlet, R., Bortolus, C., Barbet, M., Sendid, B., and Jawhara, S. (2018). A decrease in anaerobic bacteria promotes candida glabrata overgrowth while beta-glucan treatment restores the gut microbiota and attenuates colitis. *Gut Pathog.* 10:50. doi: 10.1186/s13099-018-0277-2

Chen, H., Du, G., Yan, X., Ye, H., Guo, Q., Wang, Z., et al. (2022). Selenium-enriched pediococcus acidilactici mrs-7 alleviates patulin-induced jejunum injuries in mice and its possible mechanisms. *J. Agric. Food. Chem.* 70, 4755–4764. doi: 10.1021/acs.jafc.2c00949

Cox, L. M., and Blaser, M. J. (2013). Pathways in microbe-induced obesity. *Cell Metab.* 17, 883–894. doi: 10.1016/j.cmet.2013.05.004

Crowther, L. M., Mathis, D., Poms, M., and Plecko, B. (2019). New insights into human lysine degradation pathways with relevance to pyridoxine-dependent epilepsy due to antiquitin deficiency. *J. Inherit. Metab. Dis.* 42, 620–628. doi: 10.1002/jimd.12076

Feng, W., Ao, H., and Peng, C. (2018). Gut microbiota, short-chain fatty acids, and herbal medicines. *Front. Pharmacol.* 9:1354. doi: 10.3389/fphar.2018.01354

Feng, W., Liu, J., Tan, Y., Ao, H., Wang, J., and Peng, C. (2020). Polysaccharides from atractylodes macrocephala koidz. Ameliorate ulcerative colitis via extensive modification of gut microbiota and host metabolism. *Food Res. Int.* 138:109777. doi: 10.1016/j.foodres.2020.109777

Fernstrom, J. D. (2013). Large neutral amino acids: Dietary effects on brain neurochemistry and function. *Amino Acids* 45, 419–430. doi: 10.1007/s00726-012-1330-y

Fu, X., Liu, Z., Zhu, C., Mou, H., and Kong, Q. (2019). Nondigestible carbohydrates, butyrate, and butyrate-producing bacteria. *Crit. Rev. Food Sci. Nutr.* 59, S130–S152. doi: 10.1080/10408398.2018.1542587

Gao, J., Xu, K., Liu, H., Liu, G., Bai, M., Peng, C., et al. (2018). Impact of the gut microbiota on intestinal immunity mediated by tryptophan metabolism. *Front. Cell. Infect. Microbiol.* 8:13. doi: 10.3389/fcimb.2018.00013

Gao, K., Pi, Y., Mu, C. L., Farzi, A., Liu, Z., and Zhu, W. Y. (2019). Increasing carbohydrate availability in the hindgut promotes hypothalamic neurotransmitter synthesis: Aromatic amino acids linking the microbiota-brain axis. *J. Neurochem.* 149, 641–659. doi: 10.1111/jnc.14709

Guo, S. H., Nighot, M., Al-Sadi, R., Alhmoud, T., Nighot, P., and Ma, T. Y. (2015). Lipopolysaccharide regulation of intestinal tight junction permeability is mediated by tlr4 signal transduction pathway activation of fak and myd88. *J. Immunol.* 195, 4999–5010. doi: 10.4049/jimmunol.1402598

Han, H., Yi, B., Zhong, R., Wang, M., Zhang, S., Ma, J., et al. (2021). From gut microbiota to host appetite: Gut microbiota-derived metabolites as key regulators. *Microbiome* 9:162. doi: 10.1186/s40168-021-01093-y

Hu, W., Huang, L., Zhou, Z., Yin, L., and Tang, J. (2021). Diallyl disulfide (dads) ameliorates intestinal candida albicans infection by modulating the gut microbiota and metabolites and providing intestinal protection in mice. *Front. Cell. Infect. Microbiol.* 11:743454. doi: 10.3389/fcimb.2021.743454

Huang, P., Jiang, A., Wang, X., Zhou, Y., Tang, W., Ren, C., et al. (2021). Nmn maintains intestinal homeostasis by regulating the gut microbiota. *Front. Nutr.* 8:714604. doi: 10.3389/fnut.2021.714604

Jayachandran, M., Chen, J., Chung, S. S. M., and Xu, B. (2018). A critical review on the impacts of beta-glucans on gut microbiota and human health. *J. Nutr. Biochem.* 61, 101–110. doi: 10.1016/j.jnutbio.2018.06.010

Johnson, W. T., Dorn, N. C., Ogbonna, D. A., Bottini, N., and Shah, N. J. (2022). Lipid-based regulators of immunity. *Bioeng. Transl. Med.* 7:e10288. doi: 10.1002/btm2.10288

Krautkramer, K. A., Fan, J., and Backhed, F. (2021). Gut microbial metabolites as multi-kingdom intermediates. *Nat. Rev. Microbiol.* 19, 77–94. doi: 10.1038/s41579-020-0438-4

Krunt, O., Zita, L., Kraus, A., Bures, D., Needham, T., and Volek, Z. (2022). The effect of housing system on rabbit growth performance, carcass traits, and meat quality characteristics of different muscles. *Meat Sci.* 193:108953. doi: 10.1016/j.meatsci.2022.108953

Leandro, J., and Houten, S. M. (2019). Saccharopine, a lysine degradation intermediate, is a mitochondrial toxin. *J. Cell Biol.* 218, 391–392. doi: 10.1083/jcb.201901033

Lee, N. Y., Yoon, S. J., Han, D. H., Gupta, H., Youn, G. S., Shin, M. J., et al. (2020). Lactobacillus and pediococcus ameliorate progression of non-alcoholic fatty liver disease through modulation of the gut microbiome. *Gut Microbes* 11, 882–899. doi: 10.1080/19490976.2020.1712984

Lin, H. M., Barnett, M. P., Roy, N. C., Joyce, N. I., Zhu, S., Armstrong, K., et al. (2010). Metabolomic analysis identifies inflammatory and noninflammatory metabolic effects of genetic modification in a mouse model of crohn's disease. *J. Proteome. Res.* 9, 1965–1975. doi: 10.1021/pr901130s

Liu, B., Cui, Y., Ali, Q., Zhu, X., Li, D., Ma, S., et al. (2022). Gut microbiota modulate rabbit meat quality in response to dietary fiber. *Front. Nutr.* 9:849429. doi: 10.3389/fnut.2022.849429

Liu, Y., Hou, Y., Wang, G., Zheng, X., and Hao, H. (2020). Gut microbial metabolites of aromatic amino acids as signals in host-microbe interplay. *Trends Endocrinol. Metab.* 31, 818–834. doi: 10.1016/j.tem.2020.02.012

Luo, J., Liu, S., Yu, B., He, J., Mao, X., Cheng, L., et al. (2019). Beta-glucan from agrobacterium sp. Zx09 improves growth performance and intestinal function in weaned piglets. *J. Anim. Physiol. Anim. Nutr.* 103, 1818–1827. doi: 10.1111/jpn.13163

Luo, Z. Z., Shen, L. H., Jiang, J., Huang, Y. X., Bai, L. P., Yu, S. M., et al. (2019). Plasma metabolite changes in dairy cows during parturition identified using untargeted metabolomics. *J. Dairy Sci.* 102, 4639–4650. doi: 10.3168/jds.2018-15601

Luo, Y., Zhang, L., Li, H., Smidt, H., Wright, A. G., Zhang, K., et al. (2017). Different types of dietary fibers trigger specific alterations in composition and predicted functions of colonic bacterial communities in balb/c mice. *Front. Microbiol.* 8:966. doi: 10.3389/fmicb.2017.00966

Luo, Z., Ma, L., Zhou, T., Huang, Y., Zhang, L., Du, Z., et al. (2022). Beta-glucan alters gut microbiota and plasma metabolites in pre-weaning dairy calves. *Metabolites* 12:697. doi: 10.3390/metabo12080687

McDonnell, R. P., Jv, O. D., Earley, B., Clarke, A. M., and Kenny, D. A. (2019). Effect of supplementation with n-3 polyunsaturated fatty acids and/or beta-glucans on performance, feeding behaviour and immune status of holstein friesian bull calves during the pre- and post-weaning periods. *J. Anim. Sci. Biotechnol.* 10:7. doi: 10.1186/s40104-019-0317-x

Meguid, M. M., Fetissov, S. O., Varma, M., Sato, T., Zhang, L., Laviano, A., et al. (2000). Hypothalamic dopamine and serotonin in the regulation of food intake. *Nutrition* 16, 843–857. doi: 10.1016/s0899-9007(00)00449-4

Mosa, A., Gerber, A., Neunzig, J., and Bernhardt, R. (2016). Products of gut-microbial tryptophan metabolism inhibit the steroid hormone-synthesizing cytochrome p450 11a1. *Endocrine* 53, 610–614. doi: 10.1007/s12020-016-0874-0

Nasr, M. A. F., Abd-Elhamid, T., and Hussein, M. A. (2017). Growth performance, carcass characteristics, meat quality and muscle amino-acid profile of different rabbits breeds and their crosses. *Meat Sci.* 134, 150–157. doi: 10.1016/j.meatsci.2017.07.027

Nixon, G. F. (2009). Sphingolipids in inflammation: Pathological implications and potential therapeutic targets. *Br. J. Pharmacol.* 158, 982–993.

Oceandy, D., Amanda, B., Ashari, F. Y., Faizah, Z., Azis, M. A., and Stafford, N. (2019). The cross-talk between the tnf-alpha and rassf-hippo signalling pathways. *Int. J. Mol. Sci.* 20:2346. doi: 10.3390/ijms20092346

Ren, W., Wang, P., Yan, J., Liu, G., Zeng, B., Hussain, T., et al. (2018). Melatonin alleviates weanling stress in mice: Involvement of intestinal microbiota. *J. Pineal. Res.* 64:e12448. doi: 10.1111/jpi.12448

Safi-Stibler, S., Thevenot, E. A., Jouneau, L., Jouin, M., Seyer, A., Jammes, H., et al. (2020). Differential effects of post-weaning diet and maternal obesity on mouse liver and brain metabolomes. *Nutrients* 12:1572. doi: 10.3390/nu12061572

Sam-On, M. F. S., Mustafa, S., Yusof, M. T., Mohd Hashim, A., Abbasiliasi, S., Zulkifly, S., et al. (2022). Evaluation of three bacillus spp. Isolated from the gut of giant freshwater prawn as potential probiotics against pathogens causing vibriosis and aeromonosis. *Microb. Pathog.* 164:105417. doi: 10.1016/j.micpath.2022.105417

Sanders, M. E., Merenstein, D. J., Reid, G., Gibson, G. R., and Rastall, R. A. (2019). Probiotics and prebiotics in intestinal health and disease: From biology to the clinic. *Nat. Rev. Gastroenterol. Hepatol.* 16, 605–616. doi: 10.1038/s41575-019-0173-3

Schneider, C., Rath, G. M., Delorme, N., El Btaouri, H., Hornebeck, W., and Martiny, L. (2005). Interleukin-1beta (il-1beta) induces a crosstalk between camp and ceramide signaling pathways in thyroid epithelial cells. *Biochimie* 87, 1121–1126. doi: 10.1016/j.biochi.2005.05.004

Sedger, L. M., and McDermott, M. F. (2014). Tnf and tnf-receptors: From mediators of cell death and inflammation to therapeutic giants - past, present and future. *Cytokine Growth Factor Rev.* 25, 453–472. doi: 10.1016/j.cytogfr.2014.07.016

Shao, X., Sun, C., Tang, X., Zhang, X., Han, D., Liang, S., et al. (2020). Anti-inflammatory and intestinal microbiota modulation properties of jinxiang garlic (*Allium sativum* l.) polysaccharides toward dextran sodium sulfate-induced colitis. *J. Agric. Food Chem.* 68, 12295–12309. doi: 10.1021/acs.jafc.0c04773

Shen, R. L., Dang, X. Y., Dong, J. L., and Hu, X. Z. (2012). Effects of oat beta-glucan and barley beta-glucan on fecal characteristics, intestinal microflora, and intestinal bacterial metabolites in rats. *J. Agric. Food Chem.* 60, 11301–11308. doi: 10.1021/jf302824h

Song, X., Zhong, L., Lyu, N., Liu, F., Li, B., Hao, Y., et al. (2019). Inulin can alleviate metabolism disorders in ob/ob mice by partially restoring leptin-related pathways mediated by gut microbiota. *Genom. Proteom. Bioinform.* 17, 64–75. doi: 10.1016/j.gpb.2019.03.001

Suzuki, T. (2013). Regulation of intestinal epithelial permeability by tight junctions. *Cell Mol. Life Sci.* 70, 631–659. doi: 10.1007/s00018-012-1070-x

Thompson, R. S., Gaffney, M., Hopkins, S., Kelley, T., Gonzalez, A., Bowers, S. J., et al. (2021). Ruminiclostridium 5, *Parabacteroides distasonis*, and bile acid profile ar.e modulated by prebiotic diet and associate with facilitated sleep/clock realignment after chronic disruption of rhythms. *Brain Behav. Immun.* 97, 150–166. doi: 10.1016/j.bbi.2021.07.006

Tian, G., Wu, X., Chen, D., Yu, B., and He, J. (2017). Adaptation of gut microbiome to different dietary nonstarch polysaccharide fractions in a porcine model. *Mol. Nutr. Food Res.* 61:1700012. doi: 10.1002/mnfr.201700012

Valdes, A. M., Walter, J., Segal, E., and Spector, T. D. (2018). Role of the gut microbiota in nutrition and health. *BMJ* 361:k2179.

van der Hee, B., and Wells, J. M. (2021). Microbial regulation of host physiology by short-chain fatty acids. *Trends Microbiol.* 29, 700–712. doi: 10.1016/j.tim.2021.02.001

Wang, J., Ni, X., Wen, B., Zhou, Y., Liu, L., Zeng, Y., et al. (2020). Bacillus strains improve growth performance via enhancing digestive function and anti-disease ability in young and weaning rex rabbits. *Appl. Microbiol. Biotechnol.* 104, 4493–4504. doi: 10.1007/s00253-020-10536-9

Wang, R., Zhang, Z., Aihemaitijiang, S., Ye, C., Halimulati, M., Huang, X., et al. (2022). Oat beta glucan ameliorates renal function and gut microbiota in diabetic rats. *Front. Nutr.* 9:875060. doi: 10.3389/fnut.2022.875060

Wu, X., Chen, D., Yu, B., Luo, Y., Zheng, P., Mao, X., et al. (2018). Effect of different dietary non-starch fiber fractions on growth performance, nutrient digestibility, and intestinal development in weaned pigs. *Nutrition* 51-52, 20–28. doi: 10.1016/j.nut.2018.01.011

Xu, L., Wang, Y., Ma, Z., Tang, X., and Gao, Y. (2020). Urine metabolomics study on potential hepatoxic biomarkers identification in rats induced by aurantio-obtusin. *Front. Pharmacol.* 11:1237. doi: 10.3389/fphar.2020.01237

Yokoyama, M. T., and Carlson, J. R. (1979). Microbial metabolites of tryptophan in the intestinal tract with special reference to skatole. *Am. J. Clin. Nutr.* 32, 173–178. doi: 10.1093/ajcn/32.1.173

You, S., Hu, X., Zhao, Q., Chen, X., and Xu, C. (2013). Oat beta-glucan inhibits lipopolysaccharide-induced nonalcoholic steatohepatitis in mice. *Food Funct.* 4, 1360–1368. doi: 10.1039/c3fo60081e

Yu, J., Xia, J., Yang, C., Pan, D., Xu, D., Sun, G., et al. (2022). Effects of oat beta-glucan intake on lipid profiles in hypercholesterolemic adults: A systematic review and meta-analysis of randomized controlled trials. *Nutrients* 14:2043. doi: 10.3390/nu14102043

Yuan, Z., Yang, L., Zhang, X., Ji, P., Hua, Y., and Wei, Y. (2020). Mechanism of huang-lian-jie-du decoction and its effective fraction in alleviating acute ulcerative colitis in mice: Regulating arachidonic acid metabolism and glycerophospholipid metabolism. *J. Ethnopharmacol.* 259:112872. doi: 10.1016/j.jep.2020.112872

Zhang, P. P., Hu, X. Z., Zhen, H. M., Xu, C., and Fan, M. T. (2012). Oat beta-glucan increased atpases activity and energy charge in small intestine of rats. *J. Agric. Food Chem.* 60, 9822–9827. doi: 10.1021/jf3017496

Zhang, X. Y., Chen, J., Yi, K., Peng, L., Xie, J., Gou, X., et al. (2020). Phlorizin ameliorates obesity-associated endotoxemia and insulin resistance in high-fat diet-fed mice by targeting the gut microbiota and intestinal barrier integrity. *Gut Microbes* 12, 1–18. doi: 10.1080/19490976.2020.1842990

Zou, X. Y., Zhang, M., Tu, W. J., Zhang, Q., Jin, M. L., Fang, R. D., et al. (2022). *Bacillus subtilis* inhibits intestinal inflammation and oxidative stress by regulating gut flora and related metabolites in laying hens. *Animal* 16:100474. doi: 10.1016/j.animal.2022.100474

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# Comparison of coated and uncoated trace elements on growth performance, apparent digestibility, intestinal development and microbial diversity in growing sheep

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The suitable supplement pattern affects the digestion and absorption of trace minerals by ruminants. This study aimed to compare the effects of coated and uncoated trace elements on growth performance, apparent digestibility, intestinal development and microbial diversity in growing sheep. Thirty 4-month-old male Yunnan semi-fine wool sheep were randomly assigned to three treatments ($n$ = 10) and fed with following diets: basal diet without adding exogenous trace elements (CON), basal diet plus 400 mg/kg coated trace elements (CTE, the rumen passage rate was 65.87%) and basal diet plus an equal amount of trace elements in uncoated form (UTE). Compared with the CON group, the average daily weight gain and apparent digestibility of crude protein were higher ($P < 0.05$) in the CTE and UTE groups, while there was no difference between the CTE and UTE groups. The serum levels of selenium, iodine and cobalt were higher ($P < 0.05$) in the CTE and UTE groups than those in the CON group, the serum levels of selenium and cobalt were higher ($P < 0.05$) in the CTE group than those in the UTE group. Compared with the CON and UTE groups, the villus height and the ratio of villus height to crypt depth in duodenum and ileum were higher ($P < 0.05$) in the CTE groups. The addition of trace minerals in diet upregulated most of the relative gene expression of *Ocludin*, *Claudin-1*, *Claudin-2*, *ZO-1*, and *ZO-2* in the duodenum and jejunum and metal ion transporters (*FPN1* and *ZNT4*) in small intestine. The relative abundance of the genera *Christensenellaceae R-7 group*, *Ruminococcus 1*, *Lachnospiraceae NK3A20 group*, and *Ruminococcaceae* in ileum, and *Ruminococcaceae UCG-014* and *Lactobacillus* in colon was higher in the CTE group that in the CON group. These results indicated that dietary trace mineral addition improved the

growth performance and intestinal development, and altered the structure of intestinal bacteria in growing sheep. Compared to uncoated form, offering trace mineral elements to sheep in coated form had a higher absorption efficiency, however, had little effect on improving growth performance of growing sheep.

KEYWORDS

**trace elements, sheep, growth performance, intestinal development, gut microbiota**

## 1 Introduction

Trace elements such as iron (Fe), copper (Cu), manganese (Mn), zinc (Zn), selenium (Se), iodine (I), and cobalt (Co) are an essential class of substances for animals provided primarily by food sources. Although the mass proportion in animal body is less than 0.01%, trace elements play an indispensable role in maintaining their normal physiological activities, such as hemopoiesis, immune response, energy metabolism, enzyme activity and reproductive function (Rabiee et al., 2010; Overton and Yasui, 2014). A deficiency of any of these trace minerals could lead to a negative impact on the health and productivity of livestock (Spears and Weiss, 2008). Commonly, inorganic salts of trace elements, such as sulfate, oxide, carbonate and chloride are supplemented in diets to meet the requirement of livestock for trace elements. However, for ruminants, the ionic bonds in inorganic salts usually dissociate as they flow through the digestive tract, allowing undesirable interactions with other molecules and preventing absorption, thus reducing the bioavailability and efficiency of trace elements (Spears, 1996, 2003; Samarin et al., 2022). For example, sulfates are first reduced to sulfides by rumen microbes, which react with molybdate to form thiomolybdate and then react with cupric ion to generate un-digestible compounds, thereby limiting the absorption of dietary Cu (Dick et al., 1975). The efficiency of absorption of Cu and Se by ruminants is much lower than that of non-ruminants (Spears, 2003). Furthermore, in some scenarios, rumen fermentation was affected by feeding conventional mineral salts, as was ruminal digestibility of nutrients (Genther and Hansen, 2015; Faulkner and Weiss, 2017; Arce-Cordero et al., 2020). To improve the availability of trace elements, the addition of coated trace elements (slow release in the rumen) to the diet was a practicable strategy (Abdelrahman et al., 2017; Arce-Cordero et al., 2020; Potier et al., 2022).

There were a series of reports on the effective application of coated trace elements in ruminants. Previous studies reported that supplementation of coated slow-release trace elements at late gestation increased the content of Zn and Se in plasma and milk of ewes, and enhanced the birth and weaning weight of lambs (Abdelrahman et al., 2017; Aliarabi et al., 2019). Supplementation of coated sustained-release trace minerals also improved the milk performance and altered rumen microbial structure of lactating yaks (Zhao et al., 2022). Compared with the addition of copper sulfate in diets, coated copper sulfate addition improved lactation performance and apparent nutrient digestibility in dairy cows (Wang C. et al., 2021), increased the average daily gain and stimulated rumen enzyme activity in Holstein bulls (Wu et al., 2021). The addition of an equal amount of coated sodium selenite contributed to improve the milk production of dairy cows (Zhang et al., 2020), improved the ruminal fermentation and nutrient digestibility of dairy bulls (Liu et al., 2020), compared to uncoated sodium selenite. However, to our knowledge, there is very little information comparing uncoated and coated compound trace elements in ruminants.

The improved bioavailability of trace elements is beneficial to exert their biological functions. More than 50% of known enzymes have at least one metal as a cofactor, of which zinc is the most common, followed by iron and manganese (Andreini et al., 2008). Most trace minerals are also involved in redox reactions, as free radicals are able to interact with metal-binding superoxide dismutase and break down into less or less toxic substances, indicating that trace minerals play an important role in alleviating oxidative stress (Pajarillo et al., 2021). Dietary zinc oxide supplementation enhanced intestinal morphological and functional development by increasing antioxidant capacity and maintaining mucosal barrier integrity in weaned piglets (Zhu et al., 2017,?). Besides, trace elements such as Fe, Mn, Zn, Se and Cu have been revealed to regulate the gut microbiota in different animals or *in vitro* (Dostal et al., 2015; Zhang et al., 2019; Foong et al., 2020; Kociova et al., 2020; Wang et al., 2020). The regulation of the gut barrier and immune response by selenium intake has been attributed to modulation of the gut microbiota (Kasaikina et al., 2011). We suspected that the coated trace elements had better biological activity on growth performance and intestinal development in sheep compared to uncoated trace elements. Thus, the ultimate objective of this study was to compare the effects of coated and uncoated trace elements on growth performance, apparent digestibility, intestinal development, and microbial diversity in ileum and colon of growing sheep.

# 2 Materials and methods

## 2.1 Approval by the ethics committee

All animals in this study were treated according to the Chinese Guidelines for Animal Welfare and the protocol was approved by the Experimental Animal Committee of Animal Nutrition Institute, Sichuan Agricultural University (Approval number: #SCAUAC2019-36).

## 2.2 Experimental design, diets, and animals

**Exp. 1.** The experiment was performed at the farm of Kunming Yixingheng Livestock Technology Co., Ltd. (Yunnan, China; 25.07°N, 102.55°E, and 2,200 m elevation) in August 2019. Three 15-month-old male Yunnan semi-fine wool sheep (55 ± 2.3 kg of body weight) with permanent rumen cannulas were used as experimental animals. All experimental sheep were housed in individual pens and fed twice per day (0700 and 1700 h) and had free access to water and diets. The basal diet was formulated according to the recommendation of National Research Council (2007) for growing sheep except for trace elements, and the composition and nutrient levels are shown in **Table 1**. The coated trace elements (contained 120 mg Fe, 16 mg Cu, 50 mg Mn, 52 mg Zn, 0.8 mg Se, 1.0 mg I and 0.8 mg Co per gram) were commercially produced and provided by Fujian Syno Biotech Co., Ltd. (Fujian, China). The final product of coated trace elements is a molten granulate produced by proportioning an inorganic trace element premix and basic coating materials (carboxymethyl cellulose, resistant starch and maltodextrin) in the solid phase. Approximately 5.0 g of coated trace elements was added to a heat-sealed nylon bag (4 × 6 cm in bag size; 50 μm in pore size). Each sheep was considered a replicate, each sample was repeated three times, and each sample had two parallel replicates per sheep for a total of six replicates. A maximum of four bags with samples were placed in the liquid phase of the rumen contents through a polyethylene hose at the same time. The coated trace elements were sequentially incubated in different batches for 0, 2, 4, 8, 16, 24, 36, and 48 h.

**Exp. 2.** The experiment was performed at the same farm as Exp. 1 from August to September 2021. A total of thirty 4-month-old male Yunnan semi-fine wool sheep (29.7 ± 0.3 kg of body weight) were divided into three treatments in a completely randomized design as follows: CON treatment, fed with a basal diet without adding exogenous trace elements; CTE treatment, fed with a basal diet plus 400 mg/kg coated trace elements in Exp. 1; UTE treatment, fed with a basal diet plus an equal amount of trace elements with uncoated form. The basal diet was kept the same as that in Exp. 1. The addition of 400 mg/kg coated trace elements had the best growth performance of Yunnan semi-fine wool sheep, according to our previous study (unpublished data). The uncoated trace elements are constituted by ferrous sulfate heptahydrate, copper sulfate pentahydrate, manganese sulfate monohydrate, zinc sulfate heptahydrate, sodium selenite pentahydrate, potassium iodide, and cobalt chloride hexahydrate. All experimental sheep were housed in separate pens and fed twice daily at 0700 and 1700 h (allowed 5% refusal). The trial lasted for 40 days, of which 10 days were the adaptation period and 30 days were the formal trial period.

## 2.3 Sample collection

All mobile nylon bags were incubated at the same time (0630 h) prior to morning feeding and retrieved according to the expected incubation time in Exp. 1. After removal from the rumen, the nylon bags were repeatedly rinsed under cold running water to remove adhesions and subsequently placed in cold water for soaking while the nylon bags were repeatedly kneaded by hand gently until the filtered water was clear (Wang E. et al., 2021). The cleaned nylon bags were dried to constant weight at 65°C for 48 hours, and the weight after drying was recorded and the rumen disappearance rate of the coated trace elements was calculated by the difference from the initial weight.

In Exp. 2, the body weight of experimental sheep was determined before the morning feeding at the beginning (day 0) and end (day 30) of the experiment to calculate the average daily gain (ADG). The orts were collected and weighed every day, the dry matter intake (DMI) per sheep was obtained from the ration supplied and refused, and the feed conversion ratio (FCR) was calculated as dividing DMI by ADG. Four sheep were randomly selected from each group and housed in individual metabolic cages for 7 days (from day 10 to 16, the first two days were the adaptation period), and all feces were collected, weighed and recorded. A 5% aliquot of each fecal sample was removed and immediately stored at −20°C until further analysis. Jugular vein blood samples were collected on day 30 before feeding in the morning and centrifuged at 1,600 × g for 10 minutes to obtain serum, which were then aliquoted and stored at −20°C until the next analysis. On day 30, four sheep of close to group average weight in each treatment were slaughtered, and tissue samples of duodenum, jejunum and ileum and chyme samples of ileum and colon were collected. For the tissue samples, the 2- and 6-cm segments were cut at mid-duodenum, mid-jejunum and mid-ileum, respectively (Hou et al., 2010). The 2-cm intestinal segments were flushed gently with pre-cooled 0.9% sodium chloride solution (w/v, physiological saline, Sichuan Kelun Pharmaceutical Co., Ltd., Chengdu, China) and then placed in 4% paraformaldehyde fix solution (Beyotime, Shanghai, China) for morphological analysis. The 6-cm intestinal segments were opened longitudinally and the contents were flushed with pre-cooled physiological saline and immediately frozen in liquid nitrogen for total RNA extraction. Chyme from the mid-section of the ileum and colon was collected in 2 mL

TABLE 1 Ingredients and chemical composition of basal diet (DM basis).

| Ingredients | Content, % | Chemical composition[b] | |
|---|---|---|---|
| Corn | 36.00 | Metabolic energy, MJ/kg | 9.36 |
| Soybean meal | 7.80 | Crude protein, % | 10.78 |
| Wheat bran | 5.10 | Neutral detergent fiber, % | 36.24 |
| NaCl | 0.22 | Acid detergent fiber, % | 18.77 |
| $NaHCO_3$ | 0.38 | Calcium, % | 0.48 |
| Premix[a] | 0.50 | Phosphorus, % | 0.39 |
| Corn silage | 30.00 | Iron, mg/kg | 84.61 |
| Wheat straw | 20.00 | Manganese, mg/kg | 5.23 |
| Total | 100.00 | Zinc, mg/kg | 25.47 |
| | | Copper, mg/kg | 18.65 |
| | | Selenium, mg/kg | 0.02 |
| | | Iodine, mg/kg | 0.09 |
| | | Cobalt, mg/kg | 0.05 |

[a]The premix provided the following per kg of diet: vitamin A, 10,000 IU; vitamin $D_3$, 1,000 IU; vitamin E 33.4 mg.
[b]Calculated values.

sterile cryopreserved tubes (Wuxi NEST Biotechnology Co., Ltd., Wuxi, China) for bacterial DNA extraction. All sampling procedure were finished within 30 minutes after the sheep being slaughtered.

## 2.4 Total tract apparent nutrient digestibility

Fecal samples from each sheep were thawed and mixed together, then dried in a forced air oven at 55°C for 48 hours and ground to pass through a 40-mesh screen before analysis, as were the samples of diet and orts. The content of dry matter (DM), crude protein (CP), acid detergent fiber (ADF), calcium (Ca) and phosphorus (P) was analyzed according to AOAC (AOAC International, 2007), and the content of neutral detergent fiber (NDF) were determined according to van Soest et al. (1991). Consequently, the apparent digestibility of nutrients can be calculated as follow: apparent nutrient digestibility = [(feed intake × nutrient content in the diet) − (fecal output × nutrient content in the feces)]/(feed intake × nutrient content in the diet × 100% (Stein and Bohlke, 2007).

## 2.5 Intestinal morphological observation

The 2-cm intestinal segments for morphological analysis were dehydrated and embedded in paraffin, cut into three sections with 5 μm thick and stained with Hematoxylin and Eosin (H&E). Morphological observation was carried out with the Nikon ECLIPSE 80i microscope (Nikon Co., Ltd., Tokyo, Japan). Villus height (distance from villus tip to crypt ostia) and width (distance from widest villus), crypt depth (distance from crypt ostia to base) were measured with the Image-Pro Plus 6.0 software (Media Cybernetics Inc., Bethesda, MD, United States). The ratio of villus height and crypt depth (V/C) was calculated as dividing villus height by crypt depth. Only the villi heights and crypt depths from vertically oriented ten adjacent villi were measured and averaged (Hou et al., 2010).

## 2.6 Serum sample analysis

The serum concentrations of Fe, Cu, Mn, Zn, Se, I and Co were determined in an induced coupled plasma mass-spectrometry (iCAP$^{TM}$ RQ ICP-MS, Thermo Fisher Scientific, Waltham, MA, USA) as described previously with minor modifications (Liu et al., 2014). In brief, 0.1 mL of reconstituted serum samples were diluted to 2 mL with a 1: 20 (v/v) sample diluent solution [constructed from 0.01% (w/v) Triton X-100, 0.005% (w/v) L-cysteine, 0.5% (v/v) nitric acid and deionized water]. The standard solutions containing the required seven single elements were purchased from the TMRM QC standards material center (Changzhou, China), and then diluted with sample diluent solution to appropriate concentrations according to the predicted concentrations of trace elements in serum. The major ICP-MS operating parameters were set as follows: Analyzer Pressure Readback, 1E-06 mbar; Spray Chamber Temperature, 2°C; Cool Flow, 14 L/min; Auxilliary Flow, 0.79 L/min; Nebulizer Flow, 1.06 L/min; Plasma Power, 1550 W; Q Cell gas, He 4.0 mL/min; injection method, automatic injection.

## 2.7 Real-time quantitative PCR (RT-PCR) analysis

Intestinal tissue samples were ground into powder in liquid nitrogen. Total RNA was extracted from tissue samples with the RNA extraction and purification kit (Tiangen Biotech Co. Ltd., Beijing, China) according to the manufacturer's instructions. The concentration and purity of total RNA were determined with the NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, Wilmington, DE, USA). The integrity of total RNA was assessed by the 1% denatured agarose gel electrophoresis. Subsequently, the qualified total RNA was reverse transcribed to cDNA by a reverse transcription Kit (HiScript® III RT SuperMix for qPCR (+gDNA wiper), Vazyme Biotech Co., Ltd., Nanjing, China). The real-time quantitative PCR (RT-PCR) reaction was performed with a real-time quantitative PCR Kit (ChamQ SYBR Color qPCR Master Mix (low ROX premixed), Vazyme Biotech Co., Ltd., Nanjing, China) in a QuantStudio™ 6 Real-Time PCR system (Applied Biosystems, Foster City, CA, USA) following the manufacturer's instruction. Relative expression of genes was calculated by using the comparative cycle threshold ($2^{-\Delta\Delta Ct}$) method. The mean value of gene expression levels in the CON treatment was set to 1.00, and GAPDH was used as internal control. The sequence and product size of genes used in this study are shown in **Table 2**.

## 2.8 DNA Extraction, amplification, and sequencing

The microbial diversity in ileal and colonic chyme was detected by 16s rDNA sequencing. The total genome DNA (gDNA) from the chyle samples was extracted and purified with the ZymoBIOMICS™ DNA Microprep Kit (Zymo Research, Orange, CA, USA). The NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific) and 0.8% agarose gel electrophoresis were used to detect the concentration and integrity of DNA, respectively. The universal bacterial primer (515F: 5′-GTGYCAGCMGCCGCGGTAA-3′ and 806R: 5′-GGACTACHVGGGTWTCTAAT-3′) with sequencing adapter and barcode were used to amplified the V3-V4 regions of the 16S rRNA gene (Caporaso et al., 2011). The PCR reaction was conducted in the GeneAmp PCR system 9700 thermocycler (Applied Biosystem, Foster city, CA, USA) with the high-fidelity polymerase KOD-401B FX Neo (Toyobo, Osaka, Japan) and the program was as follows: 94°C for 1 min, followed by 30 cycles at 94°C for 20 s, 54°C for 30 s and 72°C for 30 s, and a final extension at 72°C for 5 min. The resulting PCR products were extracted from a 2% agarose gel and further purified with the Zymoclean Gel Recovery Kit (Zymo Research) and quantified with the Qubit 2.0 Fluorometer (Thermo Fisher Scientific, Waltham, MA, USA). Sequencing by synthesis was performed with the HiSeq Rapid SBS kit v2 (200 cycles, Illumina, Inc., San Diego, CA, USA) on the Illumina HiSeq2500 platform at Chengdu Rhonin Biosciences Co., Ltd. (Chengdu, China).

## 2.9 Sequencing data analyses

The processing of sequencing data was performed as previously described (Zhou et al., 2022). Briefly, the Fast Length Adjustment of SHort reads (FLASH v1.2.11)[1] were used to splice the paired-end sequencing reads obtained from the Illumina platform, which then assigned to samples based on the unique barcode 37. The raw data was generated by truncating barcode sequences, followed by trimming using Trimmomatic v0.36,[2] and using the Uchime algorithm[3] to identify and remove chimera sequences based on the "Gold" database[4] to obtain clean reads (Edgar et al., 2011; Bolger et al., 2014). The qualified reads were clustered into operational taxonomic units (OTUs) by using the Usearch (a free unique sequence analysis tool)[5] based on a 97% similarity threshold (Edgar, 2013). The analysis of α-diversity (including Shannon, Simpson, Chao1, ACE, the Good's coverage, and PD indexes) and β-diversity as well as principal coordinate analysis (PCoA) of the Bray-Curtis distance were performed using the R package (Kembel et al., 2010; Chen et al., 2012). The potential Kyoto Encyclopedia of Genes and Genomes (KEGG) functional profiles of microbial communities were predicted with Tax4Fun[6] (a free software package that predicts the functional capabilities of microbial communities based on 16S rRNA datasets) based on the SILVA ribosomal RNA database,[7] and the secondary metabolic pathways were clustered (Aßhauer et al., 2015).

## 2.10 Calculations and statistical analysis

The degradation kinetic parameters of coated trace elements over time were calculated with the following exponential equation (Ørskov and McDonald, 1979): $DR = a + b\ (1 - e^{-c \times t})$, where "DR" is the disappearance rate of coated trace elements at time "t" (%), "a" is the rapidly degradable fraction (%), "b" is the potentially degradable fraction (%) and "c" is the rate at which b is degraded per hour (rate constant). The values of "a," "b," and "c" were calculated with the non-linear procedure of SPSS v23.0 (SPSS Inc., Chicago, IL, USA). The effective degradability (ED, %) was calculated according

1 http://www.geneious.com/plugins/flash/
2 http://www.usadellab.org/cms/?page=trimmomatic
3 http://drive5.com/usearch/manual/uchime_algo.html
4 http://drive5.com/uchime/uchime_download.html
5 http://drive5.com/uparse/
6 http://tax4fun.gobics.de/
7 http://www.arb-silva.de/

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TABLE 2 The sequence and product size of genes.

| Gene | Sequence (5′–3′) | Accession ID | Product length (bp) |
|---|---|---|---|
| *Occludin* | F: CCGTTGACTTCACCTGTGGA | XM_012096797.4 | 195 |
| | R: TTCCCTGATCCAGTCGTCCT | | |
| *Claudin-1* | F: AAGACGACGAGGCACAGAAG | NM_001185016.1 | 186 |
| | R: CAGCCCAGCCAATGAAGAGA | | |
| *Claudin-2* | F: CCTCCCTGTTCTCCCTGGTA | XM_027963236.2 | 299 |
| | R: GCACCTTCTGACACGATCCA | | |
| *ZO-1* | F: GATGTTGCCAGGGAGAAGCT | XM_042235171.1 | 247 |
| | R: TCACACCCTGCTTTGAGTCC | | |
| *ZO-2* | F: TCCTTGTGAGTGGGATTGGC | XM_027964513.2 | 365 |
| | R: TCTTCCCGCTTTTCCTCAGC | | |
| *ZNT4* | F: GCCTTTGACTTCTCGGACGA | XM_042252522.1 | 175 |
| | R: TGTTGGCCAAGGGTAAGTCC | | |
| *FPN1* | F: CAGGGAGGATGCTGTGGATC | XM_042243635.1 | 216 |
| | R: GTCGCCAATGATAGCTCCCA | | |
| *GAPDH* | F: GGTCACCAGGGCTGCTTTTA | NM_001190390.1 | 147 |
| | R: TTCCCGTTCTCTGCCTTGAC | | |

to (Ørskov and McDonald, 1979): ED = a + [b × c/(c + k)], where "a," "b," and "c" are the same parameters as above, "k" is the rumen particle passage rate (%/h). When fed *ad libitum*, the value of "k" was 0.06, according to (Ørskov and McDonald, 1979).

The experimental data in Exp. 2 were statistically analyzed with one-way analysis of variance (ANOVA) followed Duncan's *post-hoc* test or Kruskal–Wallis-test with SPSS version 23.0. Each sheep was regarded as a statistical unit. $P \leq 0.05$ was considered statistically significant, $0.05 < P < 0.10$ was considered as trends. The results were shown as mean and standard error (SEM).

# 3 Results

## 3.1 Rumen degradation rate

The ruminal disappearance rates of coated trace elements at different times are shown in **Figure 1**. The non-linear model resulted in the degradation kinetic parameters as follow: a = 6.292, b = 81.728, c = 0.031 and $R^2$ = 0. 974. Thus, the rumen effective degradability of coated trace elements can be calculated as 34.13%, the rumen passage rate was 65.87%.

## 3.2 Growth performance

Compared with the CON treatment, the final body weight and ADG were increased ($P < 0.05$) in the CTE and UTE treatments, the FCR was improved ($P < 0.05$) in the CTE and UTE treatments, whereas there was no difference between the CTE and UTE treatments (**Table 3**).

## 3.3 Total tract apparent nutrient digestibility

The apparent digestibility of CP was higher ($P < 0.05$) in the CTE and UTE treatments than that in the CON treatment, and that of other nutrients was not affected by the addition of trace elements (**Table 4**).

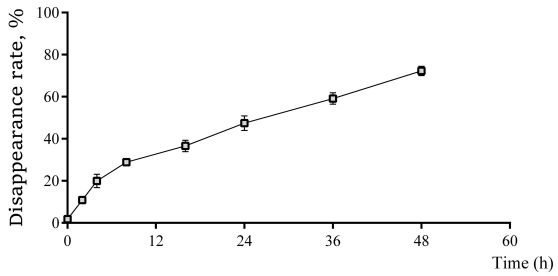


FIGURE 1
The real-time rumen disappearance rate of coated trace elements within 48 h of culture in nylon bag. Data are represented as mean and standard deviation, *n* = 3.

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TABLE 3 Effects of coated and uncoated trace elements on growth performance of sheep.

| Item | Treatments[1] | | | SEM | *P*-value |
|---|---|---|---|---|---|
| | CON | CTE | UTE | | |
| Initial body weight, kg | 29.76 | 29.78 | 29.79 | 0.45 | 1.000 |
| Final body weight, kg | 35.80$^{a}$ | 37.81$^{b}$ | 37.79$^{b}$ | 0.39 | 0.040 |
| Average daily gain, g | 201.24$^{a}$ | 267.77$^{b}$ | 266.80$^{b}$ | 11.51 | 0.020 |
| Dry matter intake, kg | 1.29 | 1.32 | 1.25 | 0.02 | 0.255 |
| FCR | 6.45$^{b}$ | 5.32$^{a}$ | 4.89$^{a}$ | 0.25 | 0.022 |

[1]CON, sheep fed with the basal diets; CTE, sheep fed with the basal diets plus 400 mg/kg coated trace elements; UTE, sheep fed with the basal diets plus an equal amount of trace elements in uncoated form; FCR, feed conversion ratio. Data with different small letter superscripts mean significantly different ($P < 0.05$), $n = 10$.

TABLE 4 Effects of coated and uncoated trace elements on total tract apparent digestibility of nutrients.

| Item | Treatments[1] | | | SEM | *P*-value |
|---|---|---|---|---|---|
| | CON | CTE | UTE | | |
| Dry matter, % | 57.28 | 63.10 | 61.53 | 1.13 | 0.078 |
| Crude protein, % | 47.87$^{a}$ | 59.88$^{b}$ | 60.64$^{b}$ | 2.04 | 0.002 |
| Neutral detergent fiber, % | 42.16 | 52.64 | 51.70 | 2.11 | 0.064 |
| Acid detergent fiber, % | 50.37 | 49.87 | 50.82 | 1.22 | 0.960 |
| Calcium, % | 54.51 | 55.56 | 56.79 | 1.04 | 0.713 |
| Phosphorus, % | 44.66 | 50.44 | 46.71 | 1.69 | 0.406 |

[1]CON, sheep fed with the basal diets; CTE, sheep fed with the basal diets plus 400 mg/kg coated trace elements; UTE, sheep fed with the basal diets plus an equal amount of trace elements in uncoated form. Data with different small letter superscripts mean significantly different ($P < 0.05$), $n = 4$.

## 3.4 Serum trace elements

Compared with the CTE treatment, the serum levels of Se and Co were lower ($P < 0.05$) in both the CON and UTE treatments, the serum level of I was lower ($P < 0.05$) in the CON treatment (Table 5).

## 3.5 Intestinal morphology

Dietary coated trace elements increased ($P < 0.05$) the villus height in the duodenum, jejunum and ileum, increased ($P < 0.05$) the crypt depth in the jejunum and the V/C in the ileum. No difference in intestinal morphology was observed between UTE and CON treatments (Table 6).

## 3.6 Relative gene expression of tight junction protein

As shown in Figure 2, compared with the CON treatment, both supplementation of coated and uncoated trace elements upregulated ($P < 0.05$) the relative mRNA abundance of *Claudin-1*, *Claudin-2*, and *Occludin* in the duodenum and jejunum. Dietary coated trace elements upregulated ($P < 0.05$) the mRNA abundance of *ZO-1* and *ZO-2* in the duodenum and jejunum, dietary uncoated trace elements upregulated ($P < 0.05$) the mRNA abundance of *ZO-2* in the duodenum.

## 3.7 Relative gene expression of metal ion transporters

The mRNA abundance of *FPN1* and *ZNT4* in the duodenum, jejunum and ileum was higher ($P < 0.05$) in the CTE treatment than that in the CON treatment, the mRNA abundance of *FPN1* and *ZNT4* in the duodenum and *FPN1* in the jejunum was higher ($P < 0.05$) in the UTE treatment than that in the CON treatment (Figure 3).

## 3.8 Sequencing data, α-diversity, and β-diversity analysis

A total of 409,756 valid sequences in ileal chyme samples and 403,426 valid sequences in colon chyme samples which met quality control were obtained from the 16S rRNA high-throughput sequencing analysis. Based on 97% sequence similarity from valid sequences, these sequences were completely clustered into 9,936 OTUs in ileal chyme samples and 13,868 OTUs in colon chyme samples. The sequencing data information of bacteria in intestinal samples was shown

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TABLE 5 Effects of coated and uncoated trace elements on serum content of trace element.

| Item | Treatments[1] | | | SEM | *P*-value |
|---|---|---|---|---|---|
| | CON | CTE | UTE | | |
| Iron, μg/L | 3368.30 | 4081.05 | 3321.20 | 207.14 | 0.357 |
| Copper, μg/L | 668.01 | 658.55 | 646.68 | 26.25 | 0.956 |
| Manganese, μg/L | 6.71 | 12.01 | 9.04 | 1.02 | 0.092 |
| Zinc, μg/L | 709.65 | 842.16 | 780.21 | 24.45 | 0.071 |
| Selenium, μg/L | 74.04$^{a}$ | 107.76$^{b}$ | 65.82$^{a}$ | 7.51 | 0.033 |
| Iodin, μg/L | 80.57$^{a}$ | 99.24$^{b}$ | 87.65$^{ab}$ | 3.31 | 0.047 |
| Cobalt, μg/L | 0.41$^{a}$ | 1.48$^{b}$ | 0.63$^{a}$ | 0.14 | <0.001 |

[1]CON, sheep fed with the basal diets; CTE, sheep fed with the basal diets plus 400 mg/kg coated trace elements; UTE, sheep fed with the basal diets plus an equal amount of trace elements in uncoated form. Data with different small letter superscripts mean significantly different ($P < 0.05$), $n = 4$.

TABLE 6 Effects of coated and uncoated trace elements on intestinal morphology.

| Item | Treatments[1] | | | SEM | *P*-value |
|---|---|---|---|---|---|
| | CON | CTE | UTE | | |
| **Duodenum** | | | | | |
| Villus height, mm | 478.35$^{a}$ | 688.66$^{b}$ | 505.33$^{a}$ | 11.85 | <0.001 |
| Crypt depth, mm | 228.91 | 242.74 | 232.17 | 3.92 | 0.325 |
| V/C | 2.12$^{a}$ | 2.90$^{b}$ | 2.23$^{a}$ | 0.06 | <0.001 |
| **Jejunum** | | | | | |
| Villus height, mm | 426.04$^{a}$ | 612.10$^{b}$ | 443.61$^{a}$ | 9.89 | <0.001 |
| Crypt depth, mm | 189.28$^{a}$ | 261.95$^{b}$ | 200.63$^{a}$ | 5.22 | <0.001 |
| V/C | 2.31 | 2.39 | 2.27 | 0.04 | 0.448 |
| **Ileum** | | | | | |
| Villus height, mm | 290.57$^{a}$ | 457.81$^{b}$ | 297.92$^{a}$ | 9.29 | <0.001 |
| Crypt depth, mm | 194.52 | 205.86 | 189.42 | 3.46 | 0.139 |
| V/C | 1.52$^{a}$ | 2.29$^{b}$ | 1.63$^{a}$ | 0.05 | <0.001 |

[1]CON, sheep fed with the basal diets; CTE, sheep fed with the basal diets plus 400 mg/kg coated trace elements; UTE, sheep fed with the basal diets plus an equal amount of trace elements in uncoated form; V/C, the ratio of villus height and crypt depth. Data with different small letter superscripts mean significantly different ($P < 0.05$), $n = 4$.

in Supplementary Table 1. A total of 504 OTUs in ileum chyme samples were shared across the three treatments, and the number of sequences in shared OTUs accounted for 44.41% of the total effective sequences (Figure 4A). A total of 663 OTUs in colon chyme samples were shared across the three treatments, and the number of sequences in shared OTUs accounted for 44.41% of the total effective sequences (Figure 4B). The effect of coated and uncoated trace elements on α-diversity index of bacteria was shown in Supplementary Table 2. The Good's coverage index was greater than 0.990, indicating that the sampling had sufficient sequence coverage to detect the majority of rumen bacteria. The indices of Shannon, Simpson, Chao1, ACE, PD in the ileal chyme samples were higher ($P < 0.05$) in the CTE treatment than those in the CON treatment, indicating that dietary coated trace elements increased bacterial diversity in the ileum. There was no difference in the α-diversity index of bacteria in the colon chyme among the three groups, indicating that dietary coating or uncoated micronutrients had no effect on bacterial diversity in colon chyme. The percent variation in the ileum chyme samples was represented by PCo1 (20.9%) and PCo2 (17.3%), the percent variation in the colon chyme samples was represented by PCo1 (21.7%) and PCo2 (15.4%); the closer the distance in the figure, the more similar the bacterial community composition of the samples (Figure 5).

## 3.9 Bacteria abundance at phylum and genus levels

There were 20 and 16 phyla taxonomically classified in the ileum and colon chyme samples, respectively. The dominant phyla of ileum bacteria were *Firmicutes* (57.28–77.24%) and *Euryarchaeota* (6.50–13.81%) (Figure 6A), and

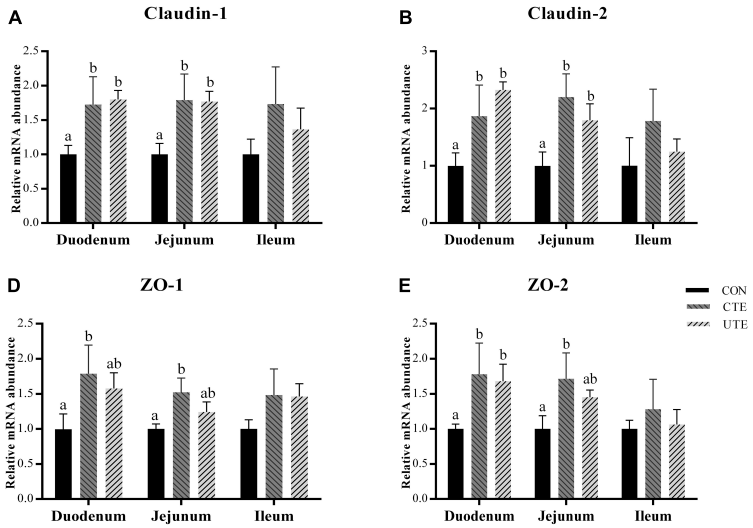


FIGURE 2
Effects of coated and uncoated trace elements on the relative gene expression of *Claudin-1* **(A)**, *Claudin-2* **(B)**, *Occludin* **(C)**, *ZO-1* **(D)**, and *ZO-2* **(E)** in small intestine. CON, sheep fed with the basal diets; CTE, sheep fed with the basal diets plus 400 mg/kg coated trace elements; UTE, sheep fed with the basal diets plus an equal amount of trace elements in uncoated form. Data are represented as mean and standard error and with different small letter superscripts mean significantly different ($P < 0.05$), $n = 4$.

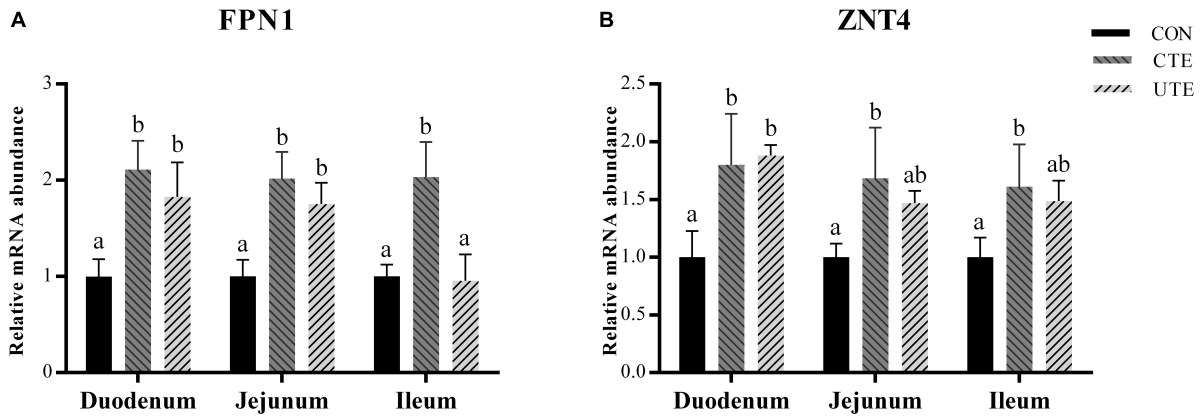


FIGURE 3
Effects of coated and uncoated trace elements on the relative gene expression of ferroportin 1 (*FPN1*, **A**) and zinc transporter 4 (*ZNT4*, **B**) in small intestine. CON, sheep fed with the basal diets; CTE, sheep fed with the basal diets plus 400 mg/kg coated trace elements; UTE, sheep fed with the basal diets plus an equal amount of trace elements in uncoated form. Data are represented as mean and standard error and with different small letter superscripts mean significantly different ($P < 0.05$), $n = 4$.

the dominant phyla of colon bacteria were *Firmicutes* (43.64–51.87%) and *Bacteroidetes* (33.39–42.06%) (Figure 6B). The relative abundance of phylum *Firmicutes* in the ileum was higher ($P < 0.05$) in the CTE treatment than that in the UTE treatment (Figure 6C). The relative abundance of phylum *Euryarchaeota* in the ileum was higher ($P < 0.05$) in the UTE treatment than that in the CON and CTE treatments (Figure 6D). The relative abundance of phylum *Proteobacteria* in the ileum was lower ($P < 0.05$) in the CTE treatment than that in the UTE treatment (Figure 6E). The relative abundance of phylum *Kiritimatiellaeota* in the ileum was higher ($P < 0.05$) in the CON treatment than that in the CTE and UTE treatments (Figure 6F).

There were 239 and 188 genera taxonomically classified in the ileum and colon chyme samples, respectively. The top three dominant genera of ileum bacteria were *Ruminococcus 2* (5.53–37.14%), *Eubacterium* (6.22–15.42%) and *Methanobrevibacter* (5.60–13.59%), as shown in Figure 7A, and the top three dominant genera of colon bacteria were *Rikenellaceae RC9 gut group* (6.72–15.97%), *Alistipes* (6.64–12.56%) and *Succinivibrio* (3.41–11.25%), as shown in Figure 7B. The

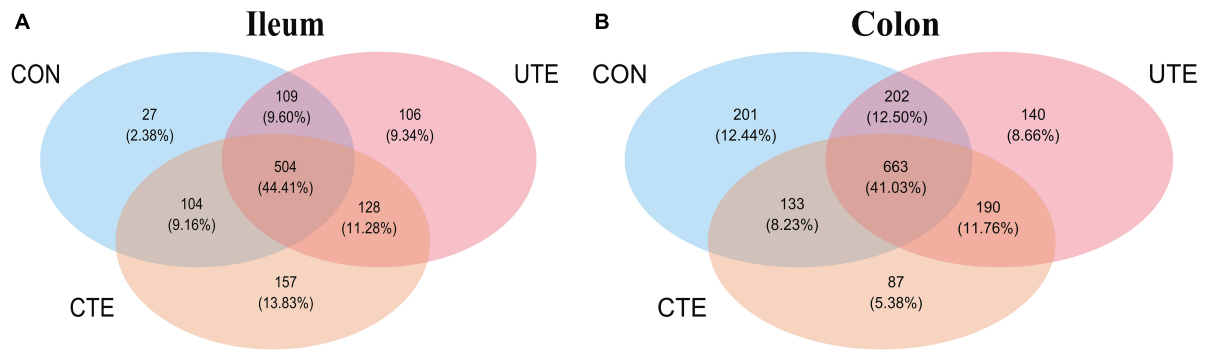


FIGURE 4
Venn diagram of the OTUs among the three treatments of bacteria in the ileum **(A)** and colon **(B)**. CON, sheep fed with the basal diets; CTE, sheep fed with the basal diets plus 400 mg/kg coated trace elements; UTE, sheep fed with the basal diets plus an equal amount of trace elements in uncoated form. *n* = 4.

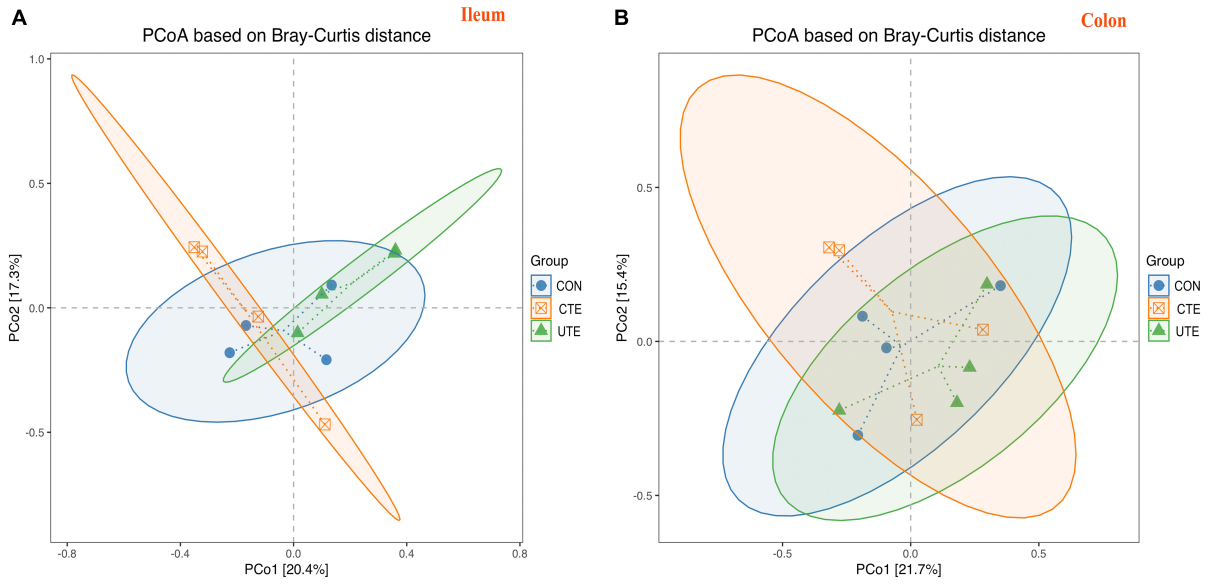


FIGURE 5
Bray–Curtis distance matrix PCoA of bacterial samples in the ileum **(A)** and colon **(B)**. CON, sheep fed with the basal diets; CTE, sheep fed with the basal diets plus 400 mg/kg coated trace elements; UTE, sheep fed with the basal diets plus an equal amount of trace elements in uncoated form. *n* = 4.

relative abundance of genus *Methanobrevibacter* in the ileum was higher ($P < 0.05$) in the UTE treatment than that in the CON treatment (Figure 7C). The relative abundance of genera *Christensenellaceae R-7group*, *Ruminococcus 1* and *f__Ruminococcaceae/g_* in the ileum was higher ($P < 0.05$) in the CTE treatment than that in the CON and UTE treatments (Figures 6D, E, G). The relative abundance of genus *Lachnospiraceae NK3A20 group* in the ileum was higher ($P < 0.05$) in the CON treatment than that in the CTE and UTE treatments (Figure 6F). The relative abundance of genus *Ruminococcaceae UCG-014* and *Lactobacillus* in the colon was higher ($P < 0.05$) in the CTE treatment than that in the CON and UTE treatments (Figures 6H, I).

## 3.10 Prediction of bacterial function

The effect of coated and uncoated trace elements on the prediction of function of bacteria in the ileum and colon were shown in Supplementary Tables 3, 4. The main gene functions of bacteria in the ileum associated with translation (~5.7%), replication and repair (~5.5%) and drug resistance: antineoplastic (~5.0%), as shown in Supplementary Table 3. The main gene functions of bacteria in the colon are associated with immune system (~5.2%), replication and repair (~5.0%) and drug resistance: antineoplastic (~5.0%), as shown in Supplementary Table 4. As shown in Figure 8, the relative

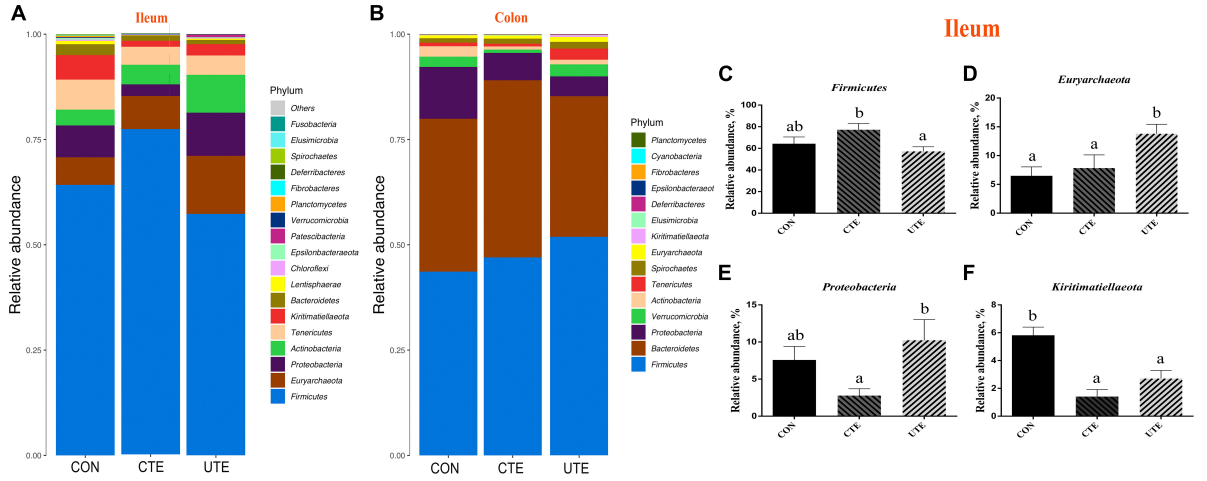


FIGURE 6

Effects of coated and uncoated trace elements on the relative abundance of bacteria at phylum level. **(A)** The bacterial composition at phylum level in the ileum; **(B)** the bacterial composition at phylum level in the colon; **(C–F)** bacteria with differences in abundance in the ileum at the phylum level. CON, sheep fed with the basal diets; CTE, sheep fed with the basal diets plus 400 mg/kg coated trace elements; UTE, sheep fed with the basal diets plus an equal amount of trace elements in uncoated form. Data are represented as mean and standard error and with different small letter superscripts mean significantly different ($P < 0.05$), $n = 4$.

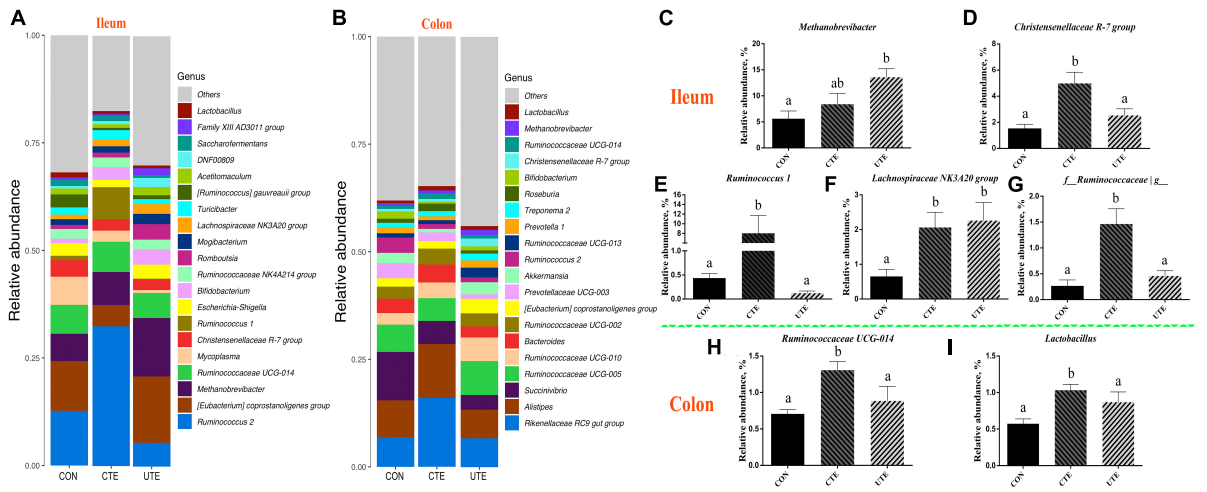


FIGURE 7

Effects of coated and uncoated trace elements on the relative abundance of bacteria at genus level. **(A)** The bacterial composition at genus level in the ileum; **(B)** the bacterial composition at genus level in the colon; **(C–G)** bacteria with differences in abundance in the ileum at the phylum level; **(H–I)** bacteria with differences in abundance in the colon at the phylum level. CON, sheep fed with the basal diets; CTE, sheep fed with the basal diets plus 400 mg/kg coated trace elements; UTE, sheep fed with the basal diets plus an equal amount of trace elements in uncoated form. Data are represented as mean and standard error and with different small letter superscripts mean significantly different ($P < 0.05$), $n = 4$.

abundance of transcription and cellular community of bacteria in the ileum was higher ($P < 0.05$) in the UTE treatment than that in the CON treatment, the relative abundance of transport and catabolism of bacteria in the ileum was higher ($P < 0.05$) in the UTE treatment than that in the CTE treatment. Compared with the CON and UTE treatments, the relative abundance of aging and substance dependence of bacteria in the ileum was lower ($P < 0.05$) in the UTE treatment, and the relative abundance of immune system and nervous system of bacteria in the ileum was higher ($P < 0.05$) in the UTE treatment. For the gene functions of bacteria in the colon, the relative abundance of development and regeneration was lower ($P < 0.05$) in the CTE treatment than that in the CON and UTE treatments, the relative abundance of infectious disease: parasitic was lower ($P < 0.05$) in the CTE treatment than that in the UTE treatment.

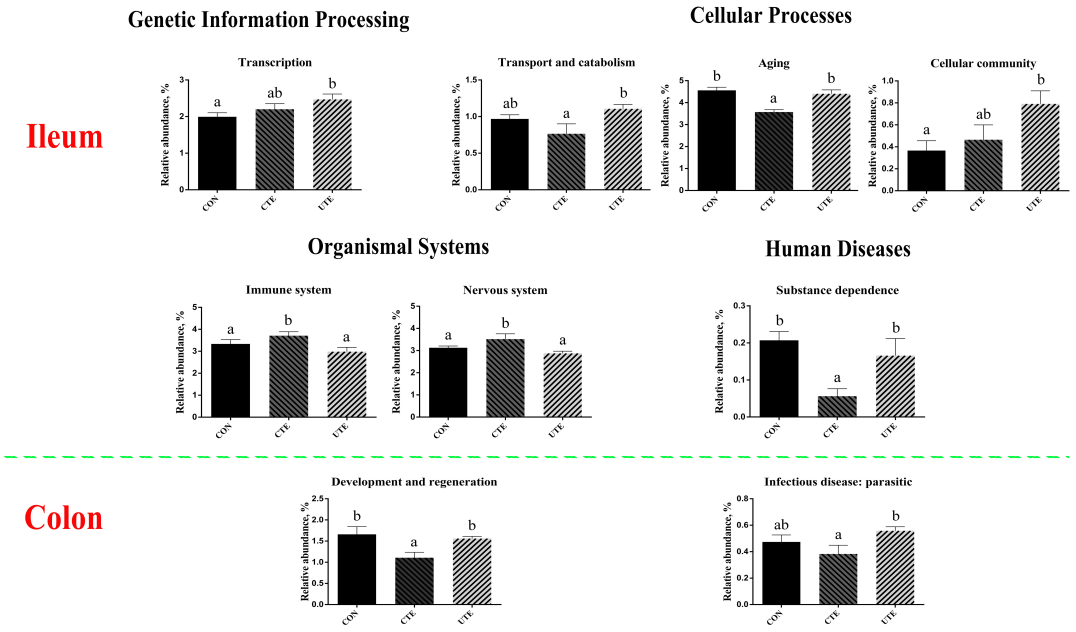


FIGURE 8
The prediction of bacterial function based on Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway at level 2. CON, sheep fed with the basal diets; CTE, sheep fed with the basal diets plus 400 mg/kg coated trace elements; UTE, sheep fed with the basal diets plus an equal amount of trace elements in uncoated form. Data are represented as mean and standard error and with different small letter superscripts mean significantly different ($P < 0.05$), $n = 4$.

## 4 Discussion

Due to its easier absorption, it is generally accepted that organic trace elements are used more efficiently than inorganic trace elements for ruminants (Byrne and Murphy, 2022). Besides organic trace elements, coating technique may be an effective measure to improve the bioavailability of inorganic trace elements. In the current study, the effective degradability of the coated trace elements was determined to be 34.13% in Exp. 1, indicating that most of them (65.87%) could escape from the rumen and enter the subsequent gastrointestinal tract. In Exp. 2, the ADG of sheep fed the basal diet without the addition of exogenous trace elements was significantly lower than that of sheep fed the basal diet with coated or uncoated trace elements. This result may be caused by insufficient trace elements in the basal diet (mainly composed of corn, soybean meal, heat bran, corn silage and wheat straw) to meet the requirements of the growing Yunnan semi-fine wool sheep. There is well-established evidence that the deficiency of trace elements can negatively affect the growth of animals (Prasad, 1995; Eder et al., 1996; Kegley et al., 2016; Huang et al., 2017), as trace elements are involved in maintaining normal intestinal absorption and microbial homeostasis (Pajarillo et al., 2021; Skalny et al., 2021). The measurement of serum mineral concentrations is a popular and potentially valuable method for evaluating the trace mineral nutritional status and the mineral bioavailability of animals (Herdt and Hoff, 2011; Burrow et al., 2020). Thus, we measured the levels of trace elements in serum and found that the levels of Se, Co and I in the serum of sheep supplemented with coated trace elements were increased compared to CON and UTE treatments. The rumen binding of abundant fiber components and minerals in ruminant feeds may affect the subsequent absorption of some trace elements in the gut (Whitehead et al., 1985). Under the present experimental conditions, increasing the rumen passage rate appears to be an effective strategy to improve the bioavailability of trace elements. Compared with the addition of coated trace elements, the growth performance of sheep fed with uncoated trace elements did not show an advantage. This may be due to the fact that trace elements added as salt also provide sufficient amounts of minerals to growing sheep, after all, they remain the main mineral providers in livestock feed, albeit with relatively low bioavailability.

Some researchers believe that in ruminants, trace elements in the form of salts are more soluble in the rumen, chemical forms of trace mineral elements can be altered, and adverse reactions between rumen contents can affect rumen fermentation patterns, potentially affecting digestibility of nutrients (Galbraith et al., 2016; Arce-Cordero et al., 2020; Miller et al., 2020). In recent years, attempts have been made to replace mineral salts with trace elements in their hydroxyl or coating form. Addition of hydroxy trace minerals improved the digestibility of nutrients in dairy cows (Miller et al., 2020; Guimaraes et al., 2022) and steers (Caldera et al., 2019;

Guimaraes et al., 2021) compared to supplementation of sulfate-sourced trace elements. However, Miller et al. (2020) reported that the source of trace mineral (sulfate or hydroxy source) had no effect on rumen turnover, and particle passage rates in Holstein cows. Arce-Cordero et al. (2020) also reported that dietary rumen-protected copper sulfate did not affect the ruminal digestibility of nutrients compared to unprotected copper sulfate. In this study, we found that the addition of coated or uncoated trace elements had no effect on apparent nutrient digestibility, with the exception of CP digestibility. The apparent digestibility of CP was higher in the CTE and UTE treatments than that in the CON treatment, which was consistent with the result of growth performance. In accordance with our findings, previous studies have shown higher digestibility of CP in dairy cows (El Ashry et al., 2012), goats (Salama et al., 2003) and growing lambs (Samarin et al., 2022) supplemented with a mix of trace minerals from different sources. Trace elements have been proven to play a critical role in regulating intestinal health and the microbiome (Shannon and Hill, 2019; Barra et al., 2021), which may be factors affecting nutrient digestibility.

The morphology of the intestinal mucosa is an important indicator of intestinal absorption function in ruminant livestock. Most absorption of trace minerals occurs in the small intestine, mainly in the duodenum, although absorption can occur anywhere in the gastrointestinal tract (Smith, 2004; Goff, 2018). The sheep in the CTE treatment had a greater villus height in the duodenum, jejunum and ileum than those sheep in the CON and UTE treatments, indicating that dietary coated trace elements were beneficial to improve the absorption function of small intestinal. Previous studies reported that a single supplement of dietary trace minerals had a positive effect on the gut health of animals, such as Fe (Zhou et al., 2020), Cu (Yin et al., 2021), Zn (Wang et al., 2018) and Se (Samo et al., 2020). Replacing inorganic trace elements with complex organic trace minerals in the diet could increase the villus height in the ileum of weaned piglets (Wang et al., 2022). The addition of coated organic trace elements could also significantly improve the villus height and villus/crypt ratio in late-phase laying hens (Chen et al., 2022). In addition to intestinal morphology, we also detected the gene expression of tight junction protein in small intestine and found that dietary coated trace elements upregulated the relative mRNA abundance of tight junction proteins (Claudin-1, Claudin-2, Occludin, ZO-1, and ZO-2) in duodenum and jejunum compared to dietary uncoated trace elements. Altering the supply form and amount of Zn could upregulate the gene expression of *Occludin* and *ZO-1* in ileal mucosa, but did not affect intestinal mucosal morphology (Wang et al., 2018). The deficiency or excess of trace elements can regulate the microenvironment of animals' intestines, including the microbiome, nutrient availability, stress and mucosal immunity (Pajarillo et al., 2021). This could be the reason that the addition of trace elements improved the apparent digestibility of the crude protein. In this study, the supply of equal amounts of uncoated trace elements limited the biological effects in the small intestine due to low bioavailability. The absorption of metal ions by intestinal epithelial cells depends on divalent metal transporters, which are then transported to the blood by transmembrane proteins such as ferroportin and zinc transporters (Hundal and Taylor, 2009; Espinoza et al., 2012). The addition of trace elements had a positive effect on the relative mRNA abundance of iron transporter and zinc transporter (FPN1 and ZNT4), especially coated trace elements. This may be related to the amount of trace elements reaching the small intestine.

Some trace minerals ingested by animals are utilized by gut microbiota to maintain basic physiological activity, although hosts have evolved mechanisms that prevent bacteria from taking advantage of them through chelation (Becker and Skaar, 2014). Meanwhile, trace minerals also regulate the composition and diversity of intestinal microorganisms. Previous studies have reported that Fe could regulate the ability of microorganisms to obtain energy from nutrients ingested by hosts (Dostal et al., 2015), Mn could regulate the detoxification involved in reactive oxygen species, carbohydrates, lipids, and protein metabolism, and DNA replication (Kehres and Maguire, 2003; Zaharik and Brett Finlay, 2004), Zn could regulate host defenses, antioxidant systems, gene expression, virulence factors (Velasco et al., 2018; Eijkelkamp et al., 2019), and Cu could reduce the relative abundance of potential pathogens (Villagómez-Estrada et al., 2020). These are all involved in the survival and adaptation of the microbiome. In this study, the most dominant bacterial phylum in ileum and colon was *Firmicutes*, which is consistent with previous studies in sheep (Wang et al., 2017; Ma et al., 2022). Compared with the CON treatment, the addition of uncoated trace elements increased the proportion of the phylum *Euryarchaeota,* and the change of the abundance of *Kiritimatiellaeota* was the most obvious with the addition of trace elements. *Euryarchaeota* is the phylum of Archaea, including some methane-producing bacteria commonly found in the gut, known for metabolizing nutrients and metabolites of other bacteria, leading to elevated levels of short-chain fatty acids, such as acetate (Horz and Conrads, 2010; Primec et al., 2019). Dietary zinc oxide supplementation significantly increased the abundance of *Euryarchaeota* in the colon, while other information about the association between *Euryarchaeota* and trace minerals has been rarely reported. *Kiritimatiellaeota* mediated pyruvate to generate acetyl-CoA and then enter the tricarboxylic acid (TCA) cycle and involve arginine biosynthesis, and also regulated fatty acid synthesis. *Kiritimatiellaeota* mediates the breakdown of pyruvate into acetyl-CoA, which then enters the tricarboxylic acid (TCA) cycle and involves arginine biosynthesis to utilize dietary N and produce energy (Guo et al., 2021). However, the cause for the addition of trace elements to reduce the proportion of *Kiritimatiellaeota* in the ileum need to be further studied. Compared to uncoated trace elements, the addition of coated trace elements increased the proportion of the phylum *Firmicutes* while decreased the proportion of the

phylum *Proteobacteria*. The proportion of dominant bacterial phyla in the ileum was significantly affected by whether or not it is coated, probably caused by the coating technique changing the amount of trace elements reaching the ileum. At the genus level, the proportion of *Christensenellaceae R-7 group*, *f_ Ruminococcaceae/g_* and *Ruminococcus 1* in the ileum was positively affected by the addition of coated trace elements. *Christensenellaceae R-7 group* play a pivotal role in the degradation of carbohydrates to acetate (Morotomi et al., 2012), and the increase in their abundance was positively correlated with the improvement of growth performance and meat quality in Hu lambs (Xiong et al., 2021). Both *f_ Ruminococcaceae/g_* and *Ruminococcus 1* belong to family *Ruminococcaceae,* which have great ability in the complex carbohydrates digestion to generate short chain fatty acids (SCFAs) (Xie et al., 2022). It has been reported that SCFAs can improve intestinal health by maintaining the integrity of intestinal barrier, secreting mucus and preventing inflammation (Peng et al., 2009; Lewis et al., 2010; Primec et al., 2017). The similar result appeared in the colon that the addition of coated trace elements increased the proportion of *Ruminococcaceae UCG-014*.

To further explore the role of microbiota in gut, the prediction of bacterial function based on KEGG pathway was performed. Findings of the present study revealed significant differences in bacterial function between sheep supplemented with coated trace elements or not. In the ileal microbiota of sheep fed with coated trace elements, there were more genes associated with the organism systems (immune system and nervous system), which critically influence the homeostasis of normal physiological functions of the host (Xing et al., 2019). Homeostasis of the gut microbiota play an important role in host health and aging (Han et al., 2017). The genes associated with aging were fewer in the CTE treatment than that in the UTE treatment, indicating that feeding coated trace elements is more beneficial for gut health in sheep. In the colon, the genes related to development and regeneration, infectious disease: parasitic were also fewer in the CTE treatment than that in the UTE treatment. Both substance dependence and infectious disease: parasitic belong to the biological pathway of human disease and have obvious associations with host health (Li and Agarwal, 2009). The decreased occurrence of these genes mirrors the decrease in sequences affiliated with intestinal injury, supporting the importance of these bacteria within the gut health. The limitation of this study is that metagenomic shotgun sequencing of gut digesta was not performed, which will provide more accurate and direct evidence for the function of gut microbiota in future studies.

## 5 Conclusion

In summary, most of the coated trace elements (65.87%) used in this experiment were able to escaped from the rumen. Under the dietary conditions of the current study, the addition of trace elements improved the growth performance and the apparent digestibility of CP of growing sheep. The addition of dietary coated trace elements had a more positive effect on serum trace element levels and intestinal development, although there was no difference in growth performance, compared to uncoated trace elements. Generally, this study partly revealed the advantages of coated trace elements over traditional trace elements for intestinal development in growing sheep, providing a theoretical basis for their further utilization on ruminants.

## 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/, PRJNA894949.

## Ethics statement

This animal study was reviewed and approved by the Experimental Animal Committee of Animal Nutrition Institute, Sichuan Agricultural University.

## Author contributions

JZ and YR wrote the original draft, performed data statistical analysis, and produced figures and tables. YR and XW performed the experiments. SY, RH, QP, and YJ provided great help in data analysis. QH, JZ, and BX performed the trial investigation and design. ZW, BX, LW, HZ, and YJ reviewed and edited the draft. 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/fmicb.2022.1080182/full#supplementary-material

## References

Abdelrahman, M. M., Aljumaah, R. S., and Khan, R. U. (2017). Effects of prepartum sustained-release trace elements ruminal bolus on performance, colustrum composition and blood metabolites in Najdi ewes. *Environ. Sci. Pollut. Res.* 24, 9675–9680. doi: 10.1007/s11356-017-8625-1

Aliarabi, H., Fadayifar, A., Alimohamady, R., and Dezfoulian, A. H. (2019). The effect of maternal supplementation of zinc, selenium, and cobalt as slow-release ruminal bolus in late pregnancy on some blood metabolites and performance of ewes and their lambs. *Biol. Trace Elem. Res.* 187, 403–410. doi: 10.1007/s12011-018-1409-8

Andreini, C., Bertini, I., Cavallaro, G., Holliday, G. L., and Thornton, J. M. (2008). Metal ions in biological catalysis: From enzyme databases to general principles. *JBIC J. Biol. Inorg. Chem.* 13, 1205–1218. doi: 10.1007/s00775-008-0404-5

AOAC International (2007). *Official methods of analysis*, 18th Edn, eds W. Hortwitz and G. W. Latimer Jr. (Rockville, MD: AOAC Int).

Arce-Cordero, J. A., Monteiro, H. F., Lelis, A. L., Lima, L. R., Restelatto, R., Brandao, V. L. N., et al. (2020). Copper sulfate and sodium selenite lipid-microencapsulation modifies ruminal microbial fermentation in a dual-flow continuous-culture system. *J. Dairy Sci.* 103, 7068–7080. doi: 10.3168/jds.2019-17913

Aßhauer, K. P., Wemheuer, B., Daniel, R., and Meinicke, P. (2015). Tax4Fun: Predicting functional profiles from metagenomic 16S rRNA data. *Bioinformatics* 31, 2882–2884. doi: 10.1093/bioinformatics/btv287

Barra, N. G., Anhê, F. F., Cavallari, J. F., Singh, A. M., Chan, D. Y., and Schertzer, J. D. (2021). Micronutrients impact the gut microbiota and blood glucose. *J. Endocrinol.* 250, R1–R21. doi: 10.1530/JOE-21-0081

Becker, K. W., and Skaar, E. P. (2014). Metal limitation and toxicity at the interface between host and pathogen. *FEMS Microbiol. Rev.* 38, 1235–1249. doi: 10.1111/1574-6976.12087

Bolger, A. M., Lohse, M., and Usadel, B. (2014). Trimmomatic: A flexible trimmer for Illumina sequence data. *Bioinformatics* 30, 2114–2120. doi: 10.1093/bioinformatics/btu170

Burrow, K., Young, W., McConnell, M., Carne, A., Barr, D., Reid, M., et al. (2020). The effect of sheep and cow milk supplementation of a low calcium diet on the distribution of macro and trace minerals in the organs of weanling rats. *Nutrients* 12:594. doi: 10.3390/nu12030594

Byrne, L., and Murphy, R. A. (2022). Relative bioavailability of trace minerals in production animal nutrition: A review. *Animals (Basel)* 12:1981. doi: 10.3390/ani12151981

Caldera, E., Weigel, B., Kucharczyk, V. N., Sellins, K. S., Archibeque, S. L., Wagner, J. J., et al. (2019). Trace mineral source influences ruminal distribution of copper and zinc and their binding strength to ruminal digesta1,2,3. *Journal Anim. Sci.* 97, 1852–1864. doi: 10.1093/jas/skz072

Caporaso, J. G., Lauber, C. L., Walters, W. A., Berg-Lyons, D., Lozupone, C. A., Turnbaugh, P. J., et al. (2011). Global patterns of 16S rRNA diversity at a depth of millions of sequences per sample. *Proc. Natl. Acad. Sci. U.S.A* 108, 4516–4522. doi: 10.1073/pnas.1000080107

Chen, J., Bittinger, K., Charlson, E. S., Hoffmann, C., Lewis, J., Wu, G. D., et al. (2012). Associating microbiome composition with environmental covariates using generalized UniFrac distances. *Bioinformatics* 28, 2106–2113. doi: 10.1093/bioinformatics/bts342

Chen, X., Ma, X.-M., Yang, C.-W., Jiang, S.-Z., Huang, L.-B., Li, Y., et al. (2022). Low level of dietary organic trace elements improve the eggshell strength. trace element utilization, and intestinal function in late-phase laying hens. *Front. Vet. Sci.* 9:903615. doi: 10.3389/fvets.2022.903615

Dick, A. T., Dewey, D. W., and Gawthorne, J. M. (1975). Thiomolybdates and the copperÔÇômolybdenumÔÇôsulphur interaction in ruminant nutrition. *J. Agric. Sci.* 85, 567–568. doi: 10.1017/S0021859600062468

Dostal, A., Lacroix, C., Bircher, L., van Pham, T., Follador, R., Zimmermann, M. B., et al. (2015). Iron modulates butyrate production by a child gut microbiota *in vitro*. *mBio* 6:e01453. doi: 10.1128/mBio.01453-15

Eder, K., Kralik, A., and Kirchgessner, M. (1996). The effect of manganese supply on thyroid hormone metabolism in the offspring of manganese-depleted dams. *Biol. Trace Elem. Res.* 55:137. doi: 10.1007/BF02784175

Edgar, R. C. (2013). UPARSE: Highly accurate OTU sequences from microbial amplicon reads. *Nat. Methods* 10, 996–998. doi: 10.1038/nmeth.2604

Edgar, R. C., Haas, B. J., Clemente, J. C., Quince, C., and Knight, R. (2011). UCHIME improves sensitivity and speed of chimera detection. *Bioinformatics* 27, 2194–2200. doi: 10.1093/bioinformatics/btr381

Eijkelkamp, B. A., Morey, J. R., Neville, S. L., Tan, A., Pederick, V. G., Cole, N., et al. (2019). Dietary zinc and the control of Streptococcus pneumoniae infection. *PLoS Pathog.* 15:e1007957. doi: 10.1371/journal.ppat.1007957

El Ashry, G. M., Hassan, A. A. M., and Soliman, S. M. (2012). Effect of feeding a combination of zinc, manganese and copper methionine chelates of early lactation high producing dairy cow. *Food Nutr. Sci.* 3, 193–203. doi: 10.1016/S0377-8401(01)00279-6

Espinoza, A., Le Blanc, S., Olivares, M., Pizarro, F., Ruz, M., and Arredondo, M. (2012). Iron, copper, and zinc transport: Inhibition of divalent metal transporter 1 (DMT1) and human copper transporter 1 (hCTR1) by shRNA. *Biol. Trace Elem. Res.* 146, 281–286. doi: 10.1007/s12011-011-9243-2

Faulkner, M. J., and Weiss, W. P. (2017). Effect of source of trace minerals in either forage-or by-product–based diets fed to dairy cows: 1. Production and macronutrient digestibility. *J. Dairy Sci.* 100, 5358–5367. doi: 10.3168/jds.2016-12095

Foong, J. P. P., Hung, L. Y., Poon, S., Savidge, T. C., and Bornstein, J. C. (2020). Early life interaction between the microbiota and the enteric nervous system. *Am. J. Physiol. Gastrointest. Liver Physiol.* 319, G541–G548. doi: 10.1152/ajpgi.00288.2020

Galbraith, M. L., Vorachek, W. R., Estill, C. T., Whanger, P. D., Bobe, G., Davis, T. Z., et al. (2016). Rumen microorganisms decrease bioavailability of inorganic selenium supplements. *Biol. Trace Elem. Res.* 171, 338–343. doi: 10.1007/s12011-015-0560-8

Genther, O. N., and Hansen, S. L. (2015). The effect of trace mineral source and concentration on ruminal digestion and mineral solubility. *J. Dairy Sci.* 98, 566–573. doi: 10.3168/jds.2014-8624

Goff, J. P. (2018). Invited review: Mineral absorption mechanisms, mineral interactions that affect acid–base and antioxidant status, and diet considerations to improve mineral status. *J. Dairy Sci.* 101, 2763–2813. doi: 10.3168/jds.2017-13112

Guimaraes, O., Jalali, S., Wagner, J. J., Spears, J. W., and Engle, T. E. (2021). Trace mineral source impacts rumen trace mineral metabolism and fiber digestion in steers fed a medium-quality grass hay diet. *J. Anim. Sci.* 99, 1–7. doi: 10.1093/jas/skab220

Guimaraes, O., Wagner, J. J., Spears, J. W., Brandao, V. L. N., and Engle, T. E. (2022). Trace mineral source influences digestion, ruminal fermentation, and ruminal copper, zinc, and manganese distribution in steers fed a diet suitable for lactating dairy cows. *Animal* 16:100500. doi: 10.1016/j.animal.2022.100500

Guo, N., Wu, Q., Shi, F., Niu, J., Zhang, T., Degen, A. A., et al. (2021). Seasonal dynamics of diet-gut microbiota interaction in adaptation of yaks to life at high altitude. *NPJ Biofilms Microbiomes* 7:38. doi: 10.1038/s41522-021-00207-6

Han, B., Sivaramakrishnan, P., Lin, C.-C. J., Neve, I. A. A., He, J., Tay, L. W. R., et al. (2017). Microbial genetic composition tunes host longevity. *Cell* 169, 1249–1262.e13. doi: 10.1016/j.cell.2017.05.036

Herdt, T. H., and Hoff, B. (2011). The use of blood analysis to evaluate trace mineral status in ruminant livestock. *Vet. Clin. North Am. Food Anim. Pract.* 27, 255–283. doi: 10.1016/j.cvfa.2011.02.004

Horz, H.-P., and Conrads, G. (2010). The discussion goes on: What is the role of Euryarchaeota in humans? *Archaea* 2010:967271. doi: 10.1155/2010/967271

Hou, Y., Wang, L., Ding, B., Liu, Y., Zhu, H., Liu, J., et al. (2010). Dietary α-ketoglutarate supplementation ameliorates intestinal injury in lipopolysaccharide-challenged piglets. *Amino Acids* 39, 555–564. doi: 10.1007/s00726-010-0473-y

Huang, X., Sun, B., Zhang, J., Gao, Y., Li, G., and Chang, Y. (2017). Selenium deficiency induced injury in chicken muscular stomach by downregulating selenoproteins. *Biol. Trace Elem. Res.* 179, 277–283. doi: 10.1007/s12011-017-0946-x

Hundal, H. S., and Taylor, P. M. (2009). Amino acid transceptors: Gate keepers of nutrient exchange and regulators of nutrient signaling. *Am. J. Physiol. Endocrinol. Metab.* 296, E603–E613. doi: 10.1152/ajpendo.91002.2008

Kasaikina, M. V., Kravtsova, M. A., Lee, B. C., Seravalli, J., Peterson, D. A., Walter, J., et al. (2011). Dietary selenium affects host selenoproteome expression by influencing the gut microbiota. *FASEB J.* 25, 2492–2499. doi: 10.1096/fj.11-181990

Kegley, E. B., Ball, J. J., and Beck, P. A. (2016). BILL E. KUNKLE INTERDISCIPLINARY BEEF SYMPOSIUM: Impact of mineral and vitamin status on beef cattle immune function and health1,2. *J. Anim. Sci.* 94, 5401–5413. doi: 10.2527/jas.2016-0720

Kehres, D. G., and Maguire, M. E. (2003). Emerging themes in manganese transport, biochemistry and pathogenesis in bacteria. *FEMS Microbiol. Rev.* 27, 263–290. doi: 10.1016/S0168-6445(03)00052-4

Kembel, S. W., Cowan, P. D., Helmus, M. R., Cornwell, W. K., Morlon, H., Ackerly, D. D., et al. (2010). Picante: R tools for integrating phylogenies and ecology. *Bioinformatics* 26, 1463–1464. doi: 10.1093/bioinformatics/btq166

Kociova, S., Dolezelikova, K., Horky, P., Skalickova, S., Baholet, D., Bozdechova, L., et al. (2020). Zinc phosphate-based nanoparticles as alternatives to zinc oxide in diet of weaned piglets. *J. Anim. Sci. Biotechnol.* 11:59. doi: 10.1186/s40104-020-00458-x

Lewis, K., Lutgendorff, F., Phan, V., Söderholm, J., Sherman, P., and McKay, D. (2010). Enhanced translocation of bacteria across metabolically stressed epithelia is reduced by butyrate. *Inflamm. Bowel Dis.* 16, 1138–1148. doi: 10.1002/ibd.21177

Li, Y., and Agarwal, P. (2009). A pathway-based view of human diseases and disease relationships. *PLoS One.* 4:e4346. doi: 10.1371/journal.pone.0004346

Liu, X., Piao, J., Huang, Z., Zhang, S.-Q., Li, W., Tian, Y., et al. (2014). Determination of 16 selected trace elements in children plasma from china economical developed rural areas using high resolution magnetic sector inductively coupled mass spectrometry. *J. Anal. Methods Chem.* 2014:975820. doi: 10.1155/2014/975820

Liu, Y. J., Zhang, Z. D., Dai, S. H., Wang, Y., Tian, X. F., Zhao, J. H., et al. (2020). Effects of sodium selenite and coated sodium selenite addition on performance, ruminal fermentation, nutrient digestibility and hepatic gene expression related to lipid metabolism in dairy bulls. *Livest. Sci.* 237:104062. doi: 10.1016/j.livsci.2020.104062

Ma, Y., Deng, X., Yang, X., Wang, J., Li, T., Hua, G., et al. (2022). Characteristics of bacterial microbiota in different intestinal segments of aohan fine-wool sheep. *Front. Microbiol.* 13:874536. doi: 10.3389/fmicb.2022.874536

Miller, M. D., Lanier, J. S., Kvidera, S. K., Dann, H. M., Ballard, C. S., and Grant, R. J. (2020). Evaluation of source of corn silage and trace minerals on rumen characteristics and passage rate of Holstein cows. *J. Dair. Sci.* 103, 8864–8879. doi: 10.3168/jds.2020-18490

Morotomi, M., Nagai, F., and Watanabe, Y. (2012). Description of *Christensenella* minuta gen. nov., sp. nov., isolated from human faeces, which forms a distinct branch in the order Clostridiales, and proposal of Christensenellaceae fam. nov. *Int. J. Syst. Evol. Microbiol.* 62, 144–149. doi: 10.1099/ijs.0.026989-0

National Research Council (2007). *Nutrient requirements of small ruminants: Sheep, goats, cervids, and new world camelids*. Washington, DC: The National Academies Press.

Ørskov, E. R., and McDonald, I. (1979). The estimation of protein degradability in the rumen from incubation measurements weighted according to rate of passage. *J. Agric. Sci.* 92, 499–503. doi: 10.1017/S0021859600063048

Overton, T. R., and Yasui, T. (2014). Practical applications of trace minerals for dairy cattle. *J. Anim. Sci.* 92, 416–426. doi: 10.2527/jas.2013-7145

Pajarillo, E. A. B., Lee, E., and Kang, D.-K. (2021). Trace metals and animal health: Interplay of the gut microbiota with iron, manganese, zinc, and copper. *Anim. Nutr.* 7, 750–761. doi: 10.1016/j.aninu.2021.03.005

Peng, L., Li, Z.-R., Green, R. S., Holzman, I. R., and Lin, J. (2009). Butyrate enhances the intestinal barrier by facilitating tight junction assembly via activation of AMP-activated protein kinase in Caco-2 cell monolayers. *J. Nutr.* 139, 1619–1625. doi: 10.3945/jn.109.104638

Potier, R., Blanc, B., Braud, C., Freret, S., and Cesbron, N. (2022). Effect of sustained-release trace element ruminal bolus on plasma trace mineral profiles in captive blesbok antelopes (*Damaliscus pygargus phillipsi*). *J. Zoo Wildl. Med.* 53, 259–265. doi: 10.1638/2021-0120

Prasad, A. S. (1995). Zinc: An overview. *Nutrition* 11, 93–99.

Primec, M., Klemenak, M., Di Gioia, D., Aloisio, I., Bozzi Cionci, N., Quagliariello, A., et al. (2019). Clinical intervention using Bifidobacterium strains in celiac disease children reveals novel microbial modulators of TNF-α and short-chain fatty acids. *Clin. Nutr.* 38, 1373–1381. doi: 10.1016/j.clnu.2018.06.931

Primec, M., Mičetić-Turk, D., and Langerholc, T. (2017). Analysis of short-chain fatty acids in human feces: A scoping review. *Anal. Biochem.* 526, 9–21. doi: 10.1016/j.ab.2017.03.007

Rabiee, A. R., Lean, I. J., Stevenson, M. A., and Socha, M. T. (2010). Effects of feeding organic trace minerals on milk production and reproductive performance in lactating dairy cows: A meta-analysis. *J. Dairy Sci.* 93, 4239–4251. doi: 10.3168/jds.2010-3058

Salama, A. A. K., Caja, G., Albanell, E., Such, X., Casals, R., and Plaixats, J. (2003). Effects of dietary supplements of zinc-methionine on milk production, udder health and zinc metabolism in dairy goats. *J. Dairy Res.* 70, 9–17. doi: 10.1017/s0022029902005708

Samarin, A. A., Norouzian, M. A., and Afzalzadeh, A. (2022). Effect of trace mineral source on biochemical and hematological parameters, digestibility, and performance in growing lambs. *Trop. Anim. Health Prod.* 54:40. doi: 10.1007/s11250-021-03042-1

Samo, S. P., Malhi, M., Kachiwal, A. B., Gadahi, J. A., Parveen, F., Kalhoro, N. H., et al. (2020). Supranutritional selenium level minimizes high concentrate diet-induced epithelial injury by alleviating oxidative stress and apoptosis in colon of goat. *BMC Vet. Res.* 16:462. doi: 10.1186/s12917-020-02653-4

Shannon, M. C., and Hill, G. M. (2019). Trace mineral supplementation for the intestinal health of young monogastric animals. *Front. Vet. Sci.* 6:73. doi: 10.3389/fvets.2019.00073

Skalny, A. V., Aschner, M., Lei, X. G., Gritsenko, V. A., Santamaria, A., Alekseenko, S. I., et al. (2021). Gut microbiota as a mediator of essential and toxic effects of zinc in the intestines and other tissues. *Int. J. Mol. Sci.* 22:13074. doi: 10.3390/ijms222313074

Smith, D. (2004). Minerals in animal and human nutrition (2003), 2nd edn. *Trop. Anim. Health Prod.* 36:774. doi: 10.1023/B:TROP.0000046014.56139.49

Spears, J. W. (1996). Organic trace minerals in ruminant nutrition. *Anim. Feed Sci. Technol.* 58, 151–163. doi: 10.1016/0377-8401(95)00881-0

Spears, J. W. (2003). Trace mineral bioavailability in ruminants. *J. Nutr.* 133, 1506S–1509S. doi: 10.1093/jn/133.5.1506S

Spears, J. W., and Weiss, W. P. (2008). Role of antioxidants and trace elements in health and immunity of transition dairy cows. *Vet. J.* 176, 70–76. doi: 10.1016/j.tvjl.2007.12.015

Stein, H. H., and Bohlke, R. A. (2007). The effects of thermal treatment of field peas (*Pisum sativum* L.) on nutrient and energy digestibility by growing pigs. *J. Anim. Sci.* 85, 1424–1431. doi: 10.2527/jas.2006-712

van Soest, P. J., Robertson, J. B., and Lewis, B. A. (1991). Methods for dietary fiber, neutral detergent fiber, and nonstarch polysaccharides in relation to animal nutrition. *J. Dairy Sci.* 74, 3583–3597. doi: 10.3168/jds.S0022-0302(91)78551-2

Velasco, E., Wang, S., Sanet, M., Fernández-Vázquez, J., Jové, D., Glaría, E., et al. (2018). A new role for Zinc limitation in bacterial pathogenicity: Modulation of α-hemolysin from uropathogenic *Escherichia coli*. *Sci. Rep.* 8:6535. doi: 10.1038/s41598-018-24964-1

Villagómez-Estrada, S., Pérez, J. F., Darwich, L., Vidal, A., van Kuijk, S., Melo-Durán, D., et al. (2020). Effects of copper and zinc sources and inclusion levels of copper on weanling pig performance and intestinal microbiota. *J. Anim. Sci.* 98:skaa117. doi: 10.1093/jas/skaa117

Wang, C., Han, L., Zhang, G. W., Du, H. S., Wu, Z. Z., Liu, Q., et al. (2021). Effects of copper sulphate and coated copper sulphate addition on lactation performance, nutrient digestibility, ruminal fermentation and blood metabolites in dairy cows. *Br. J. Nutr.* 125, 251–259. doi: 10.1017/S0007114520002986

Wang, E., Wang, J., Lv, J., Sun, X., Kong, F., Wang, S., et al. (2021). Comparison of ruminal degradability, indigestible neutral detergent fiber, and total-tract digestibility of three main crop straws with alfalfa hay and corn silage. *Animals (Basel)* 11:3218. doi: 10.3390/ani11113218

Wang, C., Zhang, L., Ying, Z., He, J., Zhou, L., Zhang, L., et al. (2018). Effects of dietary zinc oxide nanoparticles on growth, diarrhea, mineral deposition, intestinal morphology, and barrier of weaned piglets. *Biol. Trace Elem. Res.* 185, 364–374. doi: 10.1007/s12011-018-1266-5

Wang, H., Yang, F., Xin, R., Cui, D., He, J., Zhang, S., et al. (2020). The gut microbiota attenuate neuroinflammation in manganese exposure by inhibiting cerebral NLRP3 inflammasome. *Biomed. Pharm.* 129:110449. doi: 10.1016/j.biopha.2020.110449

Wang, J., Fan, H., Han, Y., Zhao, J., and Zhou, Z. (2017). Characterization of the microbial communities along the gastrointestinal tract of sheep by 454 pyrosequencing analysis. *Asian Australas. J. Anim. Sci.* 30, 100–110. doi: 10.5713/ajas.16.0166

Wang, S., Wu, S., Zhang, Y., Chen, J., and Zhou, X. (2022). Effects of different levels of organic trace minerals on oxidative status and intestinal function in weanling piglets. *Biol. Trace Elem. Res.* doi: 10.1007/s12011-022-03174-x

Whitehead, D. C., Goulden, K. M., and Hartley, R. D. (1985). The distribution of nutrient elements in cell wall and other fractions of the herbage of some grasses and legumes. *J. Sci. Food Agric.* 36, 311–318. doi: 10.1002/jsfa.2740360502

Wu, Q., La, S. K., Wang, C., Zhang, J., Liu, Q., Guo, G., et al. (2021). Effects of coated copper sulphate and coated folic acid supplementation on growth, rumen fermentation and urinary excretion of purine derivatives in Holstein bulls. *Anim. Feed Sci. Technol.* 276:114921. doi: 10.1016/j.anifeedsci.2021.114921

Xie, J., Li, L.-f, Dai, T.-Y., Qi, X., Wang, Y., Zheng, T.-z, et al. (2022). Short-chain fatty acids produced by ruminococcaceae mediate α-linolenic acid promote intestinal stem cells proliferation. *Mol. Nutr. Food Res.* 66:e2100408. doi: 10.1002/mnfr.202100408

Xing, H., Chen, J., Peng, M., Wang, Z., Liu, F., Li, S., et al. (2019). Identification of signal pathways for immunotoxicity in the spleen of common carp exposed to chlorpyrifos. *Ecotoxicol. Environ. Safety* 182:109464. doi: 10.1016/j.ecoenv.2019.109464

Xiong, Y., Guo, C., Wang, L., Chen, F., Dong, X., Li, X., et al. (2021). Effects of paper mulberry silage on the growth performance, rumen microbiota and muscle fatty acid composition in Hu Lambs. *Fermentation* 7:286. doi: 10.3390/fermentation7040286

Yin, L., Yang, Q., Zhang, Y., Wan, D., Yin, Y., Wang, Q., et al. (2021). Dietary copper improves intestinal morphology via modulating intestinal stem cell activity in Pigs. *Animals (Basel)* 11:2513. doi: 10.3390/ani11092513

Zaharik, M. L., and Brett Finlay, B. (2004). Mn2+ and bacterial pathogenesis. *FBL* 9, 1035–1042. doi: 10.2741/1317

Zhang, R., Gu, J., Wang, X., Li, Y., Liu, J., Lu, C., et al. (2019). Response of antibiotic resistance genes abundance by graphene oxide during the anaerobic digestion of swine manure with copper pollution. *Sci. Total Environ.* 654, 292–299. doi: 10.1016/j.scitotenv.2018.11.094

Zhang, Z. D., Wang, C., Du, H. S., Liu, Q., Guo, G., Huo, W. J., et al. (2020). Effects of sodium selenite and coated sodium selenite on lactation performance, total tract nutrient digestion and rumen fermentation in Holstein dairy cows. *Animal* 14, 2091–2099. doi: 10.1017/S1751731120000804

Zhao, Z. W., Ma, Z. Y., Wang, H. C., and Zhang, C. F. (2022). Effects of trace minerals supply from rumen sustained release boluses on milk yields and components, rumen fermentation and the rumen bacteria in lactating yaks (*Bos grunniens*). *Anim. Feed Sci. Technol.* 283:115184. doi: 10.1016/j.anifeedsci.2021.115184

Zhou, J., Ding, Z., Pu, Q., Xue, B., Yue, S., Guan, S., et al. (2022). Rumen fermentation and microbiome responses to enzymatic hydrolysate of cottonseed protein supplementation in continuous *in vitro* culture. *Animals (Basel)* 12:2113. doi: 10.3390/ani12162113

Zhou, J., Dong, Z., Wan, D., Wang, Q., Haung, J., Huang, P., et al. (2020). Effects of iron on intestinal development and epithelial maturation of suckling piglets. *J. Anim. Sci.* 98:skaa213. doi: 10.1093/jas/skaa213

Zhu, C., Lv, H., Chen, Z., Wang, L., Wu, X., Chen, Z., et al. (2017). Dietary zinc oxide modulates antioxidant capacity, small intestine development, and jejunal gene expression in weaned piglets. *Biol. Trace Elem. Res.* 175, 331–338. doi: 10.1007/s12011-016-0767-3

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# Gut microbiota contributes to the methionine metabolism in host

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Methionine (Met) metabolism provides methyl groups for many important physiological processes and is implicated in multiple inflammatory diseases associated with the disrupted intestinal microbiota; nevertheless, whether intestinal microbiota determines Met metabolism in the host remains largely unknown. Here, we found that gut microbiota is responsible for host Met metabolism by using various animal models, including germ-free (GF) pigs and mice. Specifically, the Met levels are elevated in both GF pigs and GF mice that mainly metabolized to S-adenosine methionine (SAM) in the liver. Furthermore, antibiotic clearance experiments demonstrate that the loss of certain ampicillin- or neomycin-sensitive gut microbiota causes decreased Met in murine colon. Overall, our study suggests that gut microbiota mediates Met metabolism in the host and is a prospective target for the treatment of Met metabolism-related diseases.

KEYWORDS

**gut microbiota, methionine, SAM, hCY, germ-free pigs**

## Introduction

Methionine (Met) is one of the essential amino acids that could participate in the biosynthesis of various nitrogen-containing substances (Liu et al., 2021). Indeed, Met metabolism in the host mainly consists of four metabolic pathways surrounding the Met-homocysteine (hCy) cycle (Yin et al., 2018; Xu et al., 2020). The host Met-hCy cycle (hereinafter referred to as the Met cycle) is associated with transmethylation and the metabolism of amino acids such as serine (Ser), glycine (Gly), glutathione (GSH), and taurine (Tau; Xu et al., 2020). Specifically, Met reacts with ATP under the catalysis of methionine adenosyltransferase (MAT) to generate S-adenosine methionine (SAM), which then provides a methyl group to generate S-adenosine homocysteine (SAH; Clare et al., 2019). Homocysteine (hCy) generated by

S-adenosylhomocysteine hydrolase (SAHH) is then remethylated by N5-methyltetrahydrofolate ($CH_3$-THF) to generate Met, and vitamin $B_{12}$ serves as a key coenzyme in this process (Lam et al., 2021). Of note, the Met cycle ensures Met level and the supply of methyl-donor SAM in liver; therefore, modulating Met cycle has therapeutic implications for many diseases, for example, alcoholic liver disease (ALD; Kharbanda, 2013). Moreover, the Met cycle in tumors has also received attention. For example, tumor initiation of lung cancer stem cells consumes large amounts of Met for histone methylation, resulting in Met depletion and exogenous Met dependence (Wang et al., 2019). Met cycle is also disrupted in liver tumors and is often associated with poor prognosis (Xu et al., 2020). Therefore, it is meaningful to fine-tune the Met metabolism in host. However, the factors affecting the Met metabolism are still unclear.

The gut has a complex microorganism community including bacteria, fungi, and viruses, which the composition is determined by natural colonization and subsequent environmental factors (Cani, 2018). Gut microbes play particularly important roles in regulating homeostasis by absorbing nutrients, producing metabolites, and cooperating or competing with other microorganisms in the intestinal tract (Osbelt et al., 2021). It is worth noting that the impact of the gut microbiota on host is not limited in gut, as supported by the findings of the gut-brain axis (Cryan et al., 2019), gut-lung axis (Dang and Marsland, 2019), and gut-liver axis (Tripathi et al., 2018). A study revealed that patients with quiescent inflammatory bowel disease (IBD) fatigue had reduced serum metabolites like Met, Ser and sarcosine (Sar), and decreased short-chain fatty acids (SCFAs)-producing gut microbiota such as *Faecalibacterium prausnitzii* (Borren et al., 2021). In a controlled study of Parkinson's disease (PD), significant differences in the trajectories of Met and homoserine were found (Hertel et al., 2019). And the PD-associated gut microbiome analysis revealed increased abundance of *Akkermansia muciniphila* (*A. muciniphila*) and *Bilophila wadsworthia* involved in sulfur metabolism (Hertel et al., 2019). Additionally, *A. muciniphila* contributed more than 70% of Met secretion potential (Hertel et al., 2019), indicating the interaction between the gut microbiota and host Met cycle. Nevertheless, whether Met metabolism in host is related to the gut microbiota remains enigmatic.

Given the importance of Met metabolism in inflammation, cancer, and neurological diseases and the impact of microbial-host interactions on host physiology, biochemistry, and immune status, an in-depth analysis of gut microbial and host Met metabolism has profound implications. Microbiota-targeted interventions mainly include antibiotic treatment, probiotic addition, and fecal microbiota transplantation (Sun and Shen, 2018). Among them, antibiotic-induced intestinal dysbiosis is a common method that can lead to drastic changes in the intestinal microbiota (Andremont et al., 2021). Notably, germ-free (GF) animal models are currently important models for studying microbiome-host interactions. Unlike pharmacological interventions such as antibiotics, GF animals achieve true "sterility" and have distinct characteristics from specific pathogen-free (SPF) animals in gut development and/or immune system (Uzbay, 2019). Therefore, GF animals are valuable experimental models in cancer and metabolism-related diseases. Moreover, studies of Met metabolism have typically used *in vitro* cell or tissue cultures and validated in rodents (Wang et al., 2019; Xu et al., 2020). Genetically inbred mice in rodents are common experimental models that help scientists accomplish many studies, but they are not ideal models of the human immune system because of the differences in immune systems of humans and mice caused by evolutionary branches (Pulendran and Davis, 2020). Therefore, the GF pig model adopted in this paper may help to provide a more valuable reference.

As experimental subjects, pigs are ideal large animal models for studying nutrition or disease in pigs and humans due to their high similarity to humans in terms of anatomy, physiology, and immune system (Miller and Ullrey, 1987; Itoh et al., 2019). The GF pig model is therefore ideal for studying gut microbe-host Met metabolism. Importantly, dietary Met plays an important role in animal nutrition and is the second/third limiting amino acid in pig and piglet diets (Yang et al., 2021). Since Met is beneficial for the growth and development of pigs, most relevant studies have focused on the effects of dietary Met or Met-restricted diets on swine meat quality, growth performance, and intestinal function (Zeitz et al., 2019; Gondret et al., 2021; Yang et al., 2021). However, there are few studies concerning Met metabolism in pigs. Currently, only limited studies on plasma Met metabolism profiles and mammalian lifespan have been retrieved focusing on the relationship between porcine plasma Met metabolism and lifespan (Mota-Martorell et al., 2021).

Therefore, we used GF pig as an experimental model to explore the impact of gut microbiota on host Met metabolism that contributes to map the Met metabolism profile of the pig gut microbiome. We then explored the link between gut microbiota and host Met metabolism in GF mice and mice

Abbreviations: *A. muciniphila*, *Akkermansia muciniphila*; ALD, Alcoholic liver disease; CH3-THF, N5-methyltetrahydrofolate; CKD: Chronic kidney disease; CVD, Cardiovascular disease; Cys, Cysteine; ESI, Electrospray ionization; GF, Germ-free; Gly, Glycine; GSH, Glutathione; HCD, High collision-induced dissociation; hCY, homocysteine; HHcy, hyperhomocysteinemia; IBD, Inflammatory bowel disease; MAT, Methionine adenosyltransferase; MAT1A, Methionine adenosyltransferase 1A; Met, Methionine; NAA, Neutral amino acid; PD, Parkinson's disease; SAH, S-adenosine homocysteine; SAHH, S-adenosylhomocysteine hydrolase; SAM, S-adenosine methionine; Sar, Sarcosine; SCFAs, Short-chain fatty acids; Ser, Serine; SPF, Specific pathogen-free; Tau, Taurine.

treated with different antibiotics, further expanding the generalizability of the experimental results and potentially providing new insights into Met metabolism-related diseases.

# Materials and methods

## Animal acquisition and breeding

### GF and SPF pigs

GF pigs and SPF pigs were harvested by uterine dissection and then transferred to sterile feeding isolators (DOSSY Experimental Animals Co., Ltd., Chengdu, China) for decontamination. Next, the piglets were taken to rearing isolators (Class Biologically Clean Ltd., Madison, Wisconsin, United States; Qi et al., 2021; Zhou et al., 2021). The isolator operates in strict accordance with operating procedures within the barrier facilities.

The feeding conditions of the two groups of experimental animals are the same, and the specific steps refer to our previous experiments (Liu et al., 2022). For the first 21 days, they were fed Co60 γ-irradiated 4.8%-fat sterile milk powder (Jiangsu Anyou Group China) diluted with sterile water (Sun et al., 2018; Liu et al., 2022). The basal diet for pigs from day 22 was provided by the Institute of Animal Nutrition, Sichuan Agricultural University (Chengdu, China). In this study, pigs were obtained from the Technical Engineering Center for the Development and Utilization of Medical Animal Resources (Chongqing, China). All experimental protocols were approved and carried out under the guidance of the Laboratory Animal Ethics Committee of South China Agricultural University [Guangzhou, China; permit number SYXK (Guangdong) 2019–0136].

Eleven GF pigs were randomly selected, five of which were kept under GF conditions (GF pigs) and their anal swabs were collected weekly for microbial testing. Another six pigs served as controls (SPF pigs) by transplanting maternal feces. Control pigs were continuously orally administered fecal bacteria suspension (1 ml/d) for 3 days at 7 days of age and kept in sterile isolators (Liu et al., 2022).

For sample collection, on the last day of the experimental period, two groups of pigs were subjected to respiratory anesthesia with isoflurane after urine collection, and other samples were collected on a clean bench after blood collection to ensure that there was no bacterial contamination throughout the entire sampling process. Liver, bile, spleen, kidney, heart, and different intestinal segments and their contents were collected, including jejunum, ileum, cecum, colon, jejunum contents, cecal contents, colonic contents, rectal contents, and feces (Figure 1A). Samples were quickly frozen in liquid nitrogen and stored in an ultra-low temperature freezer at −80°C.

### GF/SPF and antibiotic-treated mice

GF and SPF Kunming mice were bred at the Third Military Medical University (Chongqing, China). ICR mice were purchased from Liaoning Changsheng Biotechnology Co., Ltd. (Liaoning, China). GF mice were housed in sterile isolators and SPF Kunming and ICR mice were housed in sterile individual animal colonies (temperature, 25 ± 5°C; relative humidity, 55 ± 5%; dark light alternated every 12 h) with free access to drinking water and standard rodent chow.

For the Met cycle metabolite analysis experiments, GF (GF group, 4 weeks old, $n = 10$ males and 10 females) and SPF (SPF group, 4 weeks old, $n = 10$ males and 10 females) Kunming mice were treated with the same diet for 4 weeks, then were euthanized by $CO_2$ asphyxiation, and blood, duodenum, and colon were collected (Figure 1B).

To establish an antibiotic-treated mouse model, 6-week-old female ICR mice ($n = 12$) were supplemented with ampicillin (MB1407, 1 mg/ml, meilunbio), colistin (MB1064, 1 mg/ml, meilunbio), metronidazole (B300250, 1 mg/ml, Aladdin), neomycin (MB1716, 1 mg/ml, meilunbio), and vancomycin (V301569, 0.5 mg/ml, Aladdin) in drinking water for 2 weeks. Mice were then euthanized by $CO_2$ asphyxiation and colon samples were collected (Figure 1C).

## Standard and sample preparation

The key metabolites in the Met cycle involved in this study are Met, SAM, SAH, and hCY. Accurately weighed 1.0 mg of these metabolites and dissolved each standard in 10 ml methanol–water (50:50, V/V) to obtain a 100 μg/ml stock solution. The standard solution was then serially diluted to 1,000, 500, 100, 10, 1, 0.5, 0.1, 0.01, 0.001 ng/ml. All metabolites were quantified by external standard method, and all standard solutions were stored at −20°C. The processing of tissue samples and fluid samples such as serum, urine, and bile was carried out according to our previous experiments (Liu et al., 2022), and stored at −20°C prior to UPLC-Orbitrap-MS/MS analysis.

## UPLC-Orbitrap-MS/MS conditions

Met cycle-related metabolites were separated by Thermo Fisher Scientific UPLC system (Dionex UltiMate 3,000) with a C1s Hypersil Gold column (1.9 μm, 100 mm × 2.1 mm; Thermo Scientific). The main parameters and gradient elution procedure were based on our previous experiments (Liu et al., 2022).

## Identification of metabolites

Metabolite identification refers to our previous experiments (Liu et al., 2022). Briefly, information such as retention time, mass (m/z), peak and fragment ion MS or MS/MS intensity could

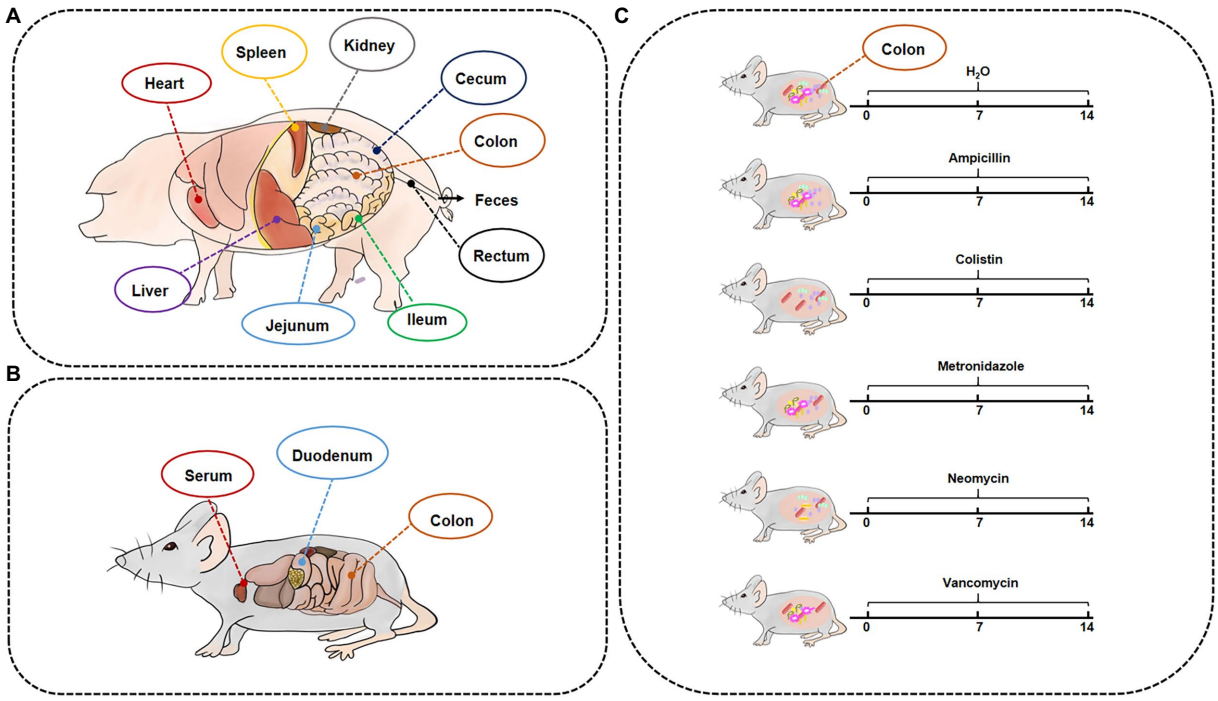


FIGURE 1
Timeline of experimental processing and sample collection information. The samples of GF/SPF pigs **(A)** and mice **(B)**. **(C)** SPF mice were treated by different antibiotics for 2weeks.

be obtained when the RAW file is opened with Xcalibur. The mass list of each MS scan was generated by the peak detection and chromatographic builder in the original data file, and then the chromatogram was built.

## Data analysis and statistics

Xcalibur converted data in an instrument-specific format (*.raw) to a common data format (.XLS) that displays information on metabolites, including calculated amounts, retention times, and peak areas. The raw data can be accessed by the Metabolights repository,[1] with the identifier MTBLS6154 (Haug et al., 2020).

All data are shown as mean ± SEM. Data between two groups were analyzed by (unpaired *t*-test, Prism 6.0) if the data exhibited Gaussian distribution and equal variance, and by (Welch-corrected unpaired *t*-test, Prism 6.0) if the data exhibited (Gaussian distribution but unequal variances). If the data were not normally distributed, a nonparametric test (Mann–Whitney U test, Prism 6.0) was performed. The Gaussian distribution of the data was analyzed by (D'Agostino-Pearson comprehensive normality test, Prism 6.0) and/or (Kolmogorov–Smirnov test, Prism 6.0). Data were analyzed for variance by (Brown-Forsythe test, Prism 6.0). Significant differences among 3 or more groups were passed (Bonferroni's multiple comparison test, one-way ANOVA assessment, and multiple *t*-test, Prism 6.0). $p < 0.05$ (*), $p < 0.01$ (**), $p < 0.001$ (***), and $p < 0.0001$ (****) were considered statistically.

# Results

## Gut microbiota alters the met cycle of pig gut

In this study, we collected different intestinal contents of GF pigs and analyzed by UPLC (Figure 1A). Four key metabolites in the Met cycle were detected, including Met, SAM, SAH, and hCY (Figure 2A). Of note, SAM, generated by Met metabolism, is an important methyl donor that cooperates with the methylation *in vivo*, such as histone methylation (Yu et al., 2019). HCY from SAH deadenosine may regulate the one-carbon metabolism of Ser and Gly, the folate cycle of THF, or the generation of cysteine (Cys) to generate GSH or Tau (Selhub, 1999; Mentch et al., 2015; Jakubowski, 2019). As a result, Met level was significantly increased by almost 10-fold in the gut contents (including cecum, colon, rectal contents, and feces) of GF pigs compared to the control group (SPF), with the exception of the jejunum contents (Figure 2B). SAM, SAH, and hCY were not significantly altered in the jejunum contents of GF pigs (Figure 2C) that may be present at very low levels even below the

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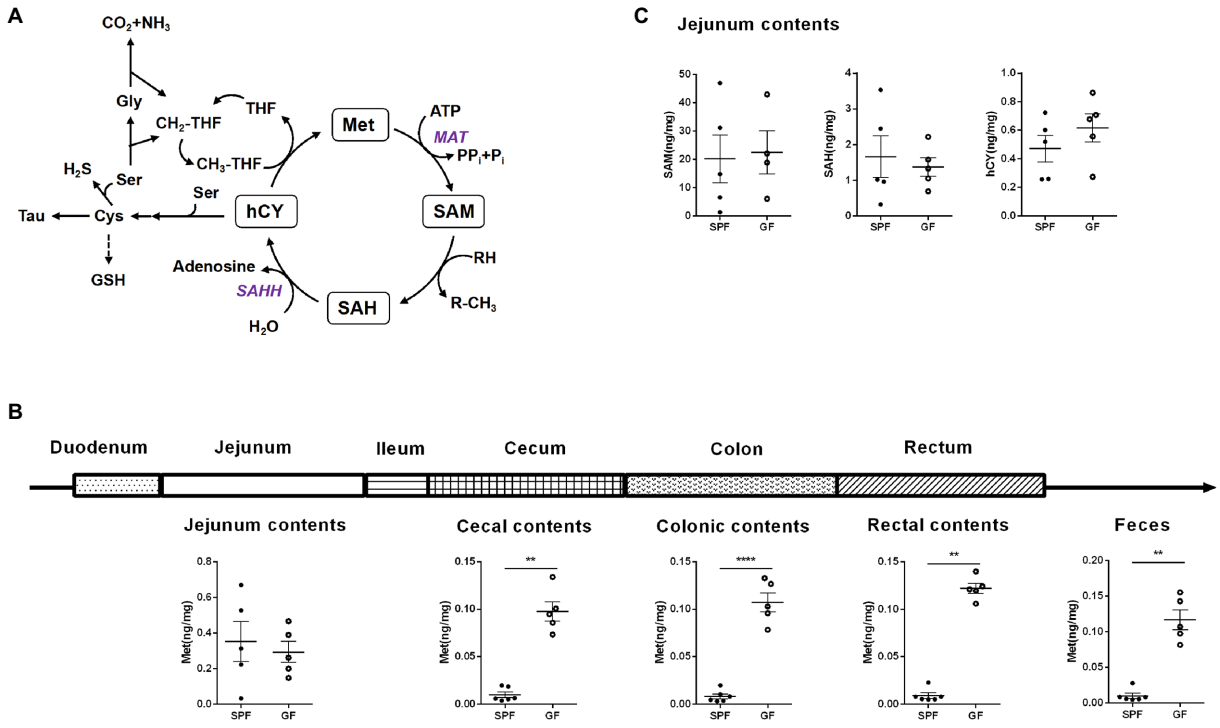


FIGURE 2
Gut microbiota alters the Met metabolism of pig gut. **(A)** Met cycle. Met consumes ATP under the catalysis of MAT to generate SAM, SAM provides methyl group to generate SAH, SAH generates hCY under the action of SAHH, and hCY obtains methyl group through $CH_3$-THF in the folic acid cycle to generate Met. hCY participates in the generation of Cys, which, in turn, generates GSH or Tau. **(B)** Met content in different intestinal contents of GF pigs ($n$=5) and SPF pigs ($n$=6). **(C)** Contents of SAM, SAH, and hCY in the jejunum contents of GF pigs ($n$=5) and SPF pigs ($n$=6). Data were analyzed by unpaired $t$-test and represented as means ± SEM. $p$<0.05 (*), $p$<0.01 (**), $p$<0.001 (***), and $p$<0.0001 (****). Met: Methionine; SAH: S-adenosine homocysteine; SAM: S-adenosine methionine; hCY: homocysteine; Ser: Serine; Cys: Cysteine; Tau: Taurine; GSH: Glutathione; Tau: Taurine; Gly: Glycine; THF: Tetrahydrofolate; MAT: Methionine adenosyltransferase; SAHH: S-adenosylhomocysteine hydrolase.

detection limit in other intestinal contents. The experimental results showed that the absence of the gut microbiota led to a significant increase of Met levels in intestinal contents, suggesting that gut microbiota was involved in the regulation of the Met cycle in pig intestines.

## Gut microbiota regulates the met cycle in different intestinal segments of pigs

About 25% of the body's trans-methylation and trans-sulfurization reactions occurred in the gastrointestinal tract, and 65% of the visceral first-pass effect of Cys occurred in the intestine (Bauchart-Thevret et al., 2009), indicating that intestinal absorption was crucial to Met cycle. Therefore, we examined Met cycle-associated metabolites in different intestinal segments. There was no significance in the four key metabolites in the jejunum (Figure 3A). Compared with SPF group, Met in the ileum of GF group was significantly decreased, and SAH showed a downward trend (Figure 3B). In the cecum and colon, Met was increased in GF group compared to SPF group, but SAM, SAH, and hCY were not significantly different (Figures 3C,D). Together, the increase of Met in GF pigs was mainly occurred in the large intestinal segment.

## Gut microbiota remodels the met cycle in extraintestinal tissue and circulation system of pigs

The Met metabolites are absorbed by the intestinal epithelium and penetrate the basement membrane and then conducted on the extraintestinal tissues to further exert their effects through blood circulation and other means (Kharbanda, 2013; Xu et al., 2020). In addition, the liver is an important organ for Met metabolism that converts Met to SAM by MAT1A (Methionine adenosyltransferase 1A; Lu and Mato, 2012). Met-related metabolites are perturbed in both cardiovascular disease (CVD) and chronic kidney disease (CKD; Xiao et al., 2015; Yang et al., 2016), and the kidneys are involved in SAH metabolism (Garibotto et al., 2009).

We collected extraintestinal tissue samples from GF pigs and SPF pigs, including liver, spleen, kidney, and heart, and detected and

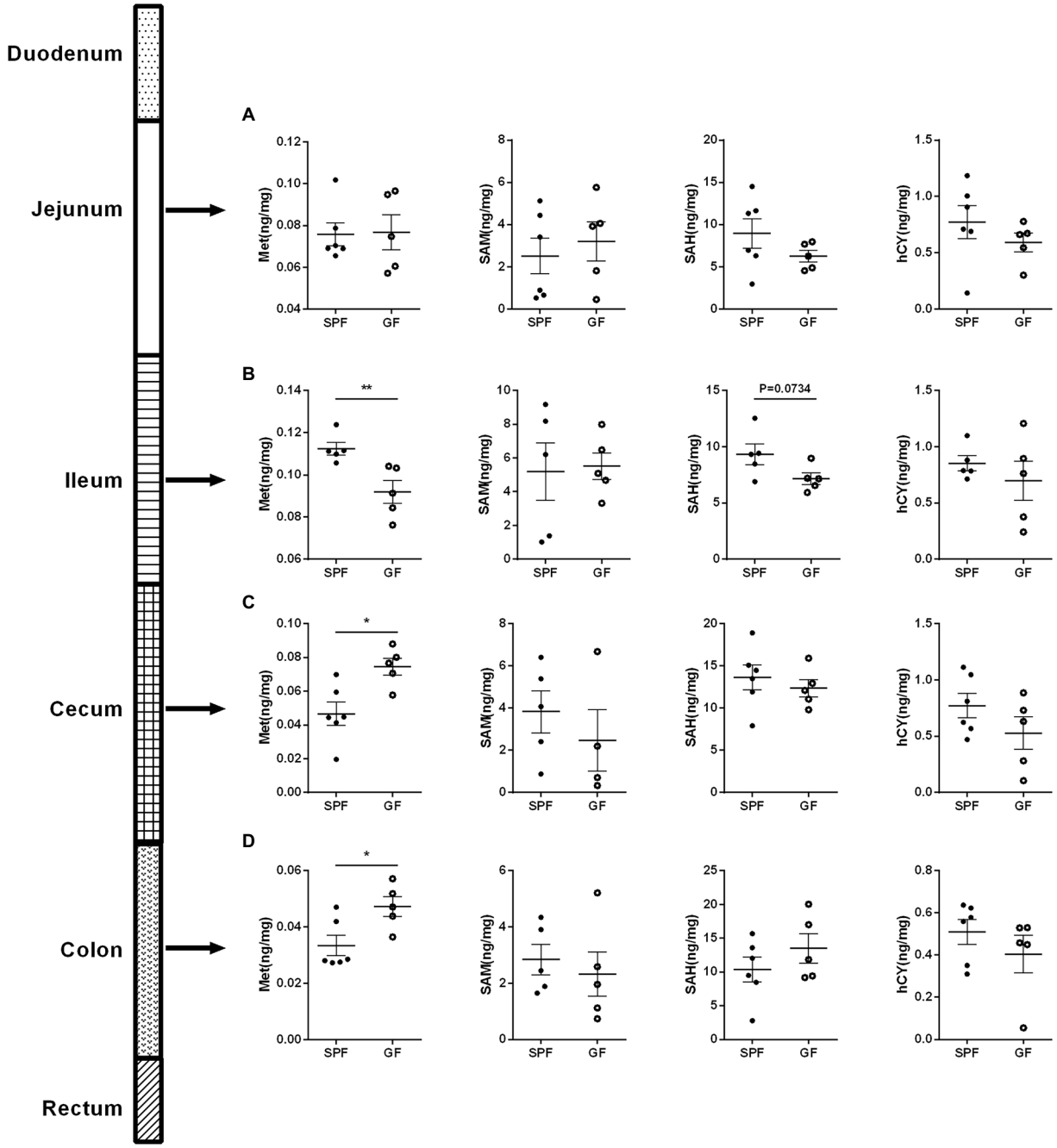


FIGURE 3
Gut microbiota regulates the Met metabolism in different intestinal segments of pigs. Contents of Met, SAM, SAH, hCY in **(A)** jejunum, **(B)** ileum, **(C)** cecum, and **(D)** colon of GF pigs ($n$=5) and SPF pigs ($n$=6). Data were analyzed by unpaired $t$-test and represented as means ± SEM. $p$<0.05 (*), $p$<0.01 (**), $p$<0.001 (***), and $p$<0.0001 (****). Met: Methionine; SAH: S-adenosine homocysteine; SAM: S-adenosine methionine; hCY: homocysteine.

analyzed the key metabolites of the Met cycle, respectively, by UPLC-Orbitrap-MS/MS. The results showed that Met in the liver of GF pigs decreased and SAM tended to increase (Figure 4A). There were no significant differences in Met, SAM, SAH, and hCY in spleen (Figure 4B), kidney (Figure 4C), and heart (Figure 4D) between GF and SPF groups. Analogously, blood, bile, and urine in the circulation system of both groups (GF and SPF) were detected by UPLC-Orbitrap-MS/MS. The results illustrated that Met was elevated in bile (Figure 5A) and serum (Figure 5B) but was no significant difference in urine (Figure 5C) in GF group. This result suggested that the lack of gut microbiota led to the increase of Met in the circulatory system, which was converted to SAM in the liver (Figure 6).

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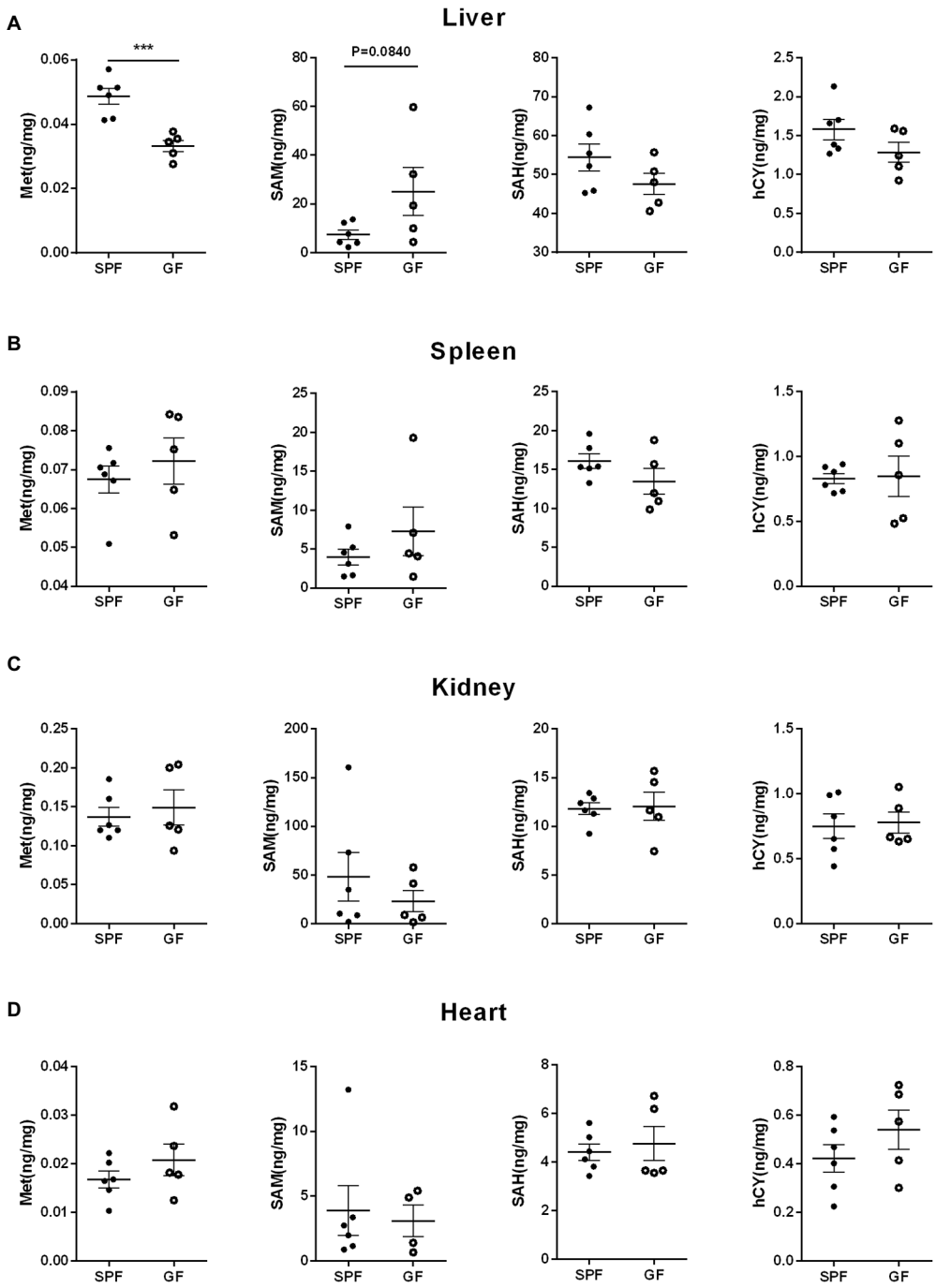


FIGURE 4
Gut microbiota remodels the Met metabolism in porcine extraintestinal tissue by gut microbiota. Contents of Met, SAM, SAH, hCY in **(A)** liver, **(B)** spleen, **(C)** kidney, **(D)** heart of GF pigs ($n$=5) and SPF pigs ($n$=6). Data were analyzed by unpaired $t$-test and represented as means ± SEM. $p$<0.05 (*), $p$<0.01 (**), $p$<0.001 (***), and $p$<0.0001 (****). Met: Methionine; SAH: S-adenosine homocysteine; SAM: S-adenosine methionine; hCY: homocysteine.

## Gut microbiota modulates the met cycle of mice

By collecting samples from GF pigs and SPF pigs, we detected Met cycle-metabolites in the intestinal contents, different intestinal segments, extraintestinal tissues, and the circulation system. It was found that the absence of gut microbiota resulted in an overall increase in circulating Met and the elevated Met might be primarily utilized in liver. Next, we sought to translate the involvement of gut microbiota on Met metabolism to rodents. We collected duodenum, colon, and serum samples from GF and

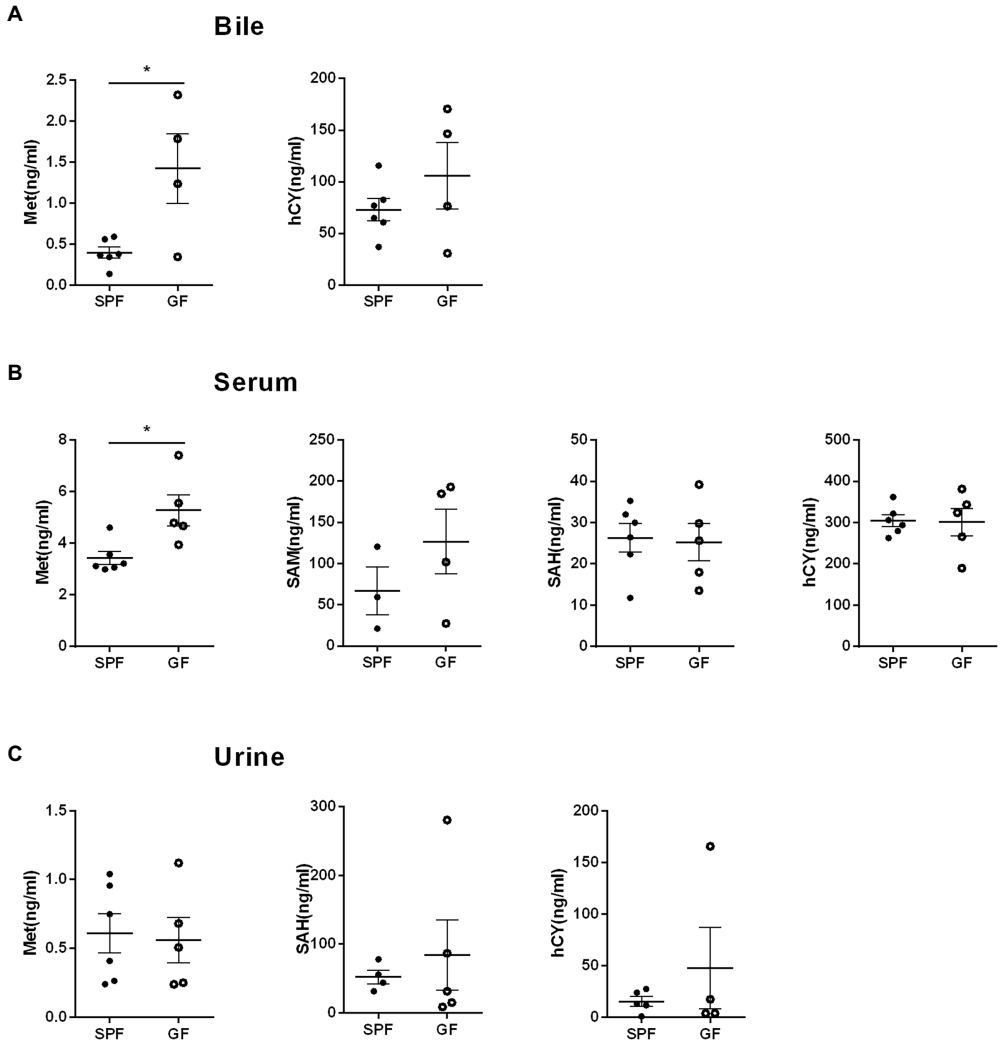


FIGURE 5
Gut microbiota modulates Met metabolism in porcine circulation system. **(A)** Met and hCY levels in bile. **(B)** Met, SAM, SAH, hCY levels in serum. **(C)** Met, SAH, hCY levels in urine of GF pigs ($n$=5) and SPF pigs ($n$=6). Data were analyzed by unpaired $t$-test and represented as means ± SEM. $p$<0.05 (*), $p$<0.01 (**), $p$<0.001 (***), and $p$<0.0001 (****). Met: Methionine; SAH: S-adenosine homocysteine; SAM: S-adenosine methionine; hCY: homocysteine.

SPF mice and examined key Met cycle metabolites. Met was significantly increased and hCY was significantly decreased in the duodenum and colon of GF mice (Figures 7A,B). SAM in the colon of GF mice was significantly decreased (Figure 7B), which was consistent with the trend in pigs. There was no significant difference on Met in the serum of GF mice, but hCY was significantly reduced (Figure 7C). Thus, the findings further confirmed that the gut microbiota can modulate the Met metabolism and the absence of gut microbiota resulted in elevated Met and lower downstream SAM and/or hCY.

In addition to host metabolism of Met, it has been demonstrated the existence of microbial metabolism of Met and Cys (Das et al., 2021). For example, gut microbes use cysteine desulfurase (CDS) to catalyze the synthesis of $H_2S$ from Cys (Wang et al., 2019). In the intestinal flora, Gram-positive *Streptococcus*, *Clostridium*, *Staphylococcus aureus*, *Mycobacterium tuberculosis*, Gram-negative *Klebsiella*, *Enterobacter*, *Salmonella*, *Akkermansia* spp., and *Desulfovibrio* are involved in the degradation of Cys (Carbonero et al., 2012). In the lower gastrointestinal tract, taurine, and isethionate are substrates for microbial metabolism, and microorganisms metabolize these organic sulfonates with the assistance of electron carriers to produce sulfide, ammonia, and other products (Laue et al., 1997). Therefore, to determine the types of microbes played pivotal roles in the regulation of host's Met metabolism, we constructed a model of microbe deletion by treating mice with different antibiotics in drinking water for 2 weeks. The results showed decreased Met and increased SAH in colon of ampicillin-treated mice (Figure 7D). Neomycin treatment caused decreased colonic Met levels (Figure 7D). However, colonic Met and SAH were not

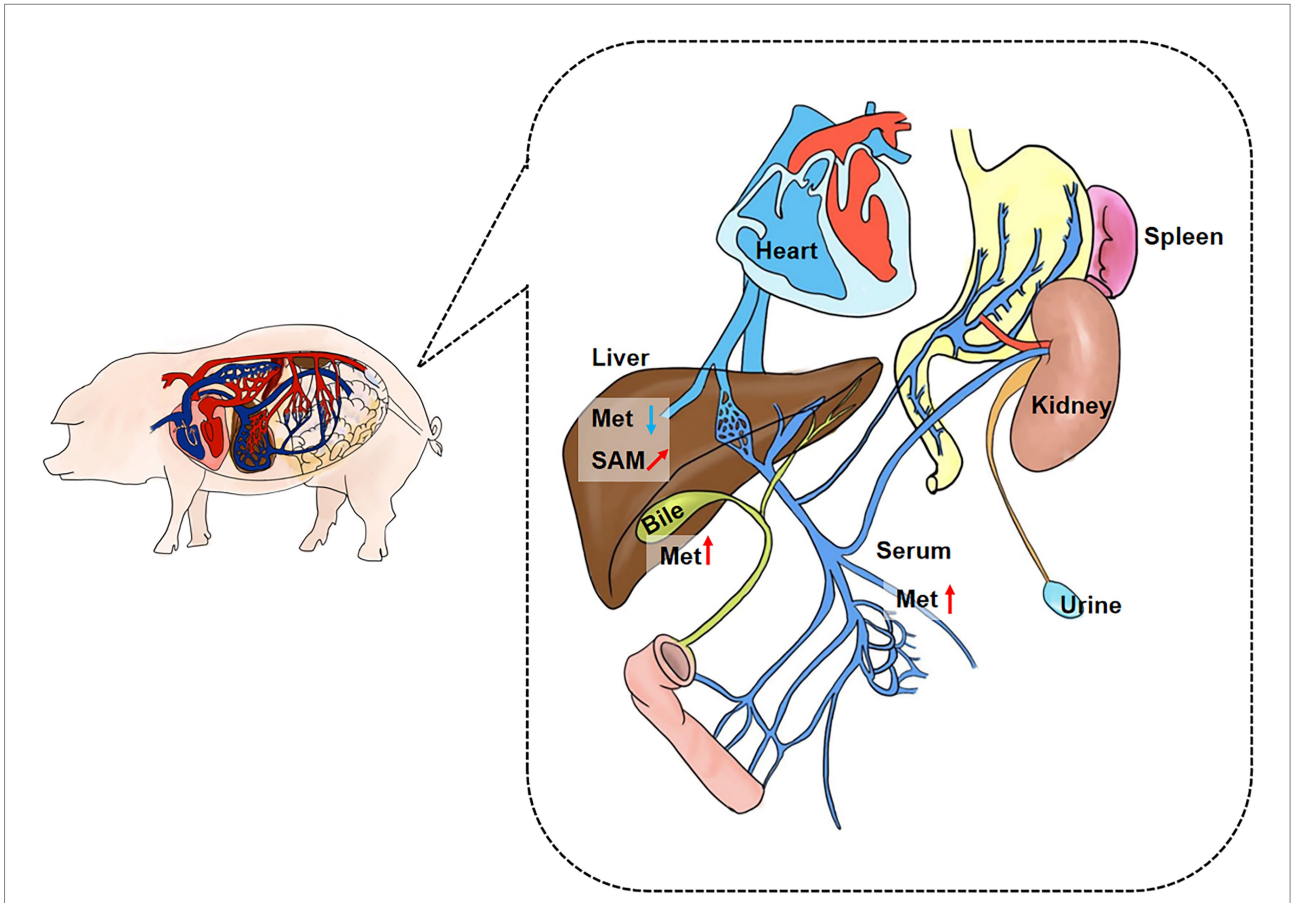


FIGURE 6
Summary plot of Met metabolic changes in GF porcine parenteral tissue and parenteral circulation. Met was increased in the overall circulation in GF pigs, and the elevated Met was mainly utilized in liver. Met: Methionine; SAH: S-adenosine homocysteine; SAM: S-adenosine methionine. *("↗"arrows represent increasing trend, "⬆" arrows represent increases, and "⬇" arrows represent decreases.)

significantly different between colistin, metronidazole, and vancomycin-treated mice (Figure 7D). Therefore, we speculated that part of the ampicillin- and/or neomycin-sensitive gut microbiota has taken a major role in regulating the Met cycle in host.

## Discussion

Met metabolism is mainly composed of Met-cycle and produces the important methyl donor SAM, which participates in the biosynthesis of various proteins and DNA (Clare et al., 2019). SAM is demethylated to generate SAH and then SAH is deadenosine to generate hCY which may participate in the folate cycle and generate Cys (Lam et al., 2021). Cys produces metabolites, such as Tau or GSH, to regulate metabolic homeostasis (Selhub, 1999; Mentch et al., 2015; Jakubowski, 2019). The intestinal tract plays a key role in Met metabolism (Bauchart-Thevret et al., 2009). In addition to host intestinal transport or metabolism of Met (Zhang et al., 2014; Li et al., 2016; Romanet et al., 2021; To V et al., 2021), the gut microbiota also plays an important role in Met metabolism in the large intestine (Carbonero et al., 2012; Wang et al., 2019). Therefore, we used GF pigs as a model to explore the metabolites of the Met cycle and found that the gut microbiota was involved in regulating Met metabolism in host and the lack of gut microbiota led to the increase of Met in the gut and circulation system (Figure 8). Furthermore, we speculated that the liver is the main site of Met metabolism and utilization in GF pigs.

The increase of Met in the gut is mainly concentrated in large intestine, while the Met in small intestine shows a decreasing trend. We speculate that it might be due to the changes in intestinal Met transporters. Amino acid absorption in the gut relies on transport across the apical and basolateral membranes of epithelial cells. As a neutral amino acid (NAA), Met is taken up across the apical membrane of intestinal epithelial cells by various $Na^+$-dependent/ independent transporters, such as *ASCT2* (*SLC1A5*), $B^0AT1$ (*SLC6A19*), $rBAT/b^{0,+}AT$ (*SLC3A1/SLC7A9*), *4F2hc/y*$^+$*LAT1* (*SLC3A2/SLC7A7*), and *LAT4* (*SLC43A2*) primarily transports Met across the basolateral membrane (To V et al., 2021). Previous studies have demonstrated that the presence of *ASCT2* and *y*$^+$*LAT1* in pig jejunum (Zhang et al., 2014; Li et al., 2016), $B^0AT1$ in pig jejunum and ileum (Yi et al., 2019; Hu et al., 2021), and $rBAT/b^{0,+}AT$ in pig ileum (Yi et al., 2019). *LAT4* is

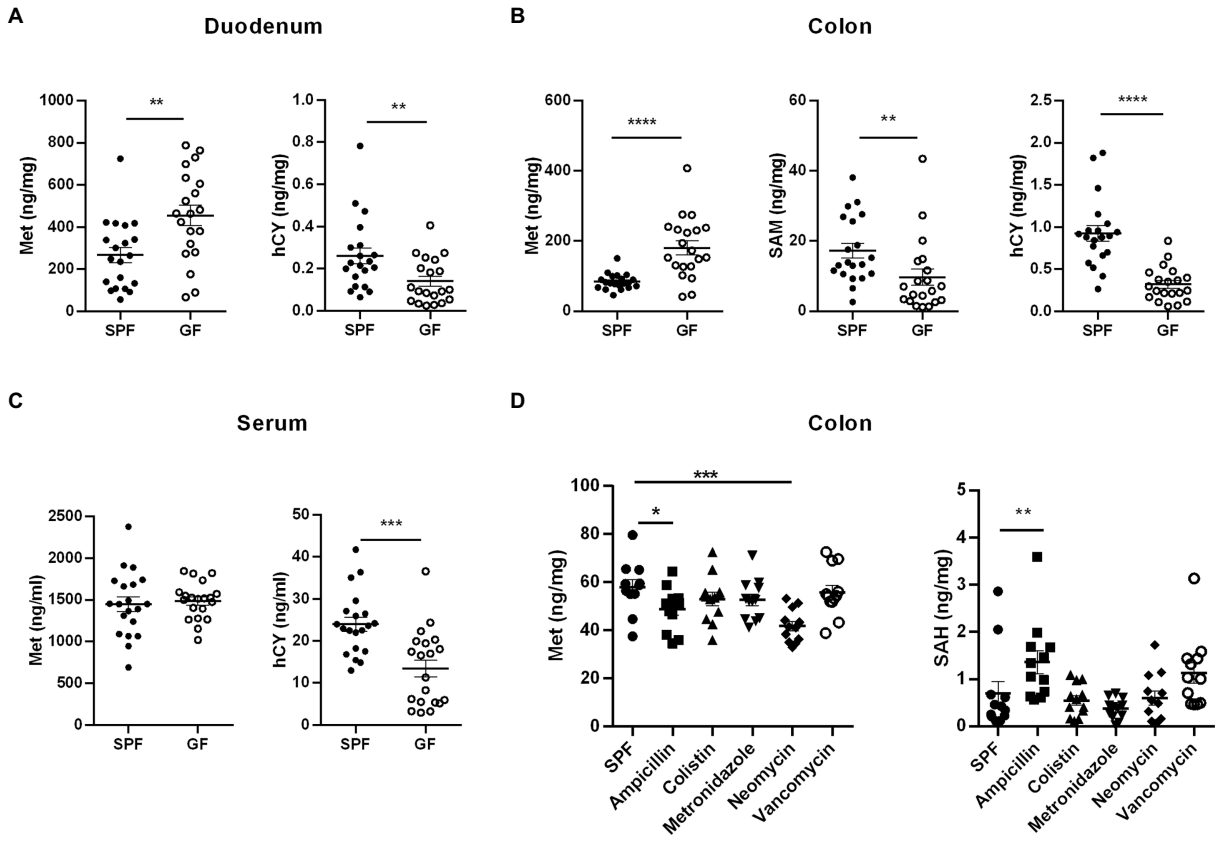


FIGURE 7
Influence of gut microbiota on Met metabolism in mice. **(A)** Met and hCY contents in duodenum of GF mice and SPF mice ($n$=20). **(B)** Met, SAM, and hCY contents in colons of GF mice and SPF mice ($n$=20). **(C)** Serum Met and hCY levels in GF mice and SPF mice ($n$=20). **(D)** Met and SAH contents in colon of SPF mice ($n$=12) after different antibiotic treatments. Data were analyzed by unpaired $t$-test (two groups) and one-way ANOVA assessment (>two groups), and represented as means ± SEM. $p$<0.05 (*), $p$<0.01 (**), $p$<0.001 (***), and $p$<0.0001 (****). Met: Methionine; SAH: S-adenosine homocysteine; SAM: S-adenosine methionine; hCY: homocysteine.

expressed in the human small intestine, especially the duodenum (Maric et al., 2021). A recent study has performed a segmented quantification of Met transporters in the porcine gastrointestinal tract and provided us with a more detailed description (Table 1). In addition to our previously mentioned Met transporters, *LAT2*, *IMINO*, *SNAT2*, and $ATB^{0,+}$ are expressed in the porcine gastrointestinal tract (Romanet et al., 2021). Moreover, the research found that in $Chd8^{+/-}$ mice model of autism spectrum disorder (ASD), increased expression of neutral amino acid transporters *Slc6a19*, *Slc7a8*, and *Slc7a15* resulted in increased serum glutamine and tryptophan levels (Yu et al., 2022). In $Chd8^{+/-}$ mice, the abundance of *Bacteroides* decreased, especially *Bacteroides uniformis*, and *Bacteroides* supplementation improved ASD-like behavior (Yu et al., 2022). In a piglet experiment, lysine restriction caused a decrease in *SLC7A1* and *SLC7A2*, limiting lysine transport (Yin et al., 2017). Sequencing found that lysine restriction increased the abundance of *Actinobacteria* and *Saccharibacteria* in the gut, and functional analysis of the microbial community revealed that the differences were concentrated in amino acid metabolism and membrane transport (Yin et al., 2017). Thus, gut microbiota has the ability to modulate different amino acid transporters. Therefore, we hypothesize that the gut microbiota might have varying degrees of influences on the Met transporters in the gut. Furthermore, based on the changes of Met-related metabolites in the gut and the expression locations of Met transporters, we suppose that $B^0AT1$, *rBAT*, and *LAT4* might be partially inhibited, and/or $ATB^{0+}$, *ASCT2*, and *SNAT2* might be promoted. However, this conjecture still needs to be verified by detecting the expression of related transporters. In addition, the gut microbiota has been shown to be involved in regulating intestinal mucosal integrity (Wang et al., 2019; Paone and Cani, 2020), angiogenesis (Franks, 2013; Andriessen et al., 2016), both of which are associated with nutrient absorption. Furthermore, gut microbes can utilize substances in the Met cycle (Carbonero et al., 2012; Wang et al., 2019; Das et al., 2021), or metabolize to produce Met-related downstream substances (Rosario et al., 2021), or interact with substances in Met cycle (Roth and Mohamadzadeh, 2021). Whether these functions of gut microbes are related to the regulation of host Met metabolism in this paper requires further experimental verification.

According to the experimental results, we found that gut microbiota could regulate host Met metabolism, but the increase in

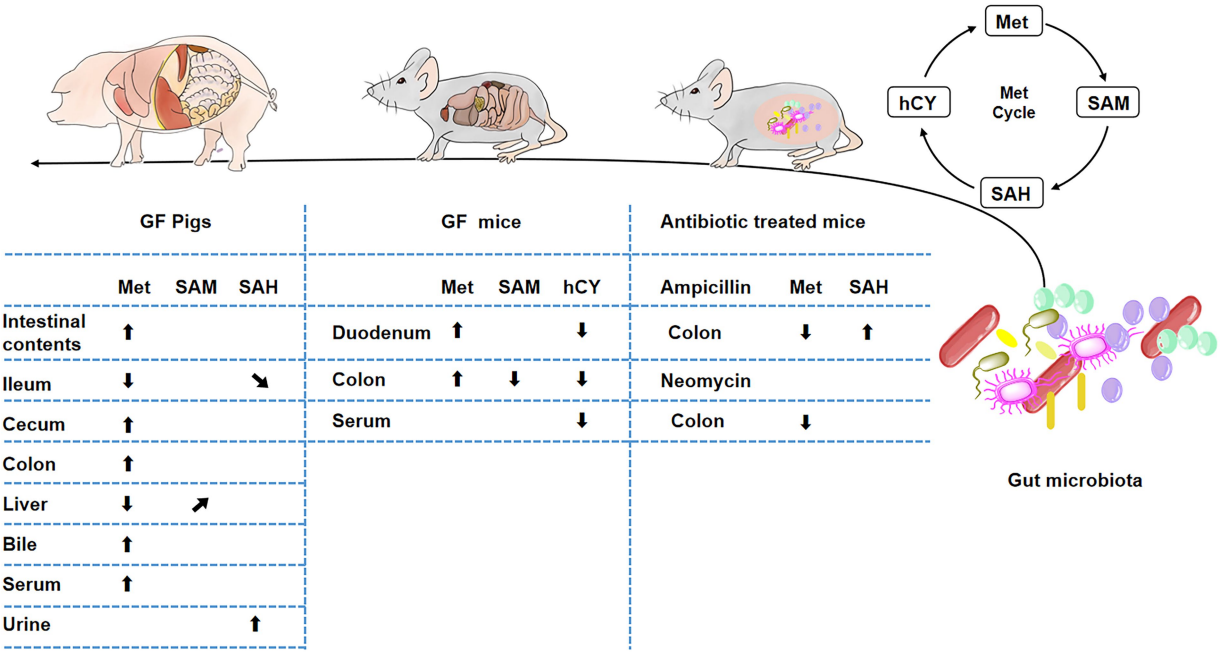


FIGURE 8
Influence of gut microbiota on Met metabolism. *("⬆" arrows represent increases, "⬇" arrows represent decreases, "↗" arrows represent increasing trend, "↘" arrows represent decreasing trend).

Met caused by lack of gut microbes was not detected in the jejunum. The possible reasons are: (1) Jejunal microbial metabolism: Jejunal microbiota is mainly involved in the degradation of lipids and sugars (Lema et al., 2020), so amino acid changes are not obvious; (2) Jejunal microbial types: the abundance and diversity of microbiota in the jejunum are very low (about $10^4 \sim 10^7$ CFU/ml), and are mainly facultative anaerobic bacteria, such as *Streptococcus* and *Lactococcus*, which are different from the large intestine (Martinez-Guryn et al., 2019; Seekatz et al., 2019); (3) Features of the jejunum: in order to facilitate the nutrient absorption, the jejunum is covered with a thin layer of loose mucus, and the intervening Paneth cells secrete antimicrobial peptides (AMPs; Guo et al., 2017; Lema et al., 2020). And the transit time is faster in the small intestine compared to the large intestine (Martinez-Guryn et al., 2019), so the environment of the jejunum is not conducive to the long-term colonization of most microorganisms.

In addition, we examined the Met cycle-metabolites in GF mice, further confirmed that gut microbiota depletion caused the increase of Met, which promoted its downstream metabolic responses to a decrease in hCY and SAM (Figure 8). A variety of gram-positive or negative bacteria are involved in Met metabolism (Carbonero et al., 2012), thus, we treated mice with five different antibiotics, including the beta-lactam antibiotic ampicillin, polymyxin antibiotic colistin, nitroimidazole derivative metronidazole, aminoglycoside antibiotic neomycin, and glycopeptide antibiotic vancomycin. However, we could not achieve a Met increase similar to GF-treated effects by treating mice with antibiotics to mimic microbe deficiency. In contrast, we found a decrease in intestinal Met in the ampicillin and neomycin-treated groups (Figure 8).

Ampicillin can prevent the synthesis of bacterial cell walls with the bacteriostatic and bactericidal effects, and its effect on Gram-positive bacteria is similar to that of penicillin. Gram-negative bacteria such as *Escherichia coli* are sensitive to ampicillin (Jõers et al., 2010), while *Pneumococcus pneumoniae* and *Pseudomonas aeruginosa* are not (Li et al., 2021; Brazel et al., 2022). Neomycin has a good bactericidal effect on Gram-negative bacteria, Gram-positive bacteria, and *Mycobacterium tuberculosis*, of which *Escherichia coli* is the most sensitive (Kadison et al., 1951; Bera et al., 2008). Combined with the experimental results, we could only conclude that the lack of certain ampicillin- or neomycin-susceptible microbes resulted in lower intestinal Met levels. Moreover, studies have shown that there are objective function differences between GF animals and SPF animals such as metabolism and immunity (Uzbay, 2019), and the elimination of a single type of microbe may cause the increase in others, thereby probably leading to more utilization of Met. Determining the key microbes in host Met metabolic remodeling requires specific inhibition of possible microbial species and detection of Met metabolites after recolonization in GF animals. In addition to the changes of Met metabolism, studies have shown that growth performance, nutrient digestibility, and skeletal muscle growth and development are affected in GF piglets (Qi et al., 2021; Zhou et al., 2021). Therefore, it may be that the effect of gut microbiota on Met metabolism in GF animals was caused by the changes of global growth characteristics to drive the regulation of nutrient metabolism. In contrast to antibiotic-treated mice, GF animals was sterile in terms of other organs. In view of recent findings,

TABLE 1 mRNA Expression of Met transporters in different intestinal tracts of pigs.

| Items | Duodenum | Proximal Jejunum | Middle jejunum | Ileum | Cecum | Proximal colon | Distal colon | References |
|---|---|---|---|---|---|---|---|---|
| *$ATB^{0+}$* | H | L | L | L | H | H | H | Romanet et al. (2021) |
| *ASCT2* | M | M | M | M | M | M | H | Zhang et al. (2014), Romanet et al. (2021) |
| *$B^0AT1$* | M | M | H | H | VL | VL | VL | Yi et al. (2019), Hu et al. (2021), Romanet et al. (2021) |
| *$rBAT/b^{0,+}AT$* | H | H | H | H | L | VL | L | Yi et al. (2019), Romanet et al. (2021) |
| *$y^+LAT1$* | H | H | H | M | L | L | L | Li et al. (2016), Romanet et al. (2021) |
| *LAT4* | H | H | H | H | L | L | L | Maric et al. (2021), Romanet et al. (2021) |
| *LAT2* | M | M | H | M | L | L | M | Romanet et al. (2021) |
| *IMINO* | M | M | M | H | M | M | H | Romanet et al. (2021) |
| *SNAT2* | M | M | M | M | M | H | M | Romanet et al. (2021) |

We artificially classify the expression of these Met transporters as: < 0.1: very low expression (VL), 0.1 ~ 0.5: low expression (L), 0.5 ~ 1: moderate expression (M), >1: high expression (H). Refers to the data in this article (Romanet et al., 2021).

the diversity of lung microbiota has a certain correlation with the risk of bronchopulmonary dysplasia (BPD) in neonates, and the lung microbiota metagenome of BPD probably changed the host metabolome (Lohmann et al., 2014; Lal et al., 2018). Therefore, it is also possible that microbiota in lung and other organs are involved in metabolic regulation in host, resulting the change trend of intestinal Met in antibiotic-treated mice is opposite to that in GF mice in this study. In order to study the influence of other organ microorganisms on Met metabolism, it may be a feasible experimental idea to redetect the changes of Met metabolism by supplementing intestinal microbiota of GF animals with whole intestinal microbiota transplantation (WIMT; Li et al., 2020).

In conclusion, the gut microbiota was involved in host Met remodeling, and Met was increased in complete GF animals (GF pigs and GF mice). However, when the microbes were cleared with antibiotics, ampicillin and neomycin treatment instead resulted in a reduction in intestinal Met. Since Met metabolism is closely related to various inflammatory diseases (Borren et al., 2021), ALD, liver tumors (Kharbanda, 2013; Xu et al., 2020), CVD, CKD (Yang et al., 2016), and other diseases, precise regulation of Met metabolism would become an effective means to treat these diseases. Therefore, our study provided new insights into the prevention and/or treatment of the associated diseases by modulating gut microbes in animals and humans.

## Data availability statement

The data presented in the study are deposited in the Metabolights repository, and the accession number is MTBLS6154.

## Ethics statement

The animal study was reviewed and approved by the Laboratory Animal Ethical Commission of the South China Agricultural University [permit number SYXK (Guangdong) 2019–0136].

## Author contributions

WT and SL designed the research and analyzed data. DY, JS, and LG assisted in the establishment of GF pigs and GF mice. XW, ZH,

and BL participated in antibiotic-animal experiments. XW and BL prepared the figures and draft the manuscript. XW, ZH, WT, and SL revised and approved the final manuscript. All authors contributed to the article and approved the submitted version.

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## Conflict of interest

WT is employed by Sichuan Animtech Feed 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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## References

Andremont, A., Cervesi, J., Bandinelli, P. A., Vitry, F., and de Gunzburg, J. (2021). Spare and repair the gut microbiota from antibiotic-induced Dysbiosis: state-of-the-art. *Drug Discov. Today* 26, 2159–2163. doi: 10.1016/j.drudis.2021.02.022

Andriessen, E. M., Wilson, A. M., Mawambo, G., Dejda, A., Miloudi, K., Sennlaub, F., et al. (2016). Gut microbiota influences pathological angiogenesis in obesity-driven Choroidal neovascularization. *EMBO Mol. Med.* 8, 1366–1379. doi: 10.15252/emmm.201606531

Bauchart-Thevret, C., Stoll, B., and Burrin, D. G. (2009). Intestinal metabolism of sulfur amino acids. *Nutr. Res. Rev.* 22, 175–187. doi: 10.1017/s0954422409990138

Bera, S., Zhanel, G. G., and Schweizer, F. (2008). Design, synthesis, and antibacterial activities of neomycin-lipid conjugates: Polycationic lipids with potent gram-positive activity. *J. Med. Chem.* 51, 6160–6164. doi: 10.1021/jm800345u

Borren, N. Z., Plichta, D., Joshi, A. D., Bonilla, G., Peng, V., Colizzo, F. P., et al. (2021). Alterations in fecal microbiomes and serum Metabolomes of fatigued patients with quiescent inflammatory bowel diseases. *Clinical gastroenterology and hepatology: the official clinical practice journal of the American Gastroenterological Association* 19, 519–527. doi: 10.1016/j.cgh.2020.03.013

Brazel, E. B., Tan, A., Neville, S. L., Iverson, A. R., Udagedara, S. R., Cunningham, B. A., et al. (2022). Dysregulation of streptococcus Pneumoniae zinc homeostasis breaks ampicillin resistance in a pneumonia infection model. *Cell Rep.* 38:110202. doi: 10.1016/j.celrep.2021.110202

Cani, P. D. (2018). Human gut microbiome: hopes. *Threats and Promises. Gut* 67, 1716–1725. doi: 10.1136/gutjnl-2018-316723

Carbonero, F., Benefiel, A. C., Alizadeh-Ghamsari, A. H., and Gaskins, H. R. (2012). Microbial pathways in colonic sulfur metabolism and links with health and disease. *Front. Physiol.* 3:448. doi: 10.3389/fphys.2012.00448

Clare, C. E., Brassington, A. H., Kwong, W. Y., and Sinclair, K. D. (2019). One-carbon metabolism: linking nutritional biochemistry to epigenetic programming of Long-term development. *Annual rev. animal biosciences* 7, 263–287. doi: 10.1146/annurev-animal-020518-115206

Cryan, J. F., O'Riordan, K. J., Cowan, C. S. M., Sandhu, K. V., Bastiaanssen, T. F. S., Boehme, M., et al. (2019). The microbiota-gut-brain Axis. *Physiol. Rev.* 99, 1877–2013. doi: 10.1152/physrev.00018.2018

Dang, A. T., and Marsland, B. J. (2019). Microbes, metabolites, and the gut-lung Axis. *Mucosal Immunol.* 12, 843–850. doi: 10.1038/s41385-019-0160-6

Das, M., Dewan, A., Shee, S., and Singh, A. (2021). The multifaceted bacterial cysteine Desulfurases: from metabolism to pathogenesis. *Antioxidants (Basel, Switzerland)* 10:997. doi: 10.3390/antiox10070997

Franks, I. (2013). Microbiota: gut microbes might promote intestinal angiogenesis. *Nat. Rev. Gastroenterol. Hepatol.* 10:3. doi: 10.1038/nrgastro.2012.227

Garibotto, G., Valli, A., Anderstam, B., Eriksson, M., Suliman, M. E., Balbi, M., et al. (2009). The kidney is the major site of S-Adenosylhomocysteine disposal in humans. *Kidney Int.* 76, 293–296. doi: 10.1038/ki.2009.117

Gondret, F., Le Floc'h, N., Batonon-Alavo, D. I., Perruchot, M. H., Mercier, Y., and Lebret, B. (2021). Flash dietary methionine supply over growth requirements in pigs: multi-facetted effects on skeletal muscle metabolism. *Animal: an international journal of animal bioscience* 15:100268. doi: 10.1016/j.animal.2021.100268

Guo, X., Li, J., Tang, R., Zhang, G., Zeng, H., Wood, R. J., et al. (2017). High fat diet alters gut microbiota and the expression of Paneth cell-antimicrobial peptides preceding changes of circulating inflammatory cytokines. *Mediat. Inflamm.* 2017, 9474896–9474899. doi: 10.1155/2017/9474896

Haug, K., Cochrane, K., Nainala, V. C., Williams, M., Chang, J., Jayaseelan, K. V., et al. (2020). Metabolights: a resource evolving in response to the needs of its scientific community. *Nucleic Acids Res.* 48, D440–D444. doi: 10.1093/nar/gkz1019

Hertel, J., Harms, A. C., Heinken, A., Baldini, F., Thinnes, C. C., Glaab, E., et al. (2019). Integrated analyses of microbiome and longitudinal Metabolome data reveal microbial-host interactions on sulfur metabolism in Parkinson's disease. *Cell Rep.* 29, 1767–1777. doi: 10.1016/j.celrep.2019.10.035

Hu, P., Zhao, F., Wang, J., and Zhu, W. (2021). Metabolomic profiling reveals the effects of early-life Lactoferrin intervention on protein synthesis, energy production and Antioxidative capacity in the liver of suckling piglets. *Food Funct.* 12, 3405–3419. doi: 10.1039/d0fo01747g

Itoh, M., Mukae, Y., Kitsuka, T., Arai, K., Nakamura, A., Uchihashi, K., et al. (2019). Development of an Immunodeficient pig model allowing Long-term accommodation of artificial human vascular tubes. *Nat. Commun.* 10:2244. doi: 10.1038/s41467-019-10107-1

Jakubowski, H. (2019). Homocysteine modification in protein structure/function and human disease. *Physiol. Rev.* 99, 555–604. doi: 10.1152/physrev.00003.2018

Jõers, A., Kaldalu, N., and Tenson, T. (2010). The frequency of Persisters in Escherichia Coli reflects the kinetics of awakening from dormancy. *J. Bacteriol.* 192, 3379–3384. doi: 10.1128/jb.00056-10

Kadison, E. R., Volini, I. F., Hoffman, S. J., and Felsenfeld, O. (1951). Neomycin therapy; its use in virus pneumonia, tuberculosis and diseases caused by gram-negative bacteria. *J. Am. Med. Assoc.* 145, 1307–1312. doi: 10.1001/jama.1951.02920350001001

Kharbanda, K. K. (2013). Methionine metabolic pathway in alcoholic liver injury. *Curr. Opin. Clin. Nutr. Metab. Care* 16, 89–95. doi: 10.1097/MCO.0b013e32835a892a

Lal, C. V., Kandasamy, J., Dolma, K., Ramani, M., Kumar, R., Wilson, L., et al. (2018). Early airway microbial metagenomic and Metabolomic signatures are associated with development of severe Bronchopulmonary dysplasia. *Am. J. Physiol. Lung Cell. Mol. Physiol.* 315, L810–L815. doi: 10.1152/ajplung.00085.2018

Lam, A. B., Kervin, K., and Tanis, J. E. (2021). Vitamin B (12) impacts amyloid Beta-induced Proteotoxicity by regulating the methionine/S-Adenosylmethionine cycle. *Cell Rep.* 36:109753. doi: 10.1016/j.celrep.2021.109753

Laue, H., Denger, K., and Cook, A. M. (1997). Taurine reduction in anaerobic respiration of Bilophila Wadsworthia Rzatau. *Appl. Environ. Microbiol.* 63, 2016–2021. doi: 10.1128/aem.63.5.2016-2021.1997

Lema, I., Araújo, J. R., Rolhion, N., and Demignot, S. (2020). Jejunum: the understudied meeting place of dietary lipids and the microbiota. *Biochimie* 178, 124–136. doi: 10.1016/j.biochi.2020.09.007

Li, Y., Xia, L., Chen, J., Lian, Y., Dandekar, A. A., Xu, F., et al. (2021). Resistance elicited by sub-lethal concentrations of ampicillin is partially mediated by quorum sensing in pseudomonas Aeruginosa. *Environ. Int.* 156:106619. doi: 10.1016/j.envint.2021.106619

Li, X. G., Xu, G. F., Zhai, Z. Y., Gao, C. Q., Yan, H. C., Xi, Q. Y., et al. (2016). Cdx 2 increases Slc 7a7 expression and proliferation of pig intestinal epithelial cells. *Oncotarget* 7, 30597–30609. doi: 10.18632/oncotarget.8894

Li, N., Zuo, B., Huang, S., Zeng, B., Han, D., Li, T., et al. (2020). Spatial heterogeneity of bacterial colonization across different gut segments following inter-species microbiota transplantation. *Microbiome* 8:161. doi: 10.1186/s40168-020-00917-7

Liu, J., Huang, J., Xin, P., Liu, G., and Wu, J. (2021). Biomedical applications of methionine-based systems. *Biomater. Sci.* 9, 1961–1973. doi: 10.1039/d0bm02180f

Liu, B., Yu, D., Sun, J., Wu, X., Xin, Z., Deng, B., et al. (2022). Characterizing the influence of gut microbiota on host tryptophan metabolism with germ-free pigs. *Animal Nutrition* 11, 190–200. doi: 10.1016/j.aninu.2022.07.005

Lohmann, P., Luna, R. A., Hollister, E. B., Devaraj, S., Mistretta, T. A., Welty, S. E., et al. (2014). The airway microbiome of intubated premature infants: characteristics and changes that predict the development of Bronchopulmonary dysplasia. *Pediatr. Res.* 76, 294–301. doi: 10.1038/pr.2014.85

Lu, S. C., and Mato, J. M. (2012). S-Adenosylmethionine in liver health, injury, and cancer. *Physiol. Rev.* 92, 1515–1542. doi: 10.1152/physrev.00047.2011

Maric, S., Flüchter, P., Guglielmetti, L. C., Staerkle, R. F., Sasse, T., Restin, T., et al. (2021). Plasma Citrulline correlates with Basolateral amino acid transporter Lat 4 expression in human small intestine. *Clinical nutrition (Edinburgh, Scotland)* 40:2244. doi: 10.1016/j.clnu.2020.10.003

Martinez-Guryn, K., Leone, V., and Chang, E. B. (2019). Regional diversity of the gastrointestinal microbiome. *Cell Host Microbe* 26, 314–324. doi: 10.1016/j.chom.2019.08.011

Mentch, S. J., Mehrmohamadi, M., Huang, L., Liu, X., Gupta, D., Mattocks, D., et al. (2015). Histone methylation dynamics and gene regulation occur through the sensing of one-carbon metabolism. *Cell Metab.* 22, 861–873. doi: 10.1016/j.cmet.2015.08.024

Miller, E. R., and Ullrey, D. E. (1987). The pig as a model for human nutrition. *Annu. Rev. Nutr.* 7, 361–382. doi: 10.1146/annurev.nu.07.070187.002045

Mota-Martorell, N., Jové, M., Berdún, R., and Pamplona, R. (2021). Plasma methionine metabolic profile is associated with longevity in mammals. *Communications biology* 4:725. doi: 10.1038/s42003-021-02254-3

Osbelt, L., Wende, M., Almási, É., Derksen, E., Muthukumarasamy, U., Lesker, T. R., et al. (2021). Klebsiella Oxytoca causes colonization resistance against multidrug-Resistant K. pneumoniae in the gut via cooperative carbohydrate competition. *Cell Host Microbe* 29, 1663–1679. doi: 10.1016/j.chom.2021.09.003

Paone, P., and Cani, P. D. (2020). Mucus barrier, Mucins and gut microbiota: the expected slimy partners? *Gut* 69, 2232–2243. doi: 10.1136/gutjnl-2020-322260

Pulendran, B., and Davis, M. M. (2020). The science and medicine of human immunology. *Science* 369:eaay4014. doi: 10.1126/science.aay4014

Qi, R., Sun, J., Qiu, X., Zhang, Y., Wang, J., Wang, Q., et al. (2021). The intestinal microbiota contributes to the growth and physiological state of muscle tissue in piglets. *Sci. Rep.* 11:11237. doi: 10.1038/s41598-021-90881-5

Romanet, S., Aschenbach, J. R., Pieper, R., Zentek, J., Htoo, J. K., Whelan, R. A., et al. (2021). Expression of proposed methionine transporters along the gastrointestinal tract of pigs and their regulation by dietary methionine sources. *Genes Nutr.* 16:14. doi: 10.1186/s12263-021-00694-4

Rosario, D., Bidkhori, G., Lee, S., Bedarf, J., Hildebrand, F., Le Chatelier, E., et al. (2021). Systematic analysis of gut microbiome reveals the role of bacterial Folate and Homocysteine metabolism in Parkinson's disease. *Cell Rep.* 34:108807. doi: 10.1016/j.celrep.2021.108807

Roth, W., and Mohamadzadeh, M. (2021). Vitamin B12 and gut-brain homeostasis in the pathophysiology of ischemic stroke. *EBioMedicine* 73:103676. doi: 10.1016/j.ebiom.2021.103676

Seekatz, A. M., Schnizlein, M. K., Koenigsknecht, M. J., Baker, J. R., Hasler, W. L., Bleske, B. E., et al. (2019). Spatial and temporal analysis of the stomach and small-intestinal microbiota in fasted healthy humans. *mSphere* 4:e00126-19. doi: 10.1128/mSphere.00126-19

Selhub, J. (1999). Homocysteine metabolism. *Annu. Rev. Nutr.* 19, 217–246. doi: 10.1146/annurev.nutr.19.1.217

Sun, M. F., and Shen, Y. Q. (2018). Dysbiosis of gut microbiota and microbial metabolites in Parkinson's disease. *Ageing Res. Rev.* 45, 53–61. doi: 10.1016/j.arr.2018.04.004

Sun, J., Zhong, H., Du, L., Li, X., Ding, Y., Cao, H., et al. (2018). Gene expression profiles of germ-free and conventional piglets from the same litter. *Sci. Rep.* 8:10745. doi: 10.1038/s41598-018-29093-3

To VMasagounder, K., and Loewen, M. E. (2021). Critical transporters of methionine and methionine hydroxyl analogue supplements across the intestine: what we know so far and what can be learned to advance animal nutrition. *Comp. Biochem. Physiol. A Mol. Integr. Physiol.* 255:110908. doi: 10.1016/j.cbpa.2021.110908

Tripathi, A., Debelius, J., Brenner, D. A., Karin, M., Loomba, R., Schnabl, B., et al. (2018). The gut-liver Axis and the intersection with the microbiome. *Nat. Rev. Gastroenterol. Hepatol.* 15, 397–411. doi: 10.1038/s41575-018-0011-z

Uzbay, T. (2019). Germ-free animal experiments in the gut microbiota studies. *Curr. Opin. Pharmacol.* 49, 6–10. doi: 10.1016/j.coph.2019.03.016

Wang, J., Guo, X., Li, H., Qi, H., Qian, J., Yan, S., et al. (2019). Hydrogen Sulfide from Cysteine Desulfurase, Not 3-Mercaptopyruvate Sulfurtransferase, Contributes to Sustaining Cell Growth and Bioenergetics in E. Coli under Anaerobic Conditions. *Front Microbiol* 10:2357. doi: 10.3389/fmicb.2019.02357

Wang, Z., Yip, L. Y., Lee, J. H. J., Wu, Z., Chew, H. Y., Chong, P. K. W., et al. (2019). Methionine is a metabolic dependency of tumor-initiating cells. *Nat. Med.* 25, 825–837. doi: 10.1038/s41591-019-0423-5

Wang, L., Zhu, L., and Qin, S. (2019). Gut microbiota modulation on intestinal mucosal adaptive immunity. *J Immunol Res* 2019, 4735040–4735010. doi: 10.1155/2019/4735040

Xiao, Y., Su, X., Huang, W., Zhang, J., Peng, C., Huang, H., et al. (2015). Role of S-Adenosylhomocysteine in cardiovascular disease and its potential epigenetic mechanism. *Int. J. Biochem. Cell Biol.* 67, 158–166. doi: 10.1016/j.biocel.2015.06.015

Xu, Q., Li, Y., Gao, X., Kang, K., Williams, J. G., Tong, L., et al. (2020). Hnf4α regulates sulfur amino acid metabolism and confers sensitivity to methionine restriction in liver cancer. *Nat. Commun.* 11:3978. doi: 10.1038/s41467-020-17818-w

Yang, J., Fang, P., Yu, D., Zhang, L., Zhang, D., Jiang, X., et al. (2016). Chronic kidney disease induces inflammatory Cd40+ monocyte differentiation via Homocysteine elevation and DNA Hypomethylation. *Circ. Res.* 119, 1226–1241. doi: 10.1161/circresaha.116.308750

Yang, Z., Hasan, M. S., Humphrey, R. M., Htoo, J. K., and Liao, S. F. (2021). Changes in growth performance, plasma metabolite concentrations, and myogenic gene expression in growing pigs fed a methionine-restricted diet. *Frontiers in bioscience (Landmark edition)* 26, 413–422. doi: 10.52586/4956

Yi, H., Yang, G., Xiong, Y., Wu, Q., Xiao, H., Wen, X., et al. (2019). Integrated Metabolomic and proteomics profiling reveals the promotion of lactobacillus Reuteri Lr1 on amino acid metabolism in the gut-liver Axis of weaned pigs. *Food Funct.* 10, 7387–7396. doi: 10.1039/c9fo01781j

Yin, J., Han, H., Li, Y., Liu, Z., Zhao, Y., Fang, R., et al. (2017). Lysine restriction affects feed intake and amino acid metabolism via gut microbiome in piglets. *Cell. Physiol. Biochem.* 44, 1749–1761. doi: 10.1159/000485782

Yin, J., Ren, W., Chen, S., Li, Y., Han, H., Gao, J., et al. (2018). Metabolic regulation of methionine restriction in diabetes. *Mol. Nutr. Food Res.* 62:e1700951. doi: 10.1002/mnfr.201700951

Yu, W., Wang, Z., Zhang, K., Chi, Z., Xu, T., Jiang, D., et al. (2019). One-carbon metabolism supports S-Adenosylmethionine and histone methylation to drive inflammatory macrophages. *Mol. Cell* 75:1147. doi: 10.1016/j.molcel.2019.06.039

Yu, Y., Zhang, B., Ji, P., Zuo, Z., Huang, Y., Wang, N., et al. (2022). Changes to gut amino acid transporters and microbiome associated with increased E/I ratio in Chd 8(+/−) mouse model of Asd-like behavior. *Nat. Commun.* 13:1151. doi: 10.1038/s41467-022-28746-2

Zeitz, J. O., Kaltenböck, S., Most, E., and Eder, K. (2019). Effects of L-methionine on performance, gut morphology and antioxidant status in gut and liver of piglets in relation to dl-methionine. *J. Anim. Physiol. Anim. Nutr.* 103, 242–250. doi: 10.1111/jpn.13000

Zhang, S., Ren, M., Zeng, X., He, P., Ma, X., and Qiao, S. (2014). Leucine stimulates Asct 2 amino acid transporter expression in porcine Jejunal epithelial cell line (Ipec-J2) through Pi3k/Akt/Mtor and Erk signaling pathways. *Amino Acids* 46, 2633–2642. doi: 10.1007/s00726-014-1809-9

Zhou, H., Sun, J., Yu, B., Liu, Z., Chen, H., He, J., et al. (2021). Gut microbiota absence and transplantation affect growth and intestinal functions: an investigation in a germ-free pig model. *Animal nutrition (Zhongguo xu mu shou yi xue hui)* 7, 295–304. doi: 10.1016/j.aninu.2020.11.012

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# Mixed oats and alfalfa improved the antioxidant activity of mutton and the performance of goats by affecting intestinal microbiota

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Oat hay and alfalfa hay are important roughage resources in livestock production. However, the effect of the mixture of oat hay and alfalfa hay on the meat quality of Albas goats is unclear. This study aimed to investigate the effects of feeding different proportions of oat hay and alfalfa hay on the growth performance and meat quality of Albas goats. Therefore, 32 goats were fed for 70days and randomly divided into four treatment groups on the principle of similar weight: whole oat group (OAT), oat alfalfa ratio 3:7 group (OA73), oat alfalfa ratio 7:3 group (OA37) and whole alfalfa group (Alfalfa), with eight goats in each group. Daily feed intake records, feces, feed samples, and rumen fluid collection were made throughout the trial. The goats were weighed on the last day of the trial, and four goats per group were randomly selected for slaughter. Cecum contents, meat samples, and hot carcass weight were collected, and data were recorded. Furthermore, the relationship between the rumen and cecal microbes on performance and meat quality was clarified by analyzing the rumen and hindgut microbiomes. The results showed that feeding alfalfa could significantly reduce the daily weight gain of fattening goats. Compared with the highest group (OA37), the daily weight gain decreased by 19.21%. Although there was no significant change in feed intake in the four treatments, the feed conversion rate of the alfalfa group significantly decreased by 30.24–36.47% compared to the other groups. However, with the increased alfalfa content, MDA decreased significantly, T-AOC was up-regulated, and the antioxidant activity of the fattened goat meat fed with the high alfalfa group was significantly higher than that of the low alfalfa group. Notably, the abundance of *Bacteroidales_unclassified* and *Clostridium* were strongly correlated with T-AOC and MDA. Therefore, increasing the proportion of alfalfa in the diet can affect the antioxidant activity of goat meat by improving the gut microbiota, while an oat-hay mixture can improve the growth performance of livestock.

KEYWORDS

**Albas goat, alfalfa hay, oat hay, fattening performance, slaughter performance, antioxidant capacity**

# 1. Introduction

Between 2020 and 2050, the global average protein demand for red meat, poultry, milk, and eggs will increase by 14%, and the total will increase by 38%, with lamb being one of the essential components of red meat consumption in the world. The most important factors affecting the production efficiency of mutton sheep include the host genetics (breed), gender, growth stage or age, dietary factors, environmental factors, feeding methods, etc. Ruminants such as cattle, sheep, and goats use fiber better than animals with a single stomach. Dietary fiber can stimulate chewing and rumination, promote saliva secretion, enhance rumen fermentation function and improve the quality of animal products. However, too much fiber in the diet will affect the diet's palatability and reduce the dry matter intake of sheep (Adesogan et al., 2019). Therefore, finding a strategy for choosing high-quality roughage is a crucial way to improve the production efficiency of sheep meat to meet the needs of red meat.

Feeding high-quality roughage is beneficial to maintain rumen homeostasis and reduce the occurrence of metabolic diseases such as rumen acidosis and abomasum displacement (Kenny et al., 2018). At present, high-quality forages such as alfalfa, oat grass, and ryegrass are the primary sources of high-quality protein in animal husbandry production and can provide animals with a variety of vitamins, minerals, and other trace elements. As a high-quality forage with strong stress resistance, alfalfa has many characteristics, such as promoting digestion and absorption of ruminants and maintaining body health, so it is one of the most popular roughages in the world. At the same time, because the content of neutral detergent fiber and lignin in oat grass is lower than that in roughage such as alfalfa, it has good palatability and helps to improve the digestion, absorption, and feed utilization efficiency of ruminants (Radović et al., 2009). It has gradually become an important source of roughage in animal husbandry production in China (Abeysekara, 2003). Combining oats and alfalfa may provide energy and adequate physical fiber for fattening sheep, ensure growth efficiency, and protect rumen health.

Dietary therapy may be compared with concentrate fodder, and the forage-based system usually promotes lean meat, widely raising the accumulation of natural antioxidants. This results in better oxidative stability of pasture-raised beef than concentrate-fed beef (Van Elswyk and McNeill, 2014; McAllister et al., 2020). Several reports have confirmed that grazing beef cattle have the advantage of producing lean meat with more omega-3 fatty acids, which directly affects the balance of antioxidant and pro-oxidative components in muscles (Ponnampalam et al., 2012; Inserra et al., 2014). In addition, the increase in antioxidant properties of meat can effectively inhibit lipid oxidation of shelf-stable meat and prolong its shelf life (Hayes et al., 2011). Meanwhile, supplementing the diet with natural antioxidants can improve the meat quality and its organoleptic properties by depositing them in the muscle with other metabolites of their own, thus performing a probiotic function (Morán et al., 2012). This is essential to improve the meat quality and antioxidant properties of lamb by feeding natural substances or high-quality roughage instead of synthetic agents after slaughter to prolong its shelf life, organoleptic quality, and richness in lipids (Andrés et al., 2014). This is to avoid supplementing with exogenous substances during meat production and processing and to help them meet the market requirements for high-quality meat. Thus, livestock fed roughage rich in dietary forage combinations have the potential to improve livestock meat quality.

The synergistic supply of alfalfa hay, starter culture, and emulsion substitute significantly improved the rumen fermentation capacity of cattle and lambs and changed the rumen microbiota, which was the main reason for the improved growth performance of cattle and lambs (Wu et al., 2021). In addition to rumen fermentation, alfalfa hay can also affect starch and fiber in the rumen bypass, affecting hindgut microbiota and fermentation (Wu et al., 2019). As the main hindgut, the fermentation in the cecum is also an important factor affecting growth performance. The changes in cecal microorganisms lead to the improvement of meat quality. However, the mechanism of how the changes in roughage affect meat quality by altering the gut microbiota is still being determined.

This study hypothesized that different proportions of alfalfa and oat feed would have different performances on fattening goats, and long-term feeding with different mixed proportions of alfalfa and oat would affect rumen fermentation, thereby affecting the changes in cecal microbiota and meat quality of goats. This study aimed to investigate the effects of different alfalfa and oat mixing ratios on fattening goats' performance and meat quality. It also clarifies the relationship between rumen and cecal microbes on performance and meat quality.

# 2. Materials and methods

The experimental protocol (Protocol number: NEAU-[2011]-9) was approved by the Northeast Agricultural University Animal Care and Use Committee (Harbin, China). The ranch meets the welfare standards of the China Animal Protection and Use Commission.

## 2.1. Experimental design

This experiment was conducted at the Acheng Experimental Base of Northeast Agricultural University from February 2022 to April 2022. Thirty-two healthy and disease-free Arbas male goats aged 6 months (22.63 ± 0.20 kg) were randomly divided into 4 groups with 8 repeats in each group according to the principle of similar body weight. The experimental groups were whole oat (OAT), oat: alfalfa = 7:3 (OA73), oat: alfalfa = 3:7 (OA37), and whole alfalfa (Alfalfa) treatment (All forage were in air-dried samples). All the

experimental goats were raised in a single column and drank freely. In this experiment, concentrate and roughage were fed separately at 06:00 and 18:00 daily, and the ratio of concentrate to forage was 65:35. The experiment lasted 70 days, where the prefeeding period was 14 days, and the experimental period was 56 days.

## 2.2. Composition and nutrition level of experimental diet

The diet was composed of concentrate having corn, corn germ meal, sprayed corn husk, DDGS, expanded soybean, and different proportions of alfalfa and oat grass mixed roughage. The dietary composition and nutrition levels are shown in Table 1.

## 2.3. Collection of samples and recording of data

After a 14-day adaptation period, a 56-day feeding experiment began. The feed's raw materials and leftovers from each group of experimental goats were collected and mixed evenly every 7 days. The collected feed samples were baked at 55°C to constant weight. The dried samples were crushed with a 1 mm sieve and stored at −20°C. The collected feed and leftover samples were used to determine the chemical composition. The digestion test was carried out by total fecal collection method on the 51st, 52nd, and 53rd days of the experimental period. About 500 g (5%) of fresh intestinal samples were collected at 7 o'clock every morning from each goat. After mixing the samples for 3 consecutive days, the samples were divided into two parts on average. One sample was mixed with 10% hydrochloric acid (10 mL) for every 100 g and evenly treated with nitrogen fixation. The other two intestinal samples were baked in an oven at 55°C to a constant weight, and the dried samples were crushed with a 1 mm sieve, marked, sealed, and stored at −20°C, and used to determine the digestibility of nutrients. On the 56th day of the experimental period, 2 h post morning feeding, 50 mL of rumen fluid samples were collected using an oral tube. In order to reduce saliva dilution of rumen fluid, the first sample was discarded before each sampling, and the filtrate was collected after four layers of gauze filtration. The contents of each goat cecum were collected after slaughter using pre-sterilized 5 mL lyophilized tubes and stored in liquid nitrogen, then transferred to the laboratory and stored in a −80°C refrigerator for gut microbiological analysis.

## 2.4. Index determination

### 2.4.1. Determination of growth performance

On the 0th and 56th day of the experimental period, the experimental goats were weighed and recorded before morning feeding, and the average daily gain (ADG) of the experimental goats was calculated. Since the beginning of the formal trial, the daily feed intake and leftover feed of each experimental group were recorded in detail, and the total feed intake together with the feed efficiency were calculated according to the following formula:

$$\text{Average daily gain (g)} = \text{Initial weight of experimental goat} / \text{Test days}$$

$$\text{Total feed intake of experimental days (kg)} = \text{Total feed volume during the test period} - \text{total feed leftover during the test period}$$

$$\text{Total feed efficiency of the experimental period (\%)} = \text{total feed intake of experimental goats} / \left(\begin{array}{l}\text{final weight of the experimental goats} \\ -\text{initial weight of experimental goats}\end{array}\right)$$

TABLE 1 Composition and nutrient levels of diets (dry matter basis, %).

| Nutrient levels | Oat | OA73 | OA37 | Alfalfa | Concentrate |
|---|---|---|---|---|---|
| Dry matter | 92.07 | 92.05 | 92.03 | 92.01 | 90.96 |
| Crude protein (CP) | 11.93 | 11.97 | 12.02 | 12.06 | 16.39 |
| Ether extract (EE) | 1.28 | 1.26 | 1.23 | 1.20 | 3.82 |
| Neutral detergent fiber (NDF) | 56.47 | 56.48 | 56.50 | 56.50 | 30.94 |
| Acid detergent fiber (ADF) | 33.19 | 35.30 | 38.12 | 40.23 | 16.29 |
| Metabolizable energy (MJ/kg)[a] | 16.49 | 16.65 | 16.87 | 17.03 | 16.63 |
| Crude Ash | 9.71 | 8.57 | 7.05 | 5.92 | 6.6 |

[a] Metabolizable energy was a calculated value, while the others were measured values.

### 2.4.2. Determination of feed composition and apparent digestibility

According to the procedure of the official Association of Analytical Chemists (Aboshora, 1990), the contents of dry matter (DM), crude protein (CP), and crude fat (EE) in feed raw material, leftover and hindgut samples were determined by wet chemical analysis in the Ruminant Nutrition Laboratory of Northeast Agricultural University. The contents of neutral detergent fiber (NDF) and acid detergent fiber (ADF) were analyzed according to the method of Van Soest et al. (1991). The results of NDF and ADF were expressed based on dry matter (DM). The apparent digestibility of nutrients was determined according to the hydrochloric acid insoluble ash (AIA) in feed and hindgut samples and calculated according to the method of Thonney et al. (1979).

### 2.4.3. Determination of slaughter performance and mutton quality

On the last day of the trial period, four goats were randomly selected from each treatment group. Sixteen were transferred to the slaughter plant for the samples collected and data recorded. Each goat's live weight and carcass weight before slaughter were recorded separately and used to determine the dressing percentage. The pH meter (pH meter: HI9125; Hanna Instruments, Pa-136 dova, Italy) electrode was inserted in the incision along the direction of the muscle fiber, and the data was recorded after the reading was stabilized. In addition, samples of the longest dorsal muscle were collected from the part of the goat between the 12th and 13th ribs of the left half of the carcass without fat for determining drip loss, total antioxidant capacity (T-AOC) and malondialdehyde (MDA). T-AOC and MDA were determined using commercial kits (A015-1-3 and A003-2; Nanjing Jiancheng Institute of Bio-engineering, 155 Nanjing). The calculation formula for dressing percentage and drip loss is as follows:

Dressing percentage % = Hot carcass weight/Live weight × 100%.

24 h: Drip loss % = $(W_1\text{-}W_2)$ /$W_1$× 100%.

48 h: Drip loss % = $(W_1\text{-}W_3)$ /$W_1$× 100%.

Each goat was divided into three square-sized samples of 3 × 3 × 3 cm and weighed as $W_1$. The meat samples were suspended in sealing bags with fishing lines, stored in a refrigerator at 4°C, and weighed again after 24 h as $W_2$ and 48 h as $W_3$.

### 2.4.4. Rumen microbiome and hindgut microbiome

Rumen and hindgut samples were sequenced by 16 s rDNA in Lianchuan Biotechnology Co., Ltd. A hindgut DNA kit (D4015, Omega, Inc., United States) was used, and nonnuclear water was treated as blank. Total DNA was eluted with 50 μL elution buffer and stored at −80°C until the sequencing was performed by LC-Bio Technology Co., Ltd. Polymerase chain reaction (PCR) analysis was performed in Zhejiang, China. Design of universal primer sequences based on the V3-V4 re-162 regions of the 16S rDNA amplified fragment: 341F (5′-CCTACGGGNGGCWGCAG-3′), 163805R (5′-GACTAC HVGGGTATCTAATCC-3′). The 5′ end of the primer was marked by a special barcode and general sequencing primers. The template DNA 25 ng, PCR Premix 12.5 μL, primers 2.5 μL, and PCR grade water were adjusted to the final volume of 25 μL reaction solution before being amplified by PCR. The PCR conditions for amplification of prokaryotic 16S fragments are as follows: initial denaturation at 98°C for 30 s, denaturation at 98°C for 10 s, annealing at 54°C for 30 s, extension at 72°C for 45 s, and final extension at 72°C for 10 s. The PCR product was confirmed by 2% agarose gel electrophoresis. In the DNA extraction process, ultra-pure water was used as a negative control instead of a sample solution to rule out the possibility of false positive PCR results. PCR products were purified by AMPure XT beads (Beckman Coulter Genomics, Danvers, MA, United States) and quantified by Qubit (Invitrogen, United States). The size and quantity of the amplified sublibrary were evaluated by Agilent 2100 biological analyzer (Agilent Technologies, United States) and Illumina sequencing platform KAPA library quantitative kit (KAPA Biosciences, Woburn, MA, United States). These libraries are sequenced on the NovaSeq PE250 platform.

## 2.5. Statistical analysis

Growth parameters data were analyzed by one-way analysis of variance (ANOVA) and the Duncan range test. The data of the Alpha diversity index were expressed as mean ± standard deviation, and ANOVA analysis of the Fisher minimum significant difference (LSD) test was used. The statistical analysis was performed using the SPSS version 25.0 of Windows (SPSS Inc., Chicago, IL, United States). $p < 0.05$ is statistically significant, and $0.05 < p < 0.10$ is a significant trend.

Samples were sequenced on the Illumina NovaSeq platform according to the manufacturer's recommendations (LC-Bio Technologies). Paired-end reads were performed on the sample based on its unique barcode and truncated using the barcode and primer sequences. Peer reads were merged using FLASH. Quality filtered raw reads according to fqtrim (v0.94) under specific filter conditions to obtain high-quality clean labels. Chimeric sequences were screened using VSEARCH software (v2.3.4). The feature table and feature sequence were obtained after deduplication using DATA2. Beta diversity was normalized to the same sequencing depth by randomly removing aligned reads. The feature abundance then normalized the relative abundance of each sample according to the SILVA (release 132) classifier. Beta diversity was calculated by QIIME2 and plotted by the R package. The correlation heatmap was analyzed by plotting the Spearman correlation matrix using the R package (v3.6.3). Sequence alignment was performed using Blast, and the SILVA database was used to annotate the characteristic sequences for each representative sequence. Other graphs were generated using the R package (v3.5.2).

## 3. Results

Table 2 shows the growth performance of different roughage proportions, and variance analysis was carried out with the initial body weight as a covariate. The results showed that feeding whole alfalfa could significantly reduce the daily weight gain of fattening goats, which was the most serious, up to 19.21% compared with the OA73 group. Although there was no significant change in feed intake, the feed conversion rate of the whole alfalfa group decreased significantly by 30.24–36.47% compared with the other groups. Table 3 shows the results of nutrient metabolism in this study. In general, with the increase in alfalfa content, the digestibility of other nutrients increased significantly, except for fat digestibility. Compared with whole oats, the CP digestibility of whole alfalfa increased by 8.25%, while the DM, NDF, and ANF increased by 14.9, 23.4, and 37.2%, respectively ($p < 0.05$).

Table 4 shows the meat production traits of fattening goats fed with different proportions of roughage. The live weight was considered a covariable in the slaughtering and meat quality analysis. The results showed that the dressing percentage, pH, and drip loss of fattening goats had no significant difference ($p > 0.05$). Interestingly, T-AOC showed a trend with increased alfalfa content ($0.05 < p < 0.10$), and MDA was significantly lower than the other treatments ($p < 0.05$). The antioxidant activity of fattening goat meat fed with the high alfalfa group was significantly higher than that of the low alfalfa group.

To investigate the effect of alfalfa and oat hay diet mix on beta diversity, we used unweighted UniFrac distances to characterize bacterial communities across all rumen samples (Figure 1). PCoA map showed that the bacterial community composition of these groups was not significantly separated from each other. A total of 25 phyla and 362 genera were found in the rumen, and 22 phyla and 369 genera in the posterior intestine. At the rumen phyla level, seven phyla were referred to as detection phyla (relative abundance >1% in at least one group). The most abundant phyla were Firmicutes, followed by Bacteroidetes and Proteobacteria. The total abundance of these three phyla exceeded 90%, but there was no significant difference among all groups ($p > 0.05$). At the genus level, 26 genera were considered detectable (relative abundance >1% in at least one group). Classified mainly as *Prevotella_1*, *Muribaculaceae_unclassified*, *Ruminococcus_2*, *Succiniclasticum, VeillonellACEAE_UCG-001*, *Rikenellaceae_RC9_gut_group*, *Succinivibrio, Ruminococcaceae_NK4A214_group*, *Christensenellaceae_R-7 _group*, *Prevotella*, *Prevotellaceae_UCG-001*, *F082_unclassified, Lachnospiraceae_unclassified*, the total abundance of these 13 genera exceeded 60%.

In the results of genus level significance, this study compared the differences of the microbiota at the genus level between the alfalfa and the oat groups, and the results showed that the *Rikenellaceae_RC9_gut_group* in the rumen and posterior gut was significantly higher than that in the oat group. *Bacteroidales_unclassified* in the back intestine of the alfalfa group was significantly higher than that of the oat group, but there was no significant change in the level of other rumen microbiota genera. Pearson correlation analysis was performed to investigate further the correlation between dominant microbial genera and growth performance and meat quality (Figure 2). This study focused on the correlation between microbiota, T-AOC, and MDA of meat. In the rumen, R*uminococcaceae_UCG-14* and *Succinivbrio* are significantly positively correlated. *Olsenella* was positively correlated with the pH of meat. *Lachnospiraceae_unclassified* was positively correlated with MDA, while *Rikenellaceae_RC9_gut_group* was negatively correlated with MDA. In addition, *Olsenella* was significantly correlated with meat pH and dressing percentage, while other genera had no significant differences in growth performance, meat quality, and fatty acid profile ($p > 0.05$).

In the back intestinal flora, the results of this study showed that *Clostridium* was positively correlated with MDA. Meanwhile, T-AOC could be influenced by *Bacteroidales_unclassified*, Treponema_2, and Bacteroides, which have a negative relationship with *Eubacterium_coprostanoligenes_group*. Meat pH was significantly positively correlated with *Rikenellaceae_RC9_gut_group* and *Ruminococcaceae_unclassified* in the hindgut tract. In order to further explore the differences in growth performance and meat quality of mixed hay, the gut microbiota of the alfalfa and star wheat groups were compared. *Muribaculaceae_unclassified* and *Clostridium* of the oat group were twice as high as those of the Alfalfa. In contrast, *Campylobacter* was less than 1 time that of the alfalfa group.

TABLE 2 Effects of oat and alfalfa on the growth of fattening goats.

| | Treatments[1] | | | | SME | *P*-value |
|---|---|---|---|---|---|---|
| Item | Oat | OA73 | OA37 | Alfalfa | | |
| Initial weight, kg | 21.80 | 22.26 | 21.88 | 22.40 | 0.33 | 0.909 |
| Final weight, kg | 26.16[a] | 26.68[a] | 26.30[a] | 25.98[b] | 0.29 | <0.001 |
| Average daily gain, g | 77.90[a] | 78.80[a] | 79.02[a] | 63.84[b] | 3.51 | 0.026 |
| Daily feed intake, kg | 1.01 | 0.95 | 1.03 | 1.09 | 0.02 | 0.336 |
| Feed conversion ratio, kg/kg | 14.02[a] | 13.38[a] | 13.45[a] | 18.26[b] | 0.90 | 0.033 |

[1]Treatments: Oat, roughage composition = 100% oats; OA73, roughage composition = 70% oats & 30% alfalfas; OA37, roughage composition = 30% oats & 70% alfalfas; Alfalfa, roughage composition = 100% alfalfas. [a,b] With row, different superscripts indicate differences between treatments ($P < 0.05$).

## 4. Discussion

This study showed the effects of the different mixed ratios of oat and alfalfa on fattening goats' growth and slaughtering

TABLE 3 Effects of oat and alfalfa on apparent digestibility of fattening goats (%).

| | Treatments[1] | | | | SME | *P*-value |
|---|---|---|---|---|---|---|
| Item | Oat | OA73 | OA37 | Alfalfa | | |
| Dry matter | 77.74[a] | 76.50[a] | 84.44[ab] | 89.34[b] | 1.70 | 0.012 |
| Crude protein | 86.82[a] | 85.47[a] | 91.11[ab] | 93.98[b] | 1.11 | 0.020 |
| Ether extract | 86.73 | 84.70 | 91.56 | 92.83 | 1.29 | 0.128 |
| Neutral detergent fiber | 70.49[a] | 72.13[a] | 80.44[ab] | 86.98[b] | 2.17 | 0.010 |
| Acid detergent fiber | 64.21[a] | 67.72[ab] | 79.74[bc] | 88.08[c] | 2.82 | 0.001 |

[1]Treatments: Oat, roughage composition = 100% oats; OA73, roughage composition = 70% oats & 30% alfalfas; OA37, roughage composition = 30% oats & 70% alfalfas; Alfalfa, roughage composition = 100% alfalfas. [abc] With row, different superscripts indicate differences between treatments ($P < 0.05$).

TABLE 4 Effects of oat and alfalfa on carcass traits of fattening goats and quality of mutton.

| | Treatments[2] | | | | SME | *P*-value |
|---|---|---|---|---|---|---|
| Item[1] | Oat | OA73 | OA37 | Alfalfa | | |
| Carcass weight, kg | 14.25 | 12.37 | 13.90 | 14.07 | 0.27 | 0.106 |
| Dressing percentage, % | 52.43 | 49.22 | 52.17 | 54.81 | 0.01 | 0.672 |
| pH | 6.62 | 6.68 | 6.58 | 6.78 | 0.06 | 0.784 |
| Drip loss-24 h, % | 0.73 | 0.59 | 0.73 | 0.50 | 0.06 | 0.631 |
| Drip loss-48 h, % | 0.86 | 0.75 | 0.93 | 0.59 | 0.06 | 0.239 |
| T-AOC, U/mL | 1.33[a] | 1.89[a] | 4.08[b] | 3.25[b] | 0.14 | 0.050 |
| MDA, nmol/mL | 4.20[a] | 3.74[ab] | 3.64[ab] | 3.37[b] | 0.07 | 0.035 |

[1]Item: T-AOC, total antioxidant capacity; MDA, malondialdehyde. [2]Treatments: Oat, roughage composition = 100% oats; OA73, roughage composition = 70% oats & 30% alfalfas; OA37, roughage composition = 30% oats & 70% alfalfas; Alfalfa, roughage composition = 100% alfalfas. [ab] With row, different superscripts indicate differences between treatments ($p < 0.05$).

performance. The results showed that a high proportion of alfalfa in roughage could improve the antioxidant activity of mutton, but whole alfalfa could reduce daily gain and feed conversion rate. Like another study, the total use of alfalfa in the diet did not significantly improve the daily gain of livestock (Tang et al., 2021). In addition, this might be related to the high content of ADF in alfalfa. The high level of ADF tends to increase the ruminal activity and its fermentation retention time in the rumen, increasing its thermal power consumption. And it was associated with the production of large amounts of gas by rumen microbial fermentation, such as the increased relative abundance of *Prevotella* in this study. However, the Pordomingo study reported that alfalfa could improve the quality of beef, especially the cooking loss and sensory evaluation indexes of meat, including juiciness, tenderness, and beef flavor (Pordomingo et al., 2022). Significantly, in this study, supplementing oats based on alfalfa could increase the daily weight gain of mutton sheep.

Live weight is a crucial economic character in animal husbandry, which is the most important factor affecting the market price in China (Zhou et al., 2012). Therefore, daily gain and feed conversion rate directly determine the farmers' income. In this study, the daily gain and feed conversion rate of the whole alfalfa group were significantly lower than those of other groups, which indicated that alfalfa hay alone could not be effectively converted into weight gain, but mixing it with an oat diet could further improve the digestive function of animals. Castells's experiment found that the average daily gain of calves fed with oats was 63% higher than that of alfalfa diets (Castells et al., 2013). Interestingly, our results are similar to those of Doran (Doran et al., 2007). The digestibility of alfalfa is significantly higher than that of oats. Oats are thought to contain more non-structural carbohydrates (Sánchez-Velázquez et al., 2021), have more pectin than alfalfa, and can be quickly degraded and absorbed into energy in the rumen (Evan et al., 1990), but their growth performance is also affected by climate, environment, feeding methods, and other factors (Pereira et al., 2018; Cremilleux et al., 2022). More dry matter is converted into feces, urine, and other forms of energy loss.

The core microflora of the phylum is Bacteroidetes, Firmicutes, and Proteobacteria, which account for about 90% of the bacterial species (Anderson et al., 2017; Guo et al., 2020). Bacteroidetes and Firmicutes are the most abundant and active flora members involved in carbohydrate and protein degradation (Xue et al., 2021). The main function of Bacteroides is to degrade many kinds of plant polysaccharides, improve the efficiency of nutrient utilization, and enhance the host's immunity (Delgado et al., 2019). Phaeophyta plays another essential role in the degradation of fiber and cellulose, related to the decomposition of polysaccharides and energy utilization (Delgado et al., 2019). There was no significant difference in rumen hilum level among the groups in this study. The change of roughage did not change the bacterial richness and taxonomic composition but had a different relative abundance at the genus level, which was consistent with other reports (Schären et al., 2017; Liang et al., 2021) and may reflect the particular niche related to dietary fiber digestion.

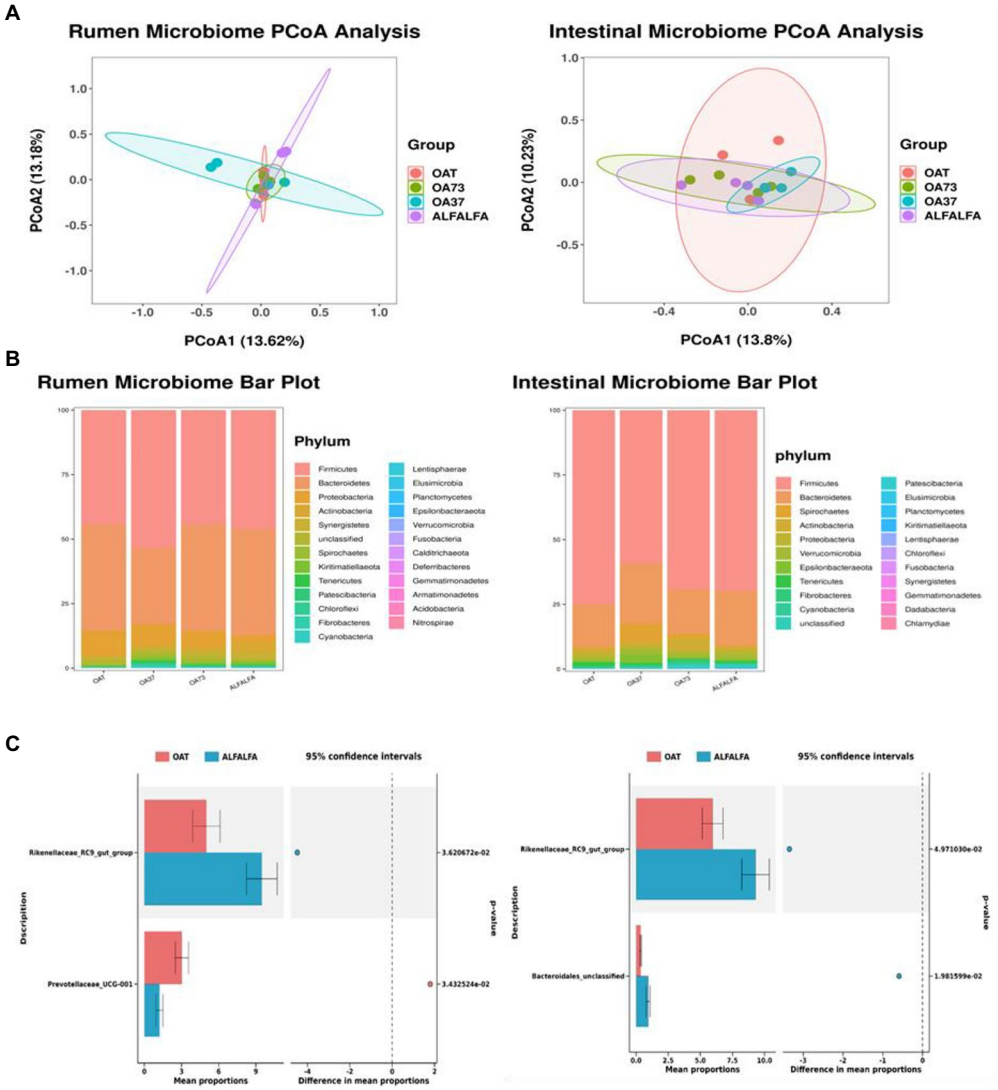


FIGURE 1
Classification of microbiome composition in posterior gut and rumen. **(A)** Principal component analysis. **(B)** Differences in microbiome phylum levels among groups. **(C)** Differences in microbiome genus levels between whole oat and whole alfalfa groups.

Many studies have reported that increasing the proportion of alfalfa in the diet can improve the meat quality traits of animals. This study confirmed that the increase of alfalfa content significantly increased the T-AOC of goat meat and decreased the MDA content, which was the same as that of Su et al. (2022). The malondialdehyde content of alfalfa powder in the Tibetan diet decreased by 16.5%. Plant active components, including flavonoids, saponins, and polysaccharides, contain antioxidant-free, radically active groups which can scavenge free radicals by supplying hydrogen to react with free radicals (Prommachart et al., 2021). This reduces the raw materials for synthesizing free radicals and increases the enzymes that decompose it to play an antioxidant role. Flavonoids, saponins, and polysaccharides all increased T-AOC and decreased MDA content in different experimental animals such as cows and mice, which means that the combined action of plant active components in alfalfa may be an important reason for improving the ability of the antioxidant defense system (Zhan et al., 2017; Xie et al., 2019).

This study discussed the correlation analysis between hindgut and rumen microorganisms with growth and slaughtering performance. The results showed that the microorganisms in the body could affect the antioxidant capacity of goat muscle. In this study, *Bacteroidales_unclassified* and *Bacteroidales* were significantly or very significantly positively correlated with T-AOC, and *Bacteroidales_unclassified* in the alfalfa group was significantly higher than in the oat group, suggesting that *Bacteroidales_unclassified* may be the key to improving meat antioxidant activity in alfalfa diet. It is well known that *Bacteroidale* plays a key role in rumen carbon transformation. Although *Bacteroidales_unclassified* lacks a clear and complete genome, its

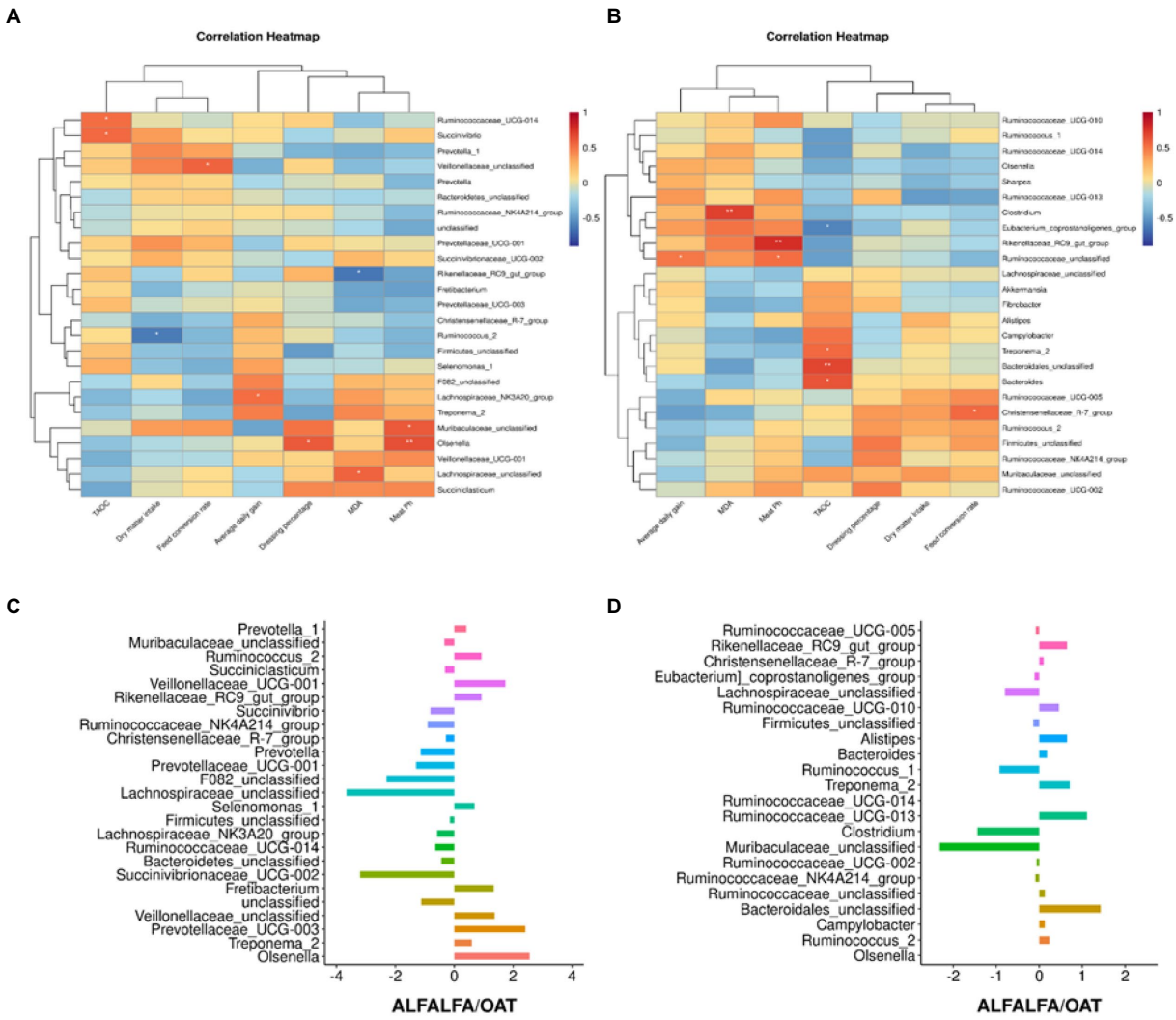


FIGURE 2
Effects of changes in the dominant microbiome of alfalfa and oat on growth performance and meat quality. **(A)** Heatmaps of Pearson's correlations between rumen dominant genera and growth performance and meat quality. **(B)** Heatmaps of Pearson's correlations between intestinal dominant genera and growth performance and meat quality. **(C)** Differential rumen genus microbiome between alfalfa and oat. **(D)** Differential intestinal genus microbiome between alfalfa and oat, *$p$<0.05, **$p$<0.01.

biological function may have the same ability to release hemicellulose monosaccharides as most other members of *Bacteroides*. Through genome metabolic analysis, *Solden* revealed various ways to ferment hemicellulose monosaccharides into short-chain fatty acids (SCFA), especially the increase in butyrate content (Solden et al., 2017). A recent study has shown that butyric acid can increase the T-AOC of the body by increasing the activity of SOD and CAT, clearing the levels of free radicals and MDA in the host (Li et al., 2022). This is an important way to improve the body's antioxidant activity in the hindgut environment.

There was a significant correlation between *Clostridium* and MDA in the hindgut. In this study, the *Clostridium* in the hindgut of the alfalfa group was significantly lower than that of the oat group. Similarly, Wu added – to the diet of yak with alfalfa hay significantly decreased the content of *Clostridium XVIII* and LPS, decreased the inflammatory reaction, and improved the liver health of yaks (Wu et al., 2019). The increase of *Clostridium* in the intestinal tract can cause Clostridial abomasitis and enteritis, mainly characterized by the necrosis of Bacillus or intestinal mucosa caused by the exotoxin produced by *Clostridium* perfringens or *Clostridium* difficulty in the gastrointestinal lumen. The proliferation of *Clostridium* perfringens in the gastrointestinal tract of ruminants is related to the increase in carbohydrates or proteins and the changes in gastrointestinal motility (Simpson et al., 2018). In this study, a high-concentrate diet may increase the abundance of *Clostridium*, while the addition of alfalfa hay decreases the abundance of *Clostridium*, which is responsible for the decrease of MDA. In addition, the results showed that the pH of goat meat was significantly correlated with *Olsenella* in the rumen and *Rikenellaceae_RC9_gut_group* in the intestine, which

meant that different proportions of alfalfa and oat changed the microflora and affected the meat quality.

## 5. Conclusion

A high proportion of alfalfa in roughage can improve the antioxidation of mutton and the digestibility of nutrients, but whole alfalfa can reduce daily weight gain and feed conversion rate. In addition, there is no significant effect of different proportions of forage on carcass traits. Feeding alfalfa could increase the abundance of *Bacteroidales_unclassified* and decrease the abundance of *Clostridium*. These microorganisms were strongly correlated with the antioxidant activity of mutton. The different roughage composition affects the intestinal microflora, thus improving livestock performance as edible products.

## Data availability statement

The data presented in the study are deposited in the NCBI Sequence Read Archive (SRA), accession numbers PRJNA880490 and PRJNA880492.

## Ethics statement

The animal study was reviewed and approved by the Northeast Agricultural University Animal Care and Use Committee (Harbin, China) (protocol code NEAU- [2011]-9) and were in accordance with the recommendations of the academy's guidelines for animal research. Written informed consent was obtained from the owners for the participation of their animals in this study.

## Author contributions

YS: methodology, visualization, investigation, formal analysis, and writing – original draft. TH: investigation. QY: visualization. CZ: project administration. YZ: supervision and writing – review and editing. YS: methodology and writing – review and editing. LX: funding acquisition, methodology, and 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.

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## References

Abeysekara, A. W. A. S. (2003). The nutritional value of oat forages for dairy cows. MS thesis Dissertation. Saskatoon, SK:University of Saskatchewan

Aboshora, W. (1990). *Association of Official Analytical Chemists. Official Methods of Analysis*. Arlington: Association of Official Analytical Chemists International. p. 405.

Adesogan, A. T., Arriola, K. G., Jiang, Y., Oyebade, A., Paula, E. M., Pech-Cervantes, A. A., et al. (2019). Symposium review: technologies for improving fiber utilization. *J. Dairy Sci.* 102, 5726–5755. doi: 10.3168/jds.2018-15334

Anderson, C. L., Sullivan, M. B., and Fernando, S. C. (2017). Dietary energy drives the dynamic response of bovine rumen viral communities. *Microbiome* 5, 155–119. doi: 10.1186/s40168-017-0374-3

Andrés, S., Huerga, L., Mateo, J., Tejido, M., Bodas, R., Morán, L., et al. (2014). The effect of quercetin dietary supplementation on meat oxidation processes and texture of fattening lambs. *Meat Sci.* 96, 806–811. doi: 10.1016/j.meatsci.2013.09.020

Castells, L., Bach, A., Aris, A., and Terré, M. (2013). Effects of forage provision to young calves on rumen fermentation and development of the gastrointestinal tract. *J. Dairy Sci.* 96, 5226–5236. doi: 10.3168/jds.2012-6419

Cremilleux, M., Coppa, M., Bouchon, M., Delaby, L., Beaure, G., Constant, I., et al. (2022). Effects of forage quantity and access-time restriction on feeding behaviour, feed efficiency, nutritional status, and dairy performance of dairy cows fed indoors. *Animal* 16:100608. doi: 10.1016/j.animal.2022.100608

Delgado, B., Bach, A., Guasch, I., González, C., Elcoso, G., Pryce, J. E., et al. (2019). Whole rumen metagenome sequencing allows classifying and predicting feed efficiency and intake levels in cattle. *Sci. Rep.* 9:11. doi: 10.1038/s41598-018-36673-w

Doran, M. P., Laca, E. A., and Sainz, R. D. (2007). Total tract and rumen digestibility of mulberry foliage (*Morus alba*), alfalfa hay and oat hay in sheep. *Anim. Fee. Sci. Tech.* 138, 239–253. doi: 10.1016/j.anifeedsci.2006.11.016

Evan, C., Mark, G., Leslie, D., and George, C. (1990). Digestion of cell wall components by dairy heifers fed diets based on alfalfa and chemically treated oat hulls. *J. Dairy Sci.* 74, 1026–1037. doi: 10.3168/jds.S0022-0302(91)78252-0

Guo, W., Zhou, M., Ma, T., Bi, S., Wang, W., Zhang, Y., et al. (2020). Survey of rumen microbiota of domestic grazing yak during different growth stages revealed novel maturation patterns of four key microbial groups and their dynamic interactions. *Anim. Microbiome.* 2:23. doi: 10.1186/s42523-020-00042-8

Hayes, J., Allen, P., Brunton, N., O'Grady, M., and Kerry, J. (2011). Phenolic composition and in vitro antioxidant capacity of four commercial phytochemical products: Olive leaf extract (Olea europaea L.), lutein, sesamol and ellagic acid. *Food Chem.* 126, 948–955. doi: 10.1016/j.foodchem.2010.11.092

Inserra, L., Priolo, A., Biondi, L., Lanza, M., Bognanno, M., Gravador, R., et al. (2014). Dietary citrus pulp reduces lipid oxidation in lamb meat. *Meat Sci.* 96, 1489–1493. doi: 10.1016/j.meatsci.2013.12.014

Kenny, D. A., Fitzsimons, C., Waters, S. M., and McGee, M. (2018). Invited review: improving feed efficiency of beef cattle - the current state of the art and future challenges. *Animal* 12, 1815–1826. doi: 10.1017/s1751731118000976

Li, W., Pan, L., Liu, H., Tan, B., Dong, X., Yang, Q., et al. (2022). Effects of the *Clostridium butyricum* on growth performance, antioxidant capacity, immunity, and disease resistance of Litopenaeus Vannamei fed with cottonseed protein concentrate (CPC) replacement of fishmeal in diet. *Fish Shellfish Immunol.* 126, 283–291. doi: 10.1016/j.fsi.2022.05.030

Liang, J., Zheng, W., Zhang, H., Zhang, P., Cai, Y., Wang, Q., et al. (2021). Transformation of bacterial community structure in rumen liquid anaerobic digestion of rice straw. *Environ. Pollut.* 269:116130. doi: 10.1016/j.envpol.2020.116130

McAllister, T. A., Stanford, K., Chaves, A. V., Evans, P. R., Eustaquio de Souza Figueiredo, E., and Ribeiro, G. (2020). Nutrition, feeding, and management of beef cattle in intensive and extensive production systems. *Anim. Agricult.*, 75–98. doi: 10.1016/B978-0-12-817052-6.00005-7

Morán, L., Andrés, S., Bodas, R., Prieto, N., and Giráldez, F. (2012). Meat texture and antioxidant status are improved when carnosic acid is included in the diet of fattening lambs. *Meat Sci.* 91, 430–434. doi: 10.1016/j.meatsci.2012.02.027

Pereira, E., Campos, A., Castelo-Branco, K., Bezerra, L., Gadelha, C., Silva, L., et al. (2018). Impact of feed restriction, sexual class and age on the growth, blood metabolites and endocrine responses of hair lambs in a tropical climate. *Small Ruminant Res.* 158, 9–14. doi: 10.1016/j.smallrumres.2017.11.007

Ponnampalam, E. N., Butler, K. L., McDonagh, M. B., Jacobs, J. L., and Hopkins, D. L. (2012). Relationship between muscle antioxidant status, forms of iron, polyunsaturated fatty acids, and functionality (retail colour) of meat in lambs. *Meat Sci.* 90, 297–303. doi: 10.1016/j.meatsci.2011.07.014

Pordomingo, A. J., Pordomingo, A. B., Grigioni, G., and Carduza, F. (2022). Performance, carcass, and meat traits of beef steers finished on small-grain winter annuals in winter or on alfalfa pasture in summer. *Livestock Sci.* 254:104759. doi: 10.1016/j.livsci.2021.104759

Prommachart, R., Cherdthong, A., Navanukraw, C., Pongdontri, P., Taron, W., Uriyapongson, J., et al. (2021). Effect of dietary anthocyanin-extracted residue on meat oxidation and fatty acid profile of male dairy cattle. *Animals* 11:322. doi: 10.3390/ani11020322

Radović, J., Sokolović, D., and Marković, J. (2009). Alfalfa-most important perennial forage legume in animal husbandry. *Biotechnol. Anim. Husbandry.* 25, 465–475. doi: 10.2298/BAH0906465R

Sánchez-Velázquez, O., Cuevas-Rodríguez, E., Mondor, M., Ribéreau, S., Arcand, Y., Mackie, A., et al. (2021). Impact of in vitro gastrointestinal digestion on peptide profile and bioactivity of cooked and non-cooked oat protein concentrates. *Curr. Res. Food Sci.* 4, 93–104. doi: 10.1016/j.crfs.2021.02.003

Schären, M., Kiri, K., Riede, S., Gardener, M., Meyer, U., Hummel, J., et al. (2017). Alterations in the rumen liquid-, particle-and epithelium-associated microbiota of dairy cows during the transition from a silage-and concentrate-based ration to pasture in spring. *Front. Micr.* 8:744. doi: 10.3389/fmicb.2017.00744

Simpson, K. M., Callan, R. J., and Van Metre, D. C. (2018). Clostridial abomasitis and enteritis in ruminants. *Vet. Clin. North Am. Food Anim. Pract.* 34, 155–184. doi: 10.1016/j.cvfa.2017.10.010

Solden, L. M., Hoyt, D. W., Collins, W. B., Plank, J. E., Daly, R. A., Hildebrand, E., et al. (2017). New roles in hemicellulosic sugar fermentation for the uncultivated Bacteroidetes family BS11. *ISME J.* 11, 691–703. doi: 10.1038/ismej.2016.150

Su, Y., Sun, X., Zhao, S., Hu, M., Li, D., Qi, S., et al. (2022). Dietary alfalfa powder supple-mentation improves growth and development, body health, and meat quality of Tibetan sheep. *Food Chem.* 396:133709. doi: 10.1016/j.foodchem.2022.133709

Tang, S., He, Y., Zhang, P., Kang, J., Yan, Q., Han, X., et al. (2021). Substitution of ramie (*Boehmeria nivea*) for alfalfa in improving the carcass and meat quality of Liuyang black goats. *Anim. Nutr.* 7, 688–694. doi: 10.1016/j.aninu.2020.11.020

Thonney, M., Duhaime, D., Moe, P., and Reid, J. T. (1979). Acid insoluble ash and permanganate lignin as indicators to determine digestibility of cattle rations. *J. Anim. Sci.* 49, 1112–1116. doi: 10.2527/jas1979.4941112x

Van Elswyk, M. E., and McNeill, S. H. (2014). Impact of grass/forage feeding versus grain finishing on beef nutrients and sensory quality: the U.S. experience. *Meat Sci.* 96, 535–540. doi: 10.1016/j.meatsci.2013.08.010

Van Soest, P. J., Robertson, J., and Lewis, B. (1991). Methods for dietary fiber, neutral detergent fiber, and nonstarch polysaccharides in relation to animal nutrition. *J. Dairy Sci.* 74, 3583–3597. doi: 10.3168/jds.S0022-0302(91)78551-2

Wu, S. R., Chen, X. D., Li, J. L., Yang, Y. T., Cui, Z. H., and Yao, J. H. (2021). Effect of alfalfa hay and starter feed supplementation on caecal mi-crobiota and fermentation, growth, and health of yak calves. *Animal* 15:100019. doi: 10.1016/j.animal.2020.100019

Wu, S., Cui, Z., Chen, X., Wang, P., and Yao, J. (2019). Changed caecal microbiota and fermentation contribute to the beneficial effects of early weaning with alfalfa hay, starter feed, and milk replacer on the growth and organ development of yak calves. *Animals* 9:921. doi: 10.3390/ani9110921

Xie, Y., Wang, L., Sun, H., Wang, Y., Yang, Z., Zhang, G., et al. (2019). Immunomodulatory, antioxidant and intestinal morpholo-gy-regulating activities of alfalfa polysaccharides in mice. *Int. J. Biol. Macromol.* 133, 1107–1114. doi: 10.1016/j.ijbiomac.2019.04.144

Xue, M. Y., Sun, H. Z., Wu, X. H., Liu, J. X., and Guan, L. L. (2021). Multi-omics reveals that the rumen microbiome and its metabolome together with the host metabolome contribute to individualized dairy cow performance. *Microbiome* 8:64. doi: 10.1186/s40168-020-00819-8

Zhan, J., Liu, M., Su, X., Zhan, K., Zhang, C., and Zhao, G. (2017). Effects of alfalfa flavonoids on the production performance, immune system, and ruminal fermentation of dairy cows. *Asian Australas J. Anim. Sci.* 30, 1416–1424. doi: 10.5713/ajas.16.0579

Zhou, G., Zhang, W., and Xu, X. (2012). China's meat industry revolution: challenges and opportunities for the future. *Meat Sci.* 92, 188–196. doi: 10.1016/j.meatsci.2012.04.016

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# Dietary *Bacillus licheniformis* shapes the foregut microbiota, improving nutrient digestibility and intestinal health in broiler chickens

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*Bacillus licheniformis* is considered a potential alternative to antibiotic growth promoters of animal growth and health. However, the effects of *Bacillus licheniformis* on the foregut and hindgut microbiota, and their relationships with nutrient digestion and health, in broiler chickens remain unclear. In this study, we aimed to identify the effects of *Bacillus licheniformis* BCG on intestinal digestion and absorption, tight junctions, inflammation, and the fore- and hind-gut microbiota. We randomly assigned 240 1-day-old male AA broilers into three treatment groups: CT (basal diet), BCG1 (basal diet + $1.0 \times 10^8$ CFU/kg *B. licheniformis* BCG), and BCG2 (basal diet + $1.0 \times 10^9$ CFU/kg *B. licheniformis* BCG). On day 42, the jejunal and ileal chyme and mucosa were subjected to analysis of digestive enzyme activity, nutrient transporters, tight junctions, and signaling molecules associated with inflammation. The ileal and cecal chyme were subjected to microbiota analysis. Compared with the CT group, the *B. licheniformis* BCG group showed significantly greater jejunal and ileal α-amylase, maltase, and sucrase activity; moreover, the α-amylase activity in the BCG2 group was higher than that in the BCG1 group ($P < 0.05$). The transcript abundance of FABP-1 and FATP-1 in the BCG2 group was significantly greater than that in the CT and BCG1 groups, and the GLUT-2 and LAT-1 relative mRNA levels were greater in the BCG2 group than the CT group ($P < 0.05$). Dietary *B. licheniformis* BCG resulted in significantly higher ileal occludin, and lower IL-8 and TLR-4 mRNA levels than observed in the CT group ($P < 0.05$). *B. licheniformis* BCG supplementation significantly decreased bacterial community richness and diversity in the ileum ($P < 0.05$). Dietary *B. licheniformis* BCG shaped the ileac microbiota by increasing the prevalence of *f_Sphingomonadaceae*, *Sphingomonas*, and *Limosilactobacillus*, and contributed to nutrient digestion and absorption; moreover, it enhanced the intestinal barrier by increasing the prevalence of *f_Lactobacillaceae*, *Lactobacillus*, and *Limosilactobacillus*. Dietary *B. licheniformis* BCG decreased microbial community diversity by diminishing *Desulfovibrio*, *Alistipes*, *Campylobacter*, *Vibrio*, *Streptococcus*, and *Escherichia coli*-Shigella levels, and

down-regulating inflammatory associated molecule expression. Therefore, dietary *B. licheniformis* BCG contributed to digestion and absorption of nutrients, enhanced the intestinal physical barrier, and decreased intestinal inflammation in broilers by decreasing microbial diversity and optimizing the microbiota structure.

KEYWORDS

***Bacillus licheniformis* BCG, broiler, digestive enzyme activity, nutrient transporter, ileac and cecum microbiota, intestinal inflammation, tight junction**

# 1. Introduction

In poultry production, broiler chickens are generally subjected to harsh and stressful conditions, particularly when they are reared at high stocking density, thus resulting in host stress and immune dysfunction (Kridtayopas et al., 2019). This subhealth status decreases the growth potential and causes intestinal dysfunction in broilers, and increases the economic costs of rearing. The intestines not only digest and absorb nutrients, but also are the largest immune organ (Zhang et al., 2022a). Trillions of microorganisms colonize the gastrointestinal tract; the total microbial number is 10 times the number of host somatic cells, and the collective number of genes is 150 times that in the host genome (Collins et al., 2012; Strandwitz, 2018). The intestinal microbiota co-develops with the host and participates in nutrient digestion, improves intestinal development and health, and regulates the body's metabolism and immunity function (Li et al., 2019; Han et al., 2020). Gut microbiota disorders have been associated with subhealth and disease. Broilers show a shift in the gut microbial profile when they experience subclinical forms of necrotic enteritis: the relative abundance of *Firmicutes*, *Lactobacillus*, and *Bacteroides* decreases, thus resulting in low host productivity (Antonissen et al., 2016). Other research has indicated that challenge with *Salmonella typhimurium* decreased *Lactobacillus* prevalence, damaged intestinal morphology, and subsequently decreased the growth performance of broilers (Jazi et al., 2019). The overall consensus is that the intestinal microbiota is interlinked with intestinal health and poultry growth.

Probiotics have been demonstrated to be an effective means of promoting animal growth and improving body health in the post-antibiotic era (Mingmongkolchai and Panbangred, 2018). *Bacillus licheniformis* (*B. licheniformis*) is a Gram-positive bacterium characterized by resistance to stresses such as high acidity and temperature. Because of these characteristics, *B. licheniformis* can be used in livestock production practices. *Bacillus* spores are metabolically dormant under harsh conditions including feed pelleting but subsequently grow in the favorable environment of the gastrointestinal tract after ingestion (Konieczka et al., 2018). *B. licheniformis* shows growth-promoting effects in poultry (Gadde et al., 2017). Moreover, *B. licheniformis* benefits broilers by protecting against heat stress and preventing necrotic enteritis (Abdelqader et al., 2020; Xu et al., 2021). These benefits might be attributable to a variety of biologically active substances produced by *B. licheniformis*, which contribute to feed digestibility, immune system regulation, and enhanced intestinal barrier function (Kim et al., 2004; Zhou et al., 2016; Kan et al., 2021). *B. licheniformis* improves the intestinal mechanical barrier and decreases intestinal permeability by up-regulating the gene expression of mucins and tight junction proteins in laying hens (Wang et al., 2017). Diets containing a mixture of *B. licheniformis* and *B. subtilis* have been found to alleviate *Escherichia coli*-induced enteritis by increasing intestinal epithelial barrier integrity (Yang et al., 2016). The potential underlying mechanism involves regulation of the composition of the intestinal microbiota to restore and maintain intestinal homeostasis (Sanders et al., 2019; Zhao et al., 2022b). For instance, probiotic *Bacillus* strains have been found to prevent or diminish gut colonization by *Chlamydia psittaci*, *Escherichia coli*, *Streptococcus*, and *Salmonella*, thus improving intestinal mucosa integrity and gut health (Zuo et al., 2020; Haque et al., 2021). Therefore, probiotic *Bacillus* appears to prevent disease or stress, and promote growth performance, possibly through an optimized intestinal microbial structure and improved gut health.

Although *B. licheniformis* has great potential application value in the broiler industry, the efficacy of probiotic *Bacillus* varies among strains and depends on the exogenous environmental conditions to which animals are exposed (Konieczka et al., 2022). In the present study, we hypothesized that dietary *B. licheniformis* BCG might alter the ileal and cecal microbiota, and contribute to broiler digestibility and gut health. To this end, we aimed to explore the protective roles of *B. licheniformis* BCG involving improved nutrient digestion and absorption, a strengthened intestinal barrier, and decreased inflammation, and to understand their relationships with the gut microbiota shifted by *B. licheniformis* BCG. Our data provided a theoretical basis for application of *B. licheniformis* BCG in the production of healthy broilers.

# 2. Materials and methods

The experimental animal protocol for this study was conducted in accordance with the recommendations of "Guidelines on Welfare and Ethical Review for Laboratory Animals" (GB/T 35892-2018), and approved by the Institutional Animal Care and Use Committee of the Institute of Feed Research of Chinese Academy of Agricultural Sciences (FRI-CAAS20210827).

## 2.1. Animals and experimental design

A total of 240 1-day-old male Arbor Acre broilers (body weight, 42.62 ± 0.82 g) were randomly allocated to three groups. Each group consisted of eight replicates (pens) with 10 broilers per pen. Two phase non-medicated basal diets in mashed form were formulated based on the nutrient requirements of the National Research Council (1994); (**Table 1**). The three groups included basal diet (CT, $n$ = 8),

and basal diet with a dose of $1.0 \times 10^8$ CFU/kg (BCG1, $n = 8$) and $1.0 \times 10^9$ CFU/kg (BCG2, $n = 8$) *B. licheniformis* BCG, respectively (Wang et al., 2017; Kan et al., 2021; Xu et al., 2021). All broilers were feed in wire-floored cages in a one-level battery on their respective diets. The study lasted 42 days, during which time broilers had *ad libitum* to feed and fresh water. Broilers were housed in an environmentally controlled room and temperature was gradually reduced from 35°C on day 1 to 26°C at day 21 and then kept roughly constant. A 20 h light-4 h dark cycle was carried out throughout the experimental period. *B. licheniformis* BCG was isolated from humus soil in the northeast forest area and preserved in the Key Laboratory of Feed Biotechnology of Ministry of Agriculture and Rural Affairs. It presents great biological characteristics in carbohydrate metabolism enzymes and stress tolerance through the whole genome sequencing and *in vitro* evaluation. *B. licheniformis* BCG with viable count = $1.08 \times 10^{10}$ CFU/g was used and mixed in the basal diet, which was prepared in bacterial mashed form after processed in activation, culture, centrifugation, freeze-drying, and grinding.

## 2.2. Sample collection

At 42 days of age, after fasting overnight, one broiler representing the average weight from each replicate was selected and humanely slaughtered. Jejunum, ileum and cecum segments were divided and fresh ileal and cecal contents were collected for α-amylase and microbiota analysis. Jejunal and ileal mucosa were scraped by autoclaved blade after precooled saline flush for maltase, sucrase and gene expression analysis. All samples were obtained as described previously (Wang et al., 2008), and immediately frozen in liquid nitrogen and stored at −80°C.

## 2.3. Biochemical analysis

Appropriately 100 mg frozen mucosa and chyme of jejunum and ileum, respectively, were taken and mixed with 1 mL cold buffer (pH7.4), containing 10 mM Tris-HCl, 0.1 EDTA-$Na_2$ and 0.8% (w/v) NaCl, and homogenized using an Ultra-Turrax homogenizer for 30 s. Homogenates were centrifuged at 3,000 × *g* for 15 min at 4°C and supernatants transferred to new tubes for protein assay and other measurements. The activities of α-amylase, maltase and sucrase were measured by colorimetry using the commercial kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) according to the manufacturer's protocols. Maltase and sucrase were normalized to tissue protein concentrations, which were measured with a bicinchoninic acid commercial kit (Thermo Fisher Scientific, Waltham, MA, USA) following the manufacturer's instructions.

## 2.4. Real-time quantitative PCR (RT-qPCR)

Selected mRNA abundance was determined by RT-qPCR, including nutrient transporters genes *FABP-1* (fatty acid binding protein 1), *FATP-1* (fatty acid transport protein 1), *GLUT-2* (glucose transporter 2), *LAT-1* (L type amino acid transporter 1), *PepT-1* (peptide transporter 1) and *SGLT-1* (sodium glucose co-transporter 1), inflammatory molecules' genes *TLR-4* (Toll-like receptor 4), *IL-1*β (interleukin 1β), *IL-8*, *IL-10*, *TNF*-α (tumor necrosis factor α), *TGF*-β (Transforming growth factor β) and *NF*-κ*B* (Nuclear factor kappa B), and tight junction genes *Claudin-1*, *Occludin*, *ZO-1* and *Mucin-2*. Total RNA was isolated from ileal mucosa samples (approximately 0.75 mg) using an RNAprep pure tissue kit (Tiangen Biotech Co. Ltd., Beijing, China) under the manufacturer's instructions. Total RNA concentrations and quality were assessed using a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). RNA integrity was evaluated using agarose gel (1%) electrophoresis. Then, cDNA was synthesized from 1 μg total RNA using a PrimeScript RT reagent kit (TaKaRa Biotechnology Co., Ltd., Otsu, Japan) following to manufacturer's protocols. Selected mRNA reactions were detected in 10 μL (Bio-Rad Laboratories, Hercules, CA, USA) using SYBR®Premix Ex TaqTM II (Tli RNaseH Plus) (TaKaRa Biotechnology Co., Otsu, Japan). The primers for nutrient transporters, inflammatory, and tight junction-related and housekeeping genes [glyceraldehyde 3-phosphate dehydrogenase (GAPDH)] were described previously (Wang et al., 2016, 2020). The $2^{-\Delta\Delta Ct}$ method was used for quantification using GAPDH as a reference gene, and relative abundance was normalized to CON group values.

## 2.5. Ileal and cecal microbiota and analysis

Bacterial genomic DNA was extracted from ileal and cecal chyme samples (Qiagen DNA stool mini kit, Qiagen, Germany). DNA quantity and quality were assessed using a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA) and 1% agarose gels, respectively. The V3–V4 hypervariable region 16S rRNA was amplified using specific primers (forward 5′-ACTCCTACGGGAGGCAGCA-3′ and reverse 5′-GGACTACHVGGGTWTCTAAT-3′), containing unique barcodes. Polymerase chain reaction (PCR) was conducted in a total volume of 20 μL, including 1 × FastPfu buffer, 250 μM dNTP, 0.2 μM each primer, 1 U FastPfu polymerase (Beijing TransGen Biotech, Beijng, China), and 10 ng template DNA. PCR products were electrophoresed on 2% agarose gels and purified using a Qiagen gel extraction kit (Qiagen, Germany). Sequencing libraries were constructed using a TruSeq®DNA PCR-Free Sample Preparation Kit (Illumina, San Diego, CA, USA) based on manufacturer's instructions, and index codes were added. Library quality was assessed using a Qubit V.2.0 Fluorometer (Thermo Fisher Scientific, Waltham, MA, USA). Qualified DNA libraries were loaded into a NovaSeq platform capable of 2 × 250 bp paired-end sequencing reads (Novogene, Beijing, China).

Paired-end reads were generated and merged using FLASH software (V1.2.7)[1]. Operational taxonomic units with 97% identity were gathered using Uparse[2](ver. 7.1). Taxonomic annotations were performed using the Mothur algorithm (70% confidence) in the Silva database[3]. Alpha-diversity was analyzed using Observed_species, Chao1, and Shannon indices. Beta-diversity was visualized using principal coordinate analysis (PCoA) plots based on weighted UniFrac distance. Bacterial biomarkers between groups were displayed using the linear discriminant analysis effect size (LEfSe, linear discriminant analysis (LDA) > 3.5).

1 http://ccb.jhu.edu/software/FLASH/
2 http://drive5.com/uparse/
3 http://www.arb-silva.de/

TABLE 1 Ingredients and chemical compositions of experimental diets (as-fed basis).

| Ingredient (%) | Content | |
|---|---|---|
| | Day 1-21 | Day 22-42 |
| Corn | 54.88 | 58.00 |
| Soybean meal | 36.27 | 32.79 |
| Fish meal | 1.75 | 1.33 |
| Soybean oil | 3.08 | 4.28 |
| Dicalcium phosphate | 1.24 | 1.08 |
| Limestone | 1.21 | 1.14 |
| Sodium chloride | 0.30 | 0.30 |
| DL-Methionine | 0.15 | 0.08 |
| L-Lysine-HCl | 0.12 | 0.00 |
| Vitamin and mineral premix[†] | 1.00 | 1.00 |
| Nutrient composition[‡] | | |
| Metabolizable energy (MJ/kg) | 12.35 | 12.80 |
| Crude protein (%) | 21.86 | 20.34 |
| Calcium (%) | 1.00 | 0.90 |
| Total Phosphorus (%) | 0.71 | 0.64 |
| Available phosphorus (%) | 0.40 | 0.35 |
| Methionine (%) | 0.46 | 0.37 |
| Methionine + Cysteine (%) | 0.75 | 0.64 |
| Lysine (%) | 1.14 | 0.95 |

[†]A vitamin-mineral premix provided the following nutrients per kg of diet: vitamin A, 10000 IU; vitamin D, 2000 IU; vitamin E, 20 IU; vitamin K, 1 mg; vitamin B1, 2 mg; riboflavin, 8 mg; vitamin $B_{12}$, 0.01 mg; pantothenic acid, 10 mg; niacin, 35 mg; pyridoxine, 3.5 mg; biotin, 0.2 mg; folic acid, 0.6 mg; Fe, 100 mg; Cu, 10 mg; Mn, 120 mg; Zn, 100 mg; I, 0.7 mg; Se, 0.3 mg.
[‡]Nutrient levels were calculated.

## 2.6. Statistical analysis

Statistical analyses were performed using one-way analysis of variance in SAS 9.4 (SAS Institute, Inc., Cary, NC, USA). Each broiler served as statistical unit. Differences between treatment means for enzyme activity and gene expression were evaluated using Duncan's multiple-range tests. Wilcox test was used for alpha-diversity index. LEfSe, *t*-test, and Metastat analyses were used to test for significant differences between microbiota relative abundance. Results were represented as mean with standard error of mean (SEM) in the tables and the mean with standard error (SE) in the figures, while $P < 0.05$ (*) and $P < 0.01$ (**) values were considered statistically and extremely significant, respectively. Bar charts were drafted in Graphpad Prism 7.0 software (GraphPad Software Inc., La Jolla, CA, USA).

# 3. Results

## 3.1. Effects of *B. licheniformis* BCG on jejunal and ileal enzyme activity in broilers

The BCG2 group showed significantly greater jejunal and ileal maltase and sucrase activity, and the BCG1 group showed significantly greater ileal sucrase activity, than the CT group ($P < 0.05$, Table 2). No differences in these two parameters were observed between the BCG1 and BCG2 groups ($P > 0.05$). Dietary *B. licheniformis* BCG resulted in significantly greater α-amylase activity than that in the CT group ($P < 0.05$), and this activity was higher in the BCG2 group than the BCG1 group ($P < 0.05$).

## 3.2. Effects of *B. licheniformis* BCG on nutrient transporter gene mRNA levels in the ileum

*FABP-1* and *FATP-1* relative mRNA levels in the BCG2 group were significantly higher than those in the CT and BCG1 groups ($P < 0.05$, Figure 1). The transcript abundance of *GLUT-2* and *LAT-1* was greater in the BCG2 group than the CT group ($P < 0.05$), and no significant difference was found between the BCG1 group and the other groups ($P > 0.05$).

## 3.3. Effects of *B. licheniformis* BCG on tissue morphology and the mRNA expression of tight junction and inflammatory molecules in the ileum

Histological examination of the ileum indicated that the villi and epithelium in the CT group, as compared with the BCG1 and BCG2 groups, showed damage; however, no clear infiltration of inflammatory cells was observed among groups (Figure 2A). Dietary *B. licheniformis* BCG significantly up-regulated ileal *occludin* mRNA levels, which were higher in the BCG2 group than the CT group ($P < 0.05$, Figure 2B). The transcript abundance of *IL-8* and *TLR-4* in the BCG1 and BCG2 groups was lower than that in the CT group ($P < 0.05$, Figure 2C), whereas no significant difference was observed between the BCG1 and BCG2 groups. No significant difference in *claudin-1*, *ZO-1*, *mucin-2*, *IL-1*β, *TNF-*α, *NF-*κ*B*, *IL-10*, and *TGF-*β transcript abundance was observed between groups ($P > 0.05$).

TABLE 2 Effects of *B. licheniformis* BCG on enzyme activity in the jejunum and ileum in broilers.

| Item | Dietary treatments[1] | | | SEM[2] | *P*-value |
|---|---|---|---|---|---|
| | CT | BCG1 | BCG2 | | |
| Jejunum | | | | | |
| Maltase (U/mg prot) | $35.60^{b}$ | $51.29^{a}$ | $49.07^{a}$ | 2.584 | 0.016 |
| Sucrase (U/mg prot) | $43.40^{b}$ | $53.11^{ab}$ | $64.63^{a}$ | 2.844 | 0.003 |
| α-amylase (U/dL) | 49.35 | 52.90 | 50.26 | 1.581 | 0.663 |
| Ileum | | | | | |
| Maltase (U/mg prot) | $134.50^{b}$ | $169.23^{a}$ | $178.36^{a}$ | 6.953 | 0.013 |
| Sucrase (U/mg prot) | $100.87^{b}$ | $129.09^{a}$ | $127.31^{a}$ | 5.088 | 0.028 |
| α-amylase (U/dL) | $24.61^{c}$ | $32.53^{b}$ | $44.76^{a}$ | 2.220 | < 0.01 |

[a–c]Different superscript letters in a row indicate a significant difference ($P < 0.05$). [1]CT, control group, basal diet; BCG1, basal diet supplemented with *B. licheniformis* BCG at $1.0 \times 10^8$ CFU/kg; BCG2, basal diet supplemented with *B. licheniformis* BCG at $1.0 \times 10^9$ CFU/kg. [2]SEM, standard error of the mean, $n = 8$.

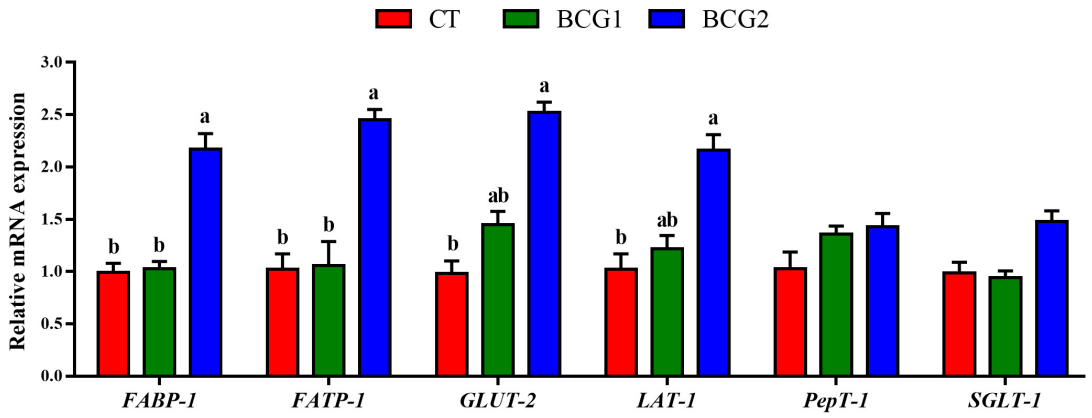


FIGURE 1
Effects of *Bacillus licheniformis* BCG on nutrient transporter gene mRNA levels in the ileum. Values are mean ± SE ($n$ = 6). [a,b]Bars with different letters within the same index indicate a significant difference between groups ($P < 0.05$).

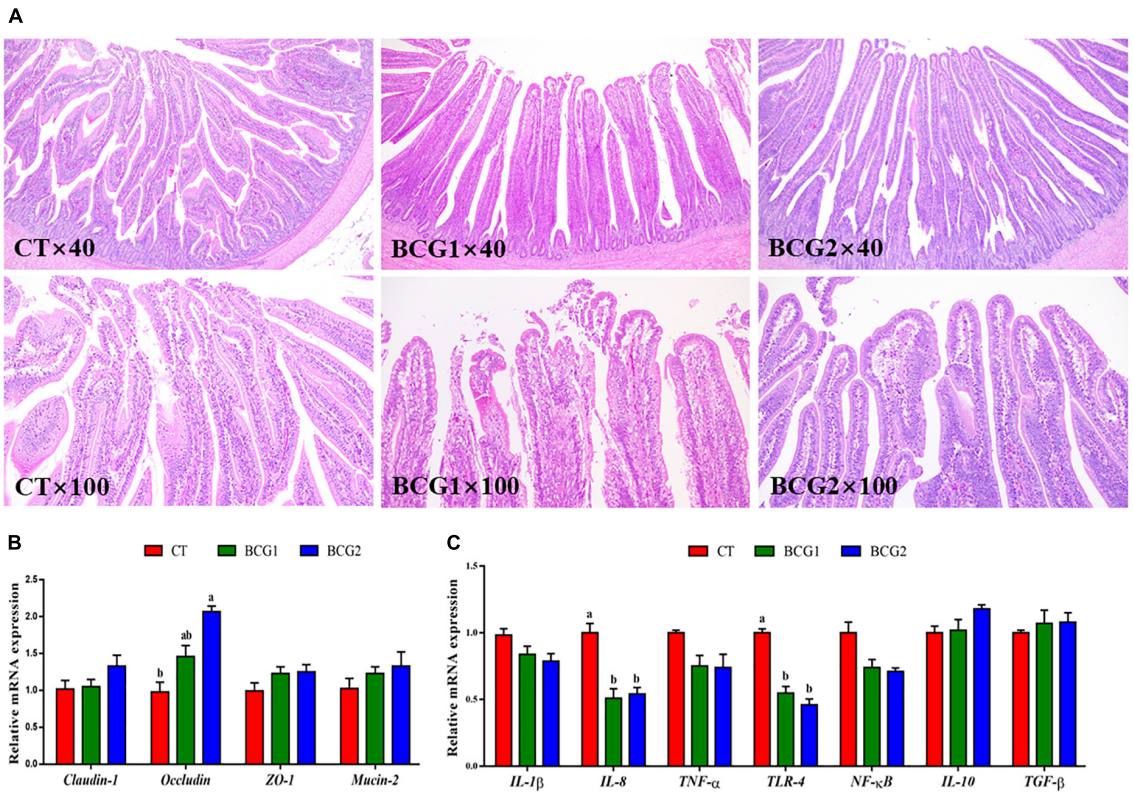


FIGURE 2
Effects of *Bacillus licheniformis* BCG on ileal morphology, and tight junction and inflammatory molecule gene mRNA levels in the ileum. **(A)** Representative ileal histological sections of broilers. **(B)** mRNA levels of the tight junction genes *claudin-1*, *occludin*, *ZO-1*, and *Mucin-2*. **(C)** The mRNA levels of the inflammatory molecule genes *TLR-4*, *IL-1β*, *IL-8*, *IL-10*, *TNF-α*, *TGF-β*, and *NF-κB*. Values are the mean ± SE ($n$ = 6). [a,b]Bars with different letters within the same index indicate a significant difference between groups ($P < 0.05$).

## 3.4. Effects of *B. licheniformis* BCG on microbiota diversity in the ileum and cecum

A total of 3,917,657 high quality sequencing reads were generated from 47 broiler gut samples, with an average of 69,233 effective sequences/sample. Alpha diversity analyses indicated varying community richness and diversity among groups in the ileal but not the cecal bacterial communities. Both the BCG1 and BCG2 groups showed significantly lower ileal Observed_species, Chao1, and Shannon indexes than the CT group ($P < 0.05$, Figures 3A–C). Differences in the microbial structure among groups and niches were evaluated with PCoA analysis based on weighted UniFrac distance. Microbial communities were well separated between the ileal microbiota and counterparts colonizing the cecum, and between groups in the ileum (Figures 3D–F). ANOSIMs also confirmed the

TABLE 3 ANOSIM analysis of differences in bacterial structure between variables, on the basis of Bray-Curtis distances.

| Varibles | *R*-value | *P*-value |
|---|---|---|
| CTI VS. CTC | 0.8549 | 0.001 |
| CTI VS. BCG1-C | 0.9971 | 0.001 |
| CTI VS. BCG2-C | 0.9967 | 0.001 |
| CTC VS. BCG1-I | 0.7238 | 0.001 |
| CTC VS. BCG2-I | 0.8644 | 0.002 |
| BCG1-I VS. BCG1-C | 0.750 | 0.001 |
| BCG1-I VS. BCG2-C | 0.7812 | 0.001 |
| BCG2-I VS. BCG1-C | 0.9555 | 0.002 |
| BCG2-I VS. BCG2-C | 0.9325 | 0.001 |
| CTI VS. BCG1-I | 0.202 | 0.029 |
| CTI VS. BCG2-I | 0.1663 | 0.041 |
| BCG1-I VS. BCG2-I | -0.0385 | 0.669 |
| CTC VS. BCG1-C | 0.1764 | 0.027 |
| CTC VS. BCG2-C | 0.1872 | 0.028 |
| BCG1-C VS. BCG2-C | 0.4191 | 0.001 |

$R > 0$ represents a significant difference between groups, whereas $R < 0$ indicates a difference within groups greater than that between groups. $P < 0.05$ indicates a significant difference in statistics. CT, control group, basal diet; BCG1, BCG1 group, basal diet supplemented with *B. licheniformis* BCG at $1.0 \times 10^8$ CFU/kg; BCG2, BCG2 group, basal diet supplemented with *B. licheniformis* BCG at $1.0 \times 10^9$ CFU/kg; I, ileum; C, cecum.

structural dissimilarity between the ileum and cecum, and between the CT group and the two BCG groups in the ileum or cecum ($R > 0$, $P < 0.05$, Table 3).

## 3.5. Effects of *B. licheniformis* BCG on ileal and cecal bacterial structures

In the ileum, Proteobacteria, Firmicutes, Bacteroidota, and Campylobacterota were the dominant bacterial phyla, with a relative abundance accounting for > 95% of the total ileal bacterial communities (Figure 4A). In contrast to the CT-ileal (CT-I) group, the BCG1-I and BCG2-I groups showed an increase in the relative abundance of Proteobacteria and Firmicutes, from 11.79 to 17.44 (16.81%), and from 64.11 to 69.37 (73.94%), respectively ($P > 0.05$). However, the BCG1-I and BCG2-I groups showed a decrease in the relative abundance of Bacteroidota and Campylobacterota, from 10.25 to 4.19 (4.62%), and from 9.08 to 6.42 (1.98%), respectively ($P > 0.05$). At the family level, in contrast to the CT group, the BCG1 and BCG2 groups showed an increase in the relative abundance of *Peptostreptococcaceae*, *Sphingomonadaceae*, and *Lactobacillaceae*, from 17.6 to 26.67 (26.92%), from 0.04 to 6.89 (5.39%), and from 12.18 to 23.17 (22.97%), respectively ($P > 0.05$). The relative abundance of *Campylobacteraceae* in the BCG1-I and BCG2-I groups was, respectively, 0.68 and 1.44%, and lower than the 7.31% in the CT-I group ($P > 0.05$, Figure 4B). Diets with BCG1 and BCG2 significantly decreased several low abundance bacteria at the family level ($P < 0.05$, Figures 4C, D). For the 35 most dominant ileal genera, the BCG1 and BCG2 diet groups showed significantly lower relative abundance of *Clostridiales bacterium CHKCI001*, *Enterococcus*, *Clostridia_vadinBB60_group*, *Faecalibacterium*, *Phascolarctobacterium*, *Barnesiella*, *Alistipes*, and *Ruminococcaceae UCG-005* than that in the CT group ($P < 0.05$, Figure 4E). The BCG1 diet group showed significantly lower relative abundance of *Lactobacillus*, *Vibrio*, *Pseudomonas*, *Bacteroides*, *Streptococcus*, *Staphylococcus*, and *Bacillus* than that in the CT group ($P < 0.05$). The relative abundance of *Helicobacter* in the BCG2-I group was significantly lower than that in the CT-I and BCG1-I groups ($P < 0.05$). In contrast to the CT-I group, the BCG1-I and BCG2-I groups showed an increase in the relative abundance of *Sphingomonas* from 0.03 to 6.34 (11.55%), whereas *Campylobacter* levels showed a decrease from 7.31 to 0.68 (1.44%) ($P > 0.05$).

In the cecum, Bacteroidota, Firmicutes, Proteobacteria, Campylobacterota, and Fusobacteriota were the major bacterial phyla, and their relative abundance was seldom affected by BCG treatments ($P > 0.05$, Figure 5A). On the basis of T-test and LEfSe results, CT-C broilers had a higher relative abundance of *f_Erysipelotrichaceae*, *f_Peptostreptococcaceae*, and *g_Romboutsia* than that in the BCG1-C and BCG2-C groups, and *f_Erysipelotrichaceae* and *f_Peptostreptococcaceae* levels in the BCG2-C group were significantly higher than those in the BCG1-C group ($P < 0.05$, Figures 5B–F). *f-Lactobacillaceae* and *g_Lactobacillus* were the dominant bacteria in the BCG2-C group, in contrast to the BCG1-C and CT-C groups, whereas *f_Enterococcaceae*, *f_Campylobacteraceae*, *f_Tannerellaceae*, *g_Escherichia-Shigella*, *g_Campylobacter*, and *g_Parabacteroides* were the dominant bacteria in the BCG1-C group, in contrast to the CT-C and BCG2-C groups. The *f_Campylobacteraceae* level in the BCG2-C group was significantly lower than that in the BCG1-C group ($P < 0.05$).

## 3.6. Correlations between ileal microbiota and enzyme activity and nutrient transporters or inflammatory and barrier parameters

A Spearman's correlation analysis was performed to explore the relationships of predominant ileal phyla, families, and genera with the nutrient digestion and absorption, or inflammatory and barrier parameters (Figure 6). *Proteobacteria*, *f_Sphingomonadaceae*, and *Sphingomonas* were significantly positively correlated with *FATP-1* expression, whereas *Alistipes* and *Barnesiella* were significantly negatively correlated with α-amylase activity ($P < 0.05$, Figure 6A). *Bacteroides* and *Limosilactobacillus* showed a significant positive correlation with *SGLT-1* expression and α-amylase activity ($P < 0.05$). *Helicobacter* was significantly positively correlated with maltase activity but negatively correlated with *SGLT-1* expression ($P < 0.05$). *Firmicutes*, *f_Lactobacillaceae*, *Lactobacillus*, *Fusobacterium*, and *Limosilactobacillus* showed significant positive correlations with *ZO-1* expression. *f_Lactobacillaceae* and *Lactobacillus* were significantly positively correlated with *occludin* and *mucin-2* expression. *f_Lactobacillaceae* showed a significantly positive correlation with *claudin-1* expression ($P < 0.05$, Figure 6B). *Bacteroidota*, *Bacteroides*, and *Phascolarctobacterium* were significantly correlated with *TLR 4* and *IL-10* expression, and *Ruminococcaceae UCG-005* was significantly correlated with *TNF-α*, *NF-κB*, and *IL-8* expression ($P < 0.05$). *Proteobacteria*, *f_Sphingomonadaceae*, and *Sphingomonas* showed significant negative correlations with *IL-1β* and *NF-κB* expression ($P < 0.05$).

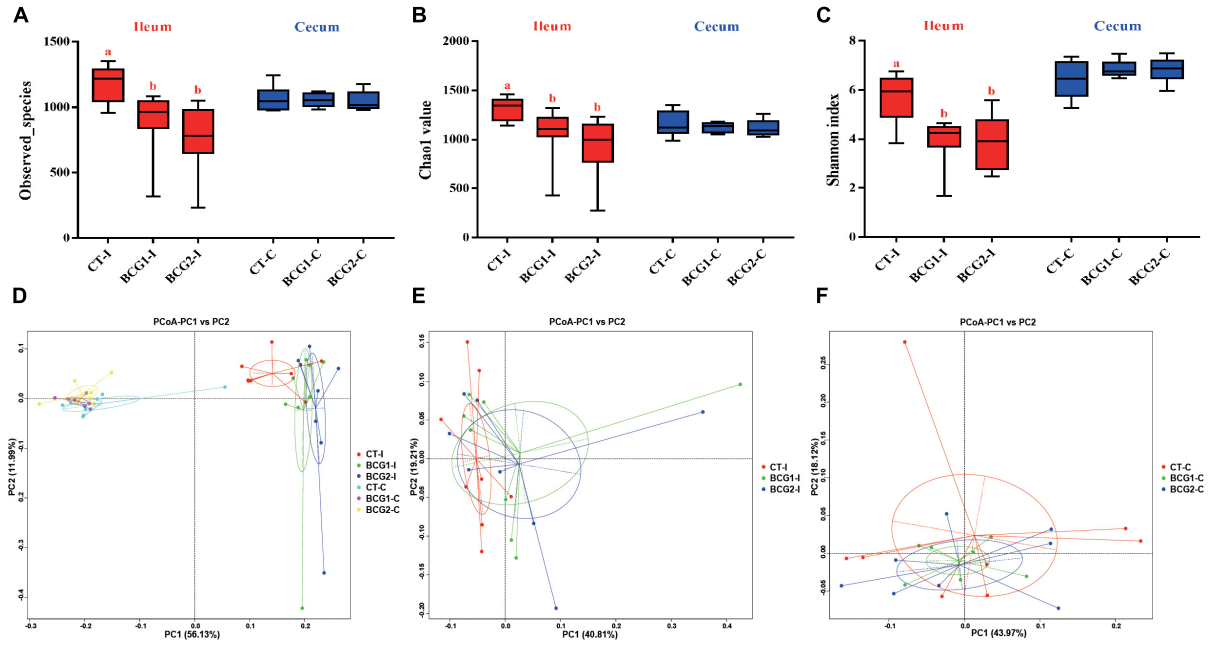


FIGURE 3
Effects of *Bacillus licheniformis* BCG on alpha- and beta-diversity of the microbiota inhabiting the ileum and cecum in broilers. **(A–C)** Bacterial richness and diversity, estimated with Observed_species, Chao1 value, and Shannon index. **(D–F)** Principal coordinate analysis based on weighted UniFrac distances, showing separation in the microbiota between the ileum and cecum, and between groups in the ileum or cecum. Values are mean ± SE ($n$ = 8; BCG1-C, $n$=7). $^{a,b}$Boxes with different letters within the same index indicate a significant difference between groups ($P < 0.05$).

## 4. Discussion

*Bacillus* spp. bacteria serve as a potential alternative to antibiotic growth promoters in livestock production, owing to their stress resistance and probiotic characteristics. Previous studies have shown that dietary supplementation with *B. licheniformis* significantly promoted broiler growth by increasing body weight and decreasing the feed to weight ratio (Liu et al., 2012; Chen and Yu, 2020; Xu et al., 2021). In addition, several studies have indicated that *B. licheniformis* administration promoted broiler growth under heat stress and *Clostridium perfringens* challenge conditions (Song et al., 2014; Zhou et al., 2016; Musa et al., 2019). These findings were consistent with those in our previous study indicating that diets with *B. licheniformis* at a dose of $1.0 \times 10^9$ CFU/kg significantly increases broiler body weight and average daily gain (Wang et al., 2022). Thus, *B. licheniformis* improved broiler growth performance under both normal and stress conditions. The mechanism underlying the improvements in nutrient digestion and host health were likely to involve the formation of beneficial metabolites such as extracellular digestive enzymes, organic acids, and antibacterial peptides (Kim et al., 2004; Knap et al., 2010; Xu et al., 2021). Thus, the activity of α-amylase, maltase, and sucrase in the jejunum and ileum was further determined, because these enzymes participated in the digestion of nutrients.

In poultry, feed starch is generally degraded by α-amylase into smaller molecular oligomers after initial hydrolyzation by the microbiota, and is finally hydrolyzed into maltose and maltotriose in the small intestine (Dhital et al., 2017). Subsequently, maltose and maltotriose are hydrolyzed into glucose by sucrase, and maltase resides at the surfaces of the intestinal villi (Zhou et al., 2021). *B. licheniformis* secretes extracellular enzymes such as α-amylase and proteases (Kaewtapee et al., 2017). In the present study, dietary *B. licheniformis* BCG, compared with the CT diet, significantly increased jejunal and ileal α-amylase, maltase, and sucrase activity, in partial agreement with findings reported by Yang et al. (2021). The α-amylase activity is an important rate-determining factor in starch digestion, because endogenous α-amylase activity is low in broilers (Zhou et al., 2021). The increased α-amylase activity may increase the nutrient digestibility coefficients of starch and organic matter and consequently increase feed metabolizable energy, thus contributing to broiler growth performance (Jiang et al., 2008; Kaczmarek et al., 2014). In addition, nutrient transporters are crucial for nutrient absorption at the brush border membrane in the small intestine. Both FABP-1 and FATP-1 are important for intestinal absorption of lipids and fatty acids, particularly in long-chain fatty acid metabolism and intracellular transportation (Coe et al., 1999; Richieri et al., 1999). GLUT-2 is an $Na^+$-independent transporter responsible for the basolateral exit of glucose from the intestinal mucosa into the portal circulation (Wang et al., 2020). Intestinal LAT-1 participates in branched-chain and aromatic amino acid transport in an $Na^+$-independent manner (Broer, 2008). As previously reported, probiotic supplementation enhances expression of some types of nutrient transporters in the small intestines in animals (Faseleh Jahromi et al., 2016; Duan et al., 2018). In agreement with previous findings, the present study indicated that *B. licheniformis* BCG administration significantly increased *FABP-1*, *FATP-1*, *GLUT-2*, and *LAT-1* expression, to a greater extent in the BCG2 group than the BCG1 group. Thus, dietary supplementation with *B. licheniformis* BCG may aid in starch digestion and the absorption of glucose, amino acids, and fatty acids, thereby resulting in higher growth performance of broilers.

To determine whether *B. licheniformis* BCG contributed to intestinal health, we assessed the gene expression of TLR4 signaling

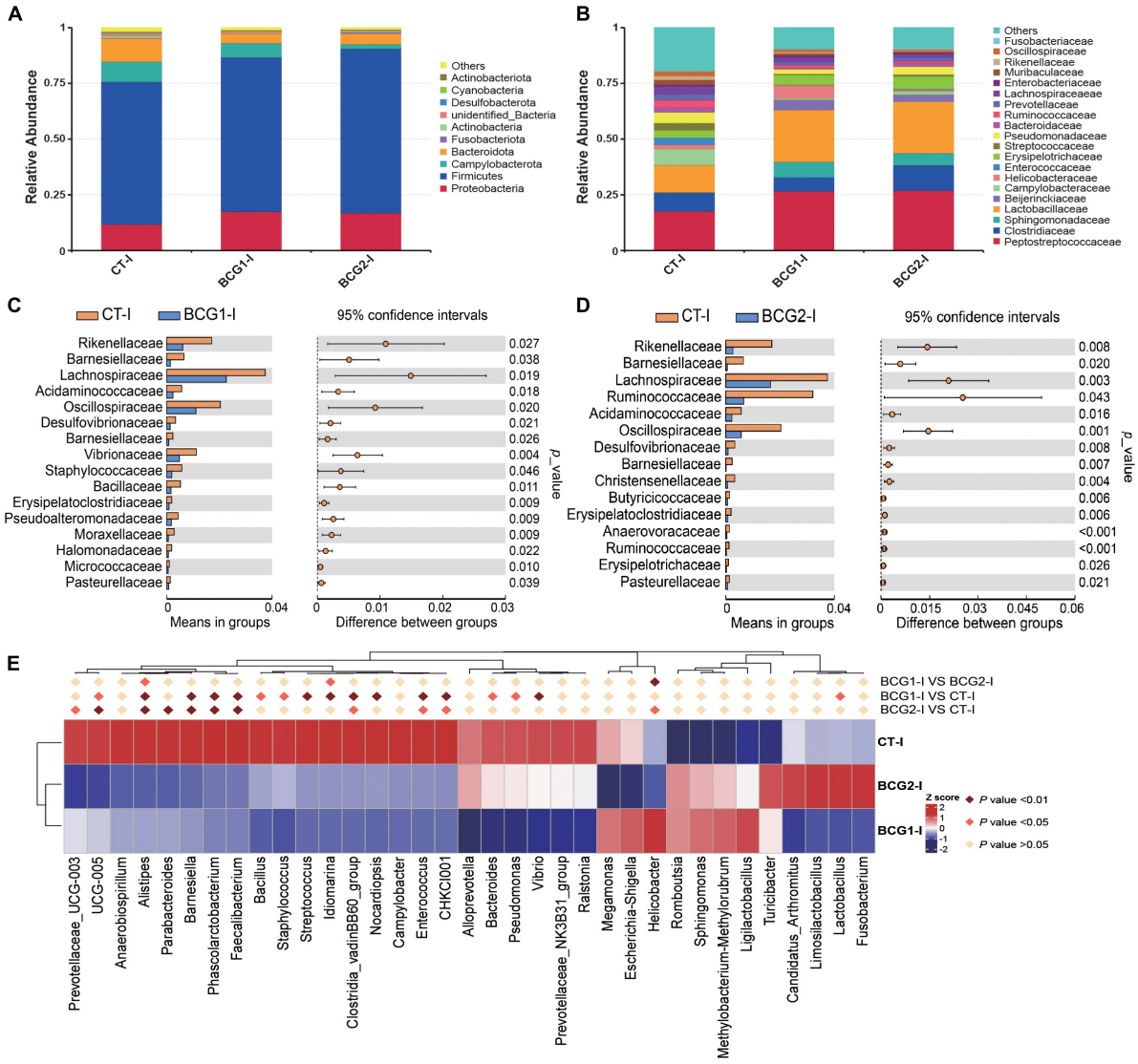


FIGURE 4
Effects of *Bacillus licheniformis* BCG on bacterial composition in the ileum. **(A,B)** Distribution of ileal bacteria at the phylum and family levels. **(C,D)** The *t*-tests were used to assess significant differences at the family level; $P < 0.05$ indicates a significant difference. **(E)** Statistical analysis of differences in the relative abundance of the top 35 genera; Metastat was used to test for significant differences; light pink diamonds indicate $P < 0.05$, and dark pink diamonds indicate $P < 0.01$ between groups ($n = 8$).

pathway related molecules, tight junction proteins, and mucin-2. TLR4, as a pathogen-associated molecular pattern, mediates downstream inflammatory signals through the linker protein MyD88, thereby activating NF-kB, which then translocates into the nucleus and elicits pro-inflammatory cytokine secretion and cellular responses of immune-associated cells (Huebener and Schwabe, 2013; Kan et al., 2021). *B. licheniformis* has been demonstrated to decrease secretion of the pro-inflammatory cytokines IL-8 and IL-6 *in vivo* (Deng et al., 2012; Roselli et al., 2017). Pretreatment with *B. licheniformis* has been found to decrease the serum TNF-α and IL-1β levels in an acetaminophen-induced acute liver injury rat model (Neag et al., 2020). Similarly, *B. licheniformis* also decreases the inflammatory response in an LPS-induced acute inflammation rat model (Deng et al., 2017). Moreover, *B. licheniformis* treatment markedly counteracts the increase in *IL-6*, *IL-8* and *TNF-α* inflammatory gene expression induced by enterotoxigenic *Escherichia coli* F4 *in vitro*. The immunity homeostasis of HT-29 cells is improved by treatment with *B. licheniformis* MCC 2514, on the basis of downregulation of IL-1α, IL-6, IL-8, IL-12, and TNF-α, and upregulation of IL-4, IL-10, TGF-2, and TGF-3 (Rohith and Halami, 2021a). These results are consistent with our observation that the ileal *TLR 4* and *IL-8* expression in the BCG1 and BCG2 groups was significantly lower than that in the CT group. The underlying mechanism was associated with *B. licheniformis* bacteriostasis. As previously reported, *B. licheniformis* inhibited the growth of pathogenic bacteria such as *Kocuria rhizophila* and enterotoxigenic *Escherichia coli* by adhering to the intestines (Rohith and Halami, 2021b; Li et al., 2022a). Another key mechanism through which *B. licheniformis* attenuates inflammation might involve intestinal barrier improvement.

The integrated intestinal barrier plays an important role in maintaining epithelial cell function, because the epithelium is permeable to feed-associated antigens, and luminal pathogens and their toxins (Awad et al., 2017). The transmembrane proteins

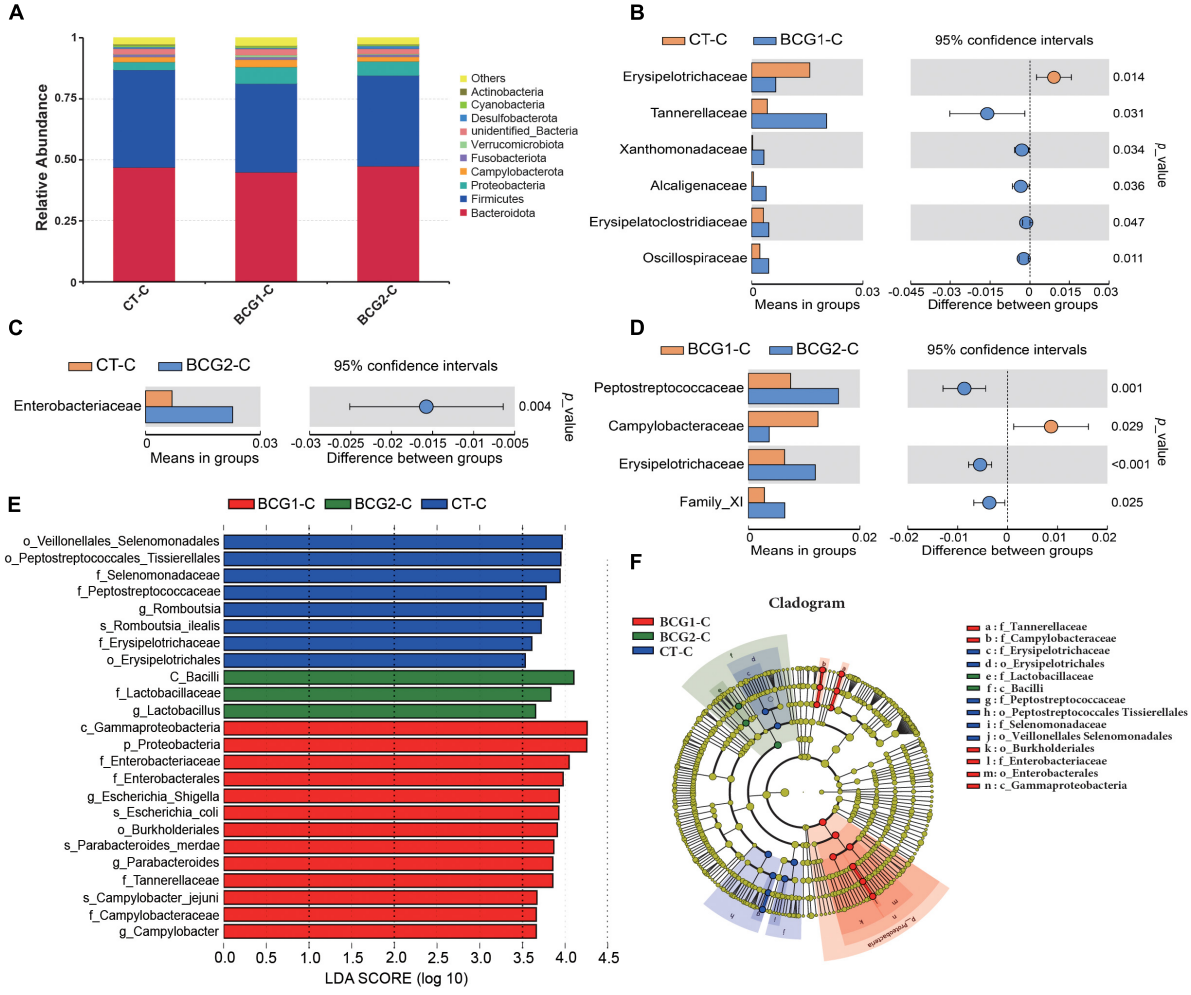


FIGURE 5
Effects of *Bacillus licheniformis* BCG on bacterial composition in the cecum. **(A)** Distribution of cecal bacteria at the phylum level. **(B–D)** The *t*-tests were used to assess significant differences at the family level; $P < 0.05$ indicates a significant difference. **(E)** LEfSe analysis of differences in taxa enrichment in microbial communities between groups; bacterial taxa with a logarithmic LDA score > 3.5 were biomarker taxa. **(F)** Cladogram showing bacteria with significant differences between groups ($n = 8$; BCG1-C, $n=7$).

claudins, occludins, and zonula occludens involved in tight junctions are responsible for connecting epithelial cells and regulating paracellular and intracellular permeability (Aijaz et al., 2006). The regular permeability and integrity of tight junctions are generally negatively affected by stress factors, such as heat stress and pathogen invasion (Song et al., 2014; Musa et al., 2019; Li et al., 2022a). Under *Clostridium perfringens* challenge, dietary *B. licheniformis* significantly increases the gene expression of *claudin-1* and *ZO-1* in the duodenum in broilers at post challenge days 7 and 21 (Musa et al., 2019). In laying hens, *B. licheniformis* administration enhances the intestinal mechanical barrier by upregulating the gene expression of mucin-2 and tight junction proteins (Wang et al., 2017). In *in vitro* experiments, *B. licheniformis* PF9 application has been found to reverse the decrease in ZO-1 and occludin expression in the cell membrane after challenge with enterotoxigenic *Escherichia coli* (Li et al., 2022a). In agreement with previous studies, the present study indicated that a diet with a high dose of *B. licheniformis* BCG resulted in significantly higher *occludin* expression than that in the CT group. Thus, *B. licheniformis* BCG alleviates ileal inflammation partly by enhancing the physical barrier. The improved barrier function resulting from *B. licheniformis* BCG might be attributable to its biologically active substances including bacteriocin and antibacterial peptides, which suppress pathogenic bacterial colonization and balance the intestinal microbiota (Zhou et al., 2016; Kan et al., 2021).

The gut microbiota provides a broad range of functions for hosts, for example, the digestion of complex dietary nutrients, defense against pathogens, enhancement of the intestinal barrier, and promotion of immune maturation (Koh et al., 2016). Owing to differences in histology and function, the broiler intestinal tract is generally divided into the fore- and hindgut. Previous studies in broilers have focused primarily on the hindgut microbiota and their functions, whereas studies on the microbiota in the foregut and their interactions with the host have been limited (Oakley et al., 2014; Huang et al., 2018). In the current study, we analyzed ileal and cecal microbial diversity and composition, and their effects under *B. licheniformis* BCG treatment. The PCoA revealed a clear separation between the ileum and cecum compartments, thus indicating a large difference between them, in agreement with findings from a previous study (van der Wielen et al., 2002). In addition, piglets fed *Bacillus* species probiotics show diminished microbial richness in feces (Kaewtapee et al., 2017; Poulsen et al., 2018). These findings are partially consistent with those in the current

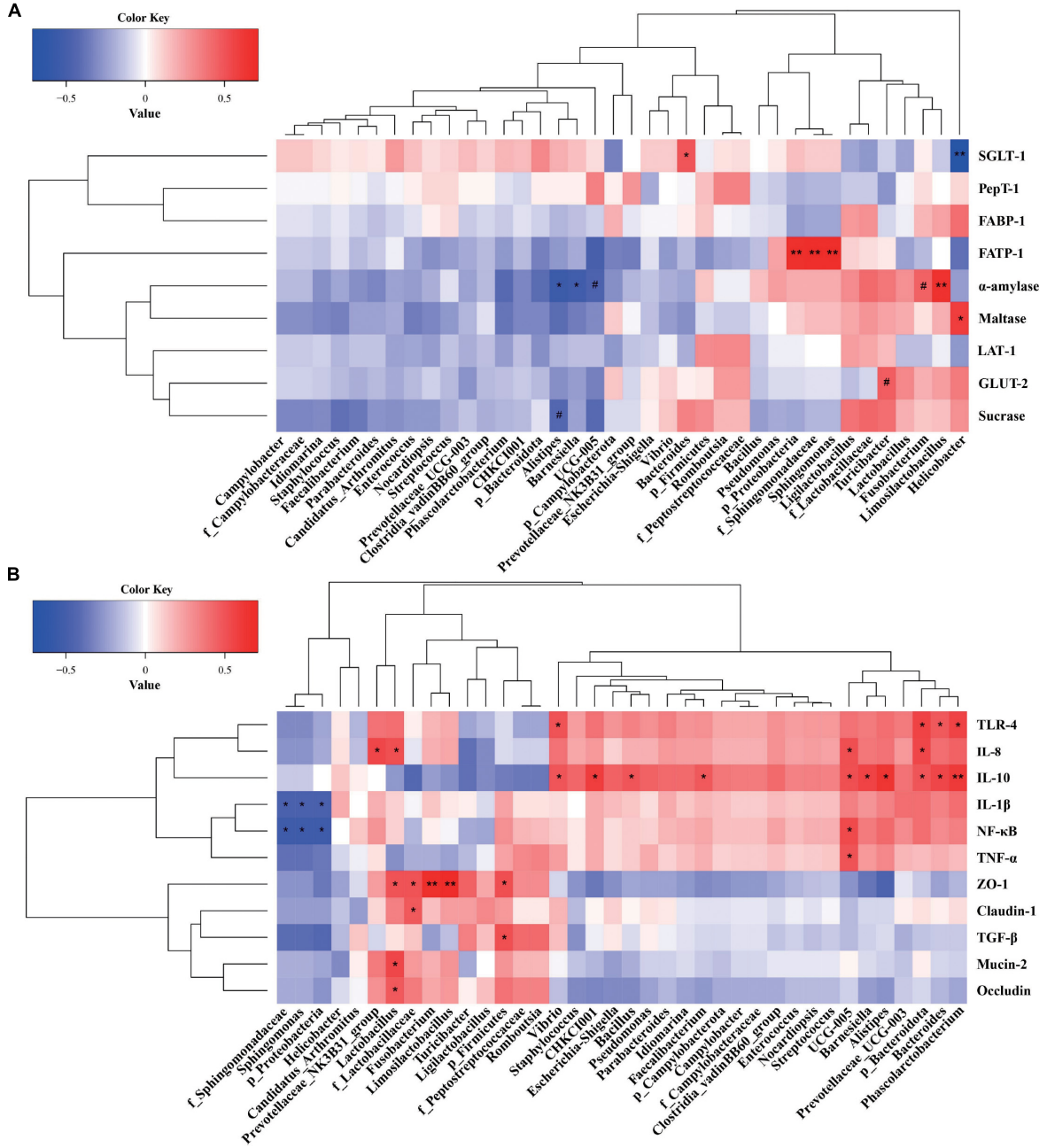


FIGURE 6
**(A)** Correlations between the ileal microbiota and enzyme activity and nutrient transporters. **(B)** Correlations between the ileal microbiota and inflammatory and barrier parameters. Red represents a positive correlation, and blue represents a negative correlation. *$P < 0.05$ and **$P < 0.01$ indicate significant and extremely significant correlations.

study indicating diminished bacterial richness and diversity in the ileum but not cecum in broilers in response to *B. licheniformis* BCG. Our findings indicated that dietary supplementation with *B. licheniformis* BCG shifted the ileal bacterial community structure in broilers. Theoretically, gut bacterial diversification is a gradual process that increases with age, and high diversity is considered a sign of gut bacterial maturity (Micah et al., 2007). Premature formation of an adult-type bacterial community negatively affects host gut immunity (Nylund et al., 2013). Dietary supplementation with *B. licheniformis* BCG significantly decreased ileal inflammation in the present study, a result partially attributed to the decrease in bacterial diversity.

Bacterial composition is also closely associated with host physiology, including digestion, metabolism, and immunity. In the present study, considerable variations in Proteobacteria, Firmicutes, Campylobacterota, and Bacteroidota were found between the ileum and cecum. Oxygen-sensitive Bacteroidota markedly increased from the ileum to the cecum, because it is adapted to a low oxygen environment. However, oxygen-tolerant Proteobacteria and Campylobacterota, such as *Enterococcaceae* and *Campylobacteraceae*, decreased from the ileum to the cecum. Our results are consistent with previous observations that spatial shifts in bacterial composition depend on microenvironment change (Huang et al., 2018; Li et al., 2022b). Firmicutes include *Peptostreptococcaceae*, *Clostridiaceae*, and

*Lactobacillaceae*, which are positively associated with energy intake and have been found to provide an additional 628 kJ of energy when their abundance increases by 20% (Hildebrandt et al., 2009; Jumpertz et al., 2011). This causal relationship has also been found in piglet models (Mach et al., 2015; Li et al., 2022a). Firmicutes fermentation is a more suitable energy source than Bacteroidota because it produces more short chain fatty acids, thus enhancing efficient heat absorption (Krajmalnik-Brown et al., 2012; Pan et al., 2022). We observed that a diet with *B. licheniformis* increased ileal Firmicutes levels but decreased Bacteroidota levels, a finding partially explained by our previous results indicating that *B. licheniformis* administration promoted broiler growth (Wang et al., 2022). In recent studies, *Sphingomonas* involved in the *f_Sphingomonadaceae* has been observed as an abundant bacterium in chicken intestines (Chen et al., 2018; Zhang et al., 2022b). The abundance of *Sphingomonas* is significantly positively correlated with fat catabolism in the liver, serum, and muscle (Li et al., 2020; Zhang et al., 2022b). In addition, *Sphingomonas* participates in lipid metabolism across the entire process of chicken embryonic development (Akinyemi et al., 2020). Diets with *B. licheniformis* resulted in significantly greater ileal *f_Sphingomonadaceae* and *Sphingomonas* levels than those in the CT group; these levels were significantly positively correlated with *FATP-1* expression but significantly negatively correlated with *IL-1*β and *NF-κB* expression. These findings suggested that the shifts in the prevalence of *f_Sphingomonadaceae* and *Sphingomonas* after *B. licheniformis* BCG treatment contributed to the intestinal absorption and oxidative decomposition of lipids and fatty acids, and alleviation of intestinal inflammation.

On the basis of the analysis of the abundance of bacteria in the present study, *Lactobacillus*, *Bacteroides*, *Alistipes*, *Escherichia-Shigella*, *Desulfovibrio*, *Streptococcus*, *Ruminococcaceae_UCG-005*, *Fusobacterium*, and *Campylobacter* predominated in broilers. In general, the presence of *Lactobacillus* is considered beneficial for intestinal health and animal health, owing to their immunomodulation, pathogen inhibition and bacteriocin production ability (Kaewtapee et al., 2017; Zhao et al., 2022b). In the current study, broilers in the BCG2 group had higher *Lactobacillus* levels in both the ileum and cecum than those in the CT or BCG1 group, in agreement with findings from previous studies (Hung et al., 2019; Zhao et al., 2022b). An increase in *Lactobacillus* might have resulted from *B. licheniformis* BCG supplementation, which creates a suitable environment for the colonization of *Lactobacillus* anaerobic bacteria by consumption of oxygen in the gut (Fazelnia et al., 2021). In previous studies, *Lactobacillus* administration has been found to contribute to nutrient absorption by significantly increasing the expression of sugar transporter genes, including *GLUT-2*, *GLUT-5*, *SGLT1*, and *SGLT4* (Faseleh Jahromi et al., 2016). *Lactobacillus* enhances occludin levels and suppresses *Escherichia coli* invasion in intestinal epithelial cells (Resta-Lenert and Barrett, 2003). Moreover, *Lactobacillus* reverses LPS-induced disruption in tight junction proteins, such as occludin, claudin-1, and ZO-1 (Zhou et al., 2014; Roselli et al., 2017). These findings were consistent with our observations indicating that *f_Lactobacillaceae* and *Lactobacillus* were significantly positively correlated with the expression of *ZO-1*, *claudin-1*, *mucin-2*, and *occludin*, and the occludin expression in the BCG2 group was significantly higher than that in the CT group. In addition, *Limosilactobacillus* spp. and *Lactobacillus* spp. have been found to significantly decrease the abundance of *Helicobacter*, one of the most common pathogens globally associated with gastritis and cancer, by 90% and 83%, respectively, in an infected mouse model (Zhao et al., 2022a). Moreover, *Limosilactobacillus* spp. administration significantly increased the prevalence of *Lactobacillus* spp. but decreased the abundance of *Desulfovibrio*. These relationships among *Limosilactobacillus*, *Lactobacillus*, *Helicobacter*, and *Desulfovibrio* were consistent with the microbial structure shift induced by *B. licheniformis* BCG administration. *Limosilactobacillus* showed a significantly positive correlation with *ZO-1* expression and α-amylase activity. These findings indicated that the increase in *f_Lactobacillaceae*, *Lactobacillus*, and *Limosilactobacillus* levels attributed to *B. licheniformis* BCG treatment improved intestinal barrier function through interaction with epithelial cells in broilers.

*Desulfovibrionaceae* is a family of opportunistic pathogens such as *Desulfovibrio*, which is a major sulfate-reducing bacterium that is ubiquitous in human intestines (Ichiishi et al., 2010). These sulfate-reducing bacteria destroy intestinal epithelial cells through generating large amounts of hydrogen sulfide (Zhang-Sun et al., 2015). In agreement with the current findings, *B. licheniformis* DSM5749 administration sustains intestinal health in laying hens by decreasing the *Desulfovibrio* level (Pan et al., 2022). *Alistipes* has been isolated from the appendicular, abdominal, perirectal, and brain abscesses, thus indicating a potential opportunistic pathogenic role in humans (Parker et al., 2020). A high relative abundance of *Alistipes* is strongly associated with gut disorders and host diseases, such as liver fibrosis and non-alcoholic steatohepatitis, which is inhibited by *Bacillus licheniformis* H2 administration (Parker et al., 2020; Zhao et al., 2022b). *Campylobacter* is well known as a major cause of acute bacterial enteritis in humans. Poultry is considered a major reservoir of *Campylobacter* and generally colonized by *Campylobacter* at the age of 2 weeks; therefore, *Campylobacter* may be as a principal vehicle of transmission to humans (Evans and Sayers, 2000; Lamb-Rosteski et al., 2008). Furthermore, *Vibrio*, a potential pathogen, should be harmful for host health (Richards et al., 2016). *Streptococcus* and *Escherichia coli* are pathogens that metabolize proteins in the small intestine (Ma et al., 2017). The above bacteria decreased after *B. licheniformis* BCG treatment, particularly in the BCG2 group. Dietary supplementation with *B. licheniformis* BCG significantly decreased the expression of pro-inflammatory molecules, and thus might decrease the risk of bacterial enteritis in broilers and its associated postinfectious sequelae in humans. However, in the present study, the relative abundance of *Bacteroides*, *Prevotellaceae*_UCG-003, and *Prevotellaceae*_NK3B31_group and *Ruminococcaceae*_UCG-005 were lower in the BCG1 and BCG2 groups than the CT group. As reported, *Prevotella* can metabolize plant cell walls and produce short chain fatty acids that benefit intestinal immunity homeostasis (Ramayo-Caldas et al., 2016). *Prevotella* and *Prevotella_9* show significantly negative correlations with levels of the pro-inflammatory cytokines IL-6 and IL-12 in the jejunum (Han et al., 2022). *Bacteroides* shows a significantly negative correlation with serum IL-6 but a positive correlation with IL-4 (Zhang et al., 2022a). The abundance of *Ruminococcaceae*, beneficial bacteria that are more commonly found in healthy people, is correlated with the production of short chain fatty acids (Koh et al., 2016; Feng et al., 2022). These reports are contrary to those from a present study reporting that bacteria whose abundance decreased after *B. licheniformis* BCG administration are positively correlated

with the pro-inflammatory molecules *IL-8*, *TLR-4*, *NF-κB*, or *TNF-α* expression; therefore, these findings must be verified in further study.

## 5. Conclusion

The present study indicated that dietary *B. licheniformis* BCG significantly increased jejunal and ileal α-amylase, maltase, and sucrase activity; up-regulated ileal *occludin* mRNA levels; and decreased the transcript abundance of *IL-8* and *TLR-4* in the ileum in broilers. Diets with *B. licheniformis* BCG significantly decreased bacterial community richness and diversity in the ileum but not the cecum. Dietary *B. licheniformis* BCG shaped the ileac microbiota; increased the prevalence of *f_Sphingomonadaceae*, *Sphingomonas*, and *Limosilactobacillus*; contributed to nutrient digestion and absorption; increased the prevalence of *f_Lactobacillaceae*, *Lactobacillus*, and *Limosilactobacillus*; and enhanced intestinal barrier function. In addition, dietary *B. licheniformis* BCG decreased microbial community diversity; decreased the abundance of *Desulfovibrio*, *Alistipes Campylobacter*, *Vibrio*, *Streptococcus*, and *Escherichia coli*-Shigella; and down-regulated expression of inflammatory molecules. Therefore, diets with *B. licheniformis* BCG contributed to broiler digestion and absorption of nutrients; enhanced the intestinal physical barrier; and decreased intestinal inflammation by decreasing microbial diversity and optimizing the microbiota structure. Our data provided a theoretical basis for *B. licheniformis* BCG application in broilers.

## 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: NCBI - PRJNA648691.

## Ethics statement

The animal study was reviewed and approved by the Institutional Animal Care and Use Committee of the Institute of Feed Research of Chinese Academy of Agricultural Sciences (FRI-CAAS20210827).

## Author contributions

YH, JW, and PY designed the experiments. XX, DL, HZ, and YH conducted experiments and collected samples. JW, XX, and HC performed sample analysis. YH analyzed the data. YH, PY, and KM wrote and 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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## References

Abdelqader, A., Abuajamieh, M., Hayajneh, F., and Al-Fataftah, A. R. (2020). Probiotic bacteria maintain normal growth mechanisms of heat stressed broiler chickens. *J. Therm. Biol.* 92:102654. doi: 10.1016/j.jtherbio.2020.102654

Aijaz, S., Balda, M. S., and Matter, K. (2006). Tight junctions: Molecular architecture and function. *Int. Rev. Cytol.* 248, 261–298. doi: 10.1016/s0074-7696(06)48005-0

Akinyemi, F. T., Ding, J., Zhou, H., Xu, K., He, C., Han, C., et al. (2020). Dynamic distribution of gut microbiota during embryonic development in chicken. *Poult. Sci.* 99, 5079–5090. doi: 10.1016/j.psj.2020.06.016

Antonissen, G., Eeckhaut, V., Van Driessche, K., Onrust, L., Haesebrouck, F., Ducatelle, R., et al. (2016). Microbial shifts associated with necrotic enteritis. *Avian. Pathol.* 45, 308–312. doi: 10.1080/03079457.2016.1152625

Awad, W. A., Hess, C., and Hess, M. (2017). Enteric pathogens and their toxin-induced disruption of the intestinal barrier through alteration of tight junctions in chickens. *Toxins* 9:60. doi: 10.3390/toxins9020060

Broer, S. (2008). Amino acid transport across mammalian intestinal and renal epithelia. *Physiol. Rev.* 88, 249–286. doi: 10.1152/physrev.00018.2006

Chen, Y. C., and Yu, Y. H. (2020). *Bacillus* licheniformis-fermented products improve growth performance and the fecal microbiota community in broilers. *Poult. Sci.* 99, 1432–1443. doi: 10.1016/j.psj.2019.10.061

Chen, Z. M., Chang, W. H., Zheng, A. J., Zhang, S., Cai, H. Y., and Liu, G. H. (2018). Comparison of gut microbial diversity in Beijing oil and arbor acres chickens. *Rev. Bras. Cienc. Avic.* 20, 37–44. doi: 10.1590/1806-9061-2017-0549

Coe, N. R., Smith, A. J., Frohnert, B. I., Watkins, P. A., and Bernlohr, D. A. (1999). The fatty acid transport protein (FATP1) is a very long chain acyl-CoA synthetase. *J. Biol. Chem.* 274, 36300–36304. doi: 10.1074/jbc.274.51.36300

Collins, S. M., Surette, M., and Bercik, P. (2012). The interplay between the intestinal microbiota and the brain. *Nat. Rev. Microbiol.* 10, 735–742. doi: 10.1038/nrmicro2876

Council, N. R. (1994). *Nutrient requirements of poultry: Ninth revised edition*. Washington, DC: The National Academies Press.

Deng, B., Wu, J., Li, X., Men, X., and Xu, Z. (2017). Probiotics and probiotic metabolic product improved intestinal function and ameliorated LPS-induced injury in rats. *Curr. Microbiol.* 74, 1306–1315. doi: 10.1007/s00284-017-1318-7

Deng, W., Dong, X. F., Tong, J. M., and Zhang, Q. (2012). The probiotic *Bacillus* licheniformis ameliorates heat stress-induced impairment of egg production, gut morphology, and intestinal mucosal immunity in laying hens. *Poult. Sci.* 91, 575–582. doi: 10.3382/ps.2010-01293

Dhital, S., Warren, F. J., Butterworth, P. J., Ellis, P. R., and Gidley, M. J. (2017). Mechanisms of starch digestion by alpha-amylase-structural basis for kinetic properties. *Crit. Rev. Food Sci. Nutr.* 57, 875–892. doi: 10.1080/10408398.2014.922043

Duan, Y., Wang, Y., Dong, H., Ding, X., Liu, Q., Li, H., et al. (2018). Changes in the intestine microbial, digestive, and immune-related genes of litopenaeus vannamei in response to dietary probiotic *Clostridium* butyricum supplementation. *Front. Microbiol.* 9:2191. doi: 10.3389/fmicb.2018.02191

Evans, S. J., and Sayers, A. R. (2000). A longitudinal study of *Campylobacter* infection of broiler flocks in Great Britain. *Prev. Vet. Med.* 46, 209–223. doi: 10.1016/s0167-5877(00)00143-4

Faseleh Jahromi, M., Wesam Altaher, Y., Shokryazdan, P., Ebrahimi, R., Ebrahimi, M., Idrus, Z., et al. (2016). Dietary supplementation of a mixture of *Lactobacillus* strains enhances performance of broiler chickens raised under heat stress conditions. *Int. J. Biometeorol.* 60, 1099–1110. doi: 10.1007/s00484-015-1103-x

Fazelnia, K., Fakhraei, J., Yarahmadi, H. M., and Amini, K. (2021). Dietary supplementation of potential probiotics *Bacillus* subtilis, *Bacillus* licheniformis, and *Saccharomyces* cerevisiae and synbiotic improves growth performance and immune responses by modulation in intestinal system in broiler chicks challenged with *Salmonella* typhimurium. *Probiotics Antimicro* 13, 1081–1092. doi: 10.1007/s12602-020-09737-5

Feng, S., Meng, C., Hao, Z., and Liu, H. (2022). *Bacillus* licheniformis reshapes the gut microbiota to alleviate the subhealth. *Nutrients* 14:1642. doi: 10.3390/nu14081642

Gadde, U., Oh, S. T., Lee, Y. S., Davis, E., Zimmerman, N., Rehberger, T., et al. (2017). The effects of direct-fed microbial supplementation, as an alternative to antibiotics, on growth performance, intestinal immune status, and epithelial barrier gene expression in broiler chickens. *Probiotics Antimicro* 9, 397–405. doi: 10.1007/s12602-017-9275-9

Han, Y., Tang, C., Zhao, Q., Fan, S., Yang, P., and Zhang, J. (2022). Butyrate mitigates lipopolysaccharide-induced intestinal morphological changes in weanling piglets by regulating the microbiota and energy metabolism, and alleviating inflammation and apoptosis. *Microorganisms* 10:2001. doi: 10.3390/microorganisms10102001

Han, Y., Zhao, Q., Tang, C., Li, Y., Zhang, K., Li, F., et al. (2020). Butyrate mitigates weanling piglets from lipopolysaccharide-induced colitis by regulating microbiota and energy metabolism of the gut-liver axis. *Front. Microbiol.* 11:588666. doi: 10.3389/fmicb.2020.588666

Haque, M. A., Wang, F., Chen, Y., Hossen, F., Islam, M. A., Hossain, M. A., et al. (2021). *Bacillus* spp. contamination: A novel risk originated from animal feed to human food chains in south-eastern Bangladesh. *Front. Microbiol.* 12:783103. doi: 10.3389/fmicb.2021.783103

Hildebrandt, M. A., Hoffmann, C., Sherrill-Mix, S. A., Keilbaugh, S. A., Hamady, M., Chen, Y. Y., et al. (2009). High-fat diet determines the composition of the murine gut microbiome independently of obesity. *Gastroenterology* 137, 1716–1724. doi: 10.1053/j.gastro.2009.08.042

Huang, P., Zhang, Y., Xiao, K., Jiang, F., Wang, H., Tang, D., et al. (2018). The chicken gut metagenome and the modulatory effects of plant-derived benzylisoquinoline alkaloids. *Microbiome* 6:211. doi: 10.1186/s40168-018-0590-5

Huebener, P., and Schwabe, R. F. (2013). Regulation of wound healing and organ fibrosis by toll-like receptors. *Biochim. Biophys. Acta.* 1832, 1005–1017. doi: 10.1016/j.bbadis.2012.11.017

Hung, D., Cheng, Y., Chen, W., Hua, K., Pietruszka, A., Dybus, A., et al. (2019). *Bacillus* licheniformis-fermented products reduce diarrhea incidence and alter the fecal microbiota community in weaning piglets. *Animals* 9:1145. doi: 10.3390/ani9121145

Ichiishi, S., Tanaka, K., Nakao, K., Izumi, K., Mikamo, H., and Watanabe, K. (2010). First isolation of *Desulfovibrio* from the human vaginal flora. *Anaerobe* 16, 229–233. doi: 10.1016/j.anaerobe.2010.02.002

Jazi, V., Mohebodini, H., Ashayerizadeh, A., Shabani, A., and Barekatain, R. (2019). Fermented soybean meal ameliorates *Salmonella* typhimurium infection in young broiler chickens. *Poult. Sci.* 98, 5648–5660. doi: 10.3382/ps/pez338

Jiang, Z., Zhou, Y., Lu, F., Han, Z., and Wang, T. (2008). Effects of different levels of supplementary alpha-amylase on digestive enzyme activities and pancreatic amylase mRNA expression of young broilers. *Asian-Austral J. Anim. Sci.* 21, 97–102. doi: 10.5713/ajas.2008.70110

Jumpertz, R., Le, D. S., Turnbaugh, P. J., Trinidad, C., Bogardus, C., Gordon, J. I., et al. (2011). Energy-balance studies reveal associations between gut microbes, caloric load, and nutrient absorption in humans. *Am. J. Clin. Nutr.* 94, 58–65. doi: 10.3945/ajcn.110.010132

Kaczmarek, S. A., Rogiewicz, A., Mogielnicka, M., Rutkowski, A., Jones, R. O., and Slominski, B. A. (2014). The effect of protease, amylase, and nonstarch polysaccharide-degrading enzyme supplementation on nutrient utilization and growth performance of broiler chickens fed corn-soybean meal-based diets. *Poult. Sci.* 93, 1745–1753. doi: 10.3382/ps.2013-03739

Kaewtapee, C., Burbach, K., Tomforde, G., Hartinger, T., Camarinha-Silva, A., Heinritz, S., et al. (2017). Effect of *Bacillus* subtilis and *Bacillus* licheniformis supplementation in diets with low- and high-protein content on ileal crude protein and amino acid digestibility and intestinal microbiota composition of growing pigs. *J. Anim. Sci. Biotechnol.* 8:37. doi: 10.1186/s40104-017-0168-2

Kan, L., Guo, F., Liu, Y., Pham, V. H., Guo, Y., and Wang, Z. (2021). Probiotics *Bacillus* licheniformis improves intestinal health of subclinical necrotic enteritis-challenged broilers. *Front. Microbiol.* 12:623739. doi: 10.3389/fmicb.2021.623739

Kim, Y., Cho, J. Y., Kuk, J. H., Moon, J. H., Cho, J. I., Kim, Y. C., et al. (2004). Identification and antimicrobial activity of phenylacetic acid produced by *Bacillus* licheniformis isolated from fermented soybean, chungkook-jang. *Curr. Microbiol.* 48, 312–317. doi: 10.1007/s00284-003-4193-3

Knap, I., Lund, B., Kehlet, A. B., Hofacre, C., and Mathis, G. (2010). *Bacillus* licheniformis prevents necrotic enteritis in broiler chickens. *Avian. Dis.* 54, 931–935. doi: 10.1637/9106-101509-ResNote.1

Koh, A., De Vadder, F., Kovatcheva-Datchary, P., and Backhed, F. (2016). From dietary fiber to host physiology: Short-chain fatty acids as key bacterial metabolites. *Cell* 165, 1332–1345. doi: 10.1016/j.cell.2016.05.041

Konieczka, P., Nowicka, K., Madar, M., Taciak, M., and Smulikowska, S. (2018). Effects of pea extrusion and enzyme and probiotic supplementation on performance, microbiota activity and biofilm formation in the broiler gastrointestinal tract. *Br. Poult. Sci.* 59, 654–662. doi: 10.1080/00071668.2018.1507017

Konieczka, P., Sandvang, D., Kinsner, M., Szkopek, D., Szyrynska, N., and Jankowski, J. (2022). *Bacillus*-based probiotics affect gut barrier integrity in different ways in chickens subjected to optimal or challenge conditions. *Vet. Microbiol.* 265:109323. doi: 10.1016/j.vetmic.2021.109323

Krajmalnik-Brown, R., Ilhan, Z. E., Kang, D. W., and DiBaise, J. K. (2012). Effects of gut microbes on nutrient absorption and energy regulation. *Nutr. Clin. Pract.* 27, 201–214. doi: 10.1177/0884533611436116

Kridtayopas, C., Rakangtong, C., Bunchasak, C., and Loongyai, W. (2019). Effect of prebiotic and synbiotic supplementation in diet on growth performance, small intestinal morphology, stress, and bacterial population under high stocking density condition of broiler chickens. *Poult. Sci.* 98, 4595–4605. doi: 10.3382/ps/pez152

Lamb-Rosteski, J. M., Kalischuk, L. D., Inglis, G. D., and Buret, A. G. (2008). Epidermal growth factor inhibits *Campylobacter* jejuni-induced claudin-4 disruption, loss of epithelial barrier function, and *Escherichia coli* translocation. *Infect. Immun.* 76, 3390–3398. doi: 10.1128/IAI.01698-07

Li, Q., Li, L., Chen, Y., Yu, C., Azevedo, P., Gong, J., et al. (2022a). *Bacillus* licheniformis PF9 improves barrier function and alleviates inflammatory responses against enterotoxigenic *Escherichia coli* F4 infection in the porcine intestinal epithelial cells. *J. Anim. Sci. Biotechnol.* 13:86. doi: 10.1186/s40104-022-00746-8

Li, Y., Han, Y., Zhao, Q., Tang, C., Zhang, J., and Qin, Y. (2022b). Fermented soy and fish protein dietary sources shape ileal and colonic microbiota, improving nutrient digestibility and host health in a piglet model. *Front. Microbiol.* 13:911500. doi: 10.3389/fmicb.2022.911500

Li, R., Hou, G., Jiang, X., Song, Z., Fan, Z., Hou, D. X., et al. (2019). Different dietary protein sources in low protein diets regulate colonic microbiota and barrier function in a piglet model. *Food Funct.* 10, 6417–6428. doi: 10.1039/c9fo01154d

Li, S., Yan, C., Liu, T., Xu, C., Wen, K., Liu, L., et al. (2020). Research note: Increase of bad bacteria and decrease of good bacteria in the gut of layers with vs. without hepatic steatosis. *Poult. Sci.* 99, 5074–5078. doi: 10.1016/j.psj.2020.07.007

Liu, X., Yan, H., Lv, L., Xu, Q., Yin, C., Zhang, K., et al. (2012). Growth performance and meat quality of broiler chickens supplemented with *Bacillus* licheniformis in drinking water. *Asian-Australas J. Anim. Sci.* 25, 682–689. doi: 10.5713/ajas.2011.11334

Ma, N., Tian, Y., Wu, Y., and Ma, X. (2017). Contributions of the interaction between dietary protein and gut microbiota to intestinal health. *Curr. Protein Pept. Sci.* 18, 795–808. doi: 10.2174/1389203718666170216153505

Mach, N., Berri, M., Estelle, J., Levenez, F., Lemonnier, G., Denis, C., et al. (2015). Early-life establishment of the swine gut microbiome and impact on host phenotypes. *Environ. Microbiol. Rep.* 7, 554–569. doi: 10.1111/1758-2229.12285

Micah, H., Claire, F.-L., Rob, K., and Gordon Jeffrey, I. (2007). The human microbiome project: Exploring the microbial part of ourselves in a changing world. *Nature* 449, 804–810. doi: 10.1038/nature06244

Mingmongkolchai, S., and Panbangred, W. (2018). *Bacillus* probiotics: An alternative to antibiotics for livestock production. *J. Appl. Microbiol.* 124, 1334–1346. doi: 10.1111/jam.13690

Musa, B. B., Duan, Y., Khawar, H., Sun, Q., Ren, Z., Elsiddig Mohamed, M. A., et al. (2019). *Bacillus* subtilis B21 and *Bacillus* licheniformis B26 improve intestinal health and performance of broiler chickens with *Clostridium* perfringens-induced necrotic enteritis. *J. Anim. Physiol. Anim. Nutr.* 103, 1039–1049. doi: 10.1111/jpn.13082

Neag, M. A., Catinean, A., Muntean, D. M., Pop, M. R., Bocsan, C. I., Botan, E. C., et al. (2020). Probiotic *Bacillus* spores protect against acetaminophen induced acute liver injury in rats. *Nutrients* 12:632. doi: 10.3390/nu12030632

Nylund, L., Satokari, R., Nikkilä, J., Rajilić-Stojanović, M., Kalliomäki, M., Isolauri, E., et al. (2013). Microarray analysis reveals marked intestinal microbiota aberrancy in infants having eczema compared to healthy children in at-risk for atopic disease. *BMC microbiol.* 13:12. doi: 10.1186/1471-2180-13-12

Oakley, B. B., Lillehoj, H. S., Kogut, M. H., Kim, W. K., Maurer, J. J., Pedroso, A., et al. (2014). The chicken gastrointestinal microbiome. *FEMS Microbiol. Lett.* 360, 100–112. doi: 10.1111/1574-6968.12608

Pan, X., Cai, Y., Kong, L., Xiao, C., Zhu, Q., and Song, Z. (2022). Probiotic effects of *Bacillus* licheniformis DSM5749 on growth performance and intestinal microecological balance of laying hens. *Front. Nutr.* 9:868093. doi: 10.3389/fnut.2022.868093

Parker, B. J., Wearsch, P. A., Veloo, A. C. M., and Rodriguez-Palacios, A. (2020). The genus *Alistipes*: Gut bacteria with emerging implications to inflammation, cancer, and mental health. *Front. Immunol.* 11:906. doi: 10.3389/fimmu.2020.00906

Poulsen, A. R., Jonge, N., Nielsen, J. L., Hojberg, O., Lauridsen, C., Cutting, S. M., et al. (2018). Impact of *Bacillus* spp. spores and gentamicin on the gastrointestinal microbiota of suckling and newly weaned piglets. *PLoS One* 13:e0207382. doi: 10.1371/journal.pone.0207382

Ramayo-Caldas, Y., Mach, N., Lepage, P., Levenez, F., Denis, C., Lemonnier, G., et al. (2016). Phylogenetic network analysis applied to pig gut microbiota identifies an ecosystem structure linked with growth traits. *ISME J.* 10, 2973–2977. doi: 10.1038/ismej.2016.77

Resta-Lenert, S., and Barrett, K. (2003). Live probiotics protect intestinal epithelial cells from the effects of infection with enteroinvasive *Escherichia coli* (EIEC). *Gut* 52, 988–997. doi: 10.1136/gut.52.7.988

Richards, G. P., Fay, J. P., Uknalis, J., Olanya, O. M., and Watson, M. A. (2016). Purification and host specificity of predatory *Halobacteriovorax* isolates from seawater. *Appl. Environ. Microbiol.* 82, 922–927. doi: 10.1128/AEM.03136-15

Richieri, G., Ogata, R., and Kleinfeld, A. (1999). Fatty acid interactions with native and mutant fatty acid binding proteins. *Mol. Cell Biochem.* 192, 77–85. doi: 10.1007/978-1-4615-4929-1_9

Rohith, H. S., and Halami, P. M. (2021a). The combined effect of potential probiotic *Bacillus* licheniformis MCC 2514 and *Bifidobacterium* breve NCIM 5671 towards anti-inflammatory activity on HT-29 cell lines. *Probiotics Antimicro* doi: 10.1007/s12602-021-09851-y [Epub ahead of print].

Rohith, H. S., and Halami, P. M. (2021b). *In vitro* validation studies for adhesion factor and adhesion efficiency of probiotic *Bacillus* licheniformis MCC 2514 and *Bifidobacterium* breve NCIM 5671 on HT-29 cell lines. *Arch. Microbiol.* 203, 2989–2998. doi: 10.1007/s00203-021-02257-y

Roselli, M., Pieper, R., Rogel-Gaillard, C., de Vries, H., Bailey, M., Smidt, H., et al. (2017). Immunomodulating effects of probiotics for microbiota modulation, gut health and disease resistance in pigs. *Anim. Feed Sci. Tech.* 233, 104–119. doi: 10.1016/j.anifeedsci.2017.07.011

Sanders, M. E., Merenstein, D. J., Reid, G., Gibson, G. R., and Rastall, R. A. (2019). Probiotics and prebiotics in intestinal health and disease: From biology to the clinic. *Nat. Rev. Gastroenterol Hepatol.* 16, 605–616. doi: 10.1038/s41575-019-0199-6

Song, J., Xiao, K., Ke, Y. L., Jiao, L. F., Hu, C. H., Diao, Q. Y., et al. (2014). Effect of a probiotic mixture on intestinal microflora, morphology, and barrier integrity of broilers subjected to heat stress. *Poult. Sci.* 93, 581–588. doi: 10.3382/ps.2013-03455

Strandwitz, P. (2018). Neurotransmitter modulation by the gut microbiota. *Brain Res.* 1693, 128–133. doi: 10.1016/j.brainres.2018.03.015

van der Wielen, P. W., Keuzenkamp, D. A., Lipman, L. J., van Knapen, F., and Biesterveld, S. (2002). Spatial and temporal variation of the intestinal bacterial community in commercially raised broiler chickens during growth. *Microb. Ecol.* 44, 286–293. doi: 10.1007/s00248-002-2015-y

Wang, J., Chen, L., Li, D., Yin, Y., Wang, X., Li, P., et al. (2008). Intrauterine growth restriction affects the proteomes of the small intestine, liver, and skeletal muscle in newborn pigs. *J. Nutr.* 138, 60–66. doi: 10.1093/jn/138.1.60

Wang, J., Han, Y., Cai, H., Wen, Z., Xu, X., Zhao, Z., et al. (2022). Effects of different levels of *Bacillus* licheniformis on growth performance, immune and intestinal morphology of broilers. *China Anim. Husb. Vet. Med.* 49, 4593–4603. doi: 10.16431/j.cnki.1671-7236.2022.12.000

Wang, W., Li, Z., Han, Q., Guo, Y., Zhang, B., and D'Inca, R. (2016). Dietary live yeast and mannan-oligosaccharide supplementation attenuate intestinal inflammation and barrier dysfunction induced by *Escherichia coli* in broilers. *Br. J. Nutr.* 116, 1878–1888. doi: 10.1017/S0007114516004116

Wang, W., Wang, J., Zhang, H., Wu, S., and Qi, G. (2020). Effects of *Clostridium* butyricum on production performance and intestinal absorption function of laying hens in the late phase of production. *Anim. Feed Sci. Tech.* 264:114476. doi: 10.1016/j.anifeedsci.2020.114476

Wang, Y., Du, W., Lei, K., Wang, B., Wang, Y., Zhou, Y., et al. (2017). Effects of dietary *Bacillus* licheniformis on gut physical barrier, immunity, and reproductive hormones of laying hens. *Probiotics Antimicro.* 9, 292–299. doi: 10.1007/s12602-017-9252-3

Xu, Y., Yu, Y., Shen, Y., Li, Q., Lan, J., Wu, Y., et al. (2021). Effects of *Bacillus* subtilis and *Bacillus* licheniformis on growth performance, immunity, short chain fatty acid production, antioxidant capacity, and cecal microflora in broilers. *Poult. Sci.* 100:101358. doi: 10.1016/j.psj.2021.101358

Yang, G. Y., Zhu, Y. H., Zhang, W., Zhou, D., Zhai, C. C., and Wang, J. F. (2016). Influence of orally fed a select mixture of *Bacillus* probiotics on intestinal T-cell migration in weaned MUC4 resistant pigs following *Escherichia coli* challenge. *Vet. Res.* 47:71. doi: 10.1186/s13567-016-0355-8

Yang, J., Huang, K., Wang, J., Wu, D., Liu, Z., Yu, P., et al. (2021). Combined use of *Bacillus* subtilis yb-114,246 and *Bacillus* licheniformis yb-214,245 improves body growth performance of chinese huainan partridge shank chickens by enhancing intestinal digestive profiles. *Probiotics Antimicro.* 13, 327–342. doi: 10.1007/s12602-020-09691-2

Zhang, R., Shi, X., Chen, Y., Liu, J., Wu, Y., and Xu, Y. (2022a). Multi-omics revealed the protective effects of phamnolipids in lipopolysaccharide challenged broilers. *Front. Immunol.* 13:824664. doi: 10.3389/fimmu.2022.824664

Zhang, X., Hu, Y., Ansari, A. R., Akhtar, M., Chen, Y., Cheng, R., et al. (2022b). Caecal microbiota could effectively increase chicken growth performance by regulating fat metabolism. *Microb. Biotechnol.* 15, 844–861. doi: 10.1111/1751-7915.13841

Zhang-Sun, W., Augusto, L. A., Zhao, L., and Caroff, M. (2015). Desulfovibrio desulfuricans isolates from the gut of a single individual: Structural and biological lipid A characterization. *FEBS Lett.* 589, 165–171. doi: 10.1016/j.febslet.2014.11.042

Zhao, Y., Li, Z., Zhao, L., Wang, J., Wang, F., Zhang, Q., et al. (2022a). Two novel lactic acid bacteria, *Limosilactobacillus* fermentum MN-LF23 and *Lactobacillus* gasseri MN-LG80, inhibited *Helicobacter* pylori infection in C57BL/6 mice. *Food Funct.* 13, 11061–11069. doi: 10.1039/d2fo02034c

Zhao, Y., Zeng, Y., Zeng, D., Wang, H., Sun, N., Xin, J., et al. (2022b). Dietary probiotic supplementation suppresses subclinical necrotic enteritis in broiler chickens in a microbiota-dependent manner. *Front. Immunol.* 13:855426. doi: 10.3389/fimmu.2022.855426

Zhou, H., Wu, Y., Sun, X., Yin, D., Wang, Y., Mahmood, T., et al. (2021). Effects of exogenous alpha-(1,4)-amylase on the utilisation of corn starch and glucose metabolism in broiler chickens. *Animal* 15:100396. doi: 10.1016/j.animal.2021.100396

Zhou, M., Zeng, D., Ni, X., Tu, T., Yin, Z., Pan, K., et al. (2016). Effects of *Bacillus* licheniformis on the growth performance and expression of lipid metabolism-related genes in broiler chickens challenged with *Clostridium* perfringens-induced necrotic enteritis. *Lipids Health Dis.* 15:48. doi: 10.1186/s12944-016-0219-2

Zhou, M., Zhu, J., Yu, H., Yin, X., Sabour, P. M., Zhao, L., et al. (2014). Investigation into *in vitro* and *in vivo* models using intestinal epithelial IPEC-J2 cells and *Caenorhabditis* elegans for selecting probiotic candidates to control porcine enterotoxigenic *Escherichia coli*. *J. Appl. Microbiol.* 117, 217–226. doi: 10.1111/jam.12505

Zuo, Z., Li, Q., Guo, Y., Li, X., Huang, S., Hegemann, J. H., et al. (2020). Feed-borne *Bacillus* cereus exacerbates respiratory distress in chickens infected with chlamydia psittaci by inducing haemorrhagic pneumonia. *Avian. Pathol.* 49, 251–260. doi: 10.1080/03079457.2020.1716940

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# Effects of inoculation with active microorganisms derived from adult goats on growth performance, gut microbiota and serum metabolome in newborn lambs

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This study evaluated the effects of inoculation with adult goat ruminal fluid on growth, health, gut microbiota and serum metabolism in lambs during the first 15days of life. Twenty four Youzhou dark newborn lambs were selected and randomly distributed across 3 treatments ($n$=8/group): autoclaved goat milk inoculated with 20mL sterilized normal saline (CON), autoclaved goat milk inoculated with 20mL fresh ruminal fluid (RF) and autoclaved goat milk inoculated with 20mL autoclaved ruminal fluid (ARF). Results showed that RF inoculation was more effective at promoting recovery of body weight. Compared with CON, greater serum concentrations of ALP, CHOL, HDL and LAC in the RF group suggested a better health status in lambs. The relative abundance of *Akkermansia* and *Escherichia-Shigella* in gut was lower in the RF group, whereas the relative abundance of *Rikenellaceae_RC9_gut_group* tended to increase. Metabolomics analysis shown that RF stimulated the metabolism of bile acids, small peptides, fatty acids and Trimethylamine-N-Oxide, which were found the correlation relationship with gut microorganisms. Overall, our study demonstrated that ruminal fluid inoculation with active microorganisms had a beneficial impact on growth, health and overall metabolism partly through modulating the gut microbial community.

KEYWORDS

**active microorganism, inoculation with rumen fluid, newborn lambs, gut microbiota, blood metabolomics**

## 1. Introduction

The proper management of the pre-ruminant animal is important for ensuring they are able to face the weaning and early post-weaning periods without experiencing excessive stress. This represents one important pillar of a successful cattle and small ruminant industry. Intestinal microorganisms are important for regulating the health and development in the pre-wean ruminant animal. For example, intestinal bacterial imbalance is associated with disordered nutrient digestion and absorption, growth retardation, diarrhea, dehydration, and death (Signorini et al., 2012; Lyoo et al., 2018). Therefore, research focused on the establishment of an optimal intestinal microbiota

would be beneficial in terms of promoting the development and health of pre-wean ruminants.

The microflora in ruminants is not fully developed at the moment of birth (Baldwin et al., 2004). Microbial colonization in pre-ruminans is divided into three stages: (1) the first 2–3 days of life are the initial colonization stage of "pioneer" bacteria, characterized by a unique profile relative to later stages of growth (Haenel, 2010). A large proportion of the bacterial community at this stage is parthenogenic and exclusively anaerobic but aerobic bacteria are also present (Minato et al., 1992); (2) the transition from colostrum to mature milk or milk replacer during which the ruminal epithelium microbial community and the ruminal environment change significantly with a dramatic decrease in aerobic and parthenogenic anaerobic bacteria (Rey et al., 2012); and (3) from day 14 to day 28 or at weaning, where bacterial communities no longer exhibit significant time-related changes, but rather change based on feeding behavior and growth (Meale et al., 2016). Bacterial species and abundance stabilize at the phylum level by the 15 day of age, and the bacterial community structure reaches a certain level of stability in about 3–4 weeks (Guzman et al., 2015). The ruminal microbiota digest fodder and other plant materials to produce volatile fatty acids (VFAs) and microbial crude protein (MCP), both of which provide energy and amino acids for ruminants (Koike and Kobayashi, 2009; Mccann et al., 2014). These are essential for maintenance of health, digestive efficiency, and growth performance in the growing ruminant animal (Morrison et al., 2010). Therefore, the first 15 days after birth are an important stage where the establishment of microbial flora can be regulated.

In a previous study, inoculation with a ruminal fluid increased the weight of calves during weaning partly due to a microbial profile that enhanced starch digestion (Ziolecki et al., 1984). Direct-fed microorganisms were reported to improve immunity of calves by balancing gut microbe profiles, thus, helping them cope with stressful conditions (Krehbiel et al., 2003). Mechanistically, the gut microbiota can stimulate immune responses through its effect on digestion and metabolism (Anand and Mande, 2018). For example, it has been demonstrated that microbiota can regulate metabolism of glutamate and creatine, which in turn impacts the immune response (Ding et al., 2021). Therefore, accumulating evidence highlights that the establishment of the microflora is important in newborn ruminants and can influence their metabolism.

Some studies reported that the original composition of the rumen returned to a pre-intervention state after exogenous microbiota interventions were discontinued (Weimer, 2015). In spite of this, there are data indicating that exogenous microbiota transplantation could improve ruminal fermentation and digestion. To our knowledge, few studies have focused on the effect of ruminal microbial transplantation within 15 days after birth on development and health (Jami et al., 2013). Thus, we hypothesized that inoculation with ruminal fluid in newborn lambs could promote growth and health through its effect on the establishment of an "ideal" gut microbiota. Such benefits are reflected in blood metabolite profiles. To address this hypothesis, Youzhou dark newborn lambs were inoculated with sterilized normal saline (CON), fresh ruminal fluid (RF) with active microorganisms or autoclaved ruminal fluid (ARF) without active microorganisms. Growth performance, health status, gut microbiota and blood metabolomics were performed to evaluate treatment effects during the first 15 days after birth.

## 2. Materials and methods

The study procedures and use animals were approved by the Ethics Committee in Chongqing Academy of animal sciences (approval number: xky-20180716, 9 June 2021).

### 2.1. Ruminal inoculum preparation

Six healthy Youzhou dark adult goats (three female and three male, ~30 kg) were used as sources of ruminal fluid. All goats were fed with the same diet for 3 weeks prior to ruminal fluid collection ~2 h post-feeding *via* oral tubing, strained into a container through four layers of cheesecloth and then pooled into a composited sample. All the composited ruminal fluid was divide into two samples. One sample was directly stored at −80°C and served as the "fresh" ruminal fluid (RF). The other portion of sample was sterilized at 205.8 KPa and 132°C for 10 min and served as the autoclaved Ruminal Fluid (ARF), which was subsequently stored at −80°C. All original samples were kept at −80°C until use as the inoculum.

### 2.2. Animals and treatments

A total of 24 Youzhou dark newborn lambs were selected and randomly assigned to one of 3 treatments for a feeding period of 15 days in individual pens. During the initial 48 h after birth, all lambs received colostrum from their mothers, and were then separated from their mothers immediately. Treatments consisted of (1) sterilized goat milk inoculated with sterilized normal saline as the control group (CON); (2) sterilized goat milk inoculated with RF (RF) and (3) sterilized goat milk inoculated with ARF (ARF). Sterilized goat milk was fed 4 times (each time ~100 mL) daily (0800, 1200, 1600 and 2000 h) to ensure that all lambs had adequate nutrition. From day 3 to 7 after birth, 20 mL ruminal fluid or autoclaved ruminal fluid was maintained at 39°C in a prewarmed thermostat water bath. It was then inoculated *via* the esophagus of each lamb using a soft stomach tube and a 20 mL syringe 2 h after the morning feeding (inoculate once a day, a total of 20 mL). The experiment lasted 15 days, and the all lambs were slaughtered on day 16.

### 2.3. Growth performance and sampling

The live weight (LW) of each lamb was recorded every 3 days before the morning feeding during the experimental period (15 days). Average daily gain (ADG) was calculated as (final LW–initial LW)/days on study. On the 16th day of the experimental period, a blood sample (~5 mL) was collected from the jugular vein and then placed into blood collection tubes. Blood samples were centrifuged at 2,500 rpm at 4°C for 15 min to obtain serum and then stored at −80°C until analysis. Fecal samples from each lamb were collected on the 16th day of the experimental period and stored at −80°C until gut microbiota profiling.

### 2.4. Sample analyses

#### 2.4.1. Blood biochemical indices determination

An automatic biochemical analyzer (Beckman Coulter AU680) was used to perform blood biochemical analysis. Briefly, assays

included colorimetric kits (modified kinetic Jaffe method), turbidimetry, latex agglutination, homogeneous EIA or indirect ISE according to the Beckman Coulter AU680 analyzer specifications.

#### 2.4.2. 16S rRNA sequencing of gut microbiota

Total DNA was extracted from 1 g feces using the MoBio PowerSoil DNA Isolation Kit (12855-50, MoBio, United States) according to the manufacturer's instructions. The quantity and quality of DNA were measured using NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, United States) Then DNA integrity was determined with 1% agarose gel electrophoresis.

Subsequently, the V3-V4 hypervariable region of the 16S rRNA gene was PCR-amplified with the universal primer pair 338F (5′-ACTCCTACGGGAGGCAGCAG-3′) and 806R (5′-GGACTACHVGGGTWTCTAAT-3′) (Liu et al., 2022). The PCR amplification was based on the protocol of Chan et al. (2015). Briefly, 10 bp barcode sequence was added to the 5′ end of the forward and reverse primers (provided by Allwegene Company, Beijing). PCR amplification was performed with a 25 μL reaction system including 12.5 μL Taq PCR MasterMix (2×), 3 μL BSA (2 ng/μL), 1 μL (5 μM) forward primer, 1 μL (5 μM) reverse primer, 2 μL dDNA (The total amount of DNA added is 30 ng) and 5.5 μL $ddH_2O$. Thermal cycling parameters were as follows: 95°C for 5 min, 28 cycles of 95°C for 45 s, 55°C for 50 s, 72°C for 45 s and final extension 72°C for 10 min. Purification of PCR products was carried out using Agencourt AMPure XP Kit (Beckman, Brea, CA, United States). Real-time PCR was used for PCR product quantification. Deep sequencing was carried out using IllluminaMiSeq PE300 platform at Allwegene Company (Beijing, China). Image analysis, base calling, and error estimation were performed using Illumina Analysis Pipeline Version 2.6.

Read qualification was performed using Illumina Analysis Pipeline Version 2.6. The low-quality sequences with length < 230 bp, average Phred scores <20 and ambiguous bases or false matches to primer sequences and barcode tags were removed. High-quality sequences were clustered into operational taxonomic units (OTUs) at a similarity level of 97% using Uparse algorithm of Vsearch (v2.7.1) software. The Ribosomal Database Project (RDP) Classifier tool was used to conduct OTU taxonomic classification into different taxonomic groups against the SILVA128 database. The rarefaction curve generation and richness and diversity indices calculation were performed using QIIME (version 1.8.0) based on the OTU information.

### 2.5. Metabolite extraction

Metabolomics was performed at Allwegene Company (Beijing, China). Briefly, a total of 20 μL of sample was transferred to an Eppendorf tube. A volume of 80 μL extraction solvent was added (acetonitrile: methanol = 1: 1, containing isotopically-labeled internal standard mixture). The mixture was sonicated in an ice-water bath for 10 min and incubated at −40°C for 1 h to precipitate proteins. The samples were centrifuged at 4°C, 12,000*g* for 15 min, then supernatant fluid was transferred into a EP tube new glass vial for UHPLC–MS–MS analysis. QC samples were created by merging equal aliquots of supernatant fluid from each sample.

### 2.6. UHPLC-MS-MS analysis

UHPLC-MS-MS analysis was performed using a UHPLC system (Vanquish, Thermo Fisher Scientific) with a UPLC BEH amide column (2.1 mm 100 mm, 1.7 m) coupled to a Q Exactive HFX mass spectrometer (Orbitrap MS, Thermal). Mobile phase consisted of 25 mmol/L ammonium acetate and 25 mmol/L ammonia (pH = 9.75). The autosampler was set to 2 L injection volume at 4°C. The QE HFX mass spectrometer with the acquisition software information-dependent acquisition (IDA) mode (Xcalibur, Thermo) was used for obtaining MS/MS spectra. The ESI conditions were: Sheath gas flow of 30 Arb, auxiliary gas flow of 25 Arb, capillary temperature of 350°C, and full MS resolution of 60,000.

Metabolomics analysis was performed based on our previous studies with slight modifications (Dong et al., 2018; Fu et al., 2021). Briefly, principle component analysis (PCA) and (orthogonal) partial least-squares-discriminant analysis (OPLS-DA) were performed using the R package metaX to monitor the reproducibility of the instrument and the differential analysis of metabolic characteristics. Parameters R2Y and Q2 were >0.5 indicating a robust model with prominent predictive ability. Metabolites with variable importance for projection (VIP) values exceeding 1 and $p < 0.05$ were selected as the important metabolites between the comparison of two groups.

### 2.7. Statistical analysis

Statistical analysis of weight indices, blood biochemical indices and diversity and relative abundance of gut microbiota were performed using GraphPad Prism 7.0 (GraphPad Software). All data are presented as means ± SEM. The KS normality test was performed to estimate data normality. Statistical differences between groups was assessed using one-way analysis of variance (ANOVA), and then multiple comparisons analysis was performed with a Tukey *post-hoc* test. Data with non-normal distribution was analyzed using the Kruskal–Wallis test and then the Dunn's multiple comparison test. $p < 0.05$ was considered as significant difference. The correlation between the gut microbiota and serum metabolites was analyzed using Spearman correlations with the R program package. The coefficients $p < 0.05$ were considered significant. The R language GGPlot package was used to draw a correlation heat map.

## 3. Results

### 3.1. Growth performance

The effects of inoculation with ruminal fluid (RF), autoclaved ruminal fluid (ARF) or sterilized physiological saline (CON) on growth performance of newborn lambs are presented in Figure 1 and Supplementary Table 1. The RF resulted in the lowest weight loss compared with CON and ARF. Moreover, for all groups the trend of weight change was to decrease first followed by an increase. Interesting, RF lambs stopped losing weigth on day 9 after birth, and then followed recovery. This was 3 days earlier than CON and ARF groups.

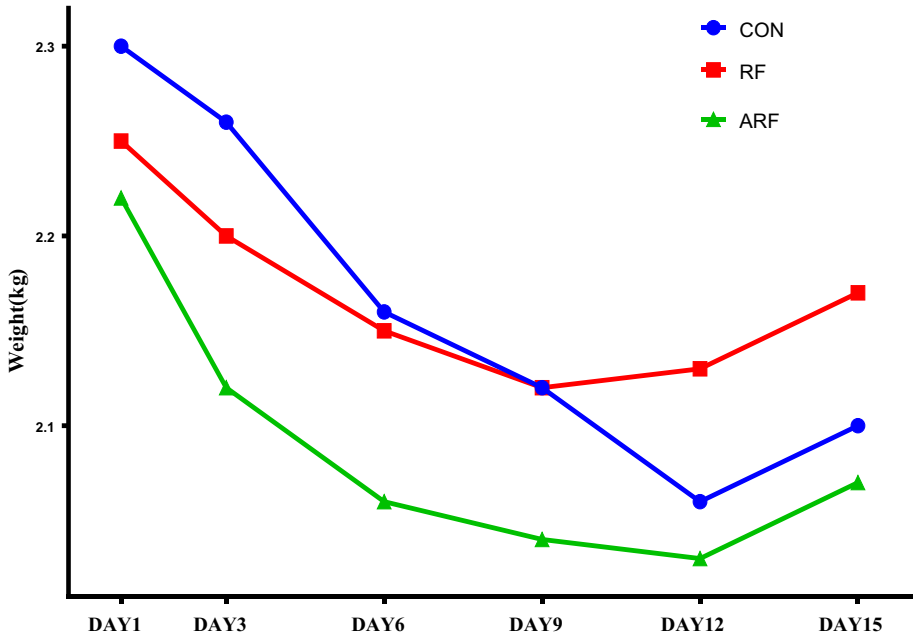


FIGURE 1
Trend of body weight change during postnatal 15days in lambs inoculated with ruminal fluid (RF), autoclaved ruminal fluid (ARF) or sterilized physiological saline (CON).

## 3.2. Blood biochemical indicators

The effects of inoculation with ruminal fluid (RF), autoclaved ruminal fluid (ARF) or sterilized physiological saline (CON) on blood biochemical indices are shown in Table 1. Compared with CON, feeding RF resulted in a greater ($p < 0.05$) blood concentration of ALP, CHOL, HDL, and LAC. Moreover, a greater ($p < 0.05$) blood concentration of ALP was also observed in RF relative to ARF. Compared with CON, ARF led to lower ($p < 0.05$) blood concentration of DBIL. No significant differences in concentrations of ALT, AST, GGT, AST/ALT, TAB, TP, ALB, GLO, A/G, TBIL, IDBIL, CHE, BUN, CREA, TG, LDL, CRP, GLU and LDH were observed among all groups.

## 3.3. Gut microbiota

Under the similarity threshold of 97%, 1,342 OTUs were obtained and the calculated good's coverage were no <99% for all samples. Venn diagrams showed that the number of OTUs in the RF group was greater compared with other groups. (Figure 2A). The Chao1 index, Shannon index and Simpson index were used to estimate the Alpha diversity of gut microbiota. Compared with the other groups, the Chao1 index indicated that feeding RF substantially increased the heterogeneity of the gut microbiota (Figure 2B). However, no significant difference among the groups emerged in the Shannon index and Simpson index (Figures 2C,D). The PCA analysis was used to compare the microbial community composition and distribution similarity of each group. According to the distance and separation of each sample in each group in the figure, it is evident that the CON group was markedly different from those of the RF group, and there were differences between the CON group and the ARF group (Figure 2E). Interestingly, the microbial composition was significantly different from that of the original samples (RF-Original and ARF-Original), and the original samples were similar in microbial composition.

We then focused on microbiota abundance at the phylum and genus levels. At the phylum level, *Bacteroidetes*, *Firmicutes*, *Verrucomicrobiota* and *Proteobacteria* were the 4 most dominant bacterial phyla in the three groups (Figure 3A). The statistical analysis showed that, at the phylum level, the gut microbiota in three groups were dominated by *Firmicutes*, *Bacteroidota*, *Proteobacteria*, *Synergistota*, *Fusobacteriota*, *Verrucomicrobiota*, and *Actinobacteriota*, while the relative abundance of other genera was below 1% (Figure 3A). The statistical analysis of abundant genera (with relative abundance >0.01%) showed that the relative abundance of *Verrucomicrobiota* was significantly lower ($p < 0.05$), while the relative abundance of *Synergistota* was significantly higher ($p < 0.05$) in the RF group than those in the CON group (Figures 3B,C). The abundance of *Synergistota* in the RF group was higher than that in other groups (Figure 3C). More details about differential microbiota abundance are shown in Supplementary Figure 1. The relative abundance of *Fusobacteriota* in the ARF group was significantly higher than that in the CON group (Figure 3D).

The analysis of the relative abundance of the bacterial genera, 29 out of the 46 genus identified showed significant differences based on the inoculation treatment. *Bacteroides*, *Christensenellaceae_R−7_group*, *Porphyromonas* and *Alloprevotella* were the common genera dominated in the gut microbiota of the lambs (Figure 4A; Supplementary Figure 2). Our results revealed that 13 genera were significantly more predominant in the RF than CON (Figure 4E; Supplementary Figure 2). They included the *Rikenellaceae_RC9_gut_group*, *Bacteroides*, *Brachymonas*, *Peptostreptococcus*, *Petrimonas*, *Phascolarctobacterium*, *Pseudomonas*, and others. The relative abundance of *Actinomyces*, the *Escherichia-Shigella*, the *Eubacterium_nodatum_group*, *Parabacteroides* and *Akkermansia* was significantly lower ($p < 0.05$) in the RF group than those in the CON group (Figures 4B–G).

TABLE 1 Effect of inoculation with ruminal fluid (RF), autoclaved ruminal fluid (ARF) or sterilized physiological saline (CON) on blood biochemical indices in lambs.

| Item | CON | RF | ARF | SEM | Value of *p* |
|---|---|---|---|---|---|
| ALT (U/L) | 9.60 | 11.25 | 10.50 | 0.764 | 0.1144 |
| AST (U/L) | 79.60 | 104.20 | 91.00 | 11.140 | 0.1077 |
| GGT (U/L) | 63.80 | 67.40 | 71.00 | 12.620 | 0.8388 |
| ALP (U/L) | 350.00[a] | 580.50[b] | 257.20[a] | 77.620 | 0.0032 |
| AST/ALT | 8.00 | 10.00 | 9.75 | 1.548 | 0.3577 |
| TBA (μmol/L) | 8.10 | 15.68 | 15.26 | 4.289 | 0.1483 |
| TP (g/L) | 71.98 | 71.76 | 71.70 | 4.358 | 0.9974 |
| ALB (g/L) | 31.95 | 32.72 | 32.46 | 1.803 | 0.9090 |
| GLO (g/L) | 40.03 | 39.04 | 39.24 | 4.504 | 0.9700 |
| A/G | 0.82 | 0.88 | 0.84 | 0.130 | 0.8785 |
| TBIL (μmol/L) | 2.50 | 2.140 | 1.94 | 0.415 | 0.3823 |
| DBIL (μmol/L) | 1.02[a] | 0.700[ab] | 0.65[b] | 0.144 | 0.0360 |
| IDBIL (μmol/L) | 1.48 | 1.56 | 1.28 | 0.322 | 0.6675 |
| CHE (U/L) | 183.30 | 177.00 | 185.60 | 7.414 | 0.5010 |
| BUN (mmol/L) | 11.21 | 13.34 | 12.88 | 1.232 | 0.1950 |
| CREA (μmol/L) | 49.53 | 51.26 | 63.36 | 7.196 | 0.1406 |
| CHOL (mmol/L) | 5.21[a] | 7.16[b] | 6.182[ab] | 0.484 | 0.0038 |
| TG (mmol/L) | 0.16 | 0.27 | 0.23 | 0.046 | 0.0896 |
| HDL (mmol/L) | 1.99[a] | 2.72[b] | 2.45[ab] | 0.265 | 0.0393 |
| LDL (mmol/L) | 2.82 | 3.81 | 4.07 | 0.601 | 0.1041 |
| CRP (mg/L) | 4.53 | 4.36 | 4.78 | 0.409 | 0.5874 |
| GLU (mmol/L) | 2.81 | 2.73 | 3.72 | 0.748 | 0.3566 |
| LAC (mmol/L) | 7.02[a] | 10.33[b] | 10.17[b] | 0.727 | 0.0004 |
| LDH (U/L) | 394.30 | 439.00 | 339.50 | 39.200 | 0.0725 |

[a,b]Letters means within a row that do not have a common superscript letter differ, $p < 0.05$. ALT, glutamate pyruvic transaminase; AST, Aspartate aminotransferase; GGT, glutamyl transferase; ALP, alkaline phosphatase; TBA, Total bile acid; TP, Total protein; ALB, albumin; GLO, Globulin; T-Bil, total bilirubin; D-Bil, Direct bilirubin; IDBIL, Indirect bilirubin; CHE, cholinesterase; BUN, blood urea nitrogen; CREA, creatinine; CHOL, total cholesterol; TG, triglyceride; HDL-C, High density lipoprotein; LDL-C, Low density lipoprotein; CRP, C-reactive protein; GLU, glucose; LAC, lactate acid concentration; LDH, lactate dehydrogenase.

## 3.4. Serum metabolic profiles

According to the results obtained, a total of 807 metabolites were identified and quantified. All samples were analyzed with a 95% confidence interval. We performed PCA score plot to visualize the overall change of metabolites, the results showed differences between the three groups (Figures 5A–C). The OPLS-DA revealed a clear separation between the CON and the RF groups in the plot, which indicated that the serum metabolic profiles of the RF group were distinct from that of the CON group (Figure 5D). The other two figures also reflect similar results (Figures 5E,F). In the OPLS-DA model, the parameter R2Y was 0.998, and the Q2 value was 0.89 (Supplementary Figure 3), indicating a good degree of reliability and predictive ability of the model used. The OPLS-DA model involved 200 random permutations and combination experiments on the data to avoid over-fitting. A good model is obtained when all replacement models of R2 and Q2 values are lower than the original values of R2 and Q2. The study results suggested that the OPLS-DA model was not over fitted as demonstrated by the R2 and Q2 values in all permutated models being lower than the value of the original R2 and Q2 models (Figures 5G–I).

The results from the screening of different metabolites were visualized in the form of volcano plots to quickly assess the difference and statistical significance in the metabolic expression levels for the CON, RF and ARF group (Figures 6A–C). Fifty-one different metabolites were observed in CON vs. RF, including 22 decreased and 29 increased metabolites (Figure 6A); 94 different metabolites in CON vs. ARF, including 9 decreased and 85 increased metabolites (Figure 6B) and 57 different metabolites in RF vs. ARF, including 9 decreased and 48 increased metabolites (Figure 6C). The top 20 important differential metabolites with VIP > 1.5 and $p < 0.05$ are depicted in VIP plots (Figures 6D–F). Compared with CON, inoculation with ruminal fluid increased the plasma relative concentrations of Chenodeoxycholic Acid, Proline-Hydroxyproline, 3-hydroxyphenylacetic acid and FFA(15:1), whereas it decreased the plasma relative concentrations of 21-Deoxycortisol, 20-COOH-AA, Glu-Met, Tetradecanedioic acid, 9(S)-HpOTrE and Trimethylamine-N-Oxide (Figure 6D). Compared with CON, inoculated with autoclaved rumen fluid increased the plasma relative concentrations of Hippuric Acid, Caffeic Acid, Arachidyl-glycine, Xanthosine, Oxypurinol and 5-Hydroxyindole-3-Acetic Acid, whereas it decreased the plasma relative concentrations of Carnitine C12:1(Figure 6E). Compared with RF, inoculated with autoclaved rumen fluid increased the plasma relative concentrations of Phenylacetyl-L-Glutamine, DL-Leucine, Cis-L-3-hydroxyproline, 6-Aminocaproic-Acid, 20-COOH-AA, Caffeic Acid, Arachidyl-glycine, 21-Deoxycortisol and Carnitine C5:0, whereas it decreased the plasma relative concentrations of Carnitine C12:1(Figure 6F).More details about differential metabolites are shown in Supplementary Tables S1–S3.

## 3.5. Correlations between serum metabolic profiles and gut microbiota

Using the Spearman rank correlation coefficients, we displayed the results in the heatmap chart and evaluated the correlation. Compared with CON, at the phylum level, we found that *Verrucomicrobiota* was strongly correlated with most metabolites and *Synergistota* was negatively correlated with Tetradecanedioic acid (Figure 7A). At the genus level, a strong correlation between 12 differential genera and 24 differential metabolites at the genera level was observed in the CON and RF groups (Figure 7B). *Akkermansia* was negatively correlated with Chenodeoxycholic Acid and various amino acids, and was positively correlated with organic acid and its derivatives. The abundance of Pathogenic bacteria such as *Escherichia-Shigella* and *Actinomyces* decreased after inoculation with ruminal fluid and was associated with

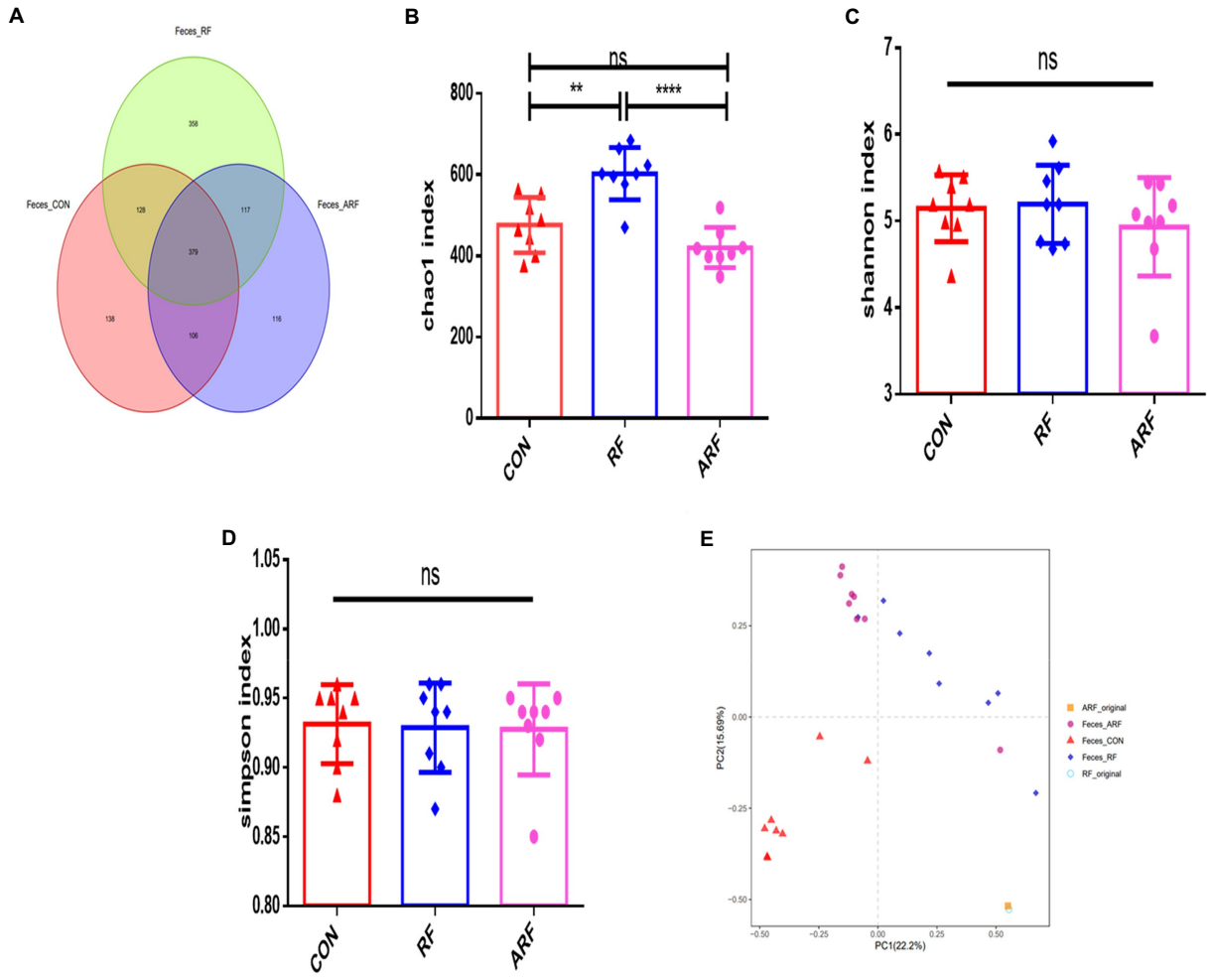


FIGURE 2
Gut Microbiota diversity analysis in 15-day old lambs after inoculation with ruminal fluid or autoclaved ruminal fluid. **(A)** Unique and shared intestinal operational taxonomic units (OTUs) for each group are shown in the Venn diagram. Alpha diversity (Chao1index, Shannon index, Simpson index) **(B–D)**; beta diversity indicated by principal component analysis (PCA) plot **(E)**. Data are presented as means±SD. $n$=8, $^{**}p<0.01$, $^{****}p<0.0001$, ns indicates no significance.

various amino acids, bile acids and other metabolites. *Rikenellaceae_RC9_gut_group* was negatively correlated with Trimethylamine-N-Oxide, and significantly positively correlated with the level of Chenodeoxycholic Acid and Lithocholic acid in serum. *Eubacterium_nodatum_group*, *Filifactor*, *Parabacteroides*, *Clostridiales_bacterium_canine_oral_taxon_082*, Porphyromonas and other genera were also correlated with many metabolites.

By taking the intersection of CON and ARF, 94 differential metabolites were obtained and narrowed down to 30 different metabolites (Figures 7C,D). At the phylum level, *Verrucomicrobiota* was negatively correlated with significantly different metabolites, *Fusobacteriota* was positively correlated with significantly different metabolites (Figure 7C). The change in *Akkermansia*, *Porphyromonas* and *NK4A214_group*, *Escherichia-Shigella* and *Parabacteroides* levels was associated with the serum levels of significantly different metabolites. Other microbiota were positively correlated with significantly different metabolites (Figure 7D). These results indicated that these differential microbiota were closely associated with, and might contribute to, the altered serum metabolic profiles in response to inoculation with ruminal fluid or autoclaved ruminal fluid. In order to better understand the metabolic regulation relationship, we have drawn a metabolic network diagram (Figure 8).

## 4. Discussion

### 4.1. Effect of ruminal fluid inoculation on weight of newborn lambs

Growth rate of lambs can potentially affect production performance in adulthood (Sato et al., 2010). Some early studies found that ruminal inoculation improved average daily gain (Pounden and Hibbs, 1949). A potential mechanism for such an effect was proposed to be a benefit of microorganism inoculation on amylase activity (Ziolecka et al., 1984). In contrast, other studies reported no significant improvements in weight gain due to ruminal fluid inoculation (De Barbieri et al., 2015). Although we did not detect a significant effect on weight gain over time, given that postpartum stress could lead to weight loss after birth in the newborn, the trend to promote a faster weight recovery in the RF group suggested a benefit of active microbial inoculation.

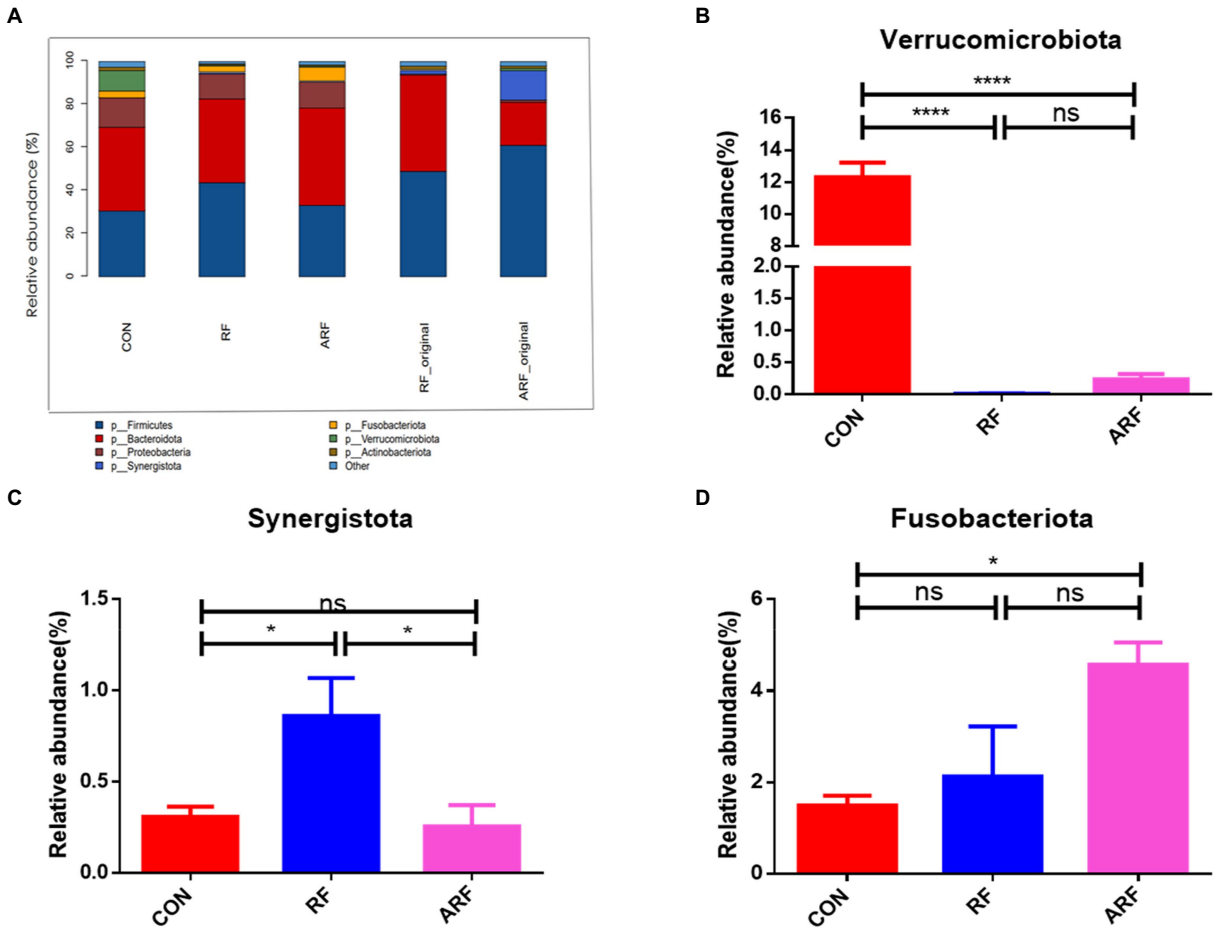


FIGURE 3
Relative abundance of microbiota at the phylum level. **(A)** Microbiota taxonomic profiling of intestinal microbiota from different groups at the phylum level with the abundance of the microbiota is more than 1%. **(B)** Ratio of *Verrucomicrobiota* in the three groups. **(C)** Ratio of *Synergistota* in the three groups. **(D)** Ratio of *Fusobacteriota* in the three groups. $n$=8, *$p$<0.05, **$p$<0.01, ***$p$<0.001, ****$p$<0.0001, ns indicates no significance.

## 4.2. Effect of ruminal fluid inoculation on blood biochemical parameters

Blood parameters are important to evaluate the health status of animals in response to nutrition (Weingand et al., 1996). The lack of difference for most of the blood parameters related to liver and kidney function and inflammation suggested that ruminal fluid inoculation had no negative influence on the health of newborn lambs.

It is noteworthy that an increase of ALP, CHOL, and HDL was observed in the RF group. A greater concentration of ALP was observed in newborn infants experiencing active bone growth (Devyatkin et al., 2021). Thus, it is plausible to speculate that fresh ruminal fluid inoculation with active microorganisms was beneficial to the development of newborn lambs. CHOL is one of the important blood indices to estimate lipoprotein metabolism. A previous study found that multi-strain probiotic increased serum CHOL in neonatal dairy calves (Guo et al., 2022), which was similar to our study. However, a low blood concentration of CHOL was also observed in a previous study with spray-dried ruminal fluid inoculation (Rezai Sarteshnizi et al., 2020). Combined with the greater HDL blood content in RF relative to CON, the greater CHOL might be helpful in preventing lipid peroxidation and stimulating the synthesis of anti-inflammatory cytokines (such as interleukin-10) (Mertens and Holvoet, 2001). We speculated that active microorganism inoculation could potential regulate lipid metabolism and inflammatory reactions to influence the health of newborn lambs.

Overall, the blood biochemical parameters suggested that microorganism inoculation had no negative effect on health, and could potentially regulate lipid metabolism and inflammatory reactions.

## 4.3. The effect of ruminal fluid inoculation on gut microbiota

Gut microbiota is significantly different from the ruminal microbiota (Godoy-Vitorino et al., 2012), but data suggest that ruminal fluid inoculation still could regulate the gut microflora (Ji et al., 2018). In the present work, a distinct microbiota cluster of CON, RF and ARF was observed using PCA analysis, suggesting that ruminal fluid inoculation could influence the establishment of microflora in newborn lambs. The greater alpha-diversity index (Chao 1) in RF compared with CON suggested that ruminal fluid inoculation with active microorganisms could improve the diversity of the gut microbiota. A similar result was also observed in a previous study with goats (Abo-Donia et al., 2011).

The establishment of microbiota after birth might involve a sophisticated process (Wang et al., 2016). In the current study,

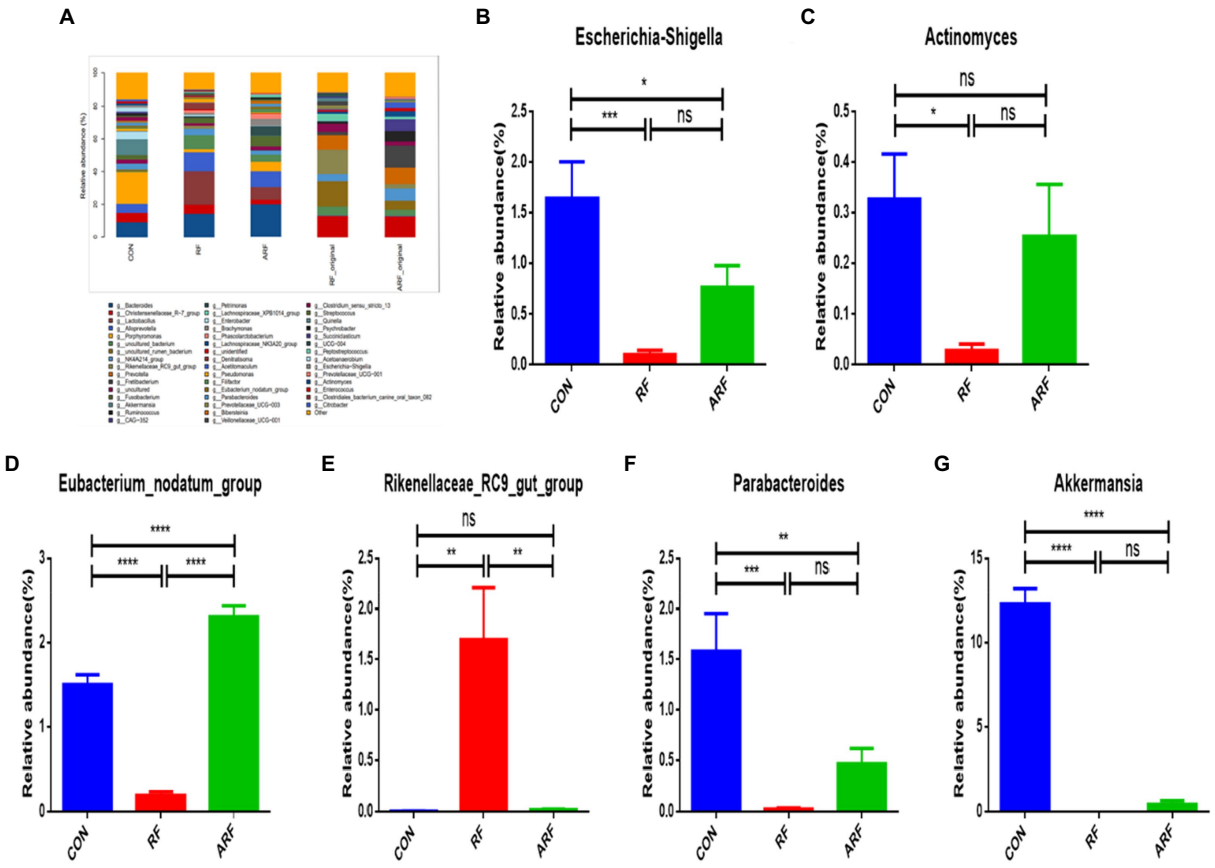


FIGURE 4
Relative abundance of microbiota at the genera level. **(A)** Microbiota taxonomic profiling of intestinal microbiota from different groups at the genera level which the abundance of the microbiota is more than 1%. **(B)** Ratio of *Escherichia-Shigella* in the three groups. **(C)** Ratio of *Actinomyces* in the three groups. **(D)** Ratio of *Eubacterium_nodatum_group* in the three groups. **(E)** Ratio of *Rikenellaceae_RC9_gut_group* in the three groups. **(F)** Ratio of *Parabacteroides* in the three groups. **(G)** Ratio of *Clostridiales_bacterium_canine_oral_taxon_082* in the three groups. **(H)** Ratio of *Akkermansia* in the three groups. $n$=8, *$p$<0.05, **$p$<0.01, ***$p$<0.001, ****$p$<0.0001, ns indicates no significance.

we illustrated the change of gut microbial taxonomical composition with ruminal fluid inoculation at the phylum and genus levels in newborn lambs. In the ruminant, *Proteobacteria*, *Bacteroidetes* and *Firmicutes* were confirmed as the dominant phyla with important functions in maintaining health and production (Sadet et al., 2007). Therefore, our results with the greatest relative abundance of *Proteobacteria*, *Bacteroidetes* and *Firmicutes* suggested that ruminal fluid inoculation could accelerate the establishment of ruminal microbiota in newborn lambs. In our study, *Verrucomicrobia* was found at a lower proportion in lambs exclusively inoculated with ruminal fluid and were represented exclusively by the genus *Akkermansia*, in agreement with earlier studies (Saleem et al., 2012). As previously reported, the relative abundance of *Verrucomicrobia* was significantly higher in milk-fed lambs (Wang et al., 2016). The present study may indirectly suggest that ruminal fluid transplantation promotes the establishment of specific microbial population in lambs in order to better adapt to the transition from milk to solid feed. *Fusobacteria* is a phylum of anaerobic Gram-negative bacilli, with a shuttle-shaped morphology, commonly found in the oral cavity (Gupta and Sethi, 2014). The view that *Fusobacterium* is involved in animal infection within a certain range has long been accepted by many researchers (Roberts, 2000). Our results indicated that inoculation with ruminal fluid in lambs may also contribute to health by reducing the number of pathogenic bacteria. *Synergistota* is a bacterial phylum consisting of gram-negative anaerobes (Belibasakis et al., 2013). End-products of *Synergistota* are mainly acetate, propionate and isobutyrate (Kang et al., 2020). SCFA are the main source of energy for ruminants to maintain normal growth.

At the genus level, most of the *Rikenellaceae_RC9_gut_group* was upregulated. The *Rikenellaceae_RC9_gut_group* is the dominant bacteria in the intestine, associated with mucin degradation (Fan et al., 2020), and is significantly in a negative correlation with obesity (Arnoriaga-Rodríguez et al., 2020; Suzuki et al., 2020). It plays an important role in intestinal mucosal health. Moreover, the relative abundance of *Rikenellaceae_RC9_gut_group* was positively correlated with host feed utilization, volatile fatty acid and short-chain fatty acid metabolism (Derakhshani et al., 2017; Li et al., 2019) and significantly negatively correlated with the expression of inflammation-related immune genes such as insulin levels and interferon IFNγ (Gomez-Arango et al., 2016). *Parabacteroides* generate acetate to mitigate heparanase-exacerbated acute pancreatitis by reducing neutrophil infiltration (Lei et al., 2021). Acetate is the main SCFA. Some species of *Parabacteroides* Significant reduce in severity of intestinal inflammation in murine models of acute and chronic colitis through dextran sulfate sodium (Kverka et al., 2011). SCFA can regulate the function of multiple

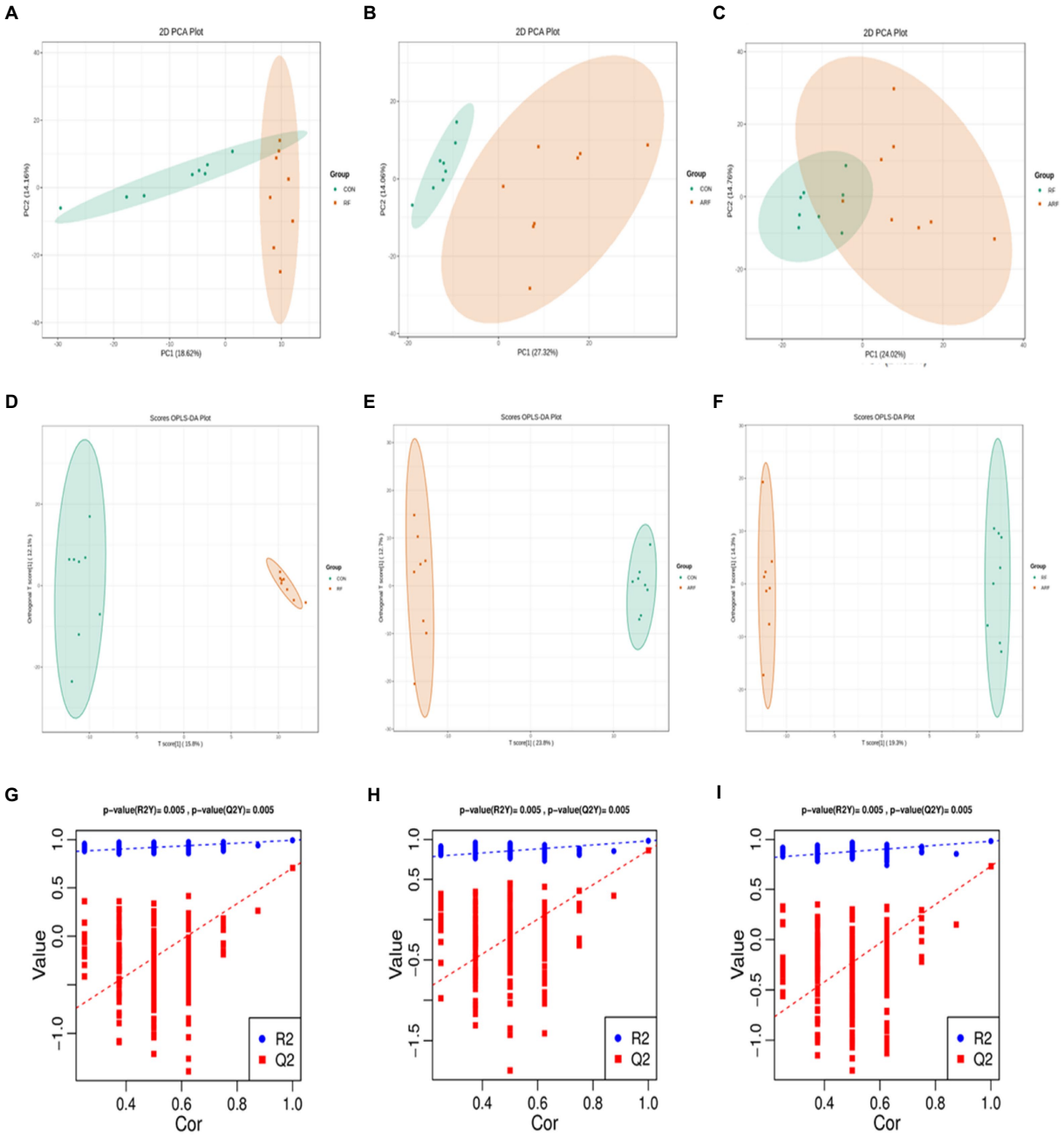


FIGURE 5
Metabolomics of PCA analysis, CON group (green) and RF group (red) **(A)**, OPLS-DA score chart, CON group (green) and ARF group (red) **(B)**, OPLS-DA score chart, RF group (green) and ARF group (red) **(C)**. OPLS-DA score chart, CON group (green) and RF group (red) **(D)**, OPLS-DA score chart, CON group (green) and ARF group (red) **(E)**, OPLS-DA score chart, RF group (green) and ARF group (red) **(F)**. Permutation test of OPLS-DA **(G–I)**.

systems, such as the intestinal, neurological, endocrine and hematological systems, and there is considerable evidence that SCFAs play an important role in the maintenance of intestinal health and the prevention and improvement of many non-communicable diseases, including cancer (Doble et al., 2016). However, due to the interaction of microflora, the treatment in this study reduced the concentration of *Parabacteroides*.

In this study, most of the *Escherichia-Shigella* was significantly downregulated, *Escherichia-Shigella* is a conditional pathogenic bacteria (Qi et al., 2021). On the first day after birth, the rectum was invaded by *Escherichia-Shigella* (Alipour et al., 2018). Recent research suggests that the artificial feeding modal can increase the number of potential pathogens such as *Escherichia-Shigella* and slow the establishment of the anaerobic environment and anaerobic microorganism (Bi et al., 2019). *Actinomyces* can use carbohydrates to produce fatty acids and are classified as conditional pathogenic bacteria (Hall, 2008). *Eubacterium_nodatum_group* has the ability to Catabolic carbohydrates to SCFAs (mainly butyrate; Barcenilla et al., 2000). *Eubacterium_ nodatum_ Group* is common in oral cavity with periodontitis and other diseases, and is considered as

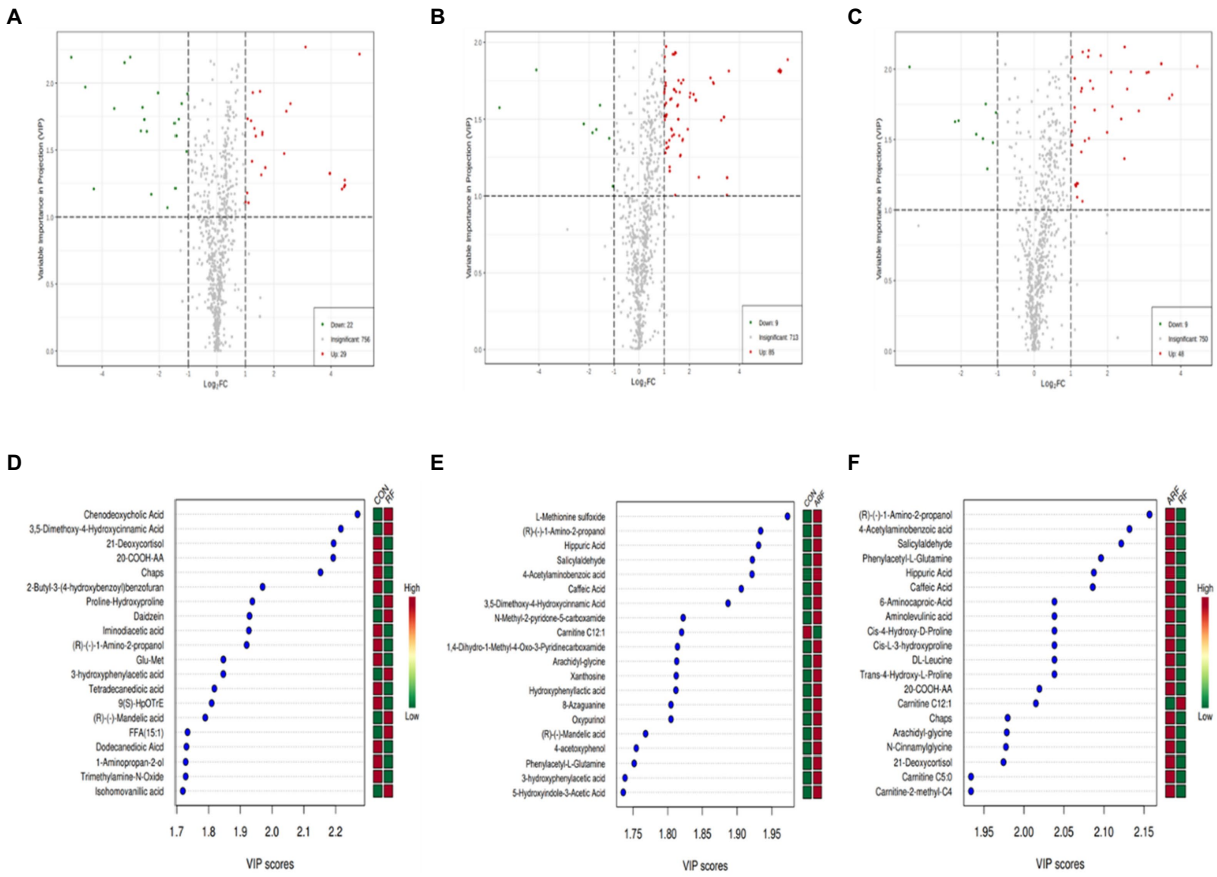


FIGURE 6
Differential metabolites in lamb serum inoculated with ruminal fluid. Volcano plots of the affected metabolites in CON *VS* RF **(A)**, CON *VS* ARF **(B)** and RF *VS* ARF **(C)**. Each point represents a metabolite. The green dots in the figure represent the down-regulated differential metabolites, the red dots represent the up-regulated differential metabolites, and the gray dots represent the metabolites without difference. Metabolites are ranked by variable importance in projection analysis (VIP) of respective groups: CON group and RF group **(D)**, CON group and ARF group **(E)** and RF group and ARF group **(F)**; the top 20 important metabolites were arranged from top to bottom according to intracellular concentration. The red box represents an up-regulated concentration of the molecule and the green box represents down-regulated concentration.

pathogenic bacteria (Hill et al., 1987). The results varied a lot from one study to another, and there is no consistent conclusion about the *Akkermansia*. *Akkermansia*, produce SCFA by degrading mucin, are closely contacted with immunity (Ganesh et al., 2013). Increasing abundance of *Akkermansia muciniphila* could decreased body fat mass, increase glucose homeostasis, and lower adipose tissue inflammation (Everard et al., 2013; Dao et al., 2016). Recent research suggests that milk positively affected their growth (Wang et al., 2018). This may further prove that inoculation with ruminal fluid promotes the maturation of lamb microbes in order to better adapt to the transition from milk to solid feed. The previous studies have found that high-doses of heme iron intake significantly increased the abundance of *Akkermansia* in the intestinal tract of mice, but damaged the intestinal mucus layer (van Hylckama Vlieg et al., 2011). The results of the present study showed that inoculation with ruminal fluid could change bacteria of the Fusobacteria, *Akkermansia*, *Eubacterium_nodatum_group*, and the like. The effect was positively correlated with microbial activity in ruminal fluid. These results support the notion that active gut microbiota may inhibit the growth of pathogens, promote the maturation of growth-promoting microbes by altering the diversity and composition of the gut microbiota.

## 4.4. The effect of ruminal fluid inoculation on serum metabolites and correlations with serum metabolic profiles

Microbiota in the gut regulate metabolic reactions such as the production of bile acids, amino acid and fatty acids, which are essential for the health of the animal (Nicholson et al., 2012). In this study, the composition of serum metabolites in the RF group was different from the CON and ARF groups. Through the serum metabolomics analysis, it was concluded that the metabolism of bile acids (Chenodeoxycholic acid, Chaps, Lithocholic acid) was greatly influenced by feeding RF. Bile acids participate in the regulation of lipid and dextrose metabolism, and play a key role in regulating hepatic metabolic pathways (Hylemon et al., 2009). The bile acids may help control growth and composition of gut microbiota through FXR and TGR-5, which also helps protect the gut microbiota from inflammation (du et al., 2020). Previous research suggested that obeticholic acid, a derivative of chenodeoxycholic acid, can increase bile acid homeostasis, lower the expression of TNF-α, IL-6, and IL-1β, suggesting that bile acids alleviate inflammation (Xiong et al., 2017). Dietary chenodeoxycholic acid can improve growth performance and intestinal health by changing blood metabolism and intestinal bacteria in weaned piglets (Song et al., 2021). Lithocholic acid is formed

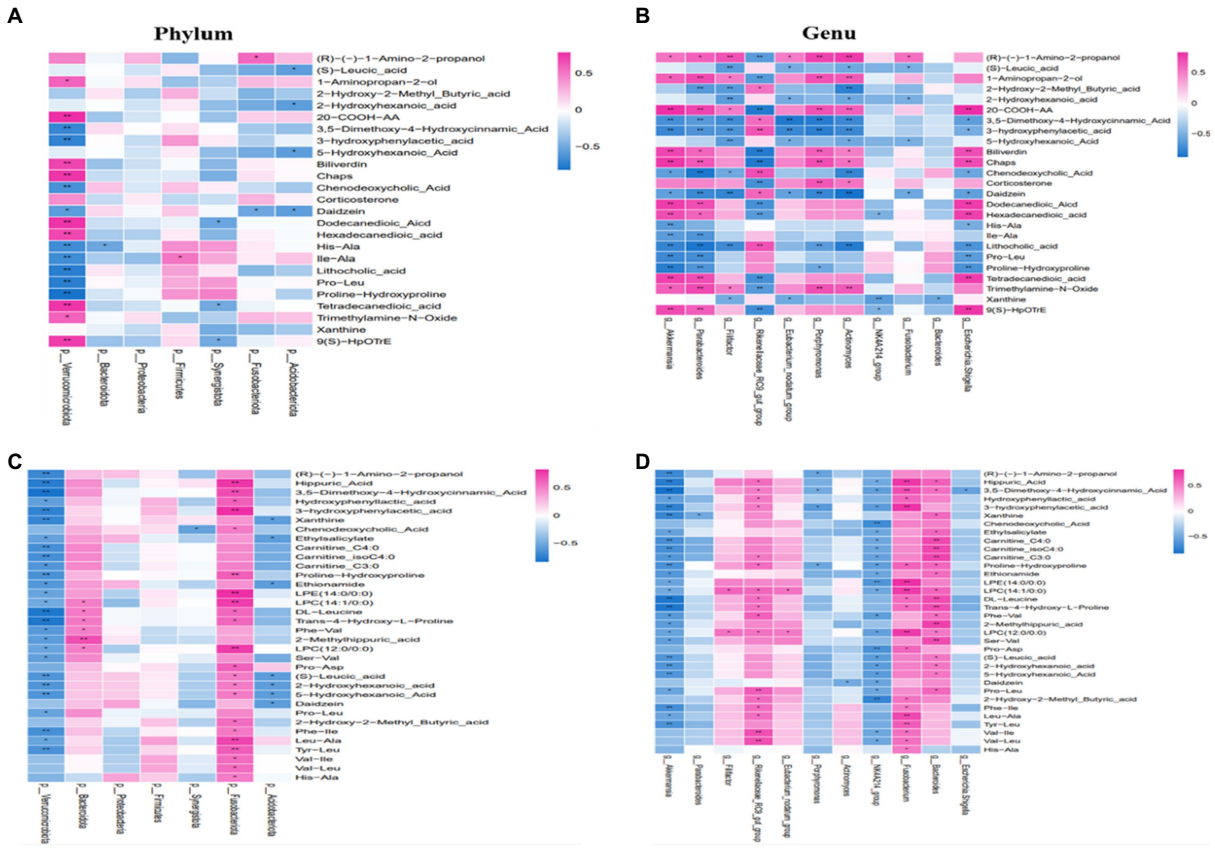


FIGURE 7
The correlation between gut microbiota and serum metabolites. At the phylum or genera level in the CON and RF groups **(A,B)**. At the phylum or genera level in the CON and ARF groups **(C,D)**. Red represents positive correlation, and green represents negative correlation. *$p$<0.05, **$p$<0.01.

by bacterial action of chenodeoxycholic acid salt and is usually combined with glycine or taurine (Bazzoli et al., 1982). Spearman correlation analysis between the affected metabolites showed that the elevated serum levels of chenodeoxycholic acid and Lithocholic acid were negatively correlated with *Escherichia-Shigella*, *Fusobacterium*, *Actinomyces*, *Porphyromonas*, *Eubacterium_ nodatum_ Group* and *Akkermansia*, and positively correlated with *Rikenellaceae_RC9_gut_ group*. The results suggested that tumor gastric juice transplantation may improve the stability of the body by regulating the level of bile acid metabolism or regulating the composition and quantity of intestinal microbiota.

The end products of protein digestion in the digestive tract are often mostly small peptides rather than free amino acids, which are absorbed intact and enter the circulation as dipeptides or tripeptides (Rérat et al., 1988). Studies have shown that insufficient peptide in the ruminal fluid of dairy cows is the main factor limiting the growth of microorganisms, and small peptides are key factors for maximum growth efficiency of microorganisms (Brooks et al., 2012). Small peptides can promote the reproduction of beneficial microorganisms in the digestive tract and improve the synthesis of microbial proteins. Our results revealed an increasing trend of small peptides (such as proline-Hydroxyproline, His-Ala, Ile-Ala, and Pro-Leu), the correlation analysis showed that the elevated serum levels of small peptides was negatively correlated with *Escherichia-Shigella*, *Fusobacterium* and *Eubacterium_nodatum_group*. The tricarboxylic acid cycle is one of the most important energy metabolism pathways, our results on correlation network analysis showed that some small peptides may indirectly participate in the tricarboxylic acid cycle, i.e., enter the cycle after hydrolysis into free amino acids. The above results demonstrated that feeding RF promoted peptide absorption by enhancing the absorption rate of small peptides or change the absorbable amount of small peptides by reducing the abundance of *Escherichia Shigella* and other bacteria, thereby increasing the concentration of small peptides in the serum, thereby enhancing the metabolism of the body and promoting *Rikenellaceae_RC9_gut_group*. Xanthine and xanthoside are related to purine and pyrimidine metabolism, and the changes of these metabolites are related to microbial apoptosis (Fujihara and Shem, 2011; Wang et al., 2012). In this study, the contents of xanthine and xanthoside in serum of lambs increased, and were negatively correlated with *Escherichia Shigella*. Therefore, we speculate that the increase of xanthine and xanthoside content is related to the decrease of *Escherichia Shigella*.

When hepatic glycogen, a quick source of glucose for muscle, is depleted, adipose tissue lipolysis into free fatty acids provides these compounds to tissues as a source of energy (Cree-Green et al., 2017). The content in serum is very low under physiological condition, but the content increases abnormally in diabetes, severe liver dysfunction, hyperthyroidism and other diseases (Wolf et al., 2016). From the weight gain results, we can speculate that the feeding RF promoted feed digestion and nutrient absorption which could have favored liver glycogen synthesis and even glucose utilization for oxidation without the need to induce adipose tissue lipolysis. Free fatty acids are also one of the substances that contribute to oxidative stress (Jiang and

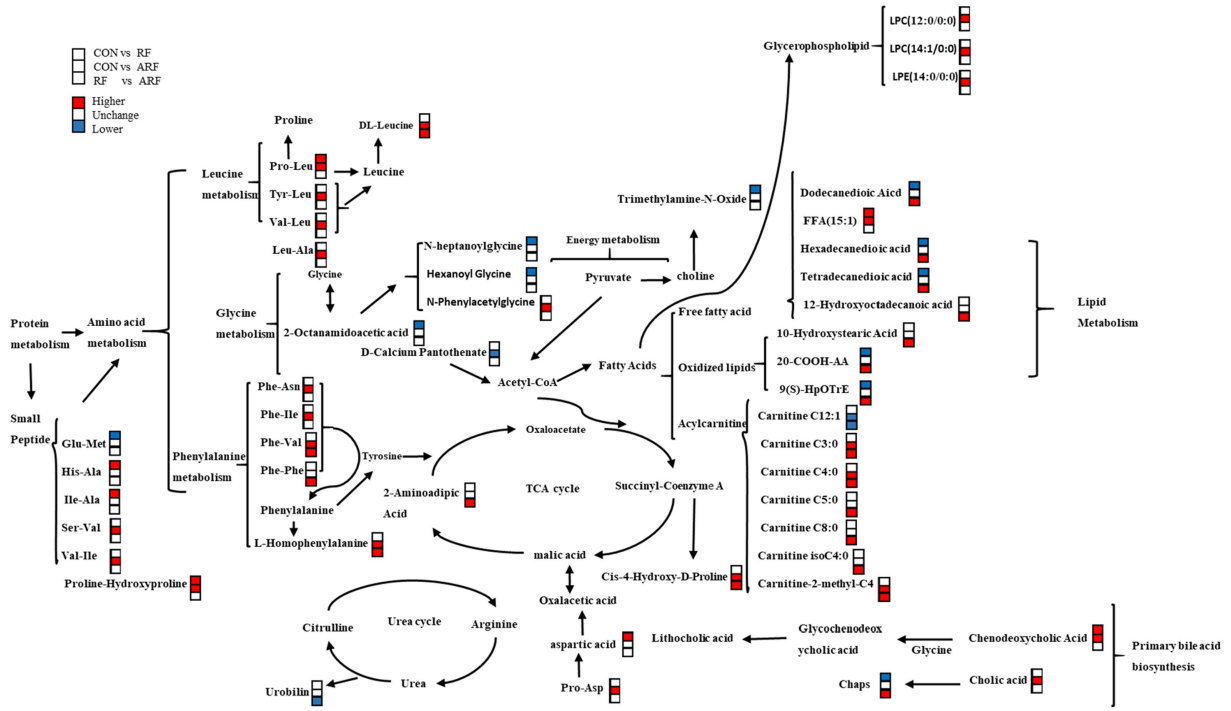


FIGURE 8
Correlation networks of fecal metabolites in CON, RF, and CON groups. Compared with the latter group, among the metabolites in the former group, the red symbol represents a significant increase, the white symbol represents no difference, and the blue symbol represents a decrease.

Yan, 2022). Oxidized lipids are metabolites of polyunsaturated fatty acids (arachidonic acid, linoleic acid, alpha-linolenic acid) that undergo auto-oxidation or are generated by the action of specific enzymes (Tang et al., 2021). 20-COOH-AA is produced by a series of enzymatic oxidation reactions of arachidonic acid. Oxidative stress is a physiological state in which there is an imbalance between oxidation and antioxidant action in the body. Oxidized lipids serve as the material base in which unsaturated fatty acids are oxidized to form oxidative metabolites under oxidative stress, thus, affecting normal functions (Tsubone et al., 2019). Trimethylamine-N-Oxide is the promoter of atherosclerosis and participates in oxidative damage, endothelial dysfunction and other diseases (Moraes et al., 2015); Injury of vascular endothelial cells can be induced by increasing vascular oxidative stress, and mitochondrial dysfunction can also be induced by oxidative stress in mice (Li et al., 2018; Brunt et al., 2020). Plasma corticosterone is thought to be the main glucocorticoid involved in the regulation of the stress response in rodents (Mizobe et al., 1997). A striking association between serum corticosterone was observed during acute or repeated restraints, chronic unpredictable stress or heat stress (Gong et al., 2015). In the current study, the concentration of Free fatty acids, Oxidized lipids, Trimethylamine-N-Oxide and corticosterone in the RF group were downregulated compared with the other groups, and the blood biochemical indices showed that the concentration of HDL, which had the effect of antagonizing lipid peroxidation, was increased. It has been reported that probiotics can alleviate lipid accumulation, regulate blood lipid levels, increase the concentration of HDL-C, slow down the formation of oxidized lipids, and antagonize oxidative lipid damage in the liver (Nido et al., 2016). In our study, Free fatty acids, Oxidized lipids, Trimethylamine-N-Oxide, Corticosterone was negatively correlated with intestinal available bacteria such as *Rikenellaceae_RC9_gut_group*, and positively correlated with pathogenic bacteria such as *Escherichia Shigella*, *Actinomyces*, *Porphyromonas*. It has been pointed out that the disturbance of intestinal microbiota is one of the stressors leading to stress, and a stable microbiota can reduce the stress response of the body to a certain extent. Based on the above results, we speculate that the transplantation of rumen juice will promote the stabilization of intestinal microbiota, thereby reducing the levels of lipid oxide, trimethylamine oxide, cortisol, etc., to some extent, it can antagonize the stress response of lambs and improve the blood lipid level of lambs.

We found that RF treatment altered the levels of metabolites in lamb serum, with the metabolites differing in expression from the ARF group, and these changes were related to intestinal microorganisms. We speculate that the sterilized ruminal fluid was rich in protein, fatty acids and other nutrients, thus, promoting the development of the microbial community of the lambs and influencing the metabolites. The difference in the results of the combined RF and ARF groups suggested that active microorganisms in the ruminal were the main factors that contributed to the differences observed.

## 5. Conclusion

This study demonstrated that ruminal fluid inoculation with active microorganisms could accelerate weight recovery and maintain health in newborn lambs through regulating the establishment of gut microbiota. The proposed mechanism of the overall effects of the ruminal fluid inoculation on gut microbiota and metabolism is schematically represented in Figure 9. In general, this management strategy could be useful for promoting the development and alleviate the postpartum stress in newborn lambs.

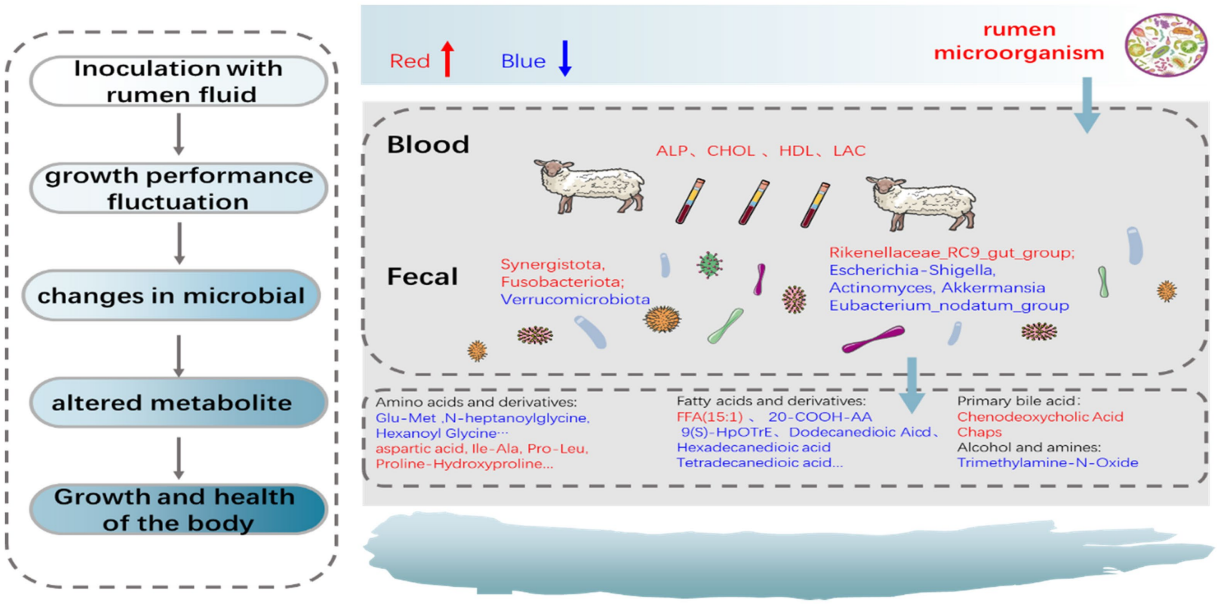


FIGURE 9
The proposed schematic diagram of the overall effects of the ruminal fluid inoculation on the gut microbial composition, function, and metabolites of lambs.

## 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: NCBI - PRJNA923048; MetaboLights - MTBLS6998.

## Ethics statement

The animal study was reviewed and approved by Ethics Committee in Chongqing Academy of animal sciences (approval number: xky-20180716, 9 June 2021).

## Author contributions

LF, LW, and LL: data analysis and original draft writing. LZ, GW, and PZ: conducted the experiments and sample collection. JL and ZZ: draft reviewing. XD: experimental design, supervision, and original draft writing and reviewing. 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/fmicb.2023.1128271/full#supplementary-material

## References

Abo-Donia, F. M., Ghada, S. I., Nadi, S., and Sayah, M. S. (2011). Effect of inoculating new born lambs with fresh or lyophilized rumen fluid on rumen activity and lamb performance. *J. Am. Sci.* 7, 409–422.

Alipour, M. J., Jalanka, J., Pessa-Morikawa, T., Kokkonen, T., Satokari, R., Hynönen, U., et al. (2018). The composition of the perinatal intestinal microbiota in cattle. *Sci. Rep.* 8:10437:10437. doi: 10.1038/s41598-018-28733-y

Anand, S., and Mande, S. S. (2018). Diet, microbiota and gut-lung connection. *Front. Microbiol.* 9:2147. doi: 10.3389/fmicb.2018.02147

Arnoriaga-Rodríguez, M., Mayneris-Perxachs, J., Burokas, A., Pérez-Brocal, V., Moya, A., Portero-Otin, M., et al. (2020). Gut bacterial Clpb-like gene function is associated with decreased body weight and a characteristic microbiota profile. *Microbiome* 8:59. doi: 10.1186/s40168-020-00837-6

Baldwin, R. L. VI, McLeod, K. R., Klotz, J. L., and Heitmann, R. N. (2004). Rumen development, intestinal growth and hepatic metabolism in the pre-and Postweaning ruminant. *J. Dairy Sci.* 87, E55–E65. doi: 10.3168/jds.S0022-0302(04)70061-2

Barcenilla, A., Pryde, S. E., Martin, J. C., Duncan, S. H., Stewart, C. S., Henderson, C., et al. (2000). Phylogenetic relationships of butyrate-producing bacteria from the human gut. *Appl. Environ. Microbiol.* 66, 1654–1661. doi: 10.1128/AEM.66.4.1654-1661.2000

Bazzoli, F., Fromm, H., Sarva, R. P., Sembrat, R. F., and Ceryak, S. (1982). Comparative formation of Lithocholic acid from Chenodeoxycholic and Ursodeoxycholic acids in the colon. *Gastroenterology* 83, 753–760. doi: 10.1016/S0016-5085(82)80003-6

Belibasakis, G. N., Öztürk, V. Ö., Emingil, G., and Bostanci, N. (2013). Synergistetes cluster a in saliva is associated with periodontitis. *J. Periodontal Res.* 48, 727–732. doi: 10.1111/jre.12061

Bi, Y., Cox, M. S., Zhang, F., Suen, G., Zhang, N., Tu, Y., et al. (2019). Feeding modes shape the acquisition and structure of the initial gut microbiota in newborn lambs. *Environ. Microbiol.* 21, 2333–2346. doi: 10.1111/1462-2920.14614

Brooks, M. A., Harvey, R. M., Johnson, N. F., and Kerley, M. S. (2012). Rumen degradable protein supply affects microbial efficiency in continuous culture and growth in steers. *J. Anim. Sci.* 90, 4985–4994. doi: 10.2527/jas.2011-4107

Brunt, V. E., Gioscia-Ryan, R. A., Casso, A. G., VanDongen, N. S., Ziemba, B. P., Sapinsley, Z. J., et al. (2020). Trimethylamine-N-oxide promotes age-related vascular oxidative stress and endothelial dysfunction in mice and healthy humans. *Hypertension* 76, 101–112. doi: 10.1161/HYPERTENSIONAHA.120.14759

Chan, C. S., Chan, K. G., Tay, Y. L., Chua, Y. H., and Goh, K. M. (2015). Diversity of thermophiles in a Malaysian hot spring determined using 16s Rrna and shotgun metagenome sequencing. *Front. Microbiol.* 6:177. doi: 10.3389/fmicb.2015.00177

Cree-Green, M., Gupta, A., Coe, G. V., Baumgartner, A. D., Pyle, L., Reusch, J. E. B., et al. (2017). Insulin resistance in type 2 diabetes youth relates to serum free fatty acids and muscle mitochondrial dysfunction. *J. Diabetes Complicat.* 31, 141–148. doi: 10.1016/j.jdiacomp.2016.10.014

Dao, M. C., Everard, A., Aron-Wisnewsky, J., Sokolovska, N., Prifti, E., Verger, E. O., et al. (2016). Akkermansia muciniphila and improved metabolic health during a dietary intervention in obesity: relationship with gut microbiome richness and ecology. *Gut* 65, 426–436. doi: 10.1136/gutjnl-2014-308778

De Barbieri, I., Hegarty, R. S., Silveira, C., and Oddy, V. H. (2015). Positive consequences of maternal diet and post-Natal rumen inoculation on rumen function and animal performance of merino lambs. *Small Ruminant Res.* 129, 37–47. doi: 10.1016/j.smallrumres.2015.05.017

Derakhshani, H., Tun, H. M., Cardoso, F. C., Plaizier, J. C., Khafipour, E., and Loor, J. J. (2017). Linking Peripartal dynamics of ruminal microbiota to dietary changes and production parameters. *Front. Microbiol.* 7:2143. doi: 10.3389/fmicb.2016.02143

Devyatkin, V., Mishurov, A., and Kolodina, E. (2021). Probiotic effect of Bacillus subtilis B-2998D, B-3057D, and bacillus licheniformis B-2999D complex on sheep and lambs. *J. Adv. Vet. Anim. Res.* 8, 146–157. doi: 10.5455/javar.2021.h497

Ding, G., Gong, Q., Ma, J., Liu, X., Wang, Y., and Cheng, X. (2021). Immunosuppressive activity is attenuated by Astragalus polysaccharides through remodeling the gut microenvironment in melanoma mice. *Cancer Sci.* 112, 4050–4063. doi: 10.1111/cas.15078

Doble, M., Raman, M., and Ambalam, P. (2016). "Probiotics and bioactive carbohydrates in colon cancer management," in *Probiotics and bioactive carbohydrates in colon cancer management*.

Dong, X., Zhou, Z., Saremi, B., Helmbrecht, A., Wang, Z., and Loor, J. J. (2018). Varying the ratio of Lys: met while maintaining the ratios of Thr: Phe, Lys: Thr, Lys: his, and Lys: Val alters mammary cellular metabolites, mammalian target of rapamycin signaling, and gene transcription. *J. Dairy Sci.* 101, 1708–1718. doi: 10.3168/jds.2017-13351

du, R., Bei, H., Jia, L., Huang, C., Chen, Q., Tao, C., et al. (2020). Danggui Buxue Tang restores antibiotic-induced metabolic disorders by remodeling the gut microbiota. *J. Ethnopharmacol.* 259:112953. doi: 10.1016/j.jep.2020.112953

Everard, A., Belzer, C., Geurts, L., Ouwerkerk, J. P., Druart, C., Bindels, L. B., et al. (2013). Cross-talk between Akkermansia muciniphila and intestinal epithelium controls diet-induced obesity. *Proc. Natl. Acad. Sci. U. S. A.* 110, 9066–9071. doi: 10.1073/pnas.1219451110

Fan, P., Bian, B., Teng, L., Nelson, C. D., Driver, J., Elzo, M. A., et al. (2020). Host genetic effects upon the early gut microbiota in a bovine model with graduated Spectrum of genetic variation. *ISME J.* 14, 302–317. doi: 10.1038/s41396-019-0529-2

Fu, L., Zhang, L., Liu, L., Yang, H., Zhou, P., Song, F., et al. (2021). Effect of heat stress on bovine mammary cellular metabolites and gene transcription related to amino acid metabolism, amino acid transportation and mammalian target of rapamycin (mTOR) Signaling. *Animals* 11:3153. doi: 10.3390/ani11113153

Fujihara, T., and Shem, M. N. (2011). Metabolism of microbial nitrogen in ruminants with special reference to nucleic acids. *Anim. Sci. J.* 82, 198–208. doi: 10.1111/j.1740-0929.2010.00871.x

Ganesh, B. P., Klopfleisch, R., Loh, G., and Blaut, M. (2013). Commensal Akkermansia muciniphila exacerbates gut inflammation in salmonella typhimurium-infected Gnotobiotic mice. *PLoS One* 8:E74963:e74963. doi: 10.1371/journal.pone.0074963

Godoy-Vitorino, F., Goldfarb, K. C., Karaoz, U., Leal, S., Garcia-Amado, M. A., Hugenholtz, P., et al. (2012). Comparative analyses of foregut and hindgut bacterial communities in hoatzins and cows. *ISME J.* 6, 531–541. doi: 10.1038/ismej.2011.131

Gomez-Arango, L. F., Barrett, H. L., McIntyre, H., Callaway, L. K., Morrison, M., Dekker Nitert, M., et al. (2016). Connections between the gut microbiome and metabolic hormones in early pregnancy in overweight and obese women. *Diabetes* 65, 2214–2223. doi: 10.2337/db16-0278

Gong, S., Miao, Y. L., Jiao, G. Z., Sun, M. J., Li, H., Lin, J., et al. (2015). Dynamics and correlation of serum cortisol and corticosterone under different physiological or stressful conditions in mice. *PLoS One* 10:E0117503. doi: 10.1371/journal.pone.0117503

Guo, Y., Li, Z., Deng, M., Li, Y., Liu, G., Liu, D., et al. (2022). Effects of a multi-strain probiotic on growth, health, and fecal bacterial Flora of neonatal dairy calves. *Anim. Biosci.* 35, 204–216. doi: 10.5713/ab.21.0084

Gupta, R. S., and Sethi, M. (2014). Phylogeny and molecular signatures for the phylum fusobacteria and its distinct subclades. *Anaerobe* 28, 182–198. doi: 10.1016/j.anaerobe.2014.06.007

Guzman, C. E., Bereza-Malcolm, L. T., de Groef, B., and Franks, A. E. (2015). Presence of selected methanogens, Fibrolytic bacteria, and Proteobacteria in the gastrointestinal tract of neonatal dairy calves from birth to 72 hours. *PLoS One* 10:e0133048:E0133048. doi: 10.1371/journal.pone.0133048

Haenel, H. (2010). M. Alexander: Microbial Ecology. VII und 511 Seiten, zahlreiche Abb. und Tab.. Verlag John Wiley and Sons, Inc., New York–London–Sidney–Toronto 1971. Preis: 5,75 £. *Mol. Nutr. Food Res.* 16, 693–694.

Hall, V. (2008). Actinomyces—gathering evidence of human colonization and infection. *Anaerobe* 14, 1–7. doi: 10.1016/j.anaerobe.2007.12.001

Hill, G. B., Ayers, O. M., and Kohan, A. P. (1987). Characteristics and sites of infection of *Eubacterium nodatum*, *Eubacterium timidum*, *Eubacterium brachy*, and other asaccharolytic eubacteria. *J. Clin. Microbiol.* 25, 1540–1545. doi: 10.1128/jcm.25.8.1540-1545.1987

Hylemon, P. B., Zhou, H., Pandak, W. M., Ren, S., Gil, G., and Dent, P. (2009). Bile acids as regulatory molecules. *J. Lipid Res.* 50, 1509–1520. doi: 10.1194/jlr.R900007-JLR200

Jami, E., Israel, A., Kotser, A., and Mizrahi, I. (2013). Exploring the bovine rumen bacterial community from birth to adulthood. *ISME J.* 7, 1069–1079. doi: 10.1038/ismej.2013.2

Ji, S., Jiang, T., Yan, H., Guo, C., Liu, J., Su, H., et al. (2018). Ecological restoration of antibiotic-disturbed gastrointestinal microbiota in foregut and hindgut of cows. *Front. Cell. Infect. Microbiol.* 8:79. doi: 10.3389/fcimb.2018.00079

Jiang, L., and Yan, J. (2022). The relationship between free fatty acids and mitochondrial oxidative stress damage to trophoblast cell in preeclampsia. *BMC Pregnancy Childbirth* 22:273. doi: 10.1186/s12884-022-04623-0

Kang, S., Khan, S., Webb, R., Denman, S., and McSweeney, C. (2020). Characterization and survey in cattle of a rumen *Pyrimadobacter* Sp. which degrades the plant toxin Fluoroacetate. *FEMS Microbiol. Ecol.* 96:fiaa077. doi: 10.1093/femsec/fiaa077

Koike, S., and Kobayashi, Y. (2009). Fibrolytic rumen bacteria: their ecology and functions. *Asian Australas. J. Anim. Sci.* 22, 131–138. doi: 10.5713/ajas.2009.r.01

Krehbiel, C. R., Rust, S. R., Zhang, G., and Gilliland, S. E. (2003). Bacterial direct-fed Microbials in ruminant diets: performance response and mode of action. *J. Anim. Sci.* 81, E120–E132.

Kverka, M., Zakostelska, Z., Klimesova, K., Sokol, D., Hudcovic, T., Hrncir, T., et al. (2011). Oral administration of Parabacteroides Distasonis antigens attenuates experimental murine colitis through modulation of immunity and microbiota composition. *Clin. Exp. Immunol.* 163, 250–259. doi: 10.1111/j.1365-2249.2010.04286.x

Lei, Y., Tang, L., Liu, S., Hu, S., Wu, L., Liu, Y., et al. (2021). Parabacteroides produces acetate to alleviate Heparanase-exacerbated acute pancreatitis through reducing neutrophil infiltration. *Microbiome* 9:115. doi: 10.1186/s40168-021-01065-2

Li, D., Ke, Y., Zhan, R., Liu, C., Zhao, M., Zeng, A., et al. (2018). Trimethylamine-N-oxide promotes brain aging and cognitive impairment in mice. *Aging Cell* 17:E12768. doi: 10.1111/acel.12768

Li, F., Li, C., Chen, Y., Liu, J., Zhang, C., Irving, B., et al. (2019). Host genetics influence the rumen microbiota and heritable rumen microbial features associate with feed efficiency in cattle. *Microbiome* 7:92. doi: 10.1186/s40168-019-0699-1

Liu, X., Hu, G., Wang, A., Long, G., Yang, Y., Wang, D., et al. (2022). Black tea reduces diet-induced obesity in mice via modulation of gut microbiota and gene expression in host tissues. *Nutrients* 14:1635. doi: 10.3390/nu14081635

Lyoo, K. S., Jung, M. C., Yoon, S. W., Kim, H. K., and Jeong, D. G. (2018). Identification of canine norovirus in dogs in South Korea. *BMC Vet. Res.* 14:413. doi: 10.1186/s12917-018-1723-6

Mccann, J. C., Wickersham, T. A., and Loor, J. J. (2014). High-throughput methods redefine the rumen microbiome and its relationship with nutrition and metabolism. *Bioinf. Biol. Insights* 8, 109–125. doi: 10.4137/BBI.S15389

Meale, S. J., Li, S., Azevedo, P., Derakhshani, H., Plaizier, J. C., Khafipour, E., et al. (2016). Development of ruminal and fecal microbiomes are affected by weaning but not weaning strategy in dairy calves. *Front. Microbiol.* 7:582. doi: 10.3389/fmicb.2016.00582

Mertens, A., and Holvoet, P. (2001). Oxidized Ldl and Hdl: antagonists in atherothrombosis. *FASEB J.* 15, 2073–2084. doi: 10.1096/fj.01-0273rev

Minato, H., Otsuka, M., Shirasaka, S., Itabashi, H., and Mitsumori, M. (1992). Colonization of microorganisms in the rumen of young calves. *J. Gen. Appl. Microbiol.* 38, 447–456. doi: 10.2323/jgam.38.447

Mizobe, K., Kishihara, K., Ezz-Din el-Naggar, R., Madkour, G. A., Kubo, C., and Nomoto, K. (1997). Restraint stress-induced elevation of endogenous glucocorticoid suppresses migration of granulocytes and macrophages to an inflammatory locus. *J. Neuroimmunol.* 73, 81–89. doi: 10.1016/S0165-5728(96)00169-5

Moraes, C., Fouque, D., Amaral, A. C., and Mafra, D. (2015). Trimethylamine N-oxide from gut microbiota in chronic kidney disease patients: focus on diet. *J. Ren. Nutr.* 25, 459–465. doi: 10.1053/j.jrn.2015.06.004

Morrison, S. J., Dawson, S., and Carson, A. F. (2010). The effects of Mannan oligosaccharide and streptococcus Faecium addition to Milk replacer on calf health and performance. *Adv. Anim. Biosci.* 131, 292–296. doi: 10.1016/j.livsci.2010.04.002

Nicholson, J. K., Holmes, E., Kinross, J., Burcelin, R., Gibson, G., Jia, W., et al. (2012). Host-gut microbiota metabolic interactions. *Science* 336, 1262–1267. doi: 10.1126/science.1223813

Nido, S. A., Shituleni, S. A., Mengistu, B. M., Liu, Y., Khan, A. Z., Gan, F., et al. (2016). Effects of selenium-enriched probiotics on lipid metabolism, Antioxidative status, histopathological lesions, and related gene expression in mice fed a high-fat diet. *Biol. Trace Elem. Res.* 171, 399–409. doi: 10.1007/s12011-015-0552-8

Pounden, W. D., and Hibbs, J. W. (1949). The influence of pasture and rumen inoculation on the establishment of certain microorganisms in the rumen of young dairy calves. *J. Dairy Sci.* 32, 1025–1031. doi: 10.3168/jds.S0022-0302(49)92157-8

Qi, M., Cao, Z., Shang, P., Zhang, H., Hussain, R., Mehmood, K., et al. (2021). Comparative analysis of fecal microbiota composition diversity in Tibetan piglets suffering from diarrheagenic *Escherichia coli* (Dec). *Microb. Pathog.* 158:105106. doi: 10.1016/j.micpath.2021.105106

Rérat, A., Nunes, C. S., Mendy, F., and Roger, L. (1988). Amino acid absorption and production of pancreatic hormones in non-anaesthetized pigs after duodenal infusions of a Milk Enzymic hydrolysate or of free amino acids. *Br. J. Nutr.* 60, 121–136. doi: 10.1079/BJN19880082

Rey, M., Enjalbert, F., and Monteils, V. (2012). Establishment of ruminal enzyme activities and fermentation capacity in dairy calves from birth through weaning. *J. Dairy Sci.* 95, 1500–1512. doi: 10.3168/jds.2011-4902

Rezai Sarteshnizi, F., Abdi-benemar, H., Seifdavati, J., Khalilvandi-Behroozyar, H., Seyedsharifi, R., and Salem, A. Z. M. (2020). Influence of spray-dried rumen fluid supplementation on performance, blood metabolites and cytokines in suckling Holstein calves. *Animal* 14, 1849–1856. doi: 10.1017/S1751731120000518

Roberts, G. L. (2000). Fusobacterial infections: an underestimated threat. *Br. J. Biomed. Sci.* 57, 156–162.

Sadet, S., Martin, C., Meunier, B., and Morgavi, D. P. (2007). PCR-DGGE analysis reveals a distinct diversity in the bacterial population attached to the rumen epithelium. *Animal* 1, 939–944. doi: 10.1017/S1751731107000304

Saleem, F., Ametaj, B. N., Bouatra, S., Mandal, R., Zebeli, Q., Dunn, S. M., et al. (2012). A metabolomics approach to uncover the effects of grain diets on rumen health in dairy cows. *J. Dairy Sci.* 95, 6606–6623. doi: 10.3168/jds.2012-5403

Sato, T., Hidaka, Y., and Kamimura, S. (2010). Sugar supplementation stimulates growth performance in calves with growth retardation. *J. Vet. Med. Sci.* 72, 29–33. doi: 10.1292/jvms.09-0180

Signorini, M. L., Soto, L. P., Zbrun, M. V., Sequeira, G. J., Rosmini, M. R., and Frizzo, L. S. (2012). Impact of probiotic administration on the health and fecal microbiota of young calves: a meta-analysis of randomized controlled trials of lactic acid bacteria. *Res. Vet. Sci.* 93, 250–258. doi: 10.1016/j.rvsc.2011.05.001

Song, M., Zhang, F., Chen, L., Yang, Q., Su, H., Yang, X., et al. (2021). Dietary Chenodeoxycholic acid improves growth performance and intestinal health by altering serum metabolic profiles and gut bacteria in weaned piglets. *Anim. Nutr.* 7, 365–375. doi: 10.1016/j.aninu.2020.07.011

Suzuki, T. A., Martins, F. M., Phifer-Rixey, M., and Nachman, M. W. (2020). The gut microbiota and Bergmann's rule in wild house mice. *Mol. Ecol.* 29, 2300–2311. doi: 10.1111/mec.15476

Tang, Y., Fang, W., Xiao, Z., Song, M., Zhuang, D., Han, B., et al. (2021). Nicotinamide ameliorates energy deficiency and improves retinal function in Cav-1(−/−) mice. *J. Neurochem.* 157, 550–560. doi: 10.1111/jnc.15266

Tsubone, T. M., Junqueira, H. C., Baptista, M. S., and Itri, R. (2019). Contrasting roles of oxidized lipids in modulating membrane microdomains. *Biochim. Biophys. Acta Biomembr.* 1861, 660–669. doi: 10.1016/j.bbamem.2018.12.017

van Hylckama Vlieg, J. E., Veiga, P., Zhang, C., Derrien, M., and Zhao, L. (2011). Impact of microbial transformation of food on health-from fermented foods to fermentation in the gastro-intestinal tract. *Curr. Opin. Biotechnol.* 22, 211–219. doi: 10.1016/j.copbio.2010.12.004

Wang, Z., Jiang, S., Ma, C., Huo, D., Peng, Q., Shao, Y., et al. (2018). Evaluation of the nutrition and function of cow and goat Milk based on intestinal microbiota by metagenomic analysis. *Food Funct.* 9, 2320–2327. doi: 10.1039/C7FO01780D

Wang, W., Li, C., Li, F., Wang, X., Zhang, X., Liu, T., et al. (2016). Effects of early feeding on the host rumen transcriptome and bacterial diversity in lambs. *Sci. Rep.* 6:32479. doi: 10.1038/srep32479

Wang, J. K., Ye, J. A., and Liu, J. X. (2012). Effects of tea saponins on rumen microbiota, rumen fermentation, methane production and growth performance—a review. *Trop. Anim. Health Prod.* 44, 697–706. doi: 10.1007/s11250-011-9960-8

Weimer, P. J. (2015). Redundancy, resilience, and host specificity of the ruminal microbiota: implications for engineering improved ruminal fermentations. *Front. Microbiol.* 6:296. doi: 10.3389/fmicb.2015.00296

Weingand, K., Brown, G., Hall, R., Davies, D., Gossett, K., Neptun, D., et al. (1996). Harmonization of animal clinical pathology testing in toxicity and safety studies. The joint scientific committee for international harmonization of clinical pathology testing. *Fundam. Appl. Toxicol. Off. J. Soc. Toxicol.* 29, 198–201. doi: 10.1093/toxsci/29.2.198

Wolf, P., Winhofer, Y., Krssak, M., Smajis, S., Harreiter, J., Kosi-Trebotic, L., et al. (2016). Suppression of plasma free fatty acids reduces myocardial lipid content and systolic function in type 2 diabetes. *Nutr Metab Cardiovasc Dis* 26, 387–392. doi: 10.1016/j.numecd.2016.03.012

Xiong, X., Ren, Y., Cui, Y., Li, R., Wang, C., and Zhang, Y. (2017). Obeticholic acid protects mice against lipopolysaccharide-induced liver injury and inflammation. *Biomed. Pharmacother.* 96, 1292–1298. doi: 10.1016/j.biopha.2017.11.083

Ziolecka, A., Osińska, Z., and Ziolecki, A. (1984). The effect of stabilized rumen extract on growth and development of calves: 1. Liveweight gain and efficiency of feed utilization. *Z. Tierphysiol. Tierernhr. Futtermittelkd.* 51, 13–20. doi: 10.1111/j.1439-0396.1984.tb01406.x

Ziolecki, A., Kwiatkowska, E., and Laskowska, H. (1984). The effect of stabilized rumen extract on growth and development of calves. 2. Digestive activity in the rumen and development of microflora in the rumen and Faeces. *Z. Tierphysiol. Tierernahr. Futtermittelkunde* 51, 20–31.

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# Metabolic and inflammatory linkage of the chicken cecal microbiome to growth performance

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**Introduction:** Chinese indigenous chicken breeds are widely used as food in China but their slow growth rate and long farming cycle has limited their industrial production.

**Methods:** In the current study we examined whether the market weights of native chicken breeds were related to specific cecal bacteria, serum metabolites and inflammatory cytokines. We examined cecal bacterial taxa using 16S rDNA analysis along with untargeted serum metabolites and serum inflammatory cytokines.

**Results:** We found that the cecal microbiota could explain 10.1% of the individual differences in chicken weights and identified key cecal bacterial genera that influenced this phenotype. The presence of *Sphaerochaeta* spp. improved growth performance via bovinic acid metabolism. In contrast, *Synergistes* and *norank_f_Desulfovibrionaceae* had a negative effect on growth by inducing expression of the inflammatory cytokine IL-6.

**Discussion:** We were able to link specific bacterial genera with growth promotion in chickens and this study will allow further development of their use as probiotics in these animals.

KEYWORDS

**indigenous chickens, market-weight, cecal microbiota, serum metabolome, inflammatory cytokine**

## Introduction

Growth performance is an important economic trait for broiler chickens and antibiotics supplied in feed have been traditionally used to increase the market weight of these animals. However, these types of intensive antibiotic-use practices have accelerated the development of antibiotic resistance in bacteria and this has become a major public global health concern (Wang et al., 2019). Therefore, many countries including China have prohibited the use of antibiotics as growth promoters in food animal production and scientists are committed to developing antibiotic alternatives for animal growth promotion (Zhang et al., 2022a). One promising alternative is the use of probiotics or associated metabolites derived from the chicken gut microbiome (Ayalew et al., 2022). The gut microbiota that are present in animal gastrointestinal tracts form a diverse, complex

and dynamic ecosystem composed of tens of millions of microorganisms (Glendinning et al., 2019). These microorganisms play essential roles in maintaining animal health and their primary site of residence in birds is the cecum (Cui et al., 2021; Zhang et al., 2022b).

There is direct evidence that the cecal microflora of chickens has a positive impact on growth performance. For example, sex differences in chicken growth performance were related to glycan and lipid metabolic functions of cecal bacteria (Cui et al., 2021). Another study demonstrated that transplantation of fecal microbiota and prebiotic supplementation promoted daily weight gains for chickens (Li et al., 2016). In particular, the use of the probiotic *Lactobacillus plantarum P-8* in broiler diets increased adsorption of recalcitrant polysaccharides that were converted to a nutrition source and thus improved feed efficiency (Borda-Molina et al., 2018). Specific bacteria have also been linked to chicken growth traits and these include *Microbacterium* spp. and *Sphingomonas* spp. in Turpan × White Leghorn hybrids that were beneficial to chicken growth while the presence of *Slackia* spp. promoted a growth-inhibiting effect on chickens (Zhang et al., 2022b). Studies such as these have indicated that gut microbiota is a potential target for regulating growth performance.

Despite established relationships between gut the microbiota and chicken growth performance, mechanistic details have yet to be formulated. However, intestinal inflammation due to colonization by bacterial pathogens has been linked to poor growth performance and probiotic treatments can counter these effects (Zhang et al., 2022a). Regulation of fat metabolism and improved growth performance have also been directly linked to specific cecal microbiota (Zhang et al., 2022b). However, more precise mechanistic details are lacking because metabolic functional pathway assignments for the key microbiota were based on 16S rDNA and metagenomic sequencing that predict, but do not prove, functional relationships (Wen et al., 2019; Elokil et al., 2020; Darwish et al., 2021; Wen et al., 2021). In contrast, metabolomics have been used to fill the information gap between gene and phenotype although metabolomics has not been directly applied to chicken growth performance (Wu et al., 2018; Liu et al., 2021). Therefore, in the current study, we integrated and interpreted metabolomic information to construct a metabolic network of chicken growth traits to gain a new perspective and identify underlying biological processes. We hypothesized that specific cecal microorganisms in chickens can regulate host serum metabolites as well as inflammatory cytokine production and thereby affect market weight. We used Qiandongnan Xiaoxiang chickens, Guizhou yellow broilers and Wumeng black-bone chickens as examples of the most common indigenous chickens used for food production in Guizhou Province, China (Xu, 2018). Our goal was to link key cecal bacterial taxa to chicken market weight and integrate this with serum metabolomic and serum cytokine analyses to identify phenotypic regulatory mechanisms. Our results can be used to guide design of microbial or probiotic intervention targets that regulate market weight. These will help establish new options for the development of antibiotic alternatives.

# Materials and methods

## Animals and sample collection

Two cohorts of indigenous chickens in Guizhou Province, China were collected in this study and the experimental cohort consisted of 60 Qiandongnan Xiaoxiang chickens that were raised to 160 ± 3 days old and 12 with the highest (1.78 ± 0.12 kg, 6 of each sex) and the lowest (1.02 ± 0.10 kg, 6 of each sex) body-weights were selected for further study. In the validation trail, 79 chickens including 55 Guizhou yellow broilers (1.89 ± 0.28 kg, $n$ = 55, 26 males and 29 females) and 24 Wumeng black-bone chickens (1.61 ± 0.22 kg, 11 males and 13 females) were collected as validation cohorts. All chickens in the same cohort were raised in the same chicken house and were provided identical commercial diets with *ad libitum* access to water and feed and were managed in the same way. Chickens were slaughtered at the age of 160 ± 3d and antibiotics were not used for 1 month prior to sample collection. We collected 24 each of blood and cecal content samples from the experimental cohort and 79 each from the validation cohort (Supplementary Table S1). Serum was collected from blood following centrifugation using standard procedures. Since serum was failed to be obtained from blood samples of three Qiandongnan Chickens, 21 serum samples (high-market-weight group, $n$ = 12; low-market-weight group, $n$ = 9 ) were involved for metabolomics analysis. Cecal content samples were collected at the same position of the intestinal tract and immediately frozen in liquid nitrogen for transport to the laboratory and then stored at −80°C.

Serum and cecal content samples from the experimental cohorts were employed for metabonomic and microbiota analysis using ultra-high-performance liquid chromatography—tandem mass spectrometry (UPLC-MS/MS) and 16S rDNA gene sequencing, respectively. Serum and cecal content samples from the validation cohort were used for cytokine determinations and microbiota analysis by enzyme-linked immunosorbent assay (ELISA) and 16S rDNA gene sequencing, respectively.

## DNA manipulations and sequencing

Total microbial DNA were extracted from 103 cecal content samples using the Magnetic Soil and Stool DNA Kit (Tiangen, Beijing, China) according to the manufacturer's protocol. DNA concentrations and purity were determined by UV spectroscopy using a NanoDrop-1000 instrument (Thermo Fisher, Pittsburg, PA, United States) and 0.8% agarose gel electrophoresis. The V3–V4 region of the bacterial 16S rRNA gene was amplified with the primer pair 338F(5′-ACTCCTACGGGAGGCAGCA-3′)and 806R(5′-GGACTACHVGGGTWTCTAAT-3′) that were combined with adapter and barcode sequences. PCR amplicons were quantified using Quant-iT dsDNA HS reagent (Thermo Fisher) and pooled.

High-throughput sequencing analysis of bacterial rDNA genes in the purified and pooled samples were performed on an Illumina Hiseq 2500 platform (Illumina, San Diego, CA, United States). Trimmomatic software (version 0.33; Bolger et al., 2014) was used to filter out primers, low-quality and ambiguous sequences. Cut adapt (version 1.9.1; Lindgreen, 2012) was used to identify and remove primer sequences. The resulting paired-end reads from the clean data sets were assembled into tags using FLASH (version 1.2.11; Magoc and Salzberg, 2011). The sequence depth of each sample was rarefied to 75,007 tags to avoid statistical bias resulting from an uneven sequencing depth. USEARCH (version 10.0; Edgar, 2013) was employed to cluster tags of >97% identity into operational taxonomic units (OTU) and the OTU filtering

threshold was set at 0.005% (Bokulich et al., 2013). RDP classifier (version 2.2; Wang et al., 2007) was used to produce OTU taxonomic assignments and representative sequences of each OTU were compared using the Silva reference database (Release 132, http://www.arb-silva.de; Quast et al., 2013) for OTU annotations.

## Determination of serum metabolomic profiles in Qiandongnan Xiaoxiang chickens

An untargeted metabolomic analysis of serum samples from the Qiandongnan Xiaoxiang chicken were conducted by a commercial company (Shanghai Biotree Biotech, Shanghai, China). The analyses were performed using UPLC-MS/MS as previously described (Dunn et al., 2011; Cai et al., 2015; Wang et al., 2016). In brief, a Vanquish high-performance liquid chromatography (HPLC) system (Thermo Fisher) equipped with a BEH amide column (2.1 mm × 100 mm, 1.7 μm) coupled to an Orbitrap MS Q Exactive HFX mass spectrometer (Thermo Fisher) was used for separations. The mobile phase consisted of (Solvent A) 25 mM ammonium acetate and 25 mM ammonia hydroxide in water pH = 9.75 and (Solvent B) acetonitrile. The auto-sampler temperature was 4°C and the injection volume was 2 μL. The QE HFX mass spectrometer was used for its ability to acquire MS/MS spectra on the information-dependent acquisition (IDA) mode and was controlled by the Xcalibur acquisition software supplied with the instrument. In this mode, the full scan MS spectrum was continuously evaluated. The ESI source conditions were set as follows: sheath gas flow rate, 30 Arb; Aux gas flow rate, 25 Arb; capillary temperature, 350°C; full MS resolution, 60,000; MS/MS resolution, 7,500; collision energy, 10/30/60 in NCE mode, spray voltage, 3.6 kV (positive) or −3.2 kV (negative).

The raw data were converted to the extensible markup language (mzXML) format using ProteoWizard and processed with an in-house program developed using R language and based on XCMS for peak detection, extraction, alignment and integration (Smith et al., 2006). An in-house $MS_2$ database (BiotreeDB) was used for metabolite annotations with a set cutoff of 0.3. Metabolites were identified using the HMDB database[1] and endogenous metabolites were reserved for further construction of metabolite feature modules. The internal standard normalization method was employed in this data analysis. The final dataset containing peak numbers, sample names and normalized peak areas were imported into SIMCA 16.0.2 (Sartorius Stedim Data Analytics AB, Umea, Sweden) for multivariate analysis (Wiklund et al., 2008).

## Serum cytokine measurements from Guizhou yellow broilers and Wumeng black-bone chickens

Commercial ELISA kits (Ziker Biological Technology, Shenzhen, China) were used to quantify levels of INF-γ, IL-1β, IL-5, IL-6, IL-17, and IL-22 in the sera of the 79 chickens in the validation cohorts according to the manufacturer's protocol. Detection limits were 5 pg./mL (IFN-γ), 40 pg./mL (IL-1β), 5 pg./mL (IL-5), 2 pg./mL (IL-6), 3 pg./mL (IL-17), and 2 pg./mL (IL-22).

## Statistical analyses

### Calculation and comparation of α- and β-diversity of cecal microbiota

OTUs with relative abundances >0.01% that were present in >10% of individuals were used for further analysis. Mothur software (version 1.31.2; Schloss et al., 2009) was employed to calculate the α-diversity of OTUs including Shannon, Simpson, Chao1, Faith's phylogenetic diversity (PD) and ACE indices (Shannon, 1948; Chao, 1984; Faith, 1992). β-diversity of chicken cecal microbial community between high-and low-market-weight chicken groups was calculated using principal coordinate analysis (PCoA) based on unweighted UniFrac distances using QIIME (Caporaso et al., 2010).

### Construction of microbial co-abundance groups

OTUs whose relative abundance was >0.05% and were present in >20% of all samples were selected for co-abundance group (CAG) construction. SPIEC-EASI package in R was employed to cluster CAGs. Interactions between OTUs were calculated based on their abundances using the SparCC algorithm with 100 bootstrap replicates followed by computing correlation matrices (Friedman and Alm, 2012). The Spearman's rank correlation coefficients of pairwise OTUs >0.55 were used to classify CAGs. The correlation coefficient values were converted to a correlation distance (1-correlation coefficient value) and the OTUs were clustered into CAGs using the Ward clustering algorithm with a 'hclust' function in the SPIEC-EASI R package. Permutational MANOVA was applied to detect the statistical significance of each CAG clustering using 999 permutations with Bray–Curtis dissimilarity. The CAG was deemed acceptable when $p < 0.005$. Wilcoxon rank sum tests were performed to identify differences of relative abundance at the CAG level between the high-market-weight chickens and low-market-weight chickens. The CAG network was visualized in Cytoscape V. 3.7.1 (Lopes et al., 2010).

### Identification of cecal microbiota associated with market-weight in the validation cohorts using two-part model

A two-part model was used to identify OTUs which that were linked with chicken market-weight in the validation trail (Fu et al., 2015). Specifically, a binomial analysis was performed to determine associations between the presence or absence of an OTU and market-weight. For a particular OTU, each sample possessed 2 binary features ($b$) that was coded as "1" and "0" when they were detected or not, respectively, to determine correlations between the presence of the microbe and the market-weight. The binary model was described as $y = \beta_1\ b + e$ where $y$, $\beta_1$, $b$ and $e$ represented the market-weight per individual, the estimated effect for the binary effect, a binary feature and the residuals, respectively. Secondly, the correlation between OTU abundance and market-weight were analyzed using the quantitative model but only the subjects that contained the cecal microbiota associated with market-weight identified in the binary model were used. The quantitative model was expressed as $y = \beta_2\ q + e$, where $q$ represented microbial abundance which are usually transformed into

1 https://hmdb.ca/

$\log_{10}$, $\beta_2$ represented the estimated effect value of the quantitative model and $e$ represented the residual. Combined with the effects of the binary model and the quantitative model, a meta-$p$-value was calculated using an unweighted Z-method. According to the minimum $p$-value for the binary model, quantitative model and meta-analysis, the corresponding allocation coefficient and final $p$-value was obtained. The Z-value was obtained from the Z distribution coefficient. $Z > 0$ represented a positive association between microbe and phenotype value and $Z < 0$ represented a negative association.

### Other statistical analyses

We normalized the metabolite dataset though $\log_{10}$ transformation of the m/z values and then the module was constructed using a soft threshold Pearson correlation analysis (Langfelder and Horvath, 2008). This combined a topological overlap distance metric and average hierarchical clustering using weighted correlation network analysis (WGCNA) in the R package. The Matthews Correlation Coefficient (MCC) method of cytoHubba plug-ins in Cytoscape was used to identify the hub bacteria in the cecal microbiota network (Chin et al., 2014). LDA Effect Size (LEfSe) analysis[2] was conducted to identify the cecal microbiota and serum metabolites that showed significant differences between high-and low-market-weight chicken groups ($p < 0.05$ and $LDA > 2.0$). All $p$-values of the multiple tests involved in this study were corrected by the Benjamini–Hochberg method. A total of 100 cross-validation tests were performed as previously described (Fu et al., 2015) to assess the proportion of cecal microbiota explaining the individual variation of market-weight. A random forest model (Ntree = 1,000) was employed to determine which OTUs could be used as microbial markers to distinguish high-and low-market-weight chickens (Guo et al., 2020). Spearman correlation analysis was used to identify relationships between cecal microbiota and serum metabolites or inflammatory cytokines.

## Results

### Experimental cohort (Qiandongnan Xiaoxiang chickens)

#### Differences in cecal microbiota diversity between high- and low-market-weight chickens

An analysis of 24 cecal content samples of chickens from the experimental cohort generated 1,800,168 high-quality reads (75,007 reads per sample) and included 727 OTUs that were clustered according to 97% sequence identity. These OTUs were annotated to 16 phyla and 12 were present in all samples and 6 were present at a relative abundance >1%. The latter included Bacteroidetes (48.4%), Firmicutes (36.9%), Proteobacteria (5.8%), Synergistetes (2.6%), Spirochaetes (1.0%) and Actinobacteria (1.6%; Figure 1). The 5 most important OTUs for the hub microbiota of the Qiandongnan Xiaoxiang chickens were all members of the *Ruminococcaceae* family (Figure 2A). We then compared the $\alpha$-*and* $\beta$-diversity of cecal microbiota between chickens with high-and low-market-weights. The $\alpha$-diversity analysis indicated that scores for the ACE (Wilcoxon rank sum test, $p = 0.03$), Shannon ($p = 0.01$) and PD ($p = 0.005$) indices in the high-market-weight chickens were significantly greater than those of low-market-weight chickens (Figures 2B,C; Supplementary Figures S1A–C). In contrast, $\beta$-diversity comparisons using PCoA indicated no significant differences between high-and low-market-weight chickens (Figure 2D).

One of our goals was the identification of cecal microbiota clusters that were related to market-weight. The gut microbiota is a huge and complex micro-ecosystem and the microbiota can directly or indirectly affect host physiological functions in the form of co-abundance groups (CAGs; Angulo et al., 2019). We therefore clustered the OTUs into CAGs based on their interaction network and obtained 261 OTUs after filtering that were clustered into 30 CAGs (Figure 3). We compared the average relative abundance of each CAG between high-and low-market-weight chickens and found that only the abundance of CAG17 ($p = 0.04$) was significantly different between the two groups and it was enriched in high-market-weight chickens. There were 5 OTUs in CAG17 and all belonged to the Clostridiales order (Supplementary Tables S2, S3). In particular, OTU234, OTU159 and OTU99 were annotated to *Eubacterium*, *Subdoligranulum*, and *Peptococcus*, respectively while OTU27 and OTU908 could not be annotated at the genus level. Significantly, differences in the average relative abundance of CAG26 between high-and low-market-weight chickens was near the level of significance ($p = 0.08$). CAG26 was a *Ruminococcaceae*-dominated CAG in which 8 / 11 OTUs belonged in this family that was enriched in high-market-weight chickens. OTU73, OTU106, OTU228, OTU829, OTU1042, and OTU79 were annotated to *Ruminococcaceae* UCG-005, *Ruminococcaceae* UCG-010, *Ruminococcaceae* UCG-014, *Ruminiclostridium*, *Negativibacillus* and *Oscillospira*, respectively. OTU950 and OTU136 were not annotated to specific bacteria at the genus level. These data suggested a beneficial effect of *Ruminococcaceae* on growth performance in chickens *via* the interaction network. The remaining 3 OTUs in CAG26 were OTU137, OTU76 and OTU1666 that were annotated to *CHKCI001* in Lachnospiraceae, Christensenellaceae and WCHB1-41, respectively.

#### Bacterial species with differential abundance between high- and low-market-weight chickens

We further identified cecal microbiota members that were enriched in high-or low-market-weight chickens at the genus level. LEfSe analysis indicated that 17 genera possessed significant differences between the two groups. In particular, *Treponema_2* and *Succinatimonas* were enriched in the low-market-weight chickens while *Ruminococcaceae UCG-009, Ruminococcaceae UCG-004* and *Ruminiclostridium 5* were at high abundance levels for the high-market-weight chickens and consistent with the CAG results above. The high-market-weight group also contained *Oribacterium*, *GCA-900066575 Defluviitaleaceae UCG-011, uncultured_bacterium f Peptococcaceae* (order Clostridiales) and *Odoribacter, Paraprevotella* and *uncultured bacterium f Rikenellaceae* (order Bacteroidales). The other 5 genera enriched in the high-market-weight chickens were represented by the orders *Brachyspira, Pseudomonas, CHKCI002* and *bacterium enrichment culture clone R4-41B* and *Sphaerochaeta* (Figure 4A).

We applied a random forest classification analysis to identify the biomarker OTUs that might accurately distinguish the high-and low-market-weight chickens. We found that the 10 most prevalent OTUs could distinguish high-market-weight chickens from low-market-weight chickens with an accuracy of 97.92% (AUC value;

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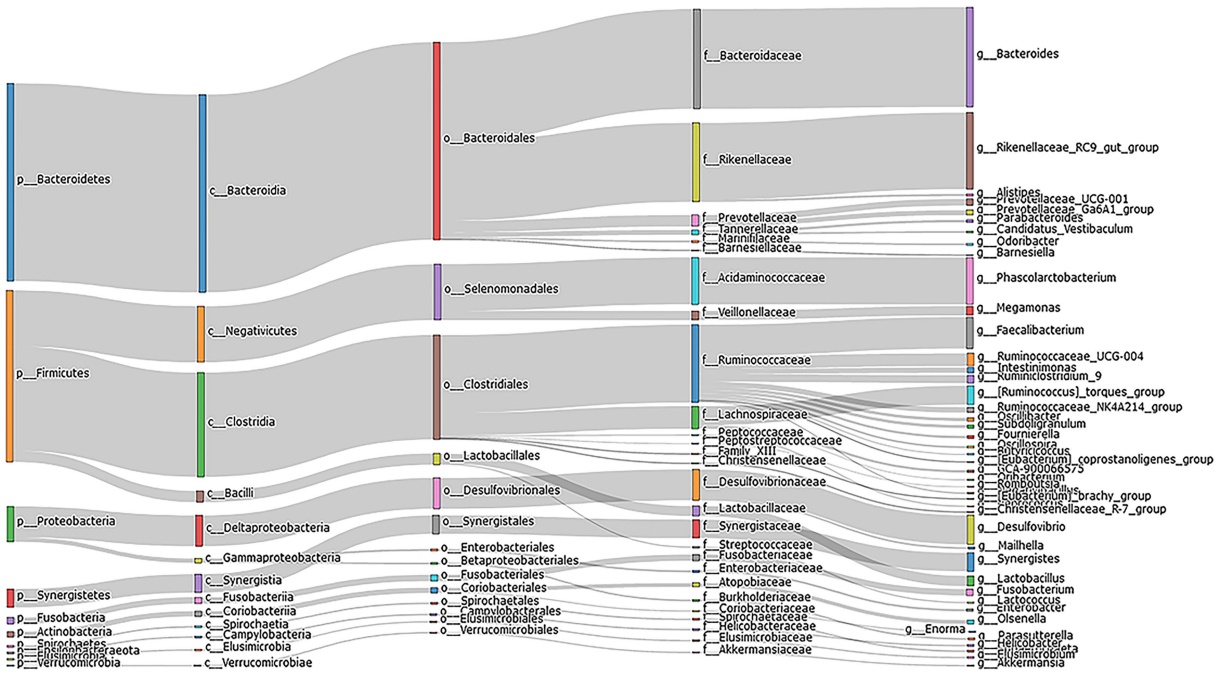


FIGURE 1
Sankey diagram depicting the bacterial composition of cecal content samples from Qiandongnan Xiaoxiang Chickens at 160 ± 3 days of age ($n$ =24). The colored columns from left to right represent the proportions of bacterial taxa from phylum to genus level.

**A**

| Rank | ID | Family | Genus |
|---|---|---|---|
| 1 | OTU154 | Ruminococcaceae | *unclassfied_f_Ruminococcaceae* |
| 2 | OTU686 | Ruminococcaceae | *unclassfied_f_Ruminococcaceae* |
| 3 | OTU149 | Ruminococcaceae | *Ruminococcaceae_NK4A214_group* |
| 4 | OTU37 | Ruminococcaceae | *Oscillibacter* |
| 5 | OTU79 | Ruminococcaceae | *Oscillospira* |
| 6 | OTU1145 | Bacteroidaceae | *Bacteroides* |
| 7 | OTU633 | Bacteroidaceae | *Bacteroides* |
| 8 | OTU29 | Atopobiaceae | *Olsenella* |
| 9 | OTU104 | Prevotellaceae | *unclassfied_f_Prevotellaceae* |
| 10 | OTU139 | unclassfied_o_Bacteroidales | *unclassfied_f_Bacteroidales* |
| 11 | OTU96 | unclassfied_o_Gastranaerophilales | *unclassfied_o_Gastranaerophilales* |
| 12 | OTU41 | Lactobacillaceae | *Lactobacillus* |
| 13 | OTU166 | Ruminococcaceae | *Ruminococcaceae_UCG-013* |
| 14 | OTU242 | Eggerthellaceae | *Enterorhabdus* |
| 15 | OTU1 | Acidaminococcaceae | *Phascolarctobacterium* |
| 16 | OTU31 | Lactobacillaceae | *Lactobacillus* |
| 17 | OTU5 | Bacteroidaceae | *Bacteroides* |
| 18 | OTU91 | Coriobacteriaceae | *Enorma* |
| 19 | OTU46 | unclassfied_o_Bacteroidales | *unclassfied_o_Bacteroidales* |
| 20 | OTU5220 | Acidaminococcaceae | *Phascolarctobacterium* |

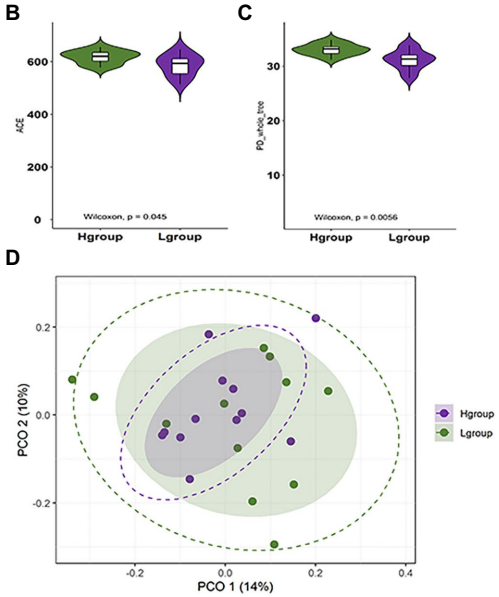


FIGURE 2
Hub cecal microbiota of Qiandongnan Xiaoxiang chickens at 160 ± 3 days of age and the difference of $\alpha$-and $\beta$-diversity between chickens in high- (H group, $n$= 12) and low-market-weight (L group, $n$= 12) groups. **(A)** The 20 most abundant OTUs in the cecal microbiota network ranked using the Matthews Correlation Coefficient (MCC) method. Comparisons of **(B)** ACE and **(C)** PD indices between high-and low-market-weight chickens. **(D)** Principal coordinates analysis (PCoA) of microbial communities in cecal content samples based on unweighted UniFrac distances between high-and low-market-weight chickens.

Figures 4B,C). This group of 10 included 5 OTUs annotated to the *Ruminococcaceae* (OTU176, OTU159, OTU950, OTU933, and OTU329) annotated to *Ruminiclostridium 5*, *Subdoligranulum*, *Ruminococcaceae*, *Faecalibacterium* and *GCA-900066225*, respectively. It is noteworthy that OTU176 (*Ruminiclostridium 5*) possessed the highest score as a marker and this OTU also significantly differed between high-and low-market-weight chickens in the LEfSe analysis (see above).

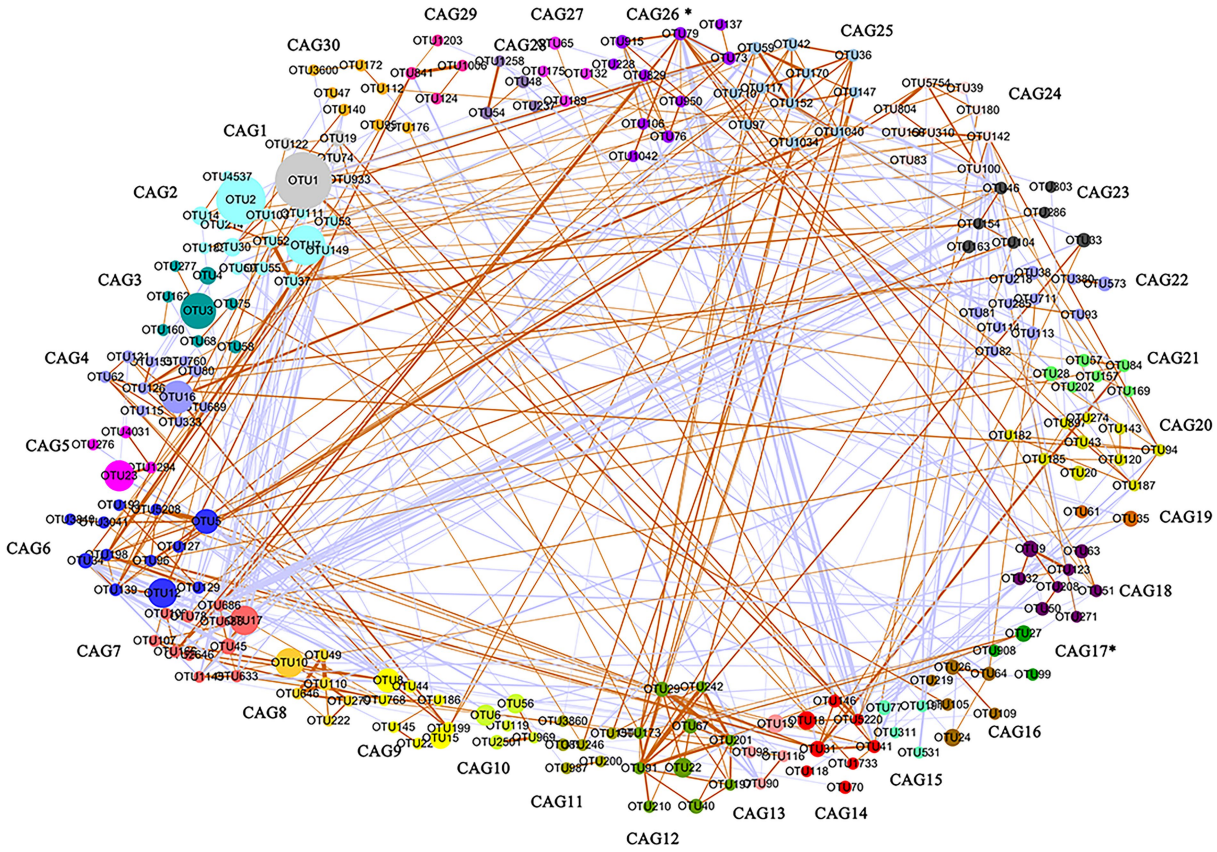


FIGURE 3
Co-abundance groups (CAG) in cecal microbiota based on the relative OTU abundance in Qiandongnan Xiaoxiang chickens at 160 ± 3 days of age ($n$ =24). A total of 261 OTUs were clustered into 30 CAGs using permutational multivariate analysis of variance. Asterisks (*) represent significant differences of relative OTU abundance in CAGs between high-and low-market-weight chickens. Node sizes are proportional to the average abundance of each OTU. The lines connecting two nodes represent SparCC correlations between the connected nodes with the line width representing the correlation magnitude. Brown and purple lines represent significantly positive and negative correlations between two OTUs, respectively, using an absolute value of correlation coefficient >0.55. Unconnected nodes were omitted.

## Differential metabolite profiles between high- and low-market-weight chickens

A total of 6,944 endogenous metabolites were obtained under ESI-and ESI$^+$ modes and following relative standard deviation de-noising and annotation, 378 metabolites remained. PCA analysis indicated significant differences in the global metabolome between high-and low-market-weight chickens (Figure 5A). Specifically, 58 metabolite features were identified as market-weight-related ($p < 0.001$, $FDR < 0.2$) and 35 metabolites were enriched in serum samples of high-market-weight chickens while 23 metabolites were enriched in low-market-weight chickens (Supplementary Table S4).

Correlations between market-weight-related bacteria and host serum metabolites indicated that in the high-market-weight group, the enriched metabolites were dominated by nucleotide metabolites including primary and secondary bile acid metabolites [clustered in the turquoise (17/35 metabolites) and brown modules]. We also identified several significant correlations between metabolite modules and market-weight-related bacteria at the genus level. The turquoise module was found to be positively correlated with *Pseudomonas* ($p = 0.01$) and *Ruminococcaceae UCG-004* ($p = 0.03$) while negatively correlated with *Treponema 2* ($p = 0.04$). The brown module comprising primary and secondary bile acid metabolites was positively correlated with *Pseudomonas* ($p = 0.04$) but negatively correlated with *Treponema 2* ($p = 0.01$). Low-market-weight related metabolites were primarily clustered in the blue and gray modules. No differential bacteria were related with blue module of metabolites while the gray module was significantly negatively correlated with *Ruminococcaceae UCG-004* ($p = 0.05$; Figures 5B, 6).

## Correlations between differential metabolites and differential cecal microbiota

We further explored whether the differential cecal microbiota possessed different metabolite profiles between high-and low-market-weight chickens. The results of differentiation analysis of serum metabolites showed that 58 differential metabolites were determined. And the results of a correlation analysis based on Spearman coefficients employed for 58 different metabolites and 17 different cecal microbiota showed that the genus *Ruminiclostridium 5* was significantly positively correlated with 10 metabolites including the amino acids prolylhydroxyproline, 4-hydroxyproline, homo-L-arginine as well as lipids but significantly negatively correlated with adrenochrome. *Ruminococcaceae UCG-011* was significantly correlated with 27 metabolites and might be one of the reasons the *Ruminococcaceae* were the hub cecal microbiota of Qiandongnan

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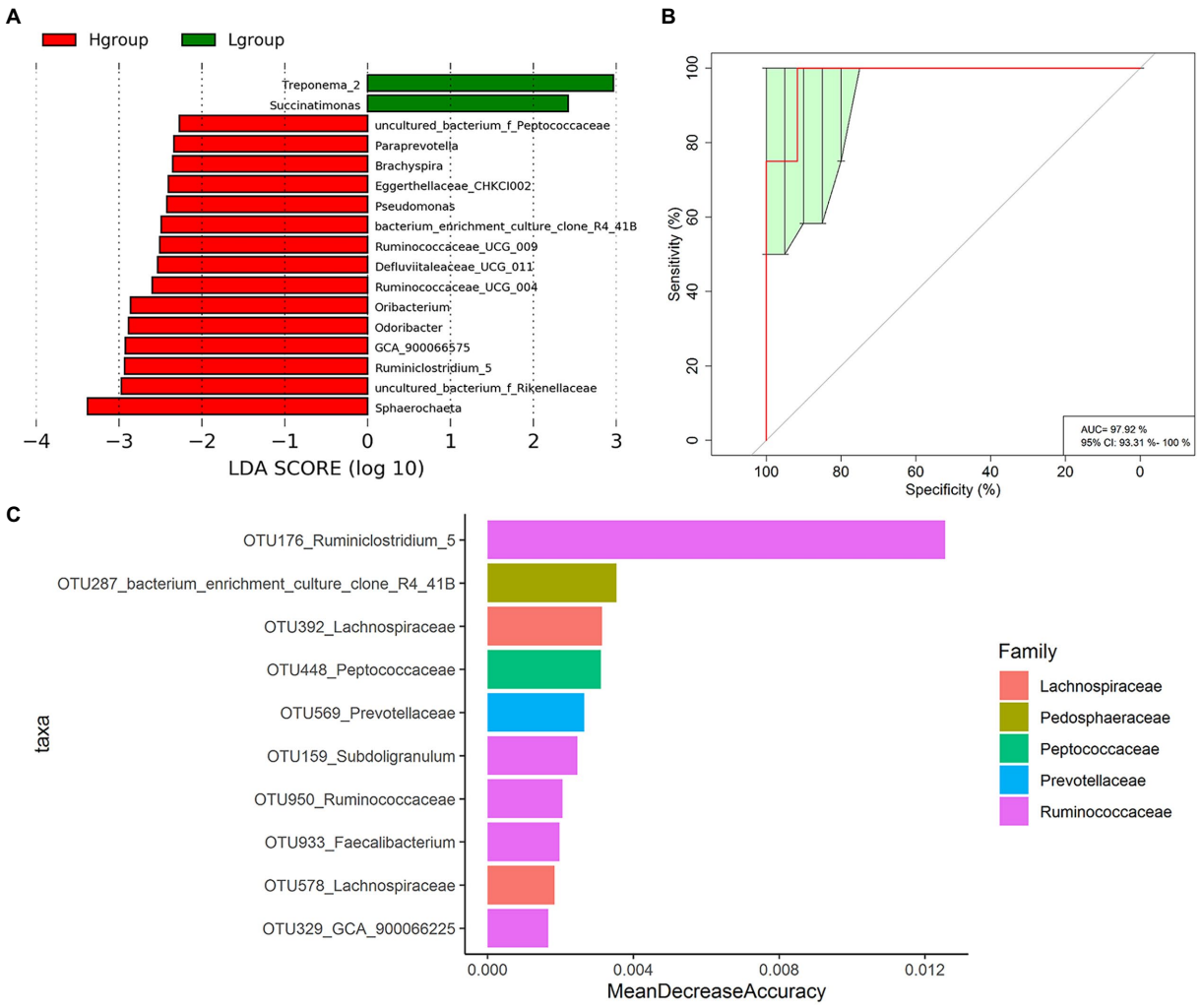


FIGURE 4
Specific OTUs of cecal microbiota related to market-weights of Qiandongnan Xiaoxiang chickens at 160 ± 3 days of age ($n$=24). **(A)** LEfSe analysis of cecal microbiota members associated with H group and L group chickens at the genus level. **(B)** Receiver operating curves (ROC) for H and L group chickens. AUC=97.92, 95% CI=93.31%–100%. **(C)** The 10 most abundant OTU biomarkers that could discriminate H and L group chickens identified using the Random Forest model. Biomarker OTUs were ranked in descending order of importance relative to the model accuracy.

Xiaoxiang chickens. Interestingly, we found that *Treponema 2* was significantly enriched in the low-market-weight chickens and was also significantly positively correlated with the sex hormone pregnanetriol (Figure 7; Supplementary Table S5).

## Validation cohort (Guizhou yellow and Wumeng black-bone chickens)

### Validating correlations of cecal microbiota and market-weight

We further verified associations between cecal microbiota and market-weight using the validation cohort of 55 Guizhou yellow and 24 Wumeng black-bone chickens (Supplementary Table S6). The cecal microbiota accounted for 10.1% of the variation among individuals with different market-weights at threshold of $5 \times 10^{-4}$ (Figure 8A-B; Supplementary Table S7). We then corrected for two influencing factors (breed and sex) and a two-part model was used to identify the cecal microbiota associated with market-weight. We found 15 bacterial taxa were significantly correlated with market-weight and *Sphaerochaeta* ($LDA = 2.53$, $p = 0.006$), *Ruminococcus* ($LDA = 1.86$, $p = 0.031$), *Marvinbryantia* ($LDA = 1.70$, $p = 0.044$) and *Paludicola* ($LDA = 1.69$, $p = 0.045$) were significantly and positively correlated (Supplementary Table S8). *Sphaerochaeta* was also enriched in the cecal microbiota of the high-market-weight chickens in the experimental cohort and was significantly associated with the conjugated linoleic acid, bovinic acid. Consistent results were obtained for *Succinatimonas* in both experimental cohort and validation cohort. *Succinatimonas* was enriched in the cecal microbiota of low-market-weight chickens and was significantly positively correlated with deoxyuridine ($r = 0.63$, $p = 0.002$) and α-linolenic acid ($r = 0.43$, $p = 0.049$). Additionally, 10 microbiotas including *norank_f_Desulfovibrionaceae* ($LDA = -4.67$, $p < 0.001$), *Parasutterella* ($LDA = -3.25$, $p < 0.001$) and *Eubacterium nodatum group* ($LDA = -2.27$, $p = 0.012$) were significantly negatively correlated with market-weight in the validation cohort (Figure 8C).

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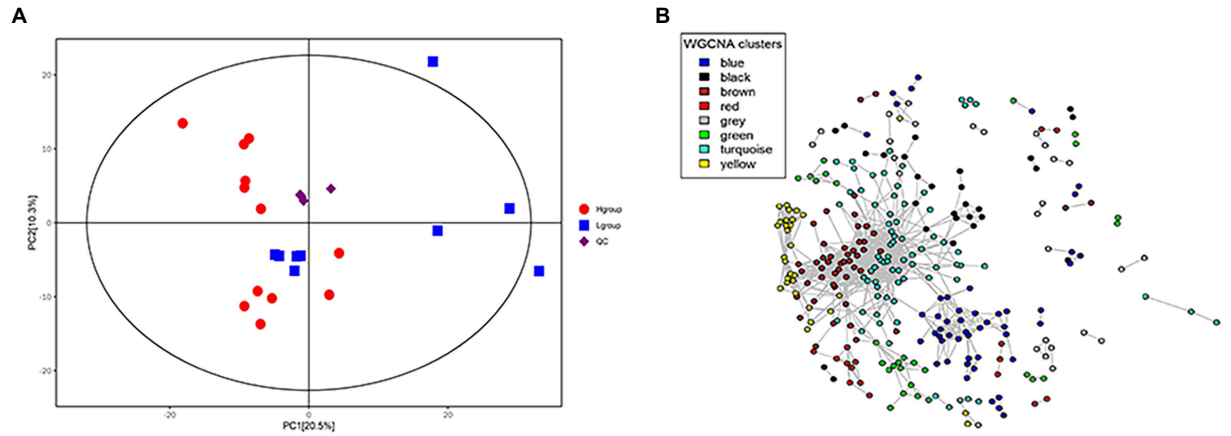


FIGURE 5
Differentiation of host serum metabolite profiles between H-group ($n$=12) and L-group ($n$=9) Qiandongnan Xiaoxiang chickens at 160 ± 3 days of age. **(A)** PCA plot of serum metabolite profiles **(B)** Co-occurrence network of serum metabolite features. The metabolites (nodes) are colored according to WGCNA module colors. Only those correlations with $|r|$>0.2 between two edges are presented.

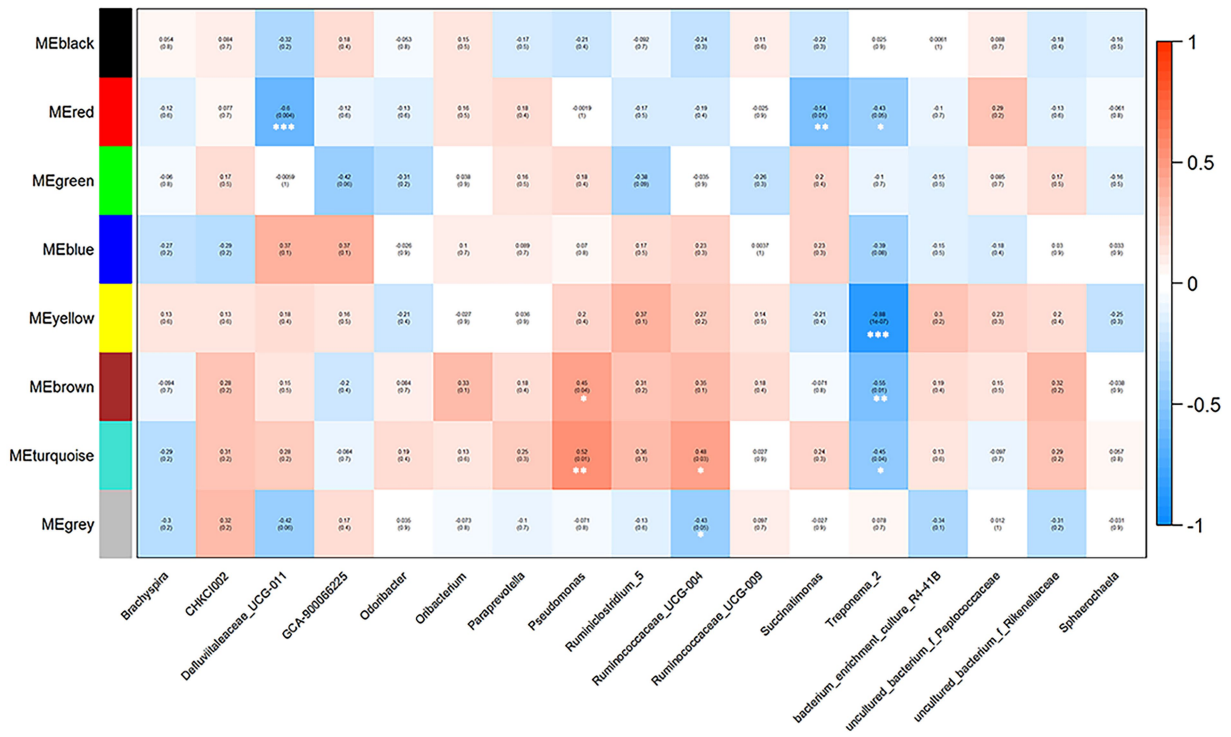


FIGURE 6
Correlations between metabolite modules and weight-related bacteria in Qiandongnan Xiaoxiang chickens at 160 ± 3 days of age ($n$=21). Each box of the matrix indicates the correlation between one metabolite module and a weight-related bacterial taxon at the genus level. The correlation coefficients ($r$) and $p$-value are listed in the small boxes (*$p$< 0.05, **$p$< 0.01, ***$p$< 0.001). The color gradient represents the values of correlation coefficients (red for positive and blue for negative correlations).

## Relationships between inflammatory cytokines and market-weight

We also examined whether market-weight was affected by the presence of the inflammatory cytokines INF-$\gamma$, IL-1$\beta$, IL-5, IL-6, IL-17, and IL-22 (Broom and Kogut, 2018; Sun et al., 2018). Correlation analysis indicated that except for IL-22 (correlation coefficient 0.052), the other 5 cytokines were negatively correlated with market-weight and only IL-6 was significant with correlation coefficients of −0.235 ($p$=*0.037*). The cecal microbiota *norank_f_Desulfovibrionaceae* ($r$= 0.33, $p$= 0.003) and *Synergistes* ($r$= 0.23, $p$= 0.04) that were negatively correlated with market-weight were extremely significantly correlated with IL-6 (Figure 8D; Supplementary Table S9).

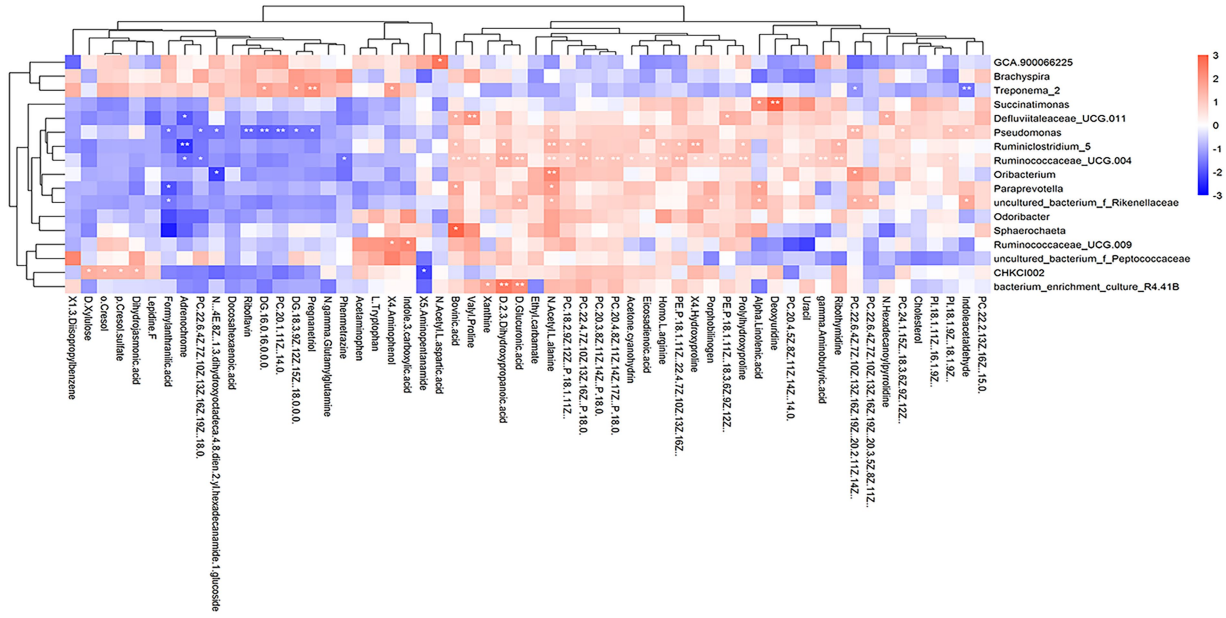


FIGURE 7
Heatmap depicting correlations between differential serum metabolites and differential species of cecal microbiota in Qiandongnan Xiaoxiang Chickens at 160 ± 3 days of age ($n$ =21). *$p$< 0.05, **$p$< 0.01, ***$p$< 0.001 were calculated using the Spearman's rank correlation test.

## Discussion

The prohibition of antibiotics as growth promotors in food animals provides the impetus to develop alternative products to control gut bacterial pathogens. The gut microbiota is a key driver of growth performance in agricultural animals so these microorganisms and their metabolites are important candidate sources of probiotics and prebiotics (Ramayo-Caldas et al., 2016; Myer et al., 2017). Their use as growth promoters do not contribute to the development and spread of antibiotic resistance (Ma et al., 2022). In the current study, our experimental cohort (60 Qiandongnan Xiaoxiang chickens) and validation cohorts (24 Wumeng black-bone and 55 Guizhou yellow chickens) were established to profile phenotypic characteristics of the cecal microbiota and link these with market-weight. These indigenous breeds are important food sources in Guizhou, China and possess distinct phenotypic characteristics. Our goal was to exploit these differences and identify common beneficial bacteria that would be probiotic candidates.

The Qiandongnan Xiaoxiang chicken was selected for its superior meat flavor, small size and strong adaptability. The Guizhou Yellow broiler is a Chinese hybrid line (Weining ♀ × New Hampshire × Plymouth Rock ♂) that possesses high market weights and tender meat while the Wumeng black bone chicken is a meat and medicinal chicken breed that is listed in the *Poultry Genetic Resources in China* and possesses black tissues and bones. These indigenous chickens are popular with Guizhou consumers so they were chosen as the research objects in this study.

We found that *Bacteroidetes, Firmicutes, Proteobacteria, Synergistetes*, and *Spirochaetes* were the predominant cecal microbiota of Qiandongnan Xiaoxiang chickens. These results were similar to findings using the commercial breed Guangdong Yellow Broiler chickens except *Actinobacteria* replaced *Synergistetes* (Wen et al., 2019). The five most important OTUs of hub cecal microbiota in Qiandongnan Xiaoxiang chickens were annotated to the *Ruminococcaceae* and implicates this family as a core member of the cecal microbiota. *Ruminococcaceae* abundance was also significantly greater in high-market-weight chickens and this data combined with the CAG results linked this family to enhanced growth performance. *Ruminococcaceae* is one of the earliest described bacteria of the bovine rumen and are highly efficient carbohydrate decomposers and central to the degradation of resistant starch *via* fermentation to glucose and xylose (Pal et al., 2021). We also found that Rumi*nococcaceae* UCG-004 was significantly correlated with 27/58 differential metabolites and 10 of these were fatty acid metabolites (listed in Figure 7 with the prefix PC). Therefore, *Ruminococcaceae* in the cecum might also perform other physiological functions in the basic life activities of indigenous chickens. This family is one of the few known microorganisms that can transform primary bile acids into secondary bile acids (Jiang et al., 2022) and depletion of the *Ruminococcaceae* in human intestinal tracts is a marker for ulcerative colitis (Sinha et al., 2020). Together, these data implicate the *Ruminococcaceae* in intestinal health maintenance *via* alleviating intestinal inflammation through metabolism of specific fatty acids. These metabolic traits would specifically promote growth performance.

In both experimental and validation cohorts, we found a positive correlation between *Sphaerochaeta* and market weight. LEfSe analysis indicated that *Sphaerochaeta* was enriched in high-market-weight chickens in the experimental cohort confirming a key role in growth promotion. *Sphaerochaeta* is involved in glucose metabolism *via* glycolysis and the pentose phosphate pathway (Caro-Quintero et al., 2012) and was significantly positively associated with bovinic acid (a polyunsaturated conjugated linoleic acid; CLA isomer; Wang et al., 2019). CLA can be absorbed and rapidly deposited onto lipids and phospholipids in membranes (Kramer et al., 1998) and it possesses

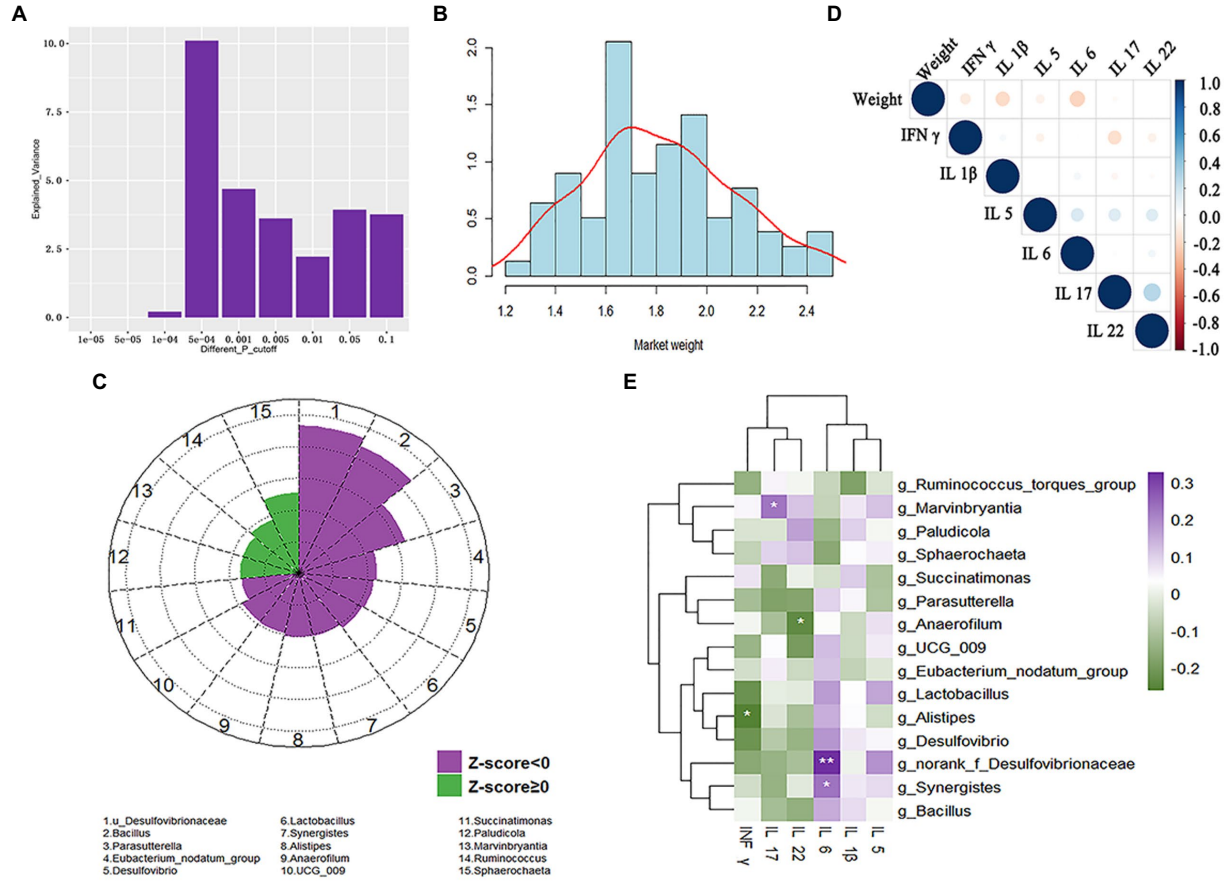


FIGURE 8
Correlation of market-weight (body-weight at 160±3 days of age) with cecal microbiota and serum inflammatory cytokines in the validation cohort composed of 24 Wumeng black-bone chickens and 55 Guizhou yellow chickens. **(A)** Variation of market-weight explained by cecal microbiota under different significant thresholds. **(B)** Inter-individual variation in market-weight. **(C)** Cecal microbiota significantly associated with chicken market-weight. Associations between cecal microbiota and market-weight are shown as Z scores. $Z < 0$ and $Z \geq 0$ indicates a negative association and a positive association, respectively. **(D)** Correlation between market-weight and 6 inflammatory cytokines. **(E)** Correlations between the abundance of cecal microbiota and host inflammatory cytokines quantified by Spearman correlation with Benjamini–Hochberg corrections. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.005$.

numerous biological functions including immunity enhancement in agricultural animals (Kuhnt et al., 2016). These beneficial effects link this genus to chicken growth performance *via* bovinic acid production.

In contrast to positive effects described above, the presence of *Treponema* and *Succinatimonas* in the cecum inhibited growth of Qiandongnan Xiaoxiang chickens. *Treponema* was previously implicated in altering the average daily gain of piglets in contrast to colonization by *Prevotella* and *Mitsuokella* (Ramayo-Caldas et al., 2016). A study of the Hadza people, a primitive hunting tribe in Africa, demonstrated that *Treponema* were significant dietary fiber degraders and this phenotype displayed significant gender differences (Schnorr et al., 2014). Other independent studies on agricultural animals have found that *Treponema* is an important marker of sex differences and might be related to alterations in sex hormone levels (He et al., 2019; Wang et al., 2021). In this study, we verified that *Treponema* was significantly positively correlated with the sex hormone pregnanetriol, indicating that a lower market-weight might be related to sexual maturity of the chickens. In the validation cohorts we also identified *Succinatimonas* as another important genus that was detrimental to Qiandongnan Xiaoxiang chicken growth. This genus of short, Gram-negative bacilli is enriched in cattle fed with grain hay (Morotomi et al., 2010). *Succinatimonas* can utilize only a few sugars such as glucose, maltose, dextrin and starch but no other carbohydrates, and its metabolites are largely succinic acid and a small amount of acetic acid (Morotomi et al., 2010). Excess succinic acid can result in diarrhea and thus lead to reduced growth.

Our combined results using the validation cohort indicated that high levels of the inflammatory cytokine IL-6 as well as the presence of *Synergistes* and *norank_f_Desulfovibrionaceae* were detrimental to growth. IL-6 levels were significantly positively correlated with these two genera and suggest they promote inflammation and this could seriously reduce growth performance due to decreased feed intake and abnormal digestion and absorption (Jiang et al., 2010; Chen et al., 2021). Probiotics such as *Lactobacillus* can mitigate inflammation in mice by regulating cytokine secretion (Lamas et al., 2016). And gut microbiota had positive effects in the chicken caecum to promote growth by mitigating intestinal inflammatory (Ayalew et al., 2022). The genus *Synergistes* is widely distributed in the natural environment and are characterized by their ability to degrade amino acids and may perform this function in natural ecosystems (Godon et al., 2005). Additionally, *Synergistes* is a normal

part of human and animal microflora and has been linked to mucosal infections (Kumar et al., 2003).

The second genus we linked to decreased growth was the sulfate-reducer *Desulfovibrio* that utilize sulfate as the terminal electron acceptor for ATP synthesis. $H_2S$ production by this genus has also been linked to increased inflammation of the intestinal epithelium in humans (Verstreken et al., 2012; Murros et al., 2021). Taken together, we identified specific cecal microbiotal members that affect the growth performance of indigenous chickens *via* inflammatory cytokine regulation.

## Conclusion

Our findings demonstrated that cecal colonization by *Sphaerochaeta* improved growth performance of Guizhou indigenous chicken by promoting bovinic acid metabolism. In contrast, colonization by *Synergistes* and *norank_f_Desulfovibrionaceae* in cecum reduced growth performance by inducing the production of the inflammatory cytokine IL-6. These results provide novel insights into the development of antibiotic alternatives to improve chicken growth performance and deepen our understanding of the physiology of native Guizhou chicken breeds.

## Data availability statement

All sequencing data has been uploaded to the Chinese gene pool database (China National GeneBank database, CNGBdb; https://db.cngb.org/cnsa/) with project number CNP0003145.

## Ethics statement

The animal study was reviewed and approved by Animal Care and Use Committee in Guizhou University.

## Author contributions

ZW and LW conceived and designed the experiments. LW, SL, ShenhY, and YY performed the experiments. ZW, FZ, HL, ShengY, and XC analyzed the data. ZW and LW 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.

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## Supplementary material

The Supplementary material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2023.1060458/full#supplementary-material

## References

Angulo, M. T., Moog, C. H., and Liu, Y. Y. (2019). A theoretical framework for controlling complex microbial communities. *Nat. Commun.* 10:1045. doi: 10.1038/s41467-019-08890-y

Ayalew, H., Zhang, H., Wang, J., Wu, S., Qiu, K., Qi, G., et al. (2022). Potential feed additives as antibiotic alternatives in broiler production. *Front. Vet. Sci.* 9:916473. doi: 10.3389/fvets.2022.916473

Bokulich, N. A., Subramanian, S., Faith, J. J., Gevers, D., Gordon, J. I., Knight, R., et al. (2013). Quality-filtering vastly improves diversity estimates from illumina amplicon sequencing. *Nat. Methods* 10, 57–59. doi: 10.1038/nmeth.2276

Bolger, A. M., Lohse, M., and Usadel, B. (2014). Trimmomatic: a flexible trimmer for illumina sequence data. *Bioinformatics* 30, 2114–2120. doi: 10.1093/bioinformatics/btu170

Borda-Molina, D., Seifert, J., and Camarinha-Silva, A. (2018). Current perspectives of the chicken gastrointestinal tract and its microbiome. *Comput. Struct. Biotechnol. J.* 16, 131–139. doi: 10.1016/j.csbj.2018.03.002

Broom, L. J., and Kogut, M. H. (2018). The role of the gut microbiome in shaping the immune system of chickens. *Vet. Immunol. Immunopathol.* 204, 44–51. doi: 10.1016/j.vetimm.2018.10.002

Cai, Y., Weng, K., Guo, Y., Peng, J., and Zhu, Z. J. (2015). An integrated targeted metabolomic platform for high-throughput metabolite profiling and automated data processing. *Metabolomics* 11, 1575–1586. doi: 10.1007/s11306-015-0809-4

Caporaso, J. G., Kuczynski, J., Stombaugh, J., Bittinger, K., Bushman, F. D., Costello, E. K., et al. (2010). Qiime allows analysis of high-throughput community sequencing data. *Nat. Methods* 7, 335–336. doi: 10.1038/nmeth.f.303

Caro-Quintero, A., Ritalahti, K. M., Cusick, K. D., Löffler, F. E., and Konstantinidis, K. T. (2012). The chimeric genome of sphaerochaeta: nonspiral spirochetes that break with the prevalent dogma in spirochete biology. *MBio* 3:e00025–12. doi: 10.1128/mBio.00025-12

Chao, A. (1984). Non-parametric estimation of the classes in a population. *Scand. J. Stat.* 11, 265–270. doi: 10.2307/4615964

Chen, C., Fang, S., Wei, H., He, M., Fu, H., Xiong, X., et al. (2021). Prevotella copri increases fat accumulation in pigs fed with formula diets. *Microbiome* 9:175. doi: 10.1186/s40168-021-01110-0

Chin, C. H., Chen, S. H., Wu, H. H., Ho, C. W., Ko, M. T., and Lin, C. Y. (2014). Cytohubba: identifying hub objects and sub-networks from complex interactome. *BMC Syst. Biol.* 8:S11. doi: 10.1186/1752-0509-8-S4-S11

Cui, L., Zhang, X., Cheng, R., Ansari, A. R., Elokil, A. A., Hu, Y., et al. (2021). Sex differences in growth performance are related to cecal microbiota in chicken. *Microb. Pathog.* 150:104710:104710. doi: 10.1016/j.micpath.2020.104710

Darwish, N., Shao, J., Schreier, L. L., and Proszkowiec-Weglarz, M. (2021). Choice of 16s ribosomal rna primers affects the microbiome analysis in chicken ceca. *Sci. Rep.* 11:11848. doi: 10.1038/s41598-021-91387-w

Dunn, W. B., Broadhurst, D., Begley, P., Zelena, E., Francis-McIntyre, S., Anderson, N., et al. (2011). Procedures for large-scale metabolic profiling of serum and plasma using gas chromatography and liquid chromatography coupled to mass spectrometry. *Nat. Protoc.* 6, 1060–1083. doi: 10.1038/nprot.2011.335

Edgar, R. C. (2013). Uparse: highly accurate otu sequences from microbial amplicon reads. *Nat. Methods* 10, 996–998. doi: 10.1038/nmeth.2604

Elokil, A. A., Magdy, M., Melak, S., Ishfaq, H., Bhuiyan, A., Cui, L., et al. (2020). Faecal microbiome sequences in relation to the egg-laying performance of hens using amplicon-based metagenomic association analysis. *Animal* 14, 706–715. doi: 10.1017/S1751731119002428

Faith, D. P. (1992). Conservation evaluation and phylogenetic diversity. *Biol. Conserv.* 61, 1–10. doi: 10.1016/0006-3207(92)91201-3

Friedman, J., and Alm, E. J. (2012). Inferring correlation networks from genomic survey data. *PLoS Comput. Biol.* 8:e1002687. doi: 10.1371/journal.pcbi.1002687

Fu, J., Bonder, M. J., Cenit, M. C., Tigchelaar, E. F., Maatman, A., Dekens, J. A. M., et al. (2015). The gut microbiome contributes to a substantial proportion of the variation in blood lipids. *Circ. Res.* 117, 817–824. doi: 10.1161/CIRCRESAHA.115.306807

Glendinning, L., Watson, K. A., and Watson, M. (2019). Development of the duodenal, ileal, jejunal and caecal microbiota in chickens. *Anim. Microbiome.* 1:17. doi: 10.1186/s42523-019-0017-z

Godon, J. J., Moriniere, J., Moletta, M., Gaillac, M., Bru, V., and Delgenes, J. P. (2005). Rarity associated with specific ecological niches in the bacterial world: the 'synergistes' example. *Environ. Microbiol.* 7, 213–224. doi: 10.1111/j.1462-2920.2004.00693.x

Guo, L., Wang, Z., Du, Y., Mao, J., Zhang, J., Yu, Z., et al. (2020). Random-forest algorithm based biomarkers in predicting prognosis in the patients with hepatocellular carcinoma. *Cancer Cell Int.* 20:251:251. doi: 10.1186/s12935-020-01274-z

He, M., Jun, G., Wu, J., Zhou, Y., Fu, H., Ke, S., et al. (2019). Host gender and androgen levels regulate gut bacterial taxa in pigs leading to sex-biased serum metabolite profiles. *Front. Microbiol.* 10:1359. doi: 10.3389/fmicb.2019.01359

Jiang, Z., Schatzmayr, G., Mohnl, M., and Applegate, T. J. (2010). Net effect of an acute phase response--partial alleviation with probiotic supplementation. *Poult. Sci.* 89, 28–33. doi: 10.3382/ps.2009-00464

Jiang, Z., Zhuo, L. B., He, Y., Fu, Y., Shen, L., Xu, F., et al. (2022). The gut microbiota-bile acid axis links the positive association between chronic insomnia and cardiometabolic diseases. *Nat. Commun.* 13:3002. doi: 10.1038/s41467-022-30712-x

Kramer, J. K., Parodi, P. W., Jensen, R. G., Mossoba, M. M., Yurawecz, M. P., and Adlof, R. O. (1998). Rumenic acid: a proposed common name for the major conjugated linoleic acid isomer found in natural products. *Lipids* 33:835. doi: 10.1007/s11745-998-0279-6

Kuhnt, K., Degen, C., and Jahreis, G. (2016). Evaluation of the impact of ruminant trans fatty acids on human health: important aspects to consider. *Crit. Rev. Food Sci. Nutr.* 56, 1964–1980. doi: 10.1080/10408398.2013.808605

Kumar, P. S., Griffen, A. L., Barton, J. A., Paster, B. J., Moeschberger, M. L., and Leys, E. J. (2003). New bacterial species associated with chronic periodontitis. *J. Dent. Res.* 82, 338–344. doi: 10.1177/154405910308200503

Lamas, B., Richard, M. L., Leducq, V., Pham, H. P., Michel, M. L., Da, C. G., et al. (2016). Card9 impacts colitis by altering gut microbiota metabolism of tryptophan into aryl hydrocarbon receptor ligands. *Nat. Med.* 22, 598–605. doi: 10.1038/nm.4102

Langfelder, P., and Horvath, S. (2008). Wgcna: an r package for weighted correlation network analysis. *BMC Bioinformatics* 9:559. doi: 10.1186/1471-2105-9-559

Li, Y., Xu, Q., Huang, Z., Lv, L., Liu, X., Yin, C., et al. (2016). Effect of bacillus subtilis cgmcc 1.1086 on the growth performance and intestinal microbiota of broilers. *J. Appl. Microbiol.* 120, 195–204. doi: 10.1111/jam.12972

Lindgreen, S. (2012). Adapterremoval: easy cleaning of next-generation sequencing reads. *BMC. Res. Notes* 5:337. doi: 10.1186/1756-0500-5-337

Liu, H., Ding, P., Tong, Y., He, X., Yin, Y., Zhang, H., et al. (2021). Metabolomic analysis of the egg yolk during the embryonic development of broilers. *Poult. Sci.* 100:101014. doi: 10.1016/j.psj.2021.01.036

Lopes, C. T., Franz, M., Kazi, F., Donaldson, S. L., Morris, Q., and Bader, G. D. (2010). Cytoscape web: an interactive web-based network browser. *Bioinformatics* 26, 2347–2348. doi: 10.1093/bioinformatics/btq430

Ma, Y., Guo, Z., Xia, B., Zhang, Y., Liu, X., Yu, Y., et al. (2022). Identification of antimicrobial peptides from the human gut microbiome using deep learning. *Nat. Biotechnol.* 40:921–931. doi: 10.1038/s41587-022-01226-0

Magoc, T., and Salzberg, S. L. (2011). Flash: fast length adjustment of short reads to improve genome assemblies. *Bioinformatics* 27, 2957–2963. doi: 10.1093/bioinformatics/btr507

Morotomi, M., Nagai, F., Watanabe, Y., and Tanaka, R. (2010). Succinatimonas hippei gen. Nov., sp. nov., isolated from human faeces. *Int. J. Syst. Evol. Microbiol.* 60, 1788–1793. doi: 10.1099/ijs.0.015958-0

Murros, K. E., Huynh, V. A., Takala, T. M., and Saris, P. (2021). Desulfovibrio bacteria are associated with parkinson's disease. *Front. Cell. Infect. Microbiol.* 11:652617. doi: 10.3389/fcimb.2021.652617

Myer, P. R., Freetly, H. C., Wells, J. E., Smith, T., and Kuehn, L. A. (2017). Analysis of the gut bacterial communities in beef cattle and their association with feed intake, growth, and efficiency. *J. Anim. Sci.* 95, 3215–3224. doi: 10.2527/jas.2016.1059

Pal, D., Naskar, M., Bera, A., and Mukhopadhyay, B. (2021). Chemical synthesis of the pentasaccharide repeating unit of the o-specific polysaccharide from ruminococcus gnavus. *Carbohydr. Res.* 507:108384. doi: 10.1016/j.carres.2021.108384

Quast, C., Pruesse, E., Yilmaz, P., Gerken, J., Schweer, T., Yarza, P., et al. (2013). The silva ribosomal rna gene database project: improved data processing and web-based tools. *Nucleic Acids Res.* 41, D590–D596. doi: 10.1093/nar/gks1219

Ramayo-Caldas, Y., Mach, N., Lepage, P., Levenez, F., Denis, C., Lemonnier, G., et al. (2016). Phylogenetic network analysis applied to pig gut microbiota identifies an ecosystem structure linked with growth traits. *ISME J.* 10, 2973–2977. doi: 10.1038/ismej.2016.77

Schloss, P. D., Westcott, S. L., Ryabin, T., Hall, J. R., Hartmann, M., Hollister, E. B., et al. (2009). Introducing mothur: open-source, platform-independent, community-supported software for describing and comparing microbial communities. *Appl. Environ. Microbiol.* 75, 7537–7541. doi: 10.1128/AEM.01541-09

Schnorr, S. L., Candela, M., Rampelli, S., Centanni, M., Consolandi, C., Basaglia, G., et al. (2014). Gut microbiome of the hadza hunter-gatherers. *Nat. Commun.* 5:3654. doi: 10.1038/ncomms4654

Shannon, B. C. E. (1948). A mathematical theory of communication. *AT T Bell Lab. Techn. J.* 27, 379–423. doi: 10.1002/j.1538-7305.1948.tb01338.x

Sinha, S. R., Haileselassie, Y., Nguyen, L. P., Tropini, C., Wang, M., Becker, L. S., et al. (2020). Dysbiosis-induced secondary bile acid deficiency promotes intestinal inflammation. *Cell Host Microbe* 27, 659–670.e5. doi: 10.1016/j.chom.2020.01.021

Smith, C. A., Want, E. J., O'Maille, G., Abagyan, R., and Siuzdak, G. (2006). Xcms: processing mass spectrometry data for metabolite profiling using nonlinear peak alignment, matching, and identification. *Anal. Chem.* 78, 779–787. doi: 10.1021/ac051437y

Sun, Y., Jiang, J., Tien, P., Liu, W., and Li, J. (2018). Ifn-λ: a new spotlight in innate immunity against influenza virus infection. *Protein Cell* 9, 832–837. doi: 10.1007/s13238-017-0503-6

Verstreken, I., Laleman, W., Wauters, G., and Verhaegen, J. (2012). Desulfovibrio desulfuricans bacteremia in an immunocompromised host with a liver graft and ulcerative colitis. *J. Clin. Microbiol.* 50, 199–201. doi: 10.1128/JCM.00987-11

Wang, Z., Fu, H., Zhou, Y., Yan, M., Chen, D., Yang, M., et al. (2021). Identification of the gut microbiota biomarkers associated with heat cycle and failure to enter oestrus in gilts. *Microbial. Biotechnol.* 14, 1316–1330. doi: 10.1111/1751-7915.13695

Wang, Q., Garrity, G. M., Tiedje, J. M., and Cole, J. R. (2007). Naive bayesian classifier for rapid assignment of rrna sequences into the new bacterial taxonomy. *Appl. Environ. Microbiol.* 73, 5261–5267. doi: 10.1128/AEM.00062-07

Wang, S. H., Wang, W. W., Zhang, H. J., Wang, J., Chen, Y., Wu, S. G., et al. (2019). Conjugated linoleic acid regulates lipid metabolism through the expression of selected hepatic genes in laying hens. *Poult. Sci.* 98, 4632–4639. doi: 10.3382/ps/pez161

Wang, J., Zhang, T., Shen, X., Liu, J., Zhao, D., Sun, Y., et al. (2016). Serum metabolomics for early diagnosis of esophageal squamous cell carcinoma by uhplc-qtof/ms. *Metabolomics* 12, 1–10. doi: 10.1007/s11306-016-1050-5

Wen, C., Yan, W., Mai, C., Duan, Z., Zheng, J., Sun, C., et al. (2021). Joint contributions of the gut microbiota and host genetics to feed efficiency in chickens. *Microbiome* 9:126. doi: 10.1186/s40168-021-01040-x

Wen, C., Yan, W., Sun, C., Ji, C., Zhou, Q., Zhang, D., et al. (2019). The gut microbiota is largely independent of host genetics in regulating fat deposition in chickens. *ISME J.* 13, 1422–1436. doi: 10.1038/s41396-019-0367-2

Wiklund, S., Johansson, E., Sjostrom, L., Mellerowicz, E. J., Edlund, U., Shockcor, J. P., et al. (2008). Visualization of gc/tof-ms-based metabolomics data for identification of biochemically interesting compounds using opls class models. *Anal. Chem.* 80, 115–122. doi: 10.1021/ac0713510

Wu, Y., Zeng, J., Zhang, F., Zhu, Z., Qi, T., Zheng, Z., et al. (2018). Integrative analysis of omics summary data reveals putative mechanisms underlying complex traits. *Nat. Commun.* 9:918. doi: 10.1038/s41467-018-03371-0

Xu, H. Q. (2018). *Animal genetic resources in Guizhou*. Beijing: China Agriculture Press.

Zhang, X., Akhtar, M., Chen, Y., Ma, Z., Liang, Y., Shi, D., et al. (2022a). Chicken jejunal microbiota improves growth performance by mitigating intestinal inflammation. *Microbiome* 10:107. doi: 10.1186/s40168-022-01299-8

Zhang, X., Hu, Y., Ansari, A. R., Akhtar, M., Chen, Y., Cheng, R., et al. (2022b). Caecal microbiota could effectively increase chicken growth performance by regulating fat metabolism. *J. Microbial. Biotechnol.* 15, 844–861. doi: 10.1111/1751-7915.13841

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# Effects of altitude on the gut microbiome and metabolomics of Sanhe heifers

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**Introduction:** Extreme environments at high altitudes pose a significant physiological challenge to animals. We evaluated the gut microbiome and fecal metabolism in Sanhe heifers from different altitudes.

**Methods:** Twenty Sanhe heifers (body weight: 334.82±13.22kg, 15-month-old) selected from two regions of China: the Xiertala Cattle Breeding Farm in Hulunbeier, Inner Mongolia [119°57′ E, 47°17′ N; approximately 700m altitude, low altitude (LA)] and Zhizhao Dairy Cow Farm in Lhasa, Tibet [91°06′ E, 29°36′ N; approximately 3,650m altitude, high altitude (HA)], were used in this study. Fecal samples were collected and differences in the gut microbiota and metabolomics of Sanhe heifers were determined using 16S rRNA gene sequencing and metabolome analysis.

**Results and discussion:** The results showed that altitude did not significantly affect the concentrations of fecal volatile fatty acids, including acetate, propionate, butyrate, and total volatile fatty acids ($p>0.05$). However, 16S rRNA gene sequencing showed that altitude significantly affected gut microbial composition. Principal coordinate analysis based on Bray–Curtis dissimilarity analysis revealed a significant difference between the two groups ($p=0.001$). At the family level, the relative abundances of Peptostreptococcaceae, Christensenellaceae, Erysipelotrichaceae, and Family_XIII were significantly lower ($p<0.05$) in LA heifers than in HA heifers. In addition, the relative abundances of Lachnospiraceae, *Domibacillus*, Bacteroidales_S24-7_group, Bacteroidales_RF16_group, Porphyromonadaceae, and Spirochaetaceae were significantly higher in HA heifers than in LA heifers ($p<0.05$). Metabolomic analysis revealed the enrichment of 10 metabolic pathways, including organismal systems, metabolism, environmental information processing, genetic information processing, and disease induction. The genera *Romboutsia*, *Paeniclostridium*, and *g_unclassified_f_Lachnospiraceae* were strongly associated with the 28 differential metabolites. This study is the first to analyze the differences in the gut microbiome and metabolome of Sanhe heifers reared at different altitudes and provides insights into the adaptation mechanism of Sanhe heifers to high-altitude areas.

KEYWORDS

**different altitudes, gut microbiota, metabolites, Sanhe heifers, high-altitude adaptation**

## 1. Introduction

Areas with an altitude higher than 2,500 m, generally defined as high altitudes (Moore et al., 2010), pose challenges for the survival, growth, and development of local animals (Moore et al., 2010; Guo et al., 2014). As the highest plateau worldwide, the Tibetan Plateau is characterized by low pressure, low oxygen, strong ultraviolet rays, and low temperatures throughout the year

(Zha et al., 2016; Sun et al., 2021). High altitude, low pressure, and hypoxia can cause various diseases such as pulmonary hypertension (Pasha and Newman, 2010; Khanna et al., 2018), high-altitude hypertension (Bilo et al., 2019), and vascular dysfunction. Additionally, numerous metabolic disorders in the digestive tract, such as enteritis and gastritis, can be caused by an imbalance in the gut microbiota under low pressure and hypoxic conditions (Khanna et al., 2018; Hill et al., 2020; Pena et al., 2022).

Bacteria play a key role in many types of feed biopolymer fermentation and degradation processes (Bickhart and Weimer, 2018), and fecal samples mostly represent the distal portion of the gut microbiotathe (de Oliveira et al., 2013). Recent studies have reported the high-altitude adaptability of various animals such as cattle (Kong et al., 2021), yak (Ayalew et al., 2021), and rats (Murray, 2016). Recently, alterations in the gastrointestinal microbiota due to altitude changes were investigated in yak (Liu et al., 2021), sheep (Holman et al., 2019), and pigs (Zeng et al., 2020). However, the changes in the gut microbiota and the physiological and metabolic mechanisms in response to high altitudes have not yet been investigated in Sanhe heifers. Moreover, variations in the metabolic adaptability of the gut microbiota of Sanhe heifers at different altitudes are not well understood.

As a dual-purpose breed, Sanhe cattle show excellent performance in both milk and meat production (Xu et al., 2017); their milk contains high concentrations of fat. In addition, Sanhe cattle exhibit strong adaptability, rough feeding tolerance (Hu et al., 2019), strong disease resistance (Usman et al., 2017), and stable genetic performance. Sanhe cattle originated from Inner Mongolia, China, and were bred to result in multiple breeds, including native Mongolian cattle, Simmental cattle, Siberian cattle, improved Russian cattle, Zabaikal cattle, Tagil cattle, Yaroslav cattle, Swedish cattle, and Hokkaido Dutch cattle. We examined whether Sanhe cattle can adapt to high-altitude environments, by evaluating the differences between Sanhe heifers in low-and high-altitude regions from a gut microbiota perspective.

In this study, the gut microbiomes of Sanhe heifers being offered the same amount of nutrients by the total mixed ration (TMR) but living in two regions of different altitudes, were compared. Using high-throughput sequencing and LS-MS-based untargeted metabolome analyzes, we aimed to reveal the effect of altitude on the gut microbiome of Sanhe cattle and to improve the understanding of the role of the gut microbiome in high-altitude adaptability. In addition, the gut microbiome and metabolomics of Sanhe heifers from low-and high-altitude regions were compared to provide insights into the high-altitude adaptability of ruminants.

# 2. Materials and methods

The study protocol was approved by the Ethical Committee of the College of Animal Science and Technology of China Agricultural University (project number AW22121202-1-2).

## 2.1. Experimental animals

Twenty Sanhe heifers (body weight: 334.82 ± 13.22 kg, 15-month-old) fed in two different altitude regions of China were selected for the experiments. Ten Sanhe heifers were selected from each trial site.

## 2.2. Study regions and management

Cattle from two regions were analyzed: those from the origin of Sanhe cattle, including Hulunbuir, Inner Mongolia Autonomous Region (119°57′ E, 47°17′ N; approximately 700 m in altitude, LA group), and Lhasa, Tibet Autonomous Region (91°06′ E, 29°36′ N; approximately 3,650 m in altitude, HA group). Lhasa has an average annual temperature of 8.6 Â °C and annual precipitation of 472.5 mm (Fan et al., 2005), whereas Hulunbuir has an average annual temperature of 3.3°C and annual precipitation of 538.3 mm.

## 2.3. Fecal sample collection and processing

To obtain representative samples, feces from Sanhe heifers were collected from the rectum using plastic gloves. The fecal samples used to analyze the gut microbiota were immediately frozen in liquid nitrogen (−80°C), and those used to analyze volatile fatty acids (VFAs) were stored at −20°C. For VFA analysis, the fecal sample from each animal was thawed, diluted, and centrifuged at 8,000 × *g* at 4°C for 10 min, and the supernatant was collected and evaluated using gas chromatography (Erwin et al., 1961).

## 2.4. Genomic DNA extraction, PCR amplification, and 16S rRNA sequencing

Total microbial genomic DNA was extracted from 1 g of fecal samples using an OMEGA kit (Omega Bio-Tek, Norcross, GA, United States), following the manufacturer's instructions. A Nanodrop 2000 spectrophotometer (Thermo Fisher Scientific, Waltham, United States) was used to confirm the purity and concentration of the extracted DNA. The V3–V4 region of the gut bacterial 16S rRNA gene was amplified using the forward primer 338F (5′-ACTCCTACGGGAGGCAGCA-3′) and the reverse primer 806R (3′-GGACTACNNGGGTATCTAAT-5′). The PCR conditions were as follows: denaturation at 95°C for 5 min, followed by 28 cycles at 95°C for 45 s, 55°C for 50 s, and 72°C for 45 s, with a final extension at 72°C for 10 min. Amplified fragments were visualized using 2% agarose gel electrophoresis, and the respective bands were purified using an Agencourt AMPure XP kit (Beckman Coulter Genomics, Brea, CA, United States) according to the manufacturer's instructions and quantified using QuantiFluor-ST (Promega, Madison, WI, USA). Purified PCR products were sequenced on an Illumina MiSeq (Illumina, San Diego, CA, United States; Caporaso et al., 2012) using a 2 × 250 bp sequencing kit.

## 2.5. Quality control and statistical analysis

Sequences with scores ≤20 (low quality), reads <200 bp, and reads containing ambiguous bases or unmatched primer sequences were filtered out using QIIME 1.8 (Caporaso et al., 2010), and barcode tags were removed. The obtained sequences were combined using PEAR 0.9.6 (Zhang et al., 2014) and demultiplexed using Flash (version 1.20; Mago and Salzberg, 2011). Reads with a combined length of <230 bp and chimeric sequences were removed using the UCHIME algorithm (Edgar et al., 2011). To reduce errors due to different sequencing depths, all samples were subsampled to an equal size of 31,719 for

downstream alpha-and beta-diversity analyzes. To ensure comparability of species diversity between samples, standardized operational taxonomic unit (OTU) documents were used to analyze the species and diversity indices.

The resulting sequences were clustered into OTUs based on a 97% sequence similarity threshold using the Ribosomal Database Project classifier (Cole et al., 2009) with a confidence threshold of 0.70 and compared against the SILVA 128 database for microbial species annotation (Quast et al., 2012). All OTUs were removed using UCLUST to generate the representative OTU table (Edgar, 2010).

The OTU level alpha diversity of the bacterial communities was determined using the Chao1, Shannon, and Simpson indices and procedures within QIIME 1.8, and visualized using the "ggplot2" package of R (version 4.0.5; Wickham, 2009). Principal coordinate analysis (PCoA) based on the Bray–Curtis dissimilarity matrix was performed in R using the "vegan" package for beta diversity analysis (Oksanen et al., 2016).

## 2.6. Metabolite extraction

The cold extraction solvent methanol/acetonitrile/$H_2O$ (2:2:1, vol/vol/vol; 1 ml) was added to an 80 mg fecal sample and vortexed for 60 s to extract metabolites. The samples were incubated on ice for 20 min and centrifuged at 14,000 × *g* for 20 min at 4°C. The supernatant was collected for liquid chromatography (LC)-MS analysis. The samples were dissolved in 100 µl of acetonitrile/water (1:1, v/v) and transferred to LC vials.

## 2.7. Liquid chromatography–MS/MS analysis and data processing

Gut microbiota metabolites were separated using an ultra-high-performance liquid chromatography system (1,290 Infinity LC, Agilent Technologies, Santa Clara, CA, United States) coupled to a quadrupole time-of-flight (TripleTOF 6,600, AB Sciex, Framingham, MA, United States). The fecal samples were analyzed using a 2.1 mm × 100 mm ACQUIY UPLC BEH 1.7 µm column (Waters, Milford, MA, United States). In both the positive and negative electrospray ionization modes, the mobile phase contained 25 mM ammonium acetate and 25 mM ammonium hydroxide in water and acetonitrile, respectively. The gradient was 85% acetonitrile for 1 min, which was linearly reduced to 65% in 11 min, reduced to 40% in 0.1 min and maintained for 4 min, and increased to 85% in 0.1 min, with a 5 min re-equilibration period. The electrospray ionization source conditions were as follows: ion source Gas1 as 60, ion source Gas2 as 60, curtain gas as 30, source temperature, 600°C; and ion spray voltage floating ±5,500 V. During MS acquisition, the instrument was set to acquire signals over an m/z range of 60–1,000 Da, and the accumulation time for the time-of-flight MS scan was set to 0.20 s/spectra. In the auto-MS/MS acquisition mode, the instrument was set to acquire signals over an m/z range of 25–1,000 Da, and the accumulation time for the production scan was set to 0.05 s/spectra. The production scan was acquired using information-dependent acquisition in high-sensitivity mode. The collision energy was fixed at 35 ± 15 eV. The declustering potential was set at ±60 V.

Raw MS data (Wiff. scan files) were converted to MzXML files using ProteoWizard MSConvert, and processed using XCMS for feature detection, retention time correction, and alignment. The metabolites were identified using accuracy mass spectrometry (<25 ppm) and MS/MS data, which were matched with the standard database.

For the extracted ion features, only variables with >50% of the nonzero measurement values in at least one group were retained. The MetaboAnalyst[1] web-based system was used for multivariate statistical analysis. After Pareto scaling, PCoA and partial least squares discriminant analysis (OPLS-DA) were performed. Leave-one-out cross-validation and response permutation testing were conducted to evaluate the robustness of the model. Metabolites showing significant differences between the LA and HA groups were identified based on the combination of a statistically significant threshold of variable influence on projection (VIP) values obtained from the OPLS-DA model and a two-tailed Student's *t*-test (*p*-value) on the raw data. The metabolites were considered significant when they had VIP values >1.0, VIP values <0.05, and *p*-values less than 0.05. Differential metabolites were identified using three databases, including the Kyoto Encyclopedia of Genes and Genomes (KEGG)[2], the human metabolome database, and the bovine metabolome database. The KEGG database was used to evaluate the enrichment analysis of KEGG metabolic pathways according to the differential metabolites (Kanehisa et al., 2012). Fisher's exact test was used to determine the significance of enriched pathways.

## 2.8. Statistical analysis

Fecal fermentation parameters were analyzed using the *t*-test in the SPSS software (version 22.0, SPSS, Inc., Chicago, IL, United States). Alpha diversity indices, which reflect the significance between the LA and HA groups, were analyzed using the Wilcoxon rank test with the "dplyr" package (authors, H. Wickham, R. François, L. Henry, K. Müller; published date, 2018; version, 0.7.6) in R. PCoA was performed based on the Bray–Curtis dissimilarity matrices in R, and "ggplot2" package in R was used to visualize the results. The differences in the relative abundance of organisms at the phylum, family, and genus levels and microbiota function between the two groups were tested using the Wilcoxon method in R (version 4.0.5). Spearman's rank correlation was used to identify the relationship between the relative abundance of the core OTUs, altitude, fecal fermentation parameters, and serum antioxidant indices using the "Psych" package (author, W. Revelle; published date, 2016; version, 1.6.9) and visualized using the "corrplot" package (author, Taiyun Wei; published date, 2017; version, 0.84) in R. All data were reported as the mean, and differences with $p < 0.05$ were considered as significant.

# 3. Results

## 3.1. Gut fermentation parameters of Sanhe heifers from different altitudes

As shown in Table 1, there was no significant difference ($p > 0.05$) in the concentrations of acetate, propionate, butyrate, and total VFAs

1 www.metaboanalyst.ca
2 http://www.kegg.jp/

TABLE 1 Fecal-sample fermentation parameters at Sanhe heifers from different altitudes.

| Items | Groups[1] | | SEM | *p*-value |
|---|---|---|---|---|
| | LA | HA | | |
| Acetate, mmol/L | 7.412 | 6.88 | 0.67 | 0.40 |
| Propionate, mmol/L | 3.92 | 3.22 | 0.43 | 0.06 |
| Butyrate, mmol/L | 1.48 | 1.48 | 0.20 | 0.88 |
| AP[2] | 1.77[b] | 2.13[a] | 0.07 | 0.002 |
| TVFAs[3], mmol/L | 12.40 | 11.60 | 1.33 | 0.52 |

[1]LA represents the low-altitude region (Hulunbuir, Inner Mongolia Autonomous Region, 119°57′ E, 47°17′ N; approximately 700 m altitude, LA); HA represents the high-altitude region (Lhasa, Tibet Autonomous Region 91°06′ E, 29°36′ N; approximately 3,750 m altitude, HA). [2]AP, the acetate-to-propionate raito (AP). [3]TVFA, total volatile fatty acids. The differences among the six groups are indicated by various letters ($p < 0.05$).

between fecal samples of Sanhe cattle reared at different altitudes. Compared with the LA group, the acetate-to-propionate ratio (A/P) increased significantly ($p < 0.05$) in the HA group.

## 3.2. Gut microbiota communities of Sanhe heifers from different altitudes

### 3.2.1. Sequencing metrics for the gut microbiota of heifers

A total of 868,445 raw sequences were generated with an average of 43,422 ± 4,392.71 (mean ± SD) per sample. An average of 2,054 ± 133.75 OTUs across all samples were identified at 3% sequence dissimilarity. Rarefaction curves showed that the number of new OTUs decreased as the number of sequences per sample increased (Additional file: Supplementary Figure S1), indicating an adequate sampling depth to cover the tested gut bacterial composition. Good's coverage for the Sanhe heifer samples showed a mean value of 0.97 across all 20 samples, indicating sufficient sequence coverage for all samples. The mean Shannon's diversity and Chao1's richness for all Sanhe heifer samples were 8.42 ± 0.49 and 2,674.69 ± 157.41 (Additional file: Supplementary Table S2), respectively.

The most highly abundant phyla in all Sanhe heifer samples were Firmicutes (64.06%), Bacteroidetes (32.33%), Tenericutes (0.93%; Figures 1A,B). Among these phyla, the most abundant families were Ruminococcaceae (36.37%), Rikenellaceae (15.43%), Peptostrepto coccaceae (12.23%), and Christensenellaceae (5.51%; Figures 1A,B). At the genus level, 11 genera showed >2% relative abundance (Figures 1A,B).

### 3.2.2. Defining the core gut microbiota for Sanhe heifers in this study

The intestinal microbiome of the Sanhe cattle varies widely. Therefore, we focused on the core OTUs found in all the Sanhe heifers. We sought to identify the core microbiota across all Sanhe heifers and found 393 shared OTUs among all samples from LA and HA Sanhe heifers, as shown in Figure 1C. These OTUs included the following bacterial families with >10% total relative abundance: Ruminococcaceae (26.10%), Rikenellaceae (11.37%, Figures 1A,B). The shared genera among all samples showing >5% of the total relative abundance were *Ruminococcaceae_UCG-005* (13.71%), *Rikenellaceae_RC9_gut_group* (7.74%; Figures 1A,B).

### 3.2.3. Differences in the gut bacterial community between LA and HA Sanhe heifers

To detect differences in the gut microbiota of LA and HA Sanhe heifers, we performed Bray–Curtis dissimilarity analysis. The results were visualized using a principal coordinate analysis (PCoA) plot, as shown in Figure 1D. The gut microbiota that differed between groups were analyzed using analysis of similarities and confirmed that the two groups significantly differed ($R^2 = 0.58$, $p = 0.001$). However, we found no significant difference ($p < 0.05$) in Chao 1 richness, Shannon diversity index, and Simpson's diversity index between the groups (Additional file: Supplementary Table S2).

At the phylum level, the relative abundances of the phyla Firmicutes, Bacteroidetes, and Verrucomicrobia did not differ significantly ($p > 0.05$) between the LA and HA groups. In contrast, compared with the LA group, the relative abundances of the phyla Proteobacteria and Actinobacteria were significantly ($p < 0.05$) lower (Table 2), whereas that of the phylum Spirochaetae was significantly ($p < 0.05$) higher in the HA group. At the family level (family of relative abundance >0.01%), lower relative abundances of Peptostreptococcaceae, Christensenellaceae, Erysipelotrichaceae, Family_XIII, Acidaminococcaceae, Peptococcaceae, Enterobacteriaceae, Spirochaetaceae and Coriobacteriaceae were observed in the HA group than in the LA group (Table 2), and the relative abundances of Lachnospiraceae, Clostridiales_vadinBB60_group, Bacteroidales_S24-7_group, Bacteroidales_RF16_group, and Porphyromonadaceae were higher in the LA group than in the HA group (Table 2). At the genus level (genera with relative abundance >0.01%), compared with the LA group, the relative abundances of 43 genera were significantly ($p < 0.05$) higher, and those of 15 genera were significantly lower in the HA group (Table 3); of these, eight genera showed a relative abundance >1%. The relative abundances of some genera differed by more than 10-fold, including *Butyrivibrio* (decreasing 20.59-fold, $p = 0.002$), *Eubacterium_xylanophilum_group* (decreasing 13.25-fold, $p < 0.001$), *Corynebacterium* (increasing 16.51-fold, $p = 0.011$), *Escherichia-Shigella* (increasing 213.31-fold, $p < 0.001$), and *Domibacillus* (increasing from 0.00 to 0.027, $p < 0.001$; Table 3).

### 3.2.4. Correlation of core gut bacteria with altitude, and gut fermentative parameters in HA and LA Sanhe heifers

To explore the role of gut bacteria in production and fermentation of VFAs, we analyzed the relationship between fecal VFA concentration (acetate, propionate, butyrate, and total VFAs) and the relative abundance of OTUs using Spearman's rank correlations, as shown in Figure 2. All OTUs with relative abundances <0.01% in all fecal samples were removed from the analysis. The relationship between OTUs and production and fermentation traits was visualized using a heat map (Figure 2). Fifty-Eight OTUs were significantly ($p < 0.05$) correlated with altitude; of these, 20 OTUs were negatively correlated with altitude, eight of which were in the family Ruminococcaceae ($p < 0.05$), four in the family Rikenellaceae ($p < 0.05$), and two in the family Lachnospiraceae ($p < 0.05$). Additionally, OTUs within unidentified_o_Clostridiales, Clostridiales_vadinBB60_group, Clostridiaceae_1, Bacteroidales_BS11_gut_group, Bacteroidales_RF16_group, and Spirochaetaceae were significantly negatively ($p < 0.05$) correlated with altitude. Thirty-eight OTUs were positively correlated with altitude, among which 14 were in the

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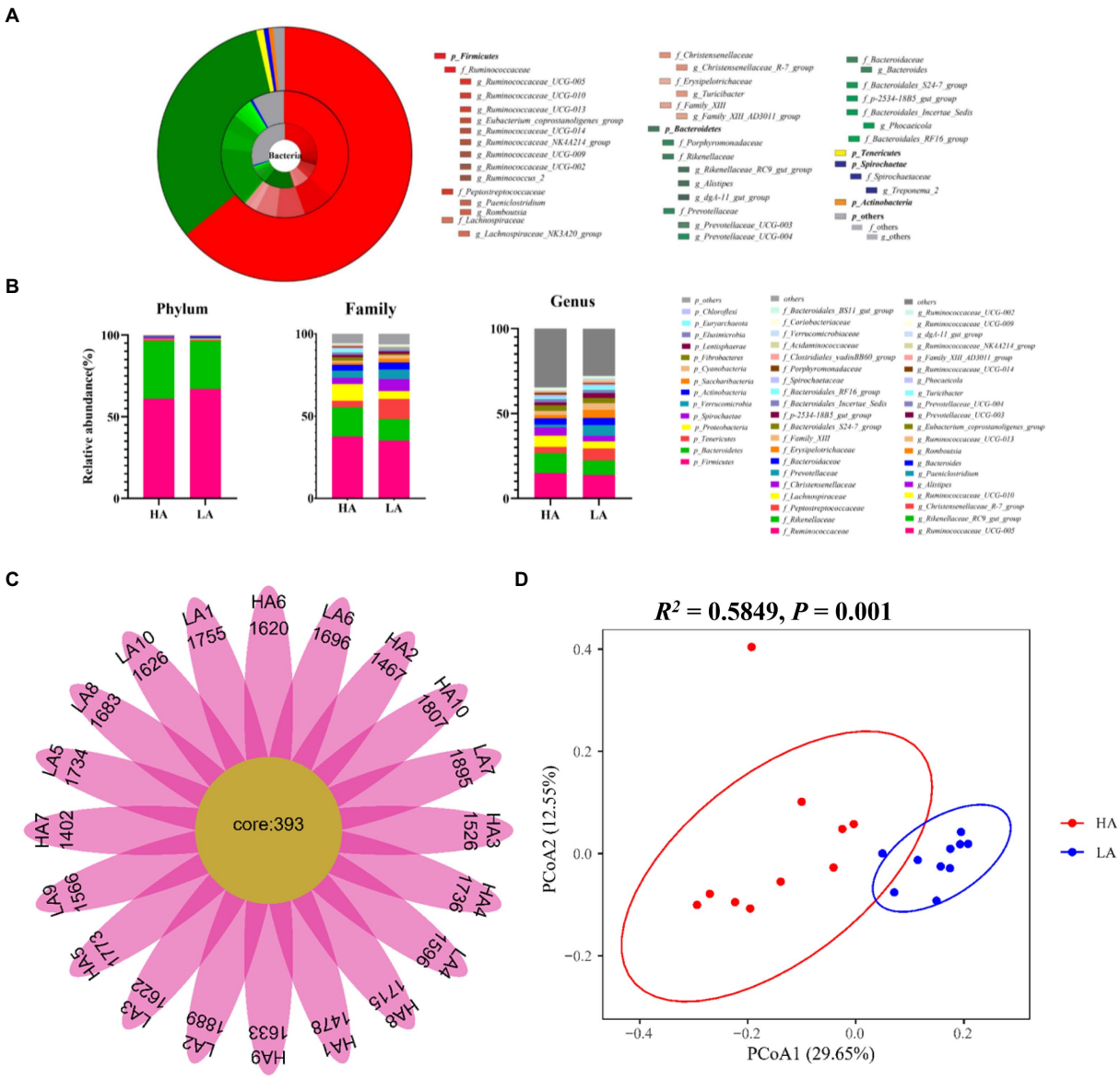


FIGURE 1
The gut bacterial composition of Sanhe heifers. **(A)** Relative distribution of the most dominant bacterial phyla, family, and genera (relative abundance >0.5% of all the 20 samples) in the low-altitude (LA) and high-altitude (HA) group samples. The inner ring in the pie chart represents the genus level, the middle ring represents the family level, and the outer ring represents the phylum level. Different color shades represent different bacteria. The numbers in brackets denote the average relative abundance of the bacteria across 20 samples. **(B)** Stacked bar graphs of the average relative abundances of phyla, family, and genus (top 20 of relative abundances) for Sanhe heifers in the LA and HA groups. **(C)** Flower diagram plot of Sanhe heifer samples. The core community in all Sanhe heifers was defined as operational taxonomic units present in all Sanhe heifers at all sampling times. **(D)** Principal coordinate analysis (PCoA) plots of bacterial communities in Sanhe heifer samples.

family Ruminococcaceae ($p < 0.05$), 11 in the family Christensenellaceae ($p < 0.05$), three in the family Family_XIII ($p < 0.05$), three in the family Lachnospiraceae ($p < 0.05$), and three in the family Peptostreptococcaceae ($p < 0.05$). In addition, OTUs within the families Erysipelotrichaceae, Acidaminococcaceae, Bacteroidaceae, and unidentified_o_Gastranaerophilales were significantly and positively correlated with altitude ($p < 0.05$).

Fourteen OTUs were negatively ($p < 0.05$) correlated with the acetate-to-propionate ratio (AP), among which six and three were in the families Ruminococcaceae and Rikenellaceae, respectively. In addition, 23 OTUs were positively correlated ($p < 0.05$) with AP, of which eight were in the family Ruminococcaceae, six in the family Christensenellaceae, three in the family Peptostreptococcaceae, and two in the family Lachnospiraceae. Furthermore, OTUs within the families Family_XIII, Erysipelotrichaceae, unidentified_o_Gastranaerophilales, and Acidaminococcaceae were significantly and positively correlated with AP ($p < 0.05$).

Analysis of VFAs showed that acetate concentration was negatively correlated with the relative abundance of OTUs in the *Eubacterium coprostanoligenes* group. Sixteen OTUs were significantly ($p < 0.05$) correlated with propionate concentration, among which 10 OTUs were negatively correlated with propionate concentration, three OTUs

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TABLE 2 Phyla and Families of relative abundance >0.1% within the gut microbiota of LA and HA heifers.

| Phyla/families | Groups[1] | | SEM | *p*-value |
|---|---|---|---|---|
| | LA | HA | | |
| Firmicutes | 67.078 | 61.035 | 1.764 | 0.105 |
| Ruminococcaceae | 35.082 | 37.653 | 1.231 | 0.190 |
| Peptostreptococcaceae | 12.234 | 4.016 | 1.142 | <0.001 |
| Lachnospiraceae | 4.796 | 10.162 | 1.855 | 0.043 |
| Christensenellaceae | 7.249 | 3.767 | 0.568 | 0.002 |
| Erysipelotrichaceae | 2.409 | 1.244 | 0.204 | 0.003 |
| Family_XIII | 2.044 | 1.151 | 0.142 | 0.002 |
| Clostridiales_vadinBB60_group | 0.223 | 0.691 | 0.067 | <0.001 |
| Acidaminococcaceae | 0.700 | 0.202 | 0.079 | 0.001 |
| Clostridiaceae_1 | 0.204 | 0.283 | 0.024 | 0.140 |
| Peptococcaceae | 0.175 | 0.104 | 0.016 | 0.010 |
| Bacteroidetes | 29.247 | 35.422 | 1.684 | 0.089 |
| Rikenellaceae | 13.138 | 17.715 | 1.129 | 0.075 |
| Prevotellaceae | 5.883 | 4.364 | 0.594 | 0.105 |
| Bacteroidaceae | 4.266 | 3.671 | 0.325 | 0.123 |
| Bacteroidales_S24-7_group | 0.794 | 2.163 | 0.345 | 0.011 |
| f_p-2,534-18B5_gut_group | 1.383 | 1.394 | 0.373 | 0.520 |
| Bacteroidales_Incertae_Sedis | 1.166 | 1.424 | 0.122 | 0.529 |
| Bacteroidales_RF16_group | 0.470 | 1.818 | 0.209 | 0.001 |
| Porphyromonadaceae | 0.343 | 0.766 | 0.089 | 0.005 |
| Bacteroidales_BS11_gut_group | 0.265 | 0.382 | 0.041 | 0.290 |
| Bacteroidales_UCG-001 | 0.194 | 0.066 | 0.031 | 0.064 |
| Proteobacteria | 0.505 | 0.192 | 0.087 | 0.016 |
| Enterobacteriaceae | 0.332 | 0.007 | 0.089 | <0.001 |
| Spirochaetae | 0.161 | 1.081 | 0.152 | <0.001 |
| Spirochaetaceae | 0.161 | 1.081 | 0.152 | <0.001 |
| Verrucomicrobia | 0.499 | 0.373 | 0.066 | 0.218 |
| Verrucomicrobiaceae | 0.432 | 0.338 | 0.058 | 0.436 |
| Actinobacteria | 0.870 | 0.300 | 0.109 | 0.001 |
| Coriobacteriaceae | 0.514 | 0.246 | 0.048 | 0.001 |
| Bifidobacteriaceae | 0.283 | 0.044 | 0.097 | 0.472 |

[1]LA represents the low-altitude region (Hulunbuir, Inner Mongolia Autonomous Region, 119°57′ E, 47°17′ N; approximately 700 m altitude, LA); HA represents the high-altitude region (Lhasa, Tibet Autonomous Region 91°06′ E, 29°36′ N; approximately 3,750 m altitude, HA).

were in the family Christensenellaceae, three OTUs were in the family Peptostreptococcaceae, two OTUs were in the family Lachnospiraceae, and one OTU was in the family Erysipelotrichaceae. Six OTUs were significantly and positively ($p < 0.05$) correlated with propionate concentration; of these, four OTUs belonged to the family Ruminococcaceae, one OTU belonged to the family Bacteroidales_RF16_group, and one OTU belonged to the family Lachnospiraceae. The total VFA concentration was negatively ($p < 0.05$) correlated with the relative abundance within the family Ruminococcaceae.

## 3.3. Gut metabolome of Sanhe heifers from different altitudes

### 3.3.1. Differential metabolites

A total of 1,727 differential metabolites were identified in the gut metabolome; of these, 1,101 and 626 metabolites were detected in positive and negative ion modes, respectively. To compare the metabolome compositions of the gut samples in the two groups, the datasets obtained from LC–MS in the positive and negative ion modes were evaluated using PCA (Figures 3A,B). The metabolites between the two groups were well-separated in the PCA score plots of the positive and negative ion mode results. Volcano plots of the positive and negative ion modes for the two groups are shown in Figures 3C,D. The OPLS-DA score plots are shown in Supplementary Figure S2. OPLS-DA revealed a clear distinction between the LA and HA groups in both the positive (R2X = 0.351, R2Ycum = 0.995, Q2cum = 0.954) and negative ion modes (R2X = 0.351, R2Ycum = 0.995, Q2cum = 0.954), which was validated by permutation analysis (positive: Q2 intercept = −0.2568; negative: Q2 intercept = −0.2413). Based on the cutoff (VIP >1 and $p < 0.05$) for differential metabolites, 368 metabolites differed significantly between the LA and HA groups, of which 231 and 137 were detected in the positive and negative ion modes, respectively.

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TABLE 3 Genera of relative abundance >0.01% within the gut microbiota of LA and HA heifers as determined using the Wilcoxon test (only $p<0.05$ is shown).

| Genera | Groups[1] | | SEM | *P*-value |
|---|---|---|---|---|
| | LR | HR | | |
| Christensenellaceae_*R*-7_group | 3.707 | 7.189 | 0.565 | 0.002 |
| Ruminococcaceae_UCG-010 | 6.610 | 4.059 | 0.435 | <0.001 |
| *Alistipes* | 4.902 | 3.306 | 0.329 | 0.023 |
| *Paeniclostridium* | 1.689 | 6.349 | 0.648 | <0.001 |
| *Romboutsia* | 1.959 | 4.773 | 0.406 | <0.001 |
| Ruminococcaceae_UCG-013 | 2.473 | 3.885 | 0.225 | 0.001 |
| Prevotellaceae_UCG-003 | 1.718 | 2.878 | 0.389 | 0.029 |
| *Turicibacter* | 1.033 | 2.172 | 0.204 | 0.006 |
| Family_XIII_AD3011_group | 0.626 | 1.035 | 0.091 | 0.029 |
| Ruminococcaceae_NK4A214_group | 0.564 | 1.054 | 0.072 | <0.001 |
| dgA-11_gut_group | 0.502 | 0.855 | 0.070 | 0.015 |
| *Treponema*_2 | 1.034 | 0.153 | 0.145 | <0.001 |
| Lachnospiraceae_NK3A20_group | 0.402 | 0.683 | 0.056 | 0.011 |
| *Phascolarctobacterium* | 0.199 | 0.696 | 0.079 | <0.001 |
| *Tyzzerella*_4 | 0.569 | 0.268 | 0.058 | 0.003 |
| *Ruminococcus*_1 | 0.432 | 0.243 | 0.041 | 0.023 |
| *Flavonifractor* | 0.177 | 0.353 | 0.027 | <0.001 |
| *Eubacterium*_brachy_group | 0.188 | 0.315 | 0.025 | 0.013 |
| *Eubacterium*_oxidoreducens_group | 0.335 | 0.118 | 0.055 | 0.007 |
| Ruminococcaceae_UCG-004 | 0.075 | 0.376 | 0.040 | <0.001 |
| *Candidatus*_Soleaferrea | 0.258 | 0.174 | 0.016 | 0.006 |
| Ruminococcaceae_UCG-011 | 0.086 | 0.324 | 0.033 | <0.001 |
| *Eubacterium_nodatum*_group | 0.081 | 0.307 | 0.032 | <0.001 |
| *Intestinibacter* | 0.106 | 0.256 | 0.024 | 0.002 |
| *Butyrivibrio* | 0.335 | 0.016 | 0.053 | 0.003 |
| Dorea | 0.133 | 0.212 | 0.018 | 0.017 |
| *Escherichia-Shigella* | 0.007 | 0.332 | 0.089 | <0.001 |
| *Marvinbryantia* | 0.097 | 0.201 | 0.016 | 0.002 |
| *Blautia* | 0.098 | 0.200 | 0.021 | 0.021 |
| Lachnospiraceae_AC2044_group | 0.154 | 0.067 | 0.015 | 0.009 |
| *Acetitomaculum* | 0.025 | 0.189 | 0.026 | <0.001 |
| Lachnospiraceae_UCG-010 | 0.074 | 0.137 | 0.013 | 0.028 |
| *Ruminiclostridium*_9 | 0.058 | 0.135 | 0.016 | 0.004 |
| *Pseudobutyrivibrio* | 0.101 | 0.061 | 0.010 | 0.049 |
| *Olsenella* | 0.038 | 0.096 | 0.009 | 0.001 |
| *Anaerorhabdus_furcosa*_group | 0.044 | 0.074 | 0.008 | 0.015 |
| *Oscillospira* | 0.045 | 0.071 | 0.007 | 0.017 |
| *Oscillibacter* | 0.033 | 0.079 | 0.008 | 0.002 |
| *Anaerovorax* | 0.034 | 0.072 | 0.009 | 0.019 |
| Lachnospiraceae_UCG-001 | 0.033 | 0.063 | 0.006 | 0.004 |
| *Anaerosporobacter* | 0.021 | 0.072 | 0.010 | 0.016 |
| *Eubacterium_hallii*_group | 0.028 | 0.052 | 0.005 | 0.012 |

*(Continued)*

TABLE 3 (Continued)

| Genera | Groups[1] | | SEM | *P*-value |
|---|---|---|---|---|
| | LR | HR | | |
| *Ruminococcus_gauvreauii*_group | 0.012 | 0.049 | 0.007 | 0.003 |
| *Gordonibacter* | 0.011 | 0.048 | 0.009 | 0.006 |
| Family_XIII_UCG-001 | 0.017 | 0.040 | 0.004 | 0.007 |
| *Atopobium* | 0.012 | 0.042 | 0.005 | 0.002 |
| *Faecalitalea* | 0.010 | 0.041 | 0.006 | 0.012 |
| *Senegalimassilia* | 0.014 | 0.037 | 0.004 | 0.003 |
| *Elusimicrobium* | 0.031 | 0.009 | 0.005 | 0.006 |
| Family_XIII_UCG-002 | 0.007 | 0.032 | 0.004 | 0.005 |
| Erysipelotrichaceae_UCG-004 | 0.033 | 0.006 | 0.006 | 0.004 |
| Lachnospiraceae_UCG-002 | 0.005 | 0.031 | 0.004 | <0.001 |
| *Corynebacterium*_1 | 0.004 | 0.027 | 0.004 | <0.001 |
| *Solobacterium* | 0.006 | 0.022 | 0.002 | <0.001 |
| *Corynebacterium* | 0.002 | 0.026 | 0.007 | 0.011 |
| *Domibacillus* | 0.000 | 0.027 | 0.004 | <0.001 |
| Anaeroplasma | 0.018 | 0.007 | 0.003 | 0.025 |
| *Eubacterium_xylanophilum*_group | 0.021 | 0.002 | 0.003 | <0.001 |

[1]LA represents the low-altitude region (Hulunbuir, Inner Mongolia Autonomous Region, 119°57′ E, 47°17′ N; approximately 700 m altitude, LA); HA represents the high-altitude region (Lhasa, Tibet Autonomous Region 91°06′ E, 29°36′ N; approximately 3,750 m altitude, HA).

#### 3.3.2. Kyoto encyclopedia of genes and genomes pathways

Metabolic pathway analysis based on the significantly different gut metabolites revealed the enrichment of 10 metabolic pathways (Figure 4A), with "nicotine addiction," "central carbon metabolism in cancer," "mineral absorption," "protein digestion and absorption," "ABC transporters," "neuroactive ligan-receptor interaction," "cAMP signaling pathway," "aminoacyl-tRNA biosynthesis," "pyrimidine metabolism," and "purine metabolism," which belong to "environmental information processing," "organismal systems," "metabolism," "human diseases," and "genetic information processing,." The differential metabolites in the differentially enriched KEGG pathways determined by hydrophilic interaction LC–MS analysis are shown in Table 4. In addition, the relationships between metabolic pathways were significantly different for the gut metabolites (Figure 4B).

### 3.4. Relationships between the core gut microbiota and metabolites

Spearman's correlation network between the core gut microbiota and gut metabolites was analyzed, that revealed 28 significant correlations (relative abundance >0.1%, $r > |0.8|$, $p < 0.05$; Figure 4C). OTUs belonging to the genus *Romboutsia* were significantly negatively and positively correlated with seven and two metabolites. Respectively. OTUs belonging to the genus *Paeniclostridium* were significantly negatively and positively correlated with 13 and five metabolites, respectively. OTUs belonging to the genus *unclassified_f_Lachnospiraceae* were significantly positively correlated with a single metabolite.

## 4. Discussion

By integrating gut 16S rRNA high-throughput sequencing and LC–MS-based untargeted metabolomic analyzes, we investigated the gut microbiome and host metabolome mechanisms involved in high-altitude adaptability. We estimated the gut microbial composition, metabolites, and variations as well as the interactions between microorganisms and metabolites in different groups.

### 4.1. Gut microbiota communities of Sanhe heifers reared in different altitudes

VFAs did not differ in ruminal samples from cattle reared in different regions, suggesting that altitude does not strongly affect the fecal fermentation parameters in Sanhe heifers.

Generally, the intestinal microbiota is stable over time in adult animals (Caporaso et al., 2011; Faith et al., 2013). In this study, we investigated the differences in the gut microbiota of Sanhe heifers reared at different altitudes. In the two groups, Firmicutes and Bacteroidetes, known to play a key role in maintaining gut homeostasis, were the most abundant phyla in the gut of Sanhe heifers, which agrees with the findings observed in yaks (Liu et al., 2021) and rats. Members of Bacteroides participate in the degradation of biopolymers and main polysaccharides, whereas bacteria from Firmicutes regulate the digestion and absorption of proteins and carbohydrates. At the phylum level, the enrichment of Proteobacteria in the gut represents an imbalanced and unstable microbiota structure or disease state in the host (Shin et al., 2015). Actinobacteria are thought to be involved in modulating gut permeability, immune system, metabolism, and the gut-brain axis, and their abundance represents the health state of the animal. The relative

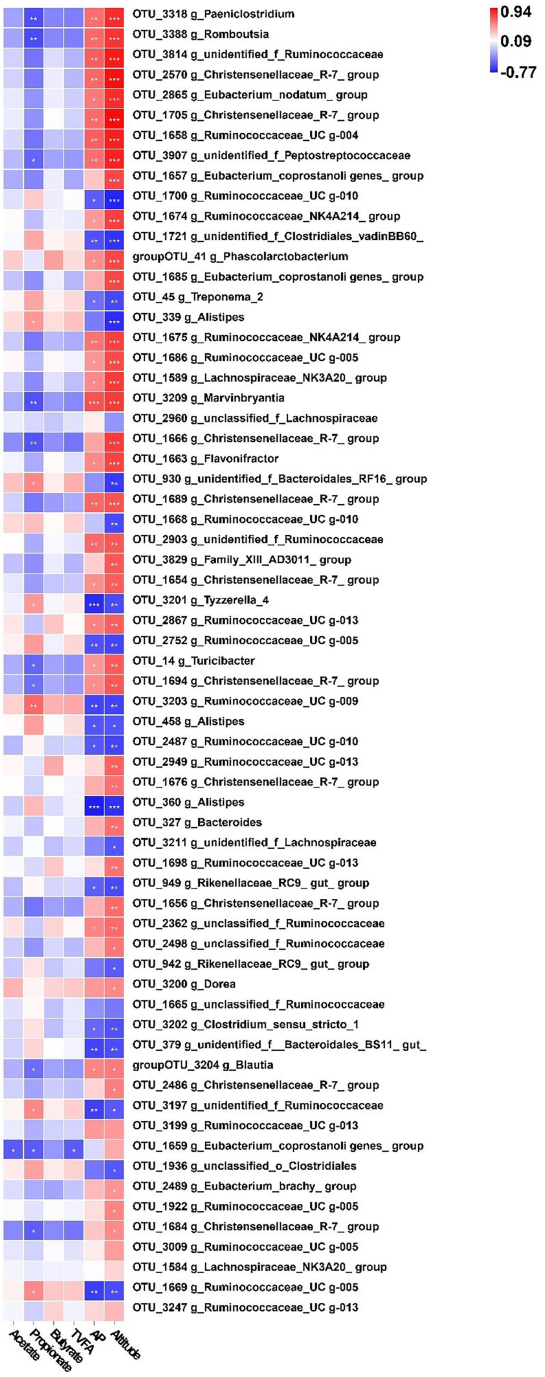


FIGURE 2
Correlation analysis of gut bacteria. **(A)** Heatmap of core operational taxonomic units (OTUs) (relative abundance >0.01% and $p<0.05$ in all samples) significantly associated with metabolite production and fecal fermentative parameters in low-altitude (LA) and high-altitude (HA) Sanhe heifers, as determined by Spearman's correlation analysis. *$0.01<p<0.05$, **$0.001<p<0.01$, ***$p\leq0.001$. **(B)** Interaction among OTUs with relative abundances >0.01% and $p<0.05$. The red and blue colors represent positive and negative correlations, respectively. The size and color of the circle represent the relative abundance of the OTUs and the genus to which they belong, respectively.

abundance of Spirochaetae was lower in the gut of HA heifers than in that of LA heifers, as observed previously in sub-adult Tibetan sheep (Li et al., 2020), which are saccharolytic and can use carbohydrates as substrates. Families showing differential abundances, including Peptostreptococcaceae, Christensenellaceae (Waters and Ley, 2019), Erysipelotrichaceae (Wu et al., 2021), Family_XIII, and Lachnospiraceae, *Domibacillus* (Sharma et al., 2014), Bacteroidales_S24-7_group (Gao et al., 2020), Bacteroidales_RF16_group, and Porphyromonadaceae (Sakamoto, 2014), most of which belonged to the phyla Firmicutes, Bacteroidetes, Actinobacteria, and Spirochaetae, are associated with fiber degradation, feed digestion, and inflammation induction. *Butyrivibrio* (Kelly et al., 2010) and *Eubacterium_xylanophilum_group* (Mukherjee et al., 2020) are butyrate-forming bacteria that play key roles in polysaccharide degradation. The relative abundances of *Eubacterium_xylanophilum_group* (Mukherjee et al., 2020), *Corynebacterium* (Salem et al., 2015), *Escherichia-Shigella* (The et al., 2016), and *Domibacillus* vary widely, and most of these organisms are pathogens, suggesting that changes in altitude affect the structure of the intestinal microbiota and the health of Sanhe heifers. Moreover, we considered the impact of altitude on the gut bacterial core OTUs of Sanhe heifers. Therefore, these OTUs may form the key bacterial community responsible for high-altitude adaptibilty in Sanhe heifers.

## 4.2. Gut metabolome of Sanhe heifers in different altitudes

The enriched differential metabolic pathways belonged to nucleotide metabolism, including pyrimidine and purine metabolism pathways. Purine and pyrimidine nucleotides are major energy carriers, subunits of nucleic acids, and precursors for the synthesis of nucleotide cofactors (Moffatt and Ashihara, 2002). The enriched differential metabolic pathways belonged to the digestive system of organismal systems, including mineral and protein digestion and absorption, suggesting that different altitudes affect the digestive system of Sanhe heifers. The enriched differential metabolic pathways belonged to environmental information processing, including "ABC transporters," "neuroactive ligan-receptor interaction," and "cAMP signaling pathway." The cAMP signaling pathway regulates critical physiological processes, including metabolism, secretion, calcium homeostasis, muscle contraction, cell fate, and gene transcription (Ould Amer and Hebert-Chatelain, 2018). The cyclic nucleotide-gated ion channel regulates downstream pathways by activating calmodulin and calcium/calmodulin-dependent protein kinase. In addition, the cAMP pathway, also known as the protein kinase A pathway, directly regulates the transmembrane transport of calcium, potassium, sodium, and chloride ions through phosphorylation of channel proteins, transporters, and receptors on the cell membrane. ABC transporters exert a variety of physiological functions, such as the removal of foreign substances, nutrient intake, resistance to foreign invasion, antigen transmission, and inhibition of transportation, and are closely related to the health of the body (Liu, 2019; Thomas and Tampé, 2020). All these pathways were upregulated in the HA group compared to those in the LA group. In addition, the KEGG pathway was enriched in human diseases, suggesting that high altitudes affect the health of Sanhe heifers. Overall, untargeted metabolomics showed that high-altitude regions could alter organismal systems, metabolism, environmental information processing, genetic information processing, and even induce disease. Altitude also affects environmental information processing, organismal systems, human diseases, and genetic information processing.

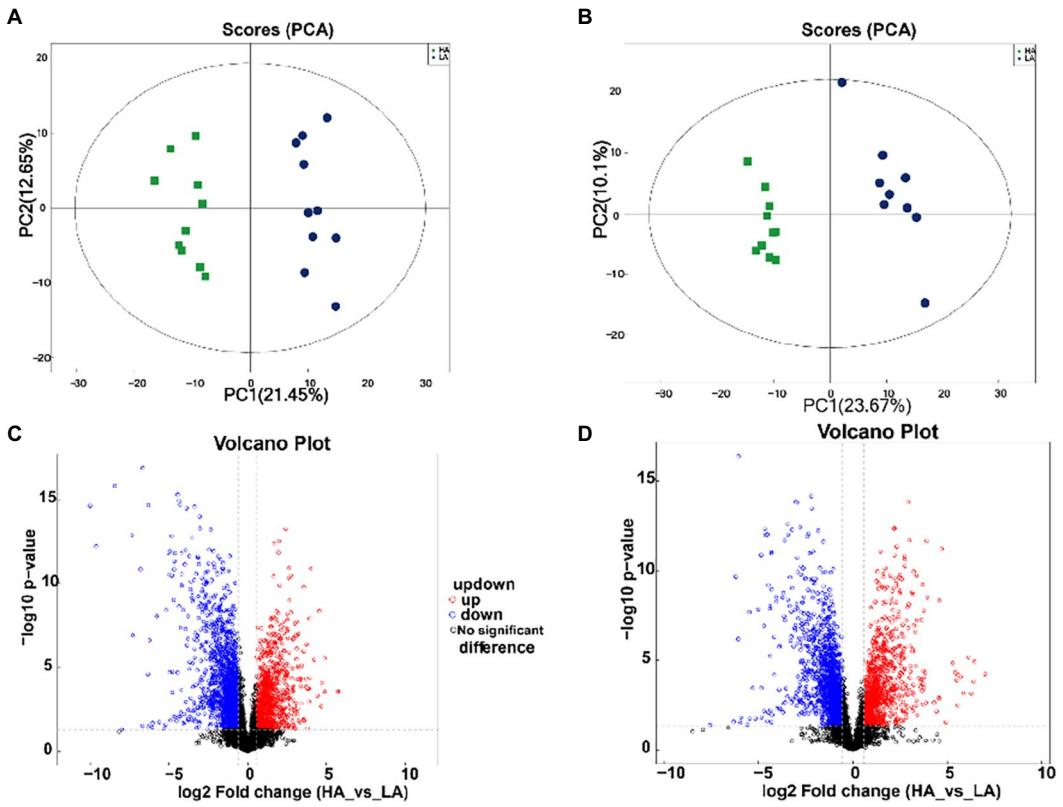


FIGURE 3
Metabolomic profiling of fecal samples. Scatter plots of the principal coordinate analysis model based on all identified metabolite features of gut samples from the two groups. [**(A)** positive mode; **(B)** negative mode]. The volcano plot of the comparison between the low-altitude (LA) and high-altitude (HA) groups [**(C)**, positive mode, **(D)**, negative mode].

## 4.3. Relationships between the core gut microbiome and gut metabolites

We found that OTUs belonging to the genus *Romboutsia* were associated with nine metabolites (melatonin, uracil, hypoxanthine, xanthine, guanine, monensin, heliotrine N-oxide, d-lyxose, and L-gulono-1,4-lactone). A previous study showed that *Romboutsia* encodes a versatile array of metabolic capabilities involved in carbohydrate utilization, fermentation of single amino acids, anaerobic respiration, and metabolic end-products (Gerritsen et al., 2019), which is consistent with our results. OTUs belonging to the genus *Paeniclostridium* were associated with 18 metabolites (L-gulono-1,4-lactone, uracil, d-lyxose, chicoric acid, guanine, 5-(2-hydroxyethyl)-4-methyl thiazole, oxeladin, hypoxanthine, 2-aminoadipic acid, melatonin, 1,2-dimyristoyl-*sn*-glycero-3-phosphate, N-omega-propyl-l-arginine, artemisinin, heliotrine N-oxide, ondansetron, Cer 20:1-d7 (d18:1-d7/20:1), deoxyinosine, and d-fructose). *Paeniclostridium* is an anaerobic pathogen in animals (Kim et al., 2017). The OTUs belonged to the genus *g_unclassified_f_Lachnospiraceae* which is a member of the family Lachnospiraceae that is positively correlated with 1-palmitoylglycerol. Previous studies have shown that insoluble fatty acid soap might reduce the growth benefits in the intestine (Yaron et al., 2013; Wang et al., 2020), suggesting that excess 1-palmitoylglycerol causes intestinal damage. Therefore, Sanhe heifers are more prone to diseases in high-altitude environments.

Overall, our results showed that the gut microbiome and metabolome of Sanhe heifers differed between the LA and HA groups. We found that the gut microbiota associated with digestion absorption of proteins and carbohydrates, including Peptostreptococcaceae, Christensenellaceae, Erysipelotrichaceae, Family_XIII, Lachnospiraceae, *Domibacillus*, Bacteroidales_S24-7_group, Bacteroidales_RF16_group, Porphyromonadaceae, and Spirochaetaceae, differed between HA heifers and LA heifers. These findings indicate that the ability of the gut microbiota to ferment dietary substrates differs between LA and HA Sanhe heifers. The core OTUs in the phyla Bacteroidetes, Firmicutes, Spirochaetes, and Cyanobacteria differed between the gut microbiota of the LA and HA groups. Therefore, these organisms may be critical bacterial communities involved in determining the high-altitude adaptabilty of Sanhe heifers. In addition, untargeted metabolomics has shown that high-altitude regions could alter organismal systems, metabolism, environmental information processing, genetic information processing, and even induce diseases. The genera *Romboutsia*, *Paeniclostridium*, and *g_unclassified_f_Lachnospiraceae* were strongly associated with the 28 differential metabolites. In summary, when Sanhe heifers encounter the stress of high-altitude environments, they respond by regulating their gut microbiome and metabolome; however, changes in altitude negatively affect the digestive ability and health of Sanhe heifers. This study contributes to the understanding of the ability of dairy cows to adapt to high-altitude regions and provides insights into

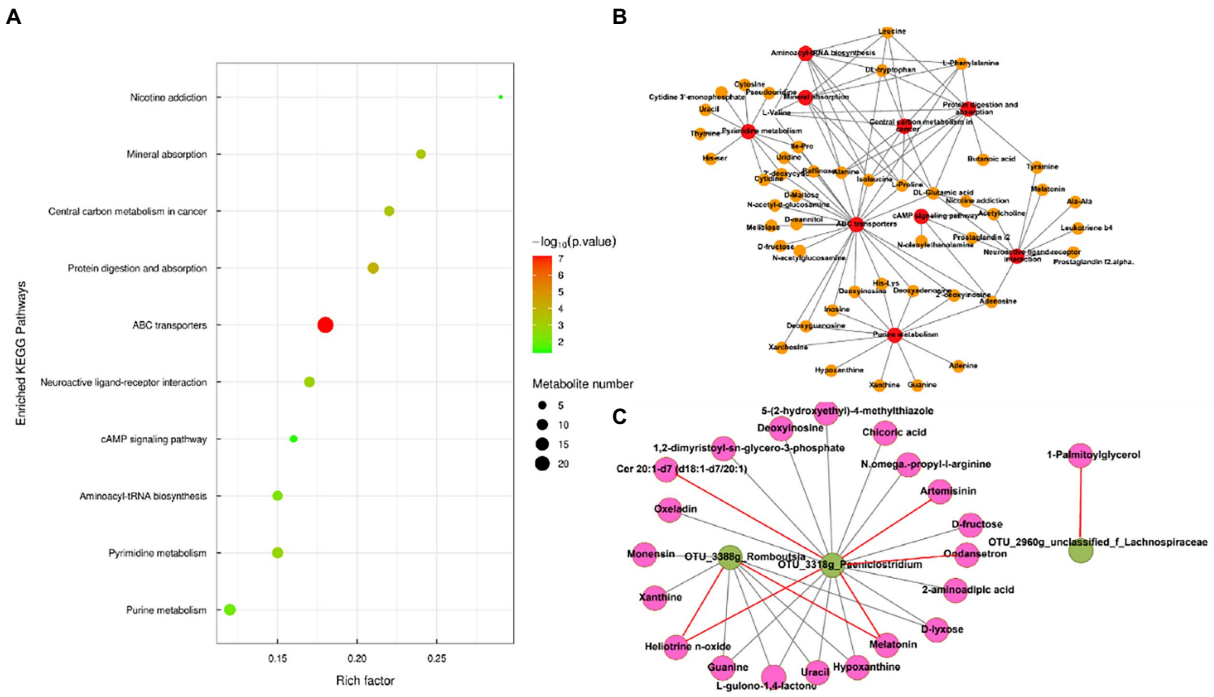


FIGURE 4
**(A)** Enriched Kyoto Encyclopedia of Genes and Genomes pathways of the comparison between the low-altitude (LA) and high-altitude (HA) groups (only those with a $p<0.05$ are shown). **(B)** Network of enriched pathways and the differential metabolites between the LA and HA groups. **(C)** Correlation between gut core OTU (relative abundance >0.1%) and differential metabolites in enrichment pathways based on **(A)** in the network (only those with $|r|>0.80$, $p<0.05$ are shown). Red and brown lines indicate positive and negative correlations, respectively.

strategies for altering the gut microbiota for high-altitude adaptation through feeding management.

## 5. Conclusion

We investigated the gut microbiome and metabolome mechanisms involved in the adaptation to high-altitude environments. Variations in the gut microbiome and metabolome, as well as the interaction of microorganisms and metabolites, were studied in the LA and HA groups by integrating gut 16S rRNA high-throughput sequencing and LC–MS-based untargeted metabolomic analyzes.

## 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. The sequence data supporting the results of this study are available in the NCBI Sequence Read Archive (SRA) under the accession number PRJNA821486.

## Ethics statement

The animal study was reviewed and approved by the study protocol was approved by the Ethical Committee of the College of Animal Science and Technology of China Agricultural University (project number AW22121202-1-2). Written informed consent was obtained from the owners for the participation of their animals in this study.

## Author contributions

XZ performed experiments and wrote the manuscript. WW, ZC, HY, YW, and SL reviewed and provided guidance for the manuscript and experiment. All authors contributed to the article and approved the submitted version.

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TABLE 4 Differential metabolites in differential enriched KEGG pathways using HILIC-MS analysis.

| HMDB[2] | Metabolites[1] | VIP | Fold-change | *P*-value | Direction[3] |
|---|---|---|---|---|---|
| HMDB0000012 | Ile-Pro | 7.242 | 9.081 | <0.001 | ↑ |
| HMDB0000085 | Deoxyguanosine | 2.842 | 4.355 | <0.001 | ↑ |
| HMDB0000296 | Uridine | 5.388 | 2.294 | <0.001 | ↑ |
| HMDB0000071 | Deoxyinosine | 3.348 | 5.345 | <0.001 | ↑ |
| HMDB0062538 | D-fructose | 2.622 | 3.145 | <0.001 | ↑ |
| HMDB0003213 | Raffinose | 1.577 | 3.502 | <0.001 | ↑ |
| HMDB0000883 | L-Valine | 1.163 | 1.955 | <0.001 | ↑ |
| HMDB0060475 | DL-Glutamic acid | 1.007 | 3.388 | <0.001 | ↑ |
| HMDB0000133 | His-Lys | 2.013 | 1.979 | <0.001 | ↑ |
| HMDB0033923 | Isoleucine | 1.720 | 1.706 | 0.002 | ↑ |
| HMDB0000163 | D-Maltose | 1.638 | 2.122 | 0.002 | ↑ |
| HMDB0000765 | D-mannitol | 1.232 | 0.083 | 0.002 | ↓ |
| HMDB0000195 | Inosine | 1.959 | 1.613 | 0.005 | ↑ |
| HMDB0000161 | Alanine | 1.280 | 4.901 | <0.001 | ↑ |
| HMDB0000101 | Deoxyadenosine | 13.257 | 4.300 | <0.001 | ↑ |
| HMDB0000162 | L-Proline | 1.002 | 2.457 | <0.001 | ↑ |
| HMDB0000299 | Xanthosine | 1.107 | 5.115 | <0.001 | ↑ |
| HMDB0000071 | 2′-deoxyinosine | 1.744 | 5.884 | <0.001 | ↑ |
| HMDB0000048 | Melibiose | 3.664 | 2.290 | <0.001 | ↑ |
| HMDB0000050 | Adenosine | 8.671 | 3.117 | 0.002 | ↑ |
| HMDB0000687 | Leucine | 2.191 | 2.023 | 0.003 | ↑ |
| HMDB0000159 | L-Phenylalanine | 1.766 | 2.397 | 0.005 | ↑ |
| HMDB0000014 | 2′-Deoxycytidine | 3.750 | 5.029 | 0.006 | ↑ |
| HMDB0000089 | Cytidine | 3.841 | 2.900 | 0.007 | ↑ |
| HMDB0000215 | N-acetyl-d-glucosamine | 2.763 | 1.969 | 0.020 | ↑ |
| HMDB0000215 | N-Acetylglucosamine | 1.524 | 4.979 | 0.023 | ↑ |
| HMDB0001085 | Leukotriene b4 | 1.131 | 1.785 | <0.001 | ↑ |
| HMDB0001335 | Prostaglandin i2 | 7.465 | 1.610 | 0.002 | ↑ |
|  | Prostaglandin f2-alpha | 1.235 | 0.608 | 0.003 | ↓ |
| HMDB0001389 | Melatonin | 1.883 | 0.389 | <0.001 | ↓ |
| HMDB0000306 | Tyramine | 6.083 | 2.265 | <0.001 | ↑ |
| HMDB0000895 | Acetylcholine | 1.136 | 0.431 | <0.001 | ↓ |
| HMDB0000303 | Ala-Ala | 2.462 | 2.064 | <0.001 | ↑ |
| HMDB0002088 | N-Oleoylethanolamine | 1.582 | 1.387 | 0.007 | ↑ |
| HMDB0030396 | DL-Tryptophan | 1.377 | 0.505 | <0.001 | ↓ |
| HMDB0000039 | Butanoic acid | 4.699 | 2.143 | 0.002 | ↑ |
| HMDB0000300 | Uracil | 4.879 | 3.933 | <0.001 | ↑ |
| HMDB0000273 | His-Ser | 8.947 | 4.890 | <0.001 | ↑ |
| HMDB0000767 | Pseudouridine | 1.198 | 2.762 | <0.001 | ↑ |
| HMDB0000262 | Thymine | 5.749 | 6.776 | <0.001 | ↑ |
| HMDB0000630 | Cytosine | 2.951 | 2.281 | <0.001 | ↑ |
|  | Cytidine 3′-monophosphate | 4.811 | 2.991 | <0.001 | ↑ |
| HMDB0000292 | Xanthine | 4.591 | 6.458 | <0.001 | ↑ |
| HMDB0000132 | Guanine | 6.161 | 4.326 | <0.001 | ↑ |
| HMDB0000034 | Adenine | 7.059 | 4.201 | <0.001 | ↑ |
| HMDB0000157 | Hypoxanthine | 6.426 | 2.727 | <0.001 | ↑ |

HILIC-MS, hydrophilic interaction liquid chromatography-mass spectrometry; VIP, variable important in projection. [1]Metabolites were filtered using significance estimates of $p < 0.05$ and VIP > 1.0 ($n$¼5). [2]HMDB of metabolites in human metabolome database. [3]'↑' indicates the metabolite in the HA group was increased when compared to those in the LA group. '↓' indicates the metabolite in the HA group was decreased when compared to those in the LA group.

## 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/fmicb.2023.1076011/full#supplementary-material

## References


Ayalew, W., Chu, M., Liang, C., Wu, X., and Yan, P. (2021). Adaptation mechanisms of yak (*Bos grunniens*) to high-altitude environmental stress. *Animals* 11:2344. doi: 10.3390/ani11082344

Bickhart, D. M., and Weimer, P. J. (2018). Symposium review: host-rumen microbe interactions may be leveraged to improve the productivity of dairy cows. *J. Dairy Sci.* 101, 7680–7689. doi: 10.3168/jds.2017-13328

Bilo, G., Caravita, S., Torlasco, C., and Parati, G. (2019). Blood pressure at high altitude: physiology and clinical implications. *Kardiol. Pol.* 77, 596–603. doi: 10.33963/KP.14832

Caporaso, J. G., Kuczynski, J., Stombaugh, J., Bittinger, K., Bushman, F. D., Costello, E. K., et al. (2010). QIIME allows analysis of high-throughput community sequencing data. *Nat. Methods* 7, 335–336. doi: 10.1038/nmeth.f.303

Caporaso, J. G., Lauber, C. L., Costello, E. K., Berg-Lyons, D., Gonzalez, A., Stombaugh, J., et al. (2011). Moving pictures of the human microbiome. *Genome Biol.* 12:R50. doi: 10.1186/gb-2011-12-5-r50

Caporaso, J. G., Lauber, C. L., Walters, W. A., Berg-Lyons, D., Huntley, J., Fierer, N., et al. (2012). Ultra-high-throughput microbial community analysis on the Illumina HiSeq and MiSeq platforms. *ISME J. Multidiscip. J. Microb. Ecol.* 6, 1621–1624. doi: 10.1038/ismej.2012.8

Cole, J. R., Wang, Q., Cardenas, E., Fish, J., Chai, B., Farris, R. J., et al. (2009). The ribosomal database project: improved alignments and new tools for rRNA analysis. *Nucleic Acids Res.* 37, D141–D145. doi: 10.1093/nar/gkn879

de Oliveira, M. N., Jewell, K. A., Freitas, F. S., Benjamin, L. A., Tótola, M. R., Borges, A. C., et al. (2013). Characterizing the microbiota across the gastrointestinal tract of a Brazilian Nelore steer. *Vet. Microbiol.* 164, 307–314. doi: 10.1016/j.vetmic.2013.02.013

Edgar, R. C. (2010). Search and clustering orders of magnitude faster than BLAST. *Bioinformatics* 26, 2460–2461. doi: 10.1093/bioinformatics/btq461

Edgar, R. C., Haas, B. J., Clemente, J. C., Quince, C., and Knight, R. (2011). UCHIME improves sensitivity and speed of chimera detection. *Bioinformatics* 27, 2194–2200. doi: 10.1093/bioinformatics/btr381

Erwin, S., Marco, G., and Emery, A. E. (1961). Volatile fatty acid analysis of blood and rumen fluid by gas chromatography. *J. Dairy Sci.* 44, 1768–1771. doi: 10.3168/jds.S0022-0302(61)89956-6

Faith, J. J., Guruge, J. L., Charbonneau, M., Subramanian, S., Seedorf, H., Goodman, A. L., et al. (2013). The long-term stability of the human gut microbiota. *Science* 341:1237439. doi: 10.1126/science.1237439

Fan, J., Liu, Q., Zhang, Y., Cheng, G., Fan, X., and Lu, W. (2005). Dynamic variations and influencing factors of groundwater levels in Lhasa city. *Wuhan Univ. J. Nat. Sci.* 10, 665–673. doi: 10.1007/BF02830378

Gao, B., Zhong, M., Shen, Q., Wu, Y., Cao, M., Ju, S., et al. (2020). Gut microbiota in early pregnancy among women with hyperglycaemia vs. normal blood glucose. *BMC Pregnancy Childbirth* 20:284. doi: 10.1186/s12884-020-02961-5

Gerrritsen, J., Hornung, B., Ritari, J., Paulin, L., and Smidt, H. (2019). A comparative and functional genomics analysis of the genus *Romboutsia* provides insight into adaptation to an intestinal lifestyle. *bioRxiv* 2019:845511. doi: 10.1101/845511

Guo, X., Long, R., Kreuzer, M., Ding, L., Shang, Z., Zhang, Y., et al. (2014). Importance of functional ingredients in yak milk-derived food on health of Tibetan nomads living under high-altitude stress: a review. *Crit. Rev. Food Sci. Nutr.* 54, 292–302. doi: 10.1080/10408398.2011.584134

Hill, G. W., Gillum, T. L., Lee, B. J., Romano, P. A., Schall, Z. J., Hamilton, A. M., et al. (2020). Prolonged treadmill running in normobaric hypoxia causes gastrointestinal barrier permeability and elevates circulating levels of pro-and anti-inflammatory cytokines. *Appl. Physiol. Nutr. Metab.* 45, 376–386. doi: 10.1139/apnm-2019-0378

Holman, D. B., Yang, W., and Alexander, T. W. (2019). Antibiotic treatment in feedlot cattle: a longitudinal study of the effect of oxytetracycline and tulathromycin on the fecal and nasopharyngeal microbiota. *Microbiome* 7:86. doi: 10.1186/s40168-019-0696-4

Hu, L., Ma, Y., Liu, L., Kang, L., Brito, L. F., Wang, D., et al. (2019). Detection of functional polymorphisms in the hsp70 gene and association with cold stress response in inner-Mongolia Sanhe cattle. *Cell Stress Chaperones* 24, 409–418. doi: 10.1007/s12192-019-00973-5

Kanehisa, M., Goto, S., Sato, Y., Furumichi, M., and Tanabe, M. (2012). KEGG for integration and interpretation of large-scale molecular data sets. *Nucleic Acids Res.* 40, D109–D114. doi: 10.1093/nar/gkr988

Kelly, W. J., Leahy, S. C., Altermann, E., Yeoman, C. J., Dunne, J. C., Kong, Z., et al. (2010). The glycobiome of the rumen bacterium *Butyrivibrio proteoclasticus* B316(T) highlights adaptation to a polysaccharide-rich environment. *PLoS One* 5:e11942. doi: 10.1371/journal.pone.0011942

Khanna, K., Mishra, K. P., Ganju, L., Kumar, B., and Singh, S. B. (2018). High-altitude-induced alterations in gut-immune Axis: a review. *Int. Rev. Immunol.* 37, 119–126. doi: 10.1080/08830185.2017.1407763

Kim, J. Y., Kim, Y. B., Song, H. S., Chung, W. H., Lee, C., Ahn, S. W., et al. (2017). Genomic analysis of a pathogenic bacterium, *Paeniclostridium sordellii* CBA7122 containing the highest number of rRNA operons, isolated from a human stool sample. *Front. Pharmacol.* 8:840. doi: 10.3389/fphar.2017.00840

Kong, Z., Li, B., Zhou, C., He, Q., Zheng, Y., and Tan, Z. (2021). Comparative analysis of metabolic differences of Jersey cattle in different high-altitude areas. *Front. Vet. Sci.* 8:713913. doi: 10.3389/fvets.2021.713913

Li, H., Yu, Q., Li, T., Shao, L., Su, M., Zhou, H., et al. (2020). Rumen microbiome and Metabolome of Tibetan sheep (*Ovis aries*) reflect animal age and nutritional requirement. *Front. Vet. Sci.* 7:609. doi: 10.3389/fvets.2020.00609

Liu, X. (2019). ABC family transporters. *Adv. Exp. Med. Biol.* 1141, 13–100. doi: 10.1007/978-981-13-7647-4_2

Liu, W., Wang, Q., Song, J., Xin, J., Zhang, S., Lei, Y., et al. (2021). Comparison of gut microbiota of yaks from different geographical regions. *Front. Microbiol.* 12:666940. doi: 10.3389/fmicb.2021.666940

Mago, T., and Salzberg, S. L. (2011). FLASH: fast length adjustment of short reads to improve genome assemblies. *Bioinformatics* 27, 2957–2963. doi: 10.1093/bioinformatics/btr507

Moffatt, B. A., and Ashihara, H. (2002). Purine and pyrimidine nucleotide synthesis and metabolism. *Arabidopsis Book* 1:e0018. doi: 10.1199/tab.0018

Moore, L. G., Niermeyer, S., and Zamudio, S. (2010). Human adaptation to high altitude: regional and life-cycle perspectives. *Am. J. Phys. Anthropol.* 107, 25–64. doi: 10.1002/(SICI)1096-8644(1998)107:27+<25::AID-AJPA3>3.0.CO;2-L

Mukherjee, A., Lordan, C., Ross, R. P., and Cotter, P. D. (2020). Gut microbes from the phylogenetically diverse genus *Eubacterium* and their various contributions to gut health. *Gut Microbes* 12:1802866. doi: 10.1080/19490976.2020.1802866

Murray, A. J. (2016). Energy metabolism and the high-altitude environment. *Exp. Physiol.* 101, 23–27. doi: 10.1113/EP085317

Oksanen, J., Blanchet, F. G., Kindt, R., Legendre, P., Minchin, P. R., O'Hara, R. B., et al. (2016). *Vegan: Community Ecology Package*. R package version 2.3–5.

Ould Amer, Y., and Hebert-Chatelain, E. (2018). Mitochondrial cAMP-PKA signaling: what do we really know? *Biochim. Biophys. Acta Bioenerg.* 1859, 868–877. doi: 10.1016/j.bbabio.2018.04.005

Pasha, M. A., and Newman, J. H. (2010). High-altitude disorders: pulmonary hypertension: pulmonary vascular disease: the global perspective. *Chest* 137, 13s–19s. doi: 10.1378/chest.09-2445

Pena, E., El Alam, S., Siques, P., and Brito, J. (2022). Oxidative stress and diseases associated with high-altitude exposure. *Antioxidants* 11:267. doi: 10.3390/antiox11020267

Quast, C., Pruesse, E., Yilmaz, P., Gerken, J., and Glckner, F. O. (2012). The SILVA ribosomal RNA gene database project: improved data processing and web-based tools. *Nucleic Acids Res.* 41, D590–D596. doi: 10.1093/nar/gks1219

Sakamoto, M.. (2014). *The Family Porphyromonadaceae*. Springer Berlin Heidelberg.

Salem, N., Salem, L., Saber, S., Ismail, G., and Bluth, M. H. (2015). *Corynebacterium urealyticum*: a comprehensive review of an understated organism. *Infect. Drug Resist.* 8, 129–145. doi: 10.2147/IDR.S74795

Sharma, A., Dhar, S. K., Prakash, O., Vemuluri, V. R., Thite, V., and Shouche, Y. S. (2014). Description of *Domibacillus indicus* sp. nov., isolated from ocean sediments and emended description of the genus *Domibacillus*. *Int. J. Syst. Evol. Microbiol.* 64, 3010–3015. doi: 10.1099/ijs.0.064295-0

Shin, N. R., Whon, T. W., and Bae, J. W. (2015). Proteobacteria: microbial signature of dysbiosis in gut microbiota. *Trends Biotechnol.* 33, 496–503. doi: 10.1016/j.tibtech.2015.06.011

Sun, Y., Liu, S., Liu, Y., Dong, Y., Li, M., An, Y., et al. (2021). Effects of the interaction among climate, terrain and human activities on biodiversity on the Qinghai-Tibet plateau. *Sci. Total Environ.* 794:148497. doi: 10.1016/j.scitotenv.2021.148497

The, H. C., Thanh, D. P., Holt, K. E., Thomson, N. R., and Baker, S. (2016). The genomic signatures of *Shigella* evolution, adaptation and geographical spread. *Nat. Rev. Microbiol.* 14, 235–250. doi: 10.1038/nrmicro.2016.10

Thomas, C., and Tampé, R. (2020). Structural and mechanistic principles of ABC transporters. *Annu. Rev. Biochem.* 89, 605–636. doi: 10.1146/annurev-biochem-011520-105201

Usman, T., Wang, Y., Liu, C., He, Y., Wang, X., Dong, Y., et al. (2017). Novel SNPs in IL-17F and IL-17A genes associated with somatic cell count in Chinese Holstein and inner-Mongolia Sanhe cattle. *J. Anim. Sci. Biotechnol.* 8:5. doi: 10.1186/s40104-016-0137-1

Wang, J., Liu, L., Liu, L., Sun, L., and Li, C. (2020). Absorption of 1,3-dioleyl-2-palmitoylglycerol and intestinal flora profiles changes in mice. *Int. J. Food Sci. Nutr.* 71, 296–306. doi: 10.1080/09637486.2019.1648386

Waters, J. L., and Ley, R. E. (2019). The human gut bacteria christensenellaceae are widespread, heritable, and associated with health. *BMC Biol.* 17:83. doi: 10.1186/s12915-019-0699-4

Wickham, H.. (2009). *Ggplot2: Elegant Graphics for Data Analysis*. Berlin: Springer Science & Business Media.

Wu, J., Liu, M., Zhou, M., Wu, L., Yang, H., Huang, L., et al. (2021). Isolation and genomic characterization of five novel strains of erysipelotrichaceae from commercial pigs. *BMC Microbiol.* 21:125. doi: 10.1186/s12866-021-02193-3

Xu, Q., Wang, Y. C., Liu, R., Brito, L. F., Kang, L., Yu, Y., et al. (2017). Differential gene expression in the peripheral blood of Chinese Sanhe cattle exposed to severe cold stress. *Genet. Mol. Res.* 16:16029593. doi: 10.4238/gmr16029593

Yaron, S., Shachar, D., Abramas, L., Riskin, A., Bader, D., Litmanovitz, I., et al. (2013). Effect of high β-palmitate content in infant formula on the intestinal microbiota of term infants. *J. Pediatr. Gastroenterol. Nutr.* 56, 376–381. doi: 10.1097/MPG.0b013e31827e1ee2

Zeng, B., Zhang, S., Xu, H., Kong, F., Yu, X., Wang, P., et al. (2020). Gut microbiota of Tibetans and Tibetan pigs varies between high and low altitude environments. *Microbiol. Res.* 235:126447. doi: 10.1016/j.micres.2020.126447

Zha, R., Sun, G., Dong, Z., and Yu, Z. (2016). Assessment of atmospheric oxygen practical pressure and plateau reaction of tourists in the Qinghai-Tibet plateau. *Ecol. Environ. Sci.* 25, 92–98. doi: 10.16258/j.cnki.1674-5906.2016.01.013

Zhang, J., Kassian, K., Tomáš, F., and Alexandros, S. (2014). PEAR: a fast and accurate Illumina paired-end reAd mergeR. *Bioinformatics* 30, 614–620. doi: 10.1093/bioinformatics/btt593

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# An integrated transcriptome and microbial community analysis reveals potential mechanisms for increased immune responses when replacing silybum marianum meal with soybean meal in growing lambs

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Silybum marianum meal is a by-product that remains silymarin complex and is perceived as a potential-protein source. The potential and its mechanism of silybum marianum meal as a protein supplement in ruminants were evaluated by testing the growth performance, biochemical parameters, cytokine levels, gut transcriptome and microbial community profiles. Forty-two male Hulunbeier growing lambs (aged about 3-month-old; averaged body weight of 21.55kg) were randomly divided into the CON (with 10% soybean meal) and SIL groups (with 10% silybum marianum meal). There was no significant difference in growth performance, feed intakes, or serum biochemical parameters between CON and SIL. The serum levels of IL-1β, TNF-α, TGF-β, HGF, and VEGF were all increased ($p$ <0.05) in the SIL group as compared with the CON group. Transcriptome gene set enrichment analysis (GSEA) revealed that the core genes in the rumen from SIL group were enriched with fructose and mannose metabolism, while the core genes in the ileum were enriched for three biological process, including digestive tract development, positive regulation of MAPK cascade, and regulation of I-kappaB kinase/NF-kappaB signaling. The 16S rDNA results showed that the relative abundance of *Bacteroidetes*, *Firmicutes*, *Synergistetes*, and *Verrucomicrobia* in the rumen from SIL group was significantly higher than that in CON group ($p$ <0.05), whereas *Proteobacteria* was significantly lower than that in CON group ($p$ <0.05). The LEfSe analysis showed that the genera *Pyramidobacter*, *Saccharofermentans*, *Anaerovibrio*, *Oscillibacter* and *Barnesiella* were enriched in the rumen from SIL group, whereas *Sharpea* was enriched in the CON group (LDA>2). In the ileum, there were no significant differences in the phylum-level classification of microbes observed. At the genus level, the relative abundances of *Bifidobacterium* and *Ruminococcus* in the ileum from SIL group were significantly higher than that in the CON group ($p$ <0.05), whereas the relative abundance of *Clostridium_XI* was lower ($p$ <0.05). Correlation analysis showed that *Clostridium_XI* was negatively correlated with VEGF, TGF-β, TNF-α and HGF ($p$ <0.05). Core genes *BMP4* and *CD4* were negatively correlated with

*Clostridium_XI* ($p$ <0.05). Our results indicated that supplementing silybum marianum meal as a replacement for soybean meal resulted in increased cytokines production without affecting growth performance in growing lambs, and the enrichment of immune-related genes and altered microbial community in the ileum were contributed to the increased immune responses.

KEYWORDS

**silybum marianum meal, soybean meal, lambs, cytokines, microbial community, transcriptome**

# 1. Introduction

The livestock industry is struggling to access quality protein feed that could satisfy animal body requirements at an affordable price, in particular in developing countries. With the current trends of rising prices of soybean meal, it is essential to develop locally available low-cost protein supplements. Silybum marianum is a kind of medicinal herb with potential health effects, which is widely grown in Asia and Latin America. The silymarin, a main bioactive component of silybum marianum, contains an isomeric mixture of unique flavonoid complexes and has hepatic protection, detoxification and anti-oxidation functions (Tajmohammadi et al., 2018). The application of silymarin in livestock has received considerable attention due to the advancement of extraction technology and its role in promoting growth. Silymarin has been shown to have a positive effect on the production and carcass plasma profiles in laying hens (Šťastník et al., 2019), horse (Dockalova et al., 2021), and pigs (Grela et al., 2020). In addition, silybum marianum meal is a by-product of silymarin production (Dehghan et al., 2011). Due to its high protein content, it has a potential to use as an unconventional feed resource (Fan et al., 2019). However, mature silybum marianum contains nitrite, which is supposed to have a toxic effect in animal (Arviv et al., 2016), resulting in increased inflammatory responses. In addition, silybum marianum meal contains a high fiber level (Stastnik et al., 2020), which may be beneficial for ruminants theoretically. The potential and its mechanism of silybum marianum meal as a protein supplement in ruminants remain uncovered.

With the development of high-throughput sequencing technology, multi-omics approach was widely used to explore the interaction between microbiota and host in response to variable phenotypes at a molecular level. The objectives of this study were: (1) to evaluate the effects of substituting soybean meal with silybum marianum meal on the growth performance, biochemical parameters and cytokine levels, (2) to explore the potential mechanism in response to altered phenotypes by using an integrated transcriptome and microbial community analysis.

# 2. Materials and methods

## 2.1. Animals, diets, and experimental design

The experimental procedures of this study were carried out in accordance with the guidelines of the Animal Ethics Committee of the Institute of Subtropical Agriculture, Chinese Academy of Sciences. A total of 42 healthy 3-month-old Hulunbeier lambs (averaged with 21.55 kg) with similar body weight were selected and randomly divided into two groups (initial weight was tested for non-significant differences between groups), with 21 sheep in each group. The experimental animals were divided into the CON (supplemented with 10% soybean meal) and SIL groups (supplemented with 10% silybum marianum meal). The compositions and nutrient levels of the experimental diets are shown in Table 1. The experiment lasted for 90 days, including the pre-feeding period of 15 days and the trial period of 75 days. During the pre-feeding period, the experimental animals were ear tagged, dewormed, weighed, and vaccinated according to the normal immunization program for lamb fattening. The sheep were fed and watered three times a day, at 6:00, 11:00 and 18:00, respectively, to ensure that there was a surplus of diet and water. The same diet was fed during the pre-feeding period and the trial period.

## 2.2. Sample collection

All the experimental sheep were weighed every 2 weeks before morning feeding from the beginning of the experimental period, the data were recorded, and the daily weight gain was calculated. The amount of feed was recorded daily, and the leftover was recorded before feeding the following day. The remaining feed was cleared afterward, and a new feed was added. Average daily feed intake = total feed intake/number of experimental days; Feed weight ratio (F/g) = average daily intake / average daily gain.

At the end of the animal test, seven tested sheep in each group were randomly selected for blood collection by jugular venipuncture before morning feeding. The blood was collected with a 10 ml BD Vacutainer serum tube and stored at 4°C for 30 min, then centrifuged at 3,000 rpm/min for 15 min to separate the serum. The collected supernatant was placed in a 1.5 ml centrifuge tube and stored at −20°C.

After dissection, the rumen was opened, and the rumen digesta collected. Then, rumen abdominal sac (5 × 5 cm) was collected. The ileum digesta and ileum tissue were collected from 50 cm proximal to the junction of the ileum and the cecum. Tissue samples were washed with pre-cooled phosphate buffer during collection. After collection, digesta and tissue samples were snap-frozen in liquid nitrogen and stored in an ultra-low temperature freezer at −80°C. Tissue samples were used for RNA extraction and sequencing, and digesta samples were used for 16S rDNA gene sequencing and bioinformatics analysis. Since the rumen and ileum were vital organs for feed digestion and

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TABLE 1 Ingredient and chemical composition (% DM) of the experimental diets.

| Item | Treatment | |
|---|---|---|
| | CON | SIL |
| Ingredients | | |
| Corn | 30.51 | 30.51 |
| Barley | 5.57 | 5.57 |
| Bran | 8.72 | 8.72 |
| Soybean meal | 10.00 | 0 |
| silybum marianum meal | 0 | 10.00 |
| Trace elements with vitamin premix[1] | 2.00 | 2.00 |
| Sodium bicarbonate | 0.70 | 0.70 |
| Salt | 0.50 | 0.50 |
| Calcium carbonate | 1.00 | 1.00 |
| Sugar beet molasses | 1.00 | 1.00 |
| Alfalfa hay | 30.00 | 30.00 |
| Wheat stalk | 10.00 | 10.00 |
| Total | 100.00 | 100.00 |
| Chemical composition,(% of DM) | | |
| DM | 94.82 | 94.06 |
| CP | 14.82 | 12.83 |
| ADF | 6.66 | 6.81 |
| NDF | 22.66 | 23.93 |
| Ca | 0.33 | 0.53 |
| P | 0.31 | 0.35 |
| ME(MJ/kg) | 10.46 | 10.11 |

CON = supplemented with 10% soybean meal; SIL = supplemented with 10% silybum marianum meal; DM = dry matter; ME = metabolizable Energy; CP = crude protein; ADF = acid detergent fiber; NDF = neutral detergent fiber; Ca = calcium; P = phosphorus.
[1]The premix provides per kilogram of ration: Fe 221.31 mg, Cu 16.23 mg, Zn 71.31 mg, Mn 71.31 mg, I 1.23 mg, Se 1.70 mg, Co 0.54 mg, VA 16250 IU, VD 5000 IU, VE 40 IU.

immune functions (Mowat and Agace, 2014; Na and Guan, 2022), their tissue and digest were selected for further transcriptome and microbial community analysis.

## 2.3. Analysis of biochemical parameters and cytokine levels in blood

Serum biochemical parameters, including total protein (TP), high-density lipoprotein (HDL), glutamic aminotransferase (ALT), glutamic oxalacetic aminotransferase (AST), lactate dehydrogenase (LDH), glucose (GLU), triglycerides (TG), total cholesterol (CHOL), amylase (AMS), blood ammonia (NH3L), total bilirubin (TBIL), and c-reactive protein (CRP), were measured using an automated biochemical instrument (Cobasc311, Roche, Basel, Switzerland). Serum cytokine indicators, including interleukin 1β (IL-1β), interleukin 2 (IL-2), tumor necrosis factor (TNF-α), transforming growth factor beta (TGF-β), hepatocyte growth factor (HGF), vascular endothelial growth factor (VEGF), insulin-like growth factor 1 (IGF-1) were measured using commercial sheep-specific ELISA kits(Jiangsu Meimian industrial Co., Ltd., Yancheng, China).

## 2.4. Rumen and ileum RNA sequencing and bioinformatic analysis

Total RNA was extracted from 14 rumen and 14 ileum tissues using Trizol (Invitrogen, Carlsbad, CA, United States) according to the manual. Subsequently, total RNA was characterized and quantified using a NanoDrop and Agilent 2,100 Bioanalyzer (Thermo Fisher Scientific, MA, United States).

Oligo (dT)-linked magnetic beads were used to purify mRNA. The purified mRNA was split into small pieces at the appropriate temperature using a fragmentation buffer. First-strand cDNA was then generated using random hexamer-triggered reverse transcription, followed by the synthesis of second-strand cDNA. After which, A-Tailing Mix and RNA Index Adapters were added by incubation to conclude the repair. The cDNA fragment obtained in the previous step was amplified by PCR and the product was purified by Ampure XP Beads and then dissolved in EB solution. The product was validated for quality control on an Agilent Technologies 2,100 Bioanalyzer. The double-stranded PCR products from the previous step were heat denatured and cyclized by splinting oligonucleotide sequences to obtain the final library. Single-stranded circular DNA (ssCirDNA) was formatted as the final library. The final library was amplified with phi29 to make DNA nanoballs (DNBs) with more than 300 copies of one molecule, DNBs were loaded into patterned nanoarrays, and single-end 50-base reads were generated on the BGIseq500 platform (BGI-Shenzhen, China).

The sequencing data was filtered with SOAPnuke (v1.5.2) (Li et al., 2008): (1) to remove reads containing sequencing adapters; (2) to remove reads with a low-quality base ratio (base quality less than or equal to 5) greater than 20%; (3) Remove reads with a ratio of unknown bases ('N' bases) greater than 5%, after which clean reads are obtained and saved in FASTQ format. These clean reads were mapped onto the sheep reference genome (GCF_002742125.1_Oar_rambouillet_v1.0) using HISAT2 (v2.0.4) (Kim et al., 2015). Bowtie2 (v2.2.5) (Langmead and Salzberg, 2012) was applied to align the clean reads to the reference coding genome, and then RSEM (v1.2.12) (Li and Dewey, 2011) was used to calculate gene expression levels. Transcripts Per Kilobase of exon model per Million mapped reads (TPM) were used to estimate the amount of gene expression in each sample. The Gene Set Enrichment Analysis (GSEA) algorithm was used to identify the pathways, biological processes, or functional components of genes with the most significant changes in expression in the CON and SIL samples (|NES| > 1, Nominal value of p:<0.05, FDR q-value:<0.25, https://www.gsea-msigdb.org/gsea/index.jsp).

## 2.5. Sequencing and bioinformatics analysis of 16SrDNA gene of rumen and ileum microorganisms

Microbial DNA was extracted from 14 rumen digest samples and 14 ileum digest samples using MagPure Stool DNA KF kit B (Magen, China) according to the manufacturer's instructions. DNA was quantified with a Qubit Fluorometer by using Qubit dsDNA BR Assay kit (Invitrogen, United States) and the quality was checked by running aliquot on 1% agarose gel. The variable region V3-V4 of the bacterial 16S rRNA gene was amplified using the condensed PCR primers 341F (5'-ACTCCTACGGGAGGCAGCAG-3′) and 806R (5'-GGACTA

TABLE 2 Effect of silybum marianum meal as a replacement for soybean meal on feed intake and growth performance of lambs.

| Item | Treatment | | SEM | *p*-value |
|---|---|---|---|---|
| | CON (*n*=21) | SIL (*n*=21) | | |
| Initial weight (Kg) | 21.09 | 22.00 | 0.26 | 0.307 |
| Final weight (Kg) | 31.61 | 32.92 | 0.39 | 0.398 |
| Average dry matter intake (Kg) | 1.29 | 1.22 | 0.02 | 0.477 |
| Average daily gain (Kg) | 0.167 | 0.173 | 0.01 | 0.771 |
| Ratio of feed to gain (F/G) | 8.20 | 7.48 | 0.18 | 0.272 |

CON = supplemented with 10% soybean meal; SIL = supplemented with 10% silybum marianum meal.
Values are expressed as means ± SEM, $n = 21$. $p < 0.05$ was regarded as statistically significant, and $0.05 < p < 0.10$ was regarded as a statistical tendency.
Average daily feed intake = total feed intake / test days; Ratio of feed to gain (F/G) = average daily feed intake/average daily gain.

CHVGGGTWTCTAAT-3′). Forward and reverse primers were labeled with Illumina adapters, pads and splice sequences. PCR enrichment was performed in a 50 μL reaction containing a 30 ng template, fusion PCR primers and PCR master mix. PCR cycling conditions were as follows: 94°C for 3 min, 94°C for 30 s, 56°C for 45 s, 72°C for 45 s, 30 cycles, 72°C for 10 min final extension for 10 min. PCR products were purified with AmpureXP beads and eluted in elution buffer. Libraries were characterized by Agilent 2,100 Bioanalyzer (Agilent, United States). Validated libraries were used for sequencing on the Illumina MiSeq platform (BGI, Shenzhen, China) following Illumina's standard procedure and generating 2 × 300 bp double-end reads.

The raw reads were filtered to remove splices and low quality and ambiguous bases, and then double-ended reads were added to the tags by the Fast Length Adjustment of Short reads program (FLASH, v1.2.11) (Magoc and Salzberg, 2011) to obtain the tags. The tags were clustered into OTUs with a cutoff of 97% using UPARSE software (v7.0.1090) (Edgar, 2013) and the chimeric sequences were compared to the Gold database for detection using UCHIME (v4.2.40) (Edgar et al., 2011). Then, OTU representative sequences were classified using the Ribosome Database Project (RDP) classifier v.2.2 with a minimum confidence threshold of 0.6 and trained on the Greengenes database v201305 using QIIME v1.8.0 (Caporaso et al., 2010). All Tags were compared with OTUs using USEARCH_global (Edgar, 2010) to obtain a statistical table of OTU abundance for each sample. The data were analyzed for diversity indices based on OTUs, including Chao1, ACE, Simpson and Shannon indices. Beta diversity analysis was performed using principal coordinate analysis (PCoA) with weighted UniFrac distances. Linear discriminant analysis (LDA) effect size (LEfSe) tools were applied to identify different taxa between CON and SIL groups by using an online software package (LC Bio-Technology Co., Ltd., Hangzhou, China) with thresholds ($p < 0.05$ and LDA > 2).

## 2.6. Statistical analysis

Firstly, Shapiro–Wilk and Levene's tests were employed to confirm the normality and homoscedasticity of data, respectively. The serum biochemical and immune indices, and alpha-diversity index results were analyzed using the independent-sample t-test in SPSS software (SPSS version 25.0, SPSS, Inc.). The relative abundance bacteria at the phylum and genus levels was assessed using Wilcoxon rank-sum test. Data are presented as means ± SEM. $p < 0.05$ was regarded as statistically significant, and $0.05 \leq p < 0.10$ was regarded as a statistical tendency. The correlation analysis was performed using Spearman's rank correlation coefficient in R package and the network was constructed on the Omics-studio (LC-Bio Technology Co., Ltd., Hangzhou, China).

TABLE 3 Effect of silybum marianum meal as a replacement for soybean meal on serum biochemical parameters in lambs.

| Item | Treatment | | SEM | *p*-value |
|---|---|---|---|---|
| | CON (*n*=7) | SIL (*n*=7) | | |
| TP (g/L) | 81.59 | 76.80 | 0.89 | 0.083 |
| HDL (mmol/L) | 1.28 | 1.37 | 0.04 | 0.729 |
| ALT (U/L) | 22.79 | 23.93 | 1.25 | 0.936 |
| AST (U/L) | 167.78 | 177.44 | 8.15 | 0.060 |
| LDH (U/L) | 816.33 | 772.25 | 20.34 | 0.658 |
| GLU (mmol/L) | 5.40 | 5.27 | 0.28 | 0.751 |
| TG (mmol/L) | 0.46 | 0.41 | 0.02 | 0.656 |
| CHOL (mmol/L) | 1.88 | 1.98 | 0.05 | 0.473 |
| AMS (U/L) | 12.11 | 11.00 | 0.72 | 0.681 |
| NH3L (mol/L) | 552.89 | 534.89 | 16.21 | 0.854 |
| TBIL (mmol/L) | 3.41 | 3.42 | 0.17 | 0.315 |
| CRPL3 (mg/L) | 3.89 | 3.86 | 0.01 | 0.729 |

CON = supplemented with 10% soybean meal; SIL = supplemented with 10% silybum marianum meal; TP = total protein; HDL = high-density lipoprotein; ALT = alanine transaminase; AST = aspartate transaminase; LDH = lactate dehydrogenase; GLU = glucose; TG = triglycerides; CHOL = total cholesterol; AMS = amylase; $NH_3L$ = blood ammonia; TBIL = total bilirubin; CRP=C reactive protein.
Values are expressed as means ± SEM, $n = 7$.

# 3. Results

## 3.1. Effects of replacing soybean meal with silybum marianum meal on growth performance, biochemical parameters, and cytokine levels in growing lambs

No difference ($p > 0.05$) was observed between SIL and CON for final body weight, feed average daily gain, or feed intake (Table 2). Most of the biochemical parameters were not affected by replacing soybean meal with silybum marianum meal except that the TP tended to decrease ($p = 0.08$) and the AST tended to increase ($p = 0.06$; Table 3).

TABLE 4 Effect of silybum marianum meal as a replacement for soybean meal on serum cytokine levels in lambs.

| Item | Treatment | | SEM | *p*-value |
|---|---|---|---|---|
| | CON (*n*=7) | SIL (*n*=7) | | |
| IL-1β (pg/mL) | 418.06[b] | 546.65[a] | 20.01 | 0.018 |
| IL-2 (pg/mL) | 683.43 | 788.85 | 19.51 | 0.083 |
| TNF-α (pg/mL) | 108.12[b] | 144.99[a] | 5.48 | 0.002 |
| TGF-β (ng/mL) | 31.21[b] | 39.41[a] | 0.91 | <0.001 |
| HGF (pg/mL) | 1242.97[b] | 1534.02[a] | 44.11 | 0.017 |
| VEGF (pg/mL) | 109.26[b] | 132.16[a] | 3.08 | <0.001 |
| IGF-1 (ng/mL) | 1236.98 | 1381.17 | 32.10 | 0.075 |

CON = supplemented with 10% soybean meal; SIL = supplemented with 10% silybum marianum meal; IL-1β = interleukin 1β; IL-2 = interleukin 2; TNF- α = tumor necrosis factor; TGF- β = transforming growth factor β; HGF=Hepatocyte growth factor; VEGF = vascular endothelial cell growth factor; IGF-1 = insulin-like growth factor 1.
Values are expressed as means ± SEM, $n=7$. $p<0.05$ was regarded as statistically significant, and $0.05<p<0.10$ was regarded as a statistical tendency.
Values in the same row with different superscript letters (a, b) differ significantly ($p < 0.05$).

Serum cytokines were determined to assess the effects of the immune response when replacing silybum marianum meal (Table 4). The levels of IL-1β and TNF-α were significantly increased in SIL as compared with CON ($p<0.05$). Similarly, lambs in SIL had higher concentrations ($p<0.05$) of TGF-β, HGF and VEGF than those in CON. The concentrations of IL-2 ($p=0.083$) and IGF-1 ($p=0.075$) tended to be higher in SIL as compared with CON.

## 3.2. Effects of replacing soybean meal with silybum marianum meal on transcriptome profiles in rumen and ileum of growing lambs

The transcriptomes of the rumen and ileum tissues were evaluated to explore the potential mechanism in response to the altered cytokines in the blood. RNA-seq of the rumen tissue in the CON and SIL groups yielded approximately 23.95 and 23.38 million clean reads, of which 95.67 and 95.49% were mapped to the sheep reference genome (Supplementary file 2; Sheet 1). About 17,682 and 17,958 expressed genes were detected in the rumen of the CON and SIL groups, respectively (Supplementary file 2; Sheet 2). RNA-seq of the ileum tissue from the CON and SIL groups yielded approximately 23.55 and 23.75 million clean reads, 94.55 and 94.37% of which were mapped to the sheep reference genome (Supplementary file 2; Sheet 3). 16,127 and 16,092 expressed genes were detected in ileum tissue from the CON and SIL groups, respectively (Supplementary file 2; Sheet 4).

Since the number of differential expressed genes observed in the rumen or ileum between groups was small (Supplementary file 4), GSEA algorithm was used to further identify the pathways, biological processes or functional components of genes with the most significant changes in expression. Results showed that the rumen of the SIL was enriched with fructose and mannose metabolism-related genes in the Kyoto Encyclopedia of Genes and Genomes (KEGG) (NES: 1.875, Nominal value of p: 0.007, FDR q-value: 0.178) (Figure 1A; Supplementary file 3; Sheet 1). Ileum of the SIL was enriched for three biological process-related genes in the Gene Ontology (GO), including digestive tract development (NES: 1.883, Nominal value of *p*: <0.001, FDR *q*-value: 0.186) (Figure 1B; Supplementary file 3; Sheet 2), positive regulation of MAPK cascade (NES: 1.667, Nominal value of p: <0.001, FDR q-value: 0.211) (Figure 1C; Supplementary file 3; Sheet 3) and regulation of I-kappaB kinase/NF-kappaB signaling (NES: 1.763, Nominal value of p: 0.002, FDR q-value: 0.236) (Figure 1D; Supplementary file 3; Sheet 4).

## 3.3. Effects of replacing soybean meal with silybum marianum meal on microbial community in rumen and ileum of growing lambs

16SrDNA gene sequencing was used to determine the microbial community composition in the rumen and ileum. As shown in Table 5, an average of 511.13 and 658.86 OTUs with 97% similarity per sample were obtained in the rumen of CON and SIL groups, respectively, which was significantly higher in SIL as compared to CON ($p<0.05$). As shown by the Coverage index of the two groups, the coverage of each sample was more than 99%, thus accurately reflecting the diversity of microbial community species and structure in the rumen. Based on the OTU clustering results for α-diversity analysis, the ACE index, Chao1 index, and Shannon index of the SIL group were significantly higher than those of the CON group ($p<0.05$). However, the Simpson index of SIL group was significantly lower than that of the CON group ($p<0.05$). The results of PCoA analysis with weighted distance metric showed that the rumen community composition of the CON group was clearly separated ($p<0.05$ and $R>0.25$) from the SIL group (Figure 2A).

Taxonomic analysis revealed 14 phyla co-identified by the two groups in the rumen (Figure 3A). The predominant phyla in the rumen digesta were *Bacteroidetes*, *Proteobacteria*, and *Firmicutes*. The relative abundances of those top three phyla were 39.71, 39.77 and 16.33% in the CON group and 52.27, 14.10, and 25.61% in the SIL group, respectively. The relative abundances of *Bacteroidetes* and *Firmicutes* in the SIL group were significantly higher than those in the CON group ($p<0.05$), but *Proteobacteria* were significantly lower than those in the CON group ($p<0.05$) (Supplementary file 1; Table 1). In addition, the relative abundances of *Synergistetes* and *Verrucomicrobia* in the SIL group

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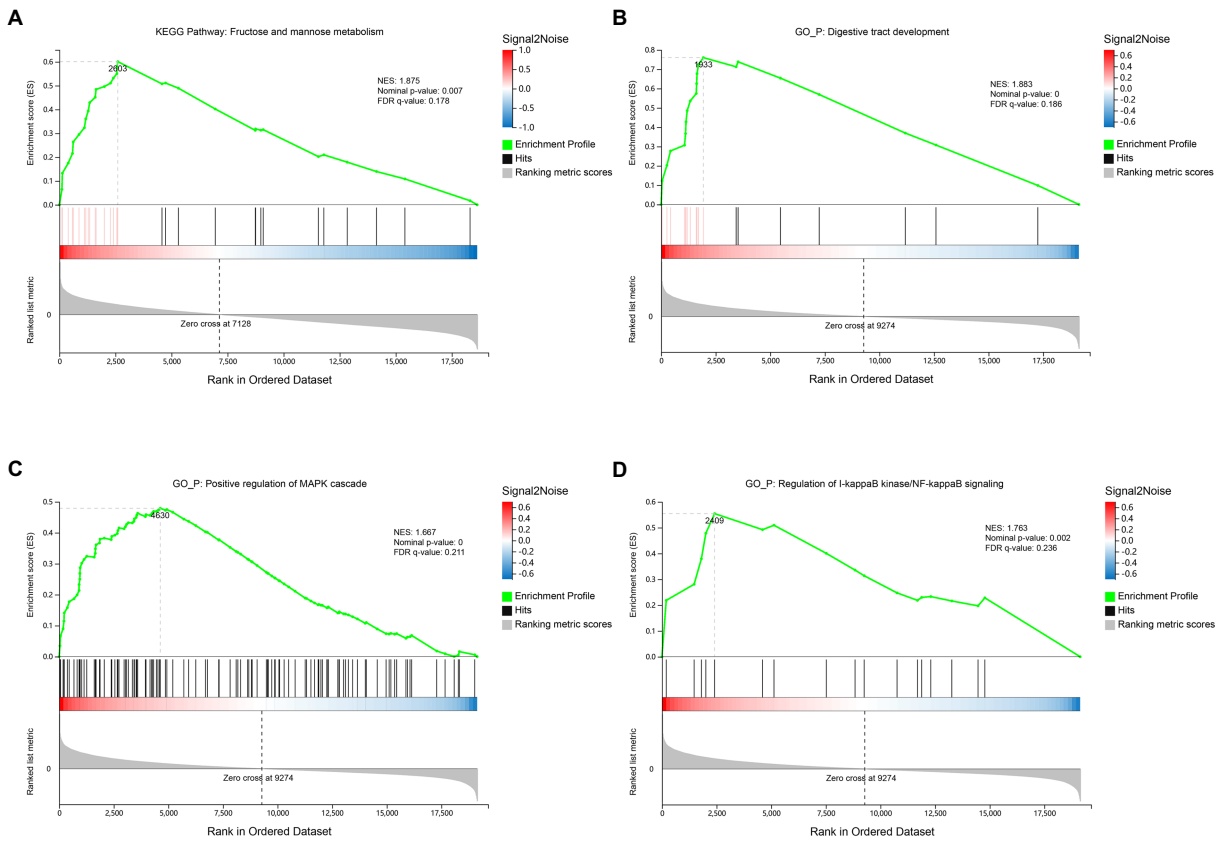


FIGURE 1
Transcriptome gene set enrichment analysis (GSEA) of rumen and ileum tissue of lambs in the CON and SIL groups. **(A)**: Gene set enriched in Fructose and mannose metabolism pathway of rumen; **(B)**: Gene set enriched in digestive tract development biological process of ileum; **(C)**: Gene set enriched in positive regulation of MAPK cascade biological process of ileum; **(D)**: Gene set enriched in regulation of I-kappaB kinase/NF-kappaB signaling biological process of ileum. Screening criteria for significant gene sets included adj. *p*-value <0.05 and FDR<0.25. NES: normalized enrichment score.

**TABLE 5** Effect of silybum marianum meal as a replacement for soybean meal on the alpha diversity index of the rumen and ileum digesta of lambs.

| Item | Treatment | | SEM | *p*-value |
|---|---|---|---|---|
| | CON (*n*=7) | SIL (*n*=7) | | |
| Rumen | | | | |
| OTUs | 511.130[b] | 658.860[a] | 21.08 | 0.001 |
| Chao1 index | 673.390[b] | 807.050[a] | 24.06 | 0.008 |
| ACE index | 694.410[b] | 818.565[a] | 22.11 | 0.004 |
| Shannon index | 2.970[b] | 3.963[a] | 0.12 | 0.004 |
| Simpson index | 0.180[a] | 0.091[b] | 0.01 | 0.040 |
| Good's coverage | 0.996[a] | 0.995[b] | 0.001 | 0.005 |
| Ileum | | | | |
| OTUs | 416.857 | 550.625 | 66.990 | 0.694 |
| Chao1 index | 600.729 | 723.454 | 72.896 | 0.613 |
| ACE index | 700.496 | 778.302 | 65.315 | 0.694 |
| Shannon index | 2.234 | 2.781 | 0.263 | 0.779 |
| Simpson index | 0.210 | 0.192 | 0.025 | 0.955 |
| Good's coverage | 0.997 | 0.996 | 0.000 | 0.536 |

CON = supplemented with 10% soybean meal; SIL = supplemented with 10% silybum marianum meal.
Values are expressed as means ± SEM, $n = 7$. $p < 0.05$ was regarded as statistically significant, and $0.05 < p < 0.10$ was regarded as a statistical tendency.
[a]Means with different superscripts differ ($p < 0.05$).
[b]Means with different superscripts differ ($p < 0.05$).

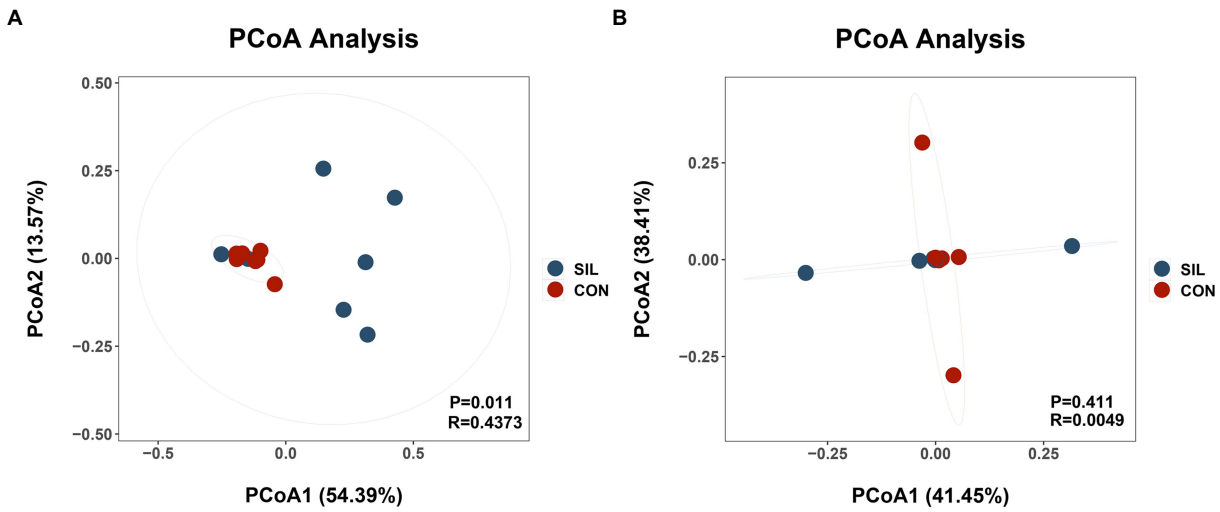


FIGURE 2
Beta diversity of the microbial communities in the rumen digesta of lambs in the CON and SIL groups. Principal coordinate analysis (PCoA) of weighted UniFrac distance metric of rumen **(A)** and ileum **(B)**. CON=supplemented with 10% soybean meal; SIL=supplemented with 10% silybum marianum meal. $p<0.05$ was regarded as statistically significant.

were also significantly higher than those in the CON group ($p < 0.05$) (Supplementary file 1; Table 1). At the genus level, the relative abundances of *Pyramidobacter* and *Saccharofermentans* were higher in the SIL group as compared with the CON group ($p < 0.05$). Conversely, the relative abundance of *Sharpea* was lower ($p < 0.05$) in the SIL group than in the CON group (Supplementary file 1; Table 2). The results of LEfSe analysis showed that the genera *Pyramidobacter*, *Saccharofermentans*, *Anaerovibrio*, *Oscillibacter* and *Barnesiella* were enriched in the SIL group, whereas *Sharpea* was enriched in the CON group (LDA > 2) (Figure 3B).

In the ileum, an average of 416.85 and 550.62 OTUs with 97% similarity per sample were obtained in the CON and SIL groups, respectively. The coverage of each sample was more than 99%, as shown by the Coverage index of both groups, thus accurately reflecting the diversity of microbial community species and structures in the ileum. Based on the OTU clustering results for α-diversity analysis, no significant differences in alpha and β diversity indexes were observed between the two groups (Figure 2B).

Taxonomic analysis revealed that ileum microbes were co-annotated into 15 phyla between the two groups (Figure 3C). *Firmicutes* was the most dominant phylum followed by *Bacteroidetes*. There were no significant differences at the phylum level between the two groups (Supplementary file 1; Table 3). At the genus level, at least one group of genera with a relative abundance greater than 0.1% in both groups was annotated to 43 (Supplementary file 1; Table 4). The relative abundances of *Bifidobacterium* and *Ruminococcus* in the SIL group were significantly higher than in the CON group ($p < 0.05$) (Supplementary file 1; Table 4). Conversely, the relative abundance of *Clostridium_XI* was lower ($p < 0.05$) in the SIL group than in the CON group (Supplementary file 1; Table 4). In addition, the LEfSe analysis results showed that the genera *Bifidobacterium* and *Ruminococcus* were dominant in the SIL group, and *Clostridium_XI* also was enriched in the CON group (LDA > 2) (Figure 3D).

### 3.4. Correlation between serum cytokines, transcriptional core genes and relative abundance of Top 20 genera in the ileum

Since the above GSEA algorithm of transcriptomes results releveled potential core genes (enriched in positive regulation of MAPK cascade and regulation of I-kappaB kinase/NF-kappaB signaling) associated with immune responses only in the ileum, rather than the rumen, the spearman's correlation coefficient was used to investigate the relationship between serum cytokines and dominant bacterial genera (top 20) in the ileum. Correlation analysis showed that *Clostridium_XI* was negatively correlated with VEGF, TGF-β, TNF-α, and HGF ($p < 0.05$). Likewise, Escherichia showed a negative correlation with IL-2, VEGF, TGF-β, TNF-α, and HGF ($p < 0.05$) (Figure 4). Moreover, the correlation between the core genes enriched by positive regulation of MAPK cascade and regulation of I-kappaB kinase/NF-kappaB signaling in GSEA and the dominant bacterial genera in ileum were analyzed. The results showed that the core genes *BMP4* and *CD4* were negatively correlated with *Clostridium_XI* ($p < 0.05$) (Figure 5).

## 4. Discussion

The rapidly growing livestock sector and the increasing demand for livestock feeds have led to a substantial upsurge in feedstuff prices, especially protein feed resources. Finding alternative protein feed resources that could replace commercial protein supplements without profoundly affecting growth performance and health is required. Herein, we performed a comprehensive evaluation of silybum marianum meal as a potential replacement for soybean meal in growing lambs using a multi-omics approach. In this study, neither growth performance nor feed intakes were affected by the replacement of soybean meal with silybum marianum meal, suggesting that silybum marianum meal could be used in the diets of growing lambs.

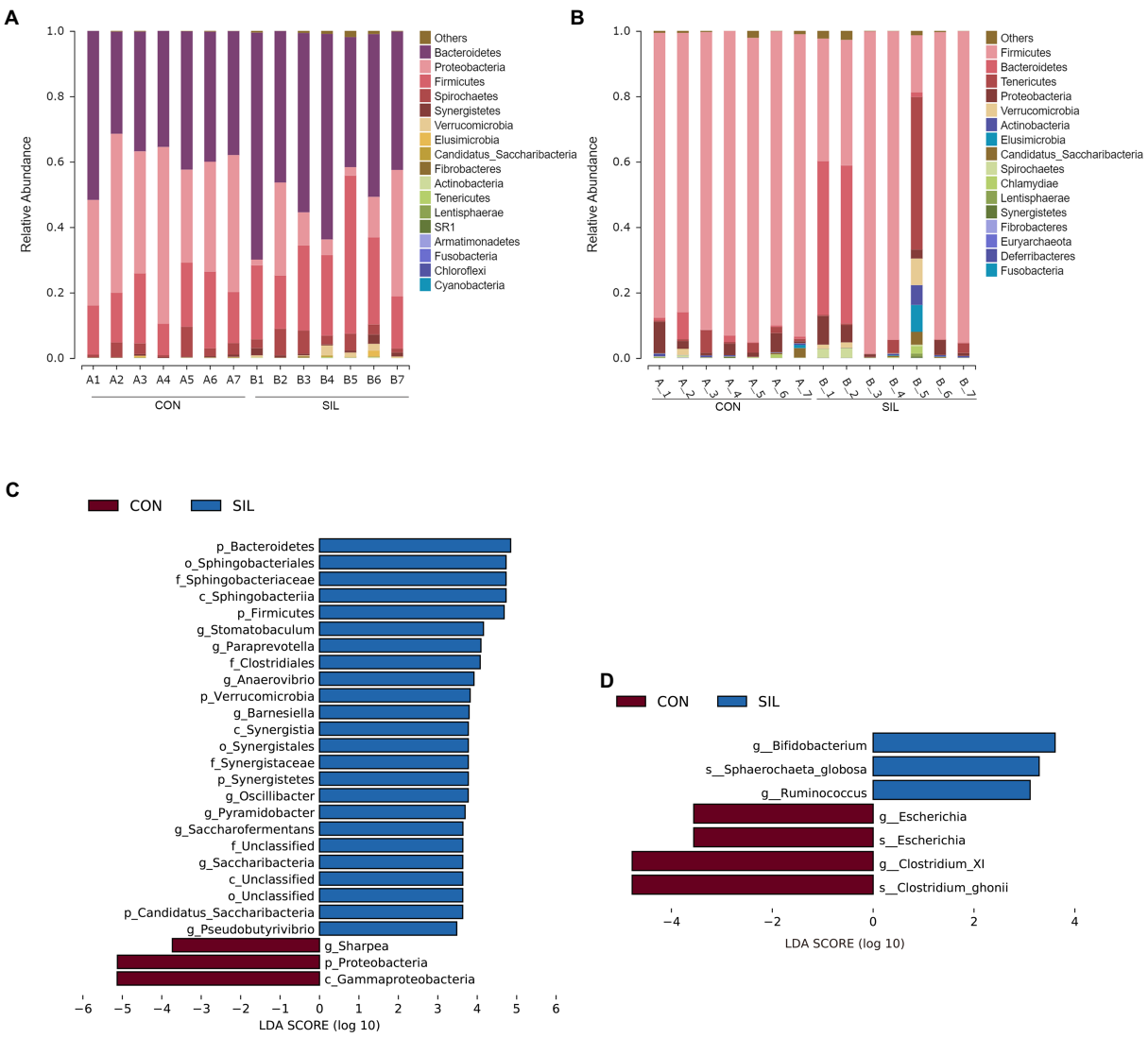


FIGURE 3
Taxonomic analysis of the microbial communities in the rumen and ileum digesta samples of lambs in the CON and SIL groups. The relative abundance of microbial composition at the phylum level in the rumen **(A)** and ileum **(B)**; Linear discriminant analysis (LDA) effect size linear discriminant analysis (LEfSe) of the rumen **(C)** and ileum **(D)** CON=supplemented with 10% soybean meal; SIL=supplemented with 10% silybum marianum meal.

The inclusion of a higher percentage of silybum marianum meal in the diets of broilers has been reported to limit the body weight gain of broilers due to the high content of fiber and nitrite in silybum marianum meal (Suchý et al., 2008). However, ruminants can effectively utilize the high fiber and nitrite content of silybum marianum meal (Niyas et al., 2019) without adversely affecting their growth. In this regard, it has been reported that adding 20% *silymarin* flour to the diet of buffaloes improved the digestibility of NDF to some extent (Nikzad et al., 2017).

The silybum marianum meal usually contains 2–4% active substances on a dry matter basis (Stastnik et al., 2020). It was expected that the *silymarin* complex or flavonoid in the silybum marianum meal could induce the immune responses of animals. Cytokines are proteins or small polypeptides with immunoregulatory and effector functions, including lymphokines, monokines, various growth factors and others (Matthews, 2002). The stimulating and releasing of pro-inflammatory cytokines are essential steps for the activation of innate host defenses and subsequently regulating adaptive immune responses (Netea et al., 2010). In this study, the elevated pro-inflammatory factors IL-1β and TNF-α in SIL group were mainly resulted from its low crude protein content, low palatability (Stastnik et al., 2020) and high nitrite (Arviv et al., 2016). However, if there is enough energy feed in the rumen (such as corn), nitrite can be easily converted into ammonia and eventually used for microbial protein synthesis for ruminants (Niyas et al., 2019). TGF- β has been reported to suppress the immune system by inhibiting the immune function of inflammatory cells and promoting the differentiation and function of Treg cells (Pourgholaminejad et al., 2016). In our study, the increased level of TGF- βin the SIL group may inhibit excessive increase of proinflammatory factors and maintain the balance of the immune function. Moreover, it has been reported that silymarin has anti-inflammatory properties (Hadolin et al., 2001) and enhances cell growth factors (Vahabzadeh et al., 2018). The unregulated HGF in the SIL group in our study was expected, since TGF-β can exhibit

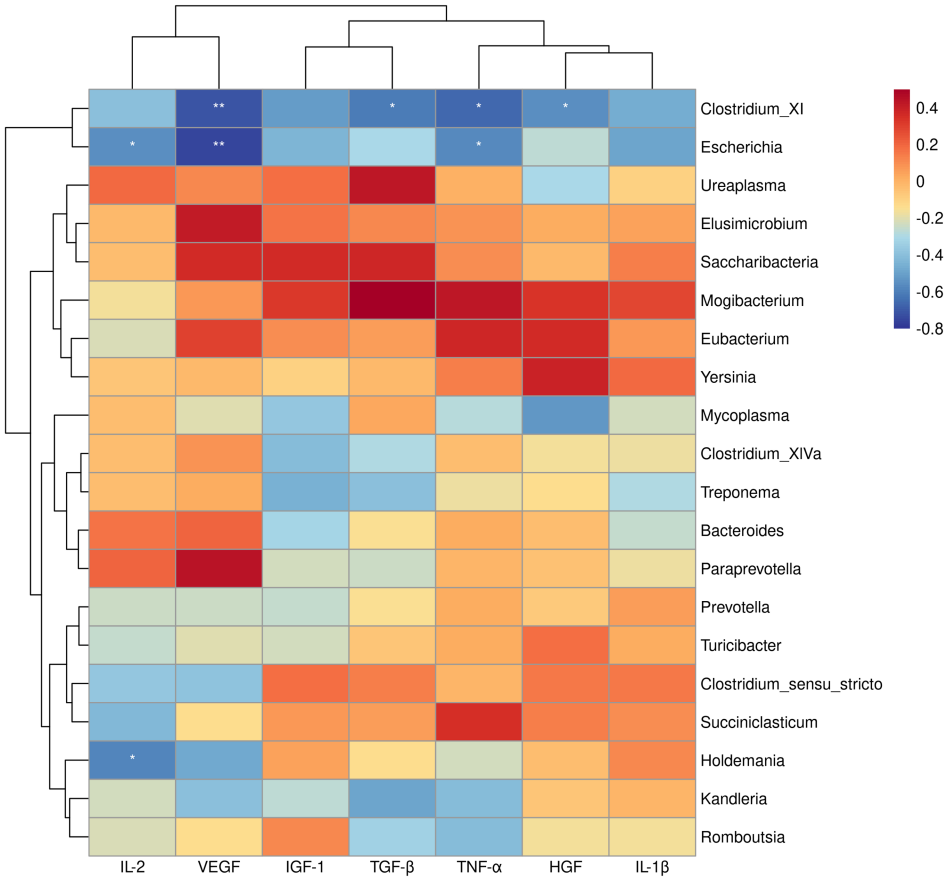


FIGURE 4
Heatmap of spearman's correlation between serum cytokines and the relative abundance of top 20 bacteria at genus level in the ileum. The blue suggests a negative correlation, and the red suggests a positive correlation. *$p$<0.05, **$p$<0.01.

immunosuppressive effects in synergy with HGF (Okunishi et al., 2005). We found that both the pro-inflammatory and anti-inflammatory cytokines were upregulated in lambs fed silybum marianum meal, which may indicate an immune system balance and result in minimal effects in growth performance and biochemical parameters of growing lambs.

Since the observed number of differential genes in the rumen and ileum was small (Supplementary file 4), a further GSEA was conducted to detect subtle gene expression changes. Fructose and mannose metabolisms are vital in cellular productivity, complex cellular processes (e.g., glycosylation) and cellular health (Lieu et al., 2021). In the present study, the GSEA analysis showed that feeding silybum marianum meal to growing lambs upregulated some genes in the fructose and mannose metabolism pathway in the rumen. This implies that the use of silybum marianum meal as a component in the growing lamb is beneficial for rumen carbohydrate metabolism. In the ileum, it was found that three biological process-related genes, including digestive tract development, positive regulation of MAPK cascade, and regulation of I-kappaB kinase/NF-kappaB signaling, were upregulated in the SIL group. MAPK cascade activation is central to various signaling pathways that regulate various important cellular physiological processes such as cell growth, differentiation, adaptation to environmental stress, and inflammatory responses (Sun et al., 2015; Kirk et al., 2020). NF-kappaB has a normal physiological function in mediating the immune response. The inflammatory response of the organism, after infection, requires the initiation of NF-kappaB signaling pathway to transcribe some cytokines to mediate the immune response to clear the invading pathogenic bacteria (Sun, 2017; Wang and Shen, 2022). Our results indicated that the ileum was the more important section for immune functions in response to feeding silybum marianum meal as compared with the rumen.

Ruminal microorganisms play a crucial role in feed degradation and production of volatile fatty acids, lipids, amino acids and hydrogen, which are essential for ruminants to maintain growth and productive performance (Kim et al., 2009; Lopes et al., 2015; Salami et al., 2021). Changes in diet composition or type can alter the microbial structure and composition in the rumen (Ramos et al., 2021). Our study found a higher microbial diversity in the rumen of lambs from the SIL group, as evidenced by the alpha and beta diversity indexes. These changes might be associated with the high fiber content in the silybum marianum meal (Koh et al., 2016). In ruminants, *Bacteroidetes* are mainly responsible for the degradation of cellulose, hemicellulose and pectin, and *Firmicutes* are mainly responsible for the decomposition of exogenous peptides and amino acids (Cholewińska et al., 2021). In our study, the relative abundance of *Bacteroidetes* and *Firmicutes* phyla in the rumen of lambs from the SIL group was higher which was in line with

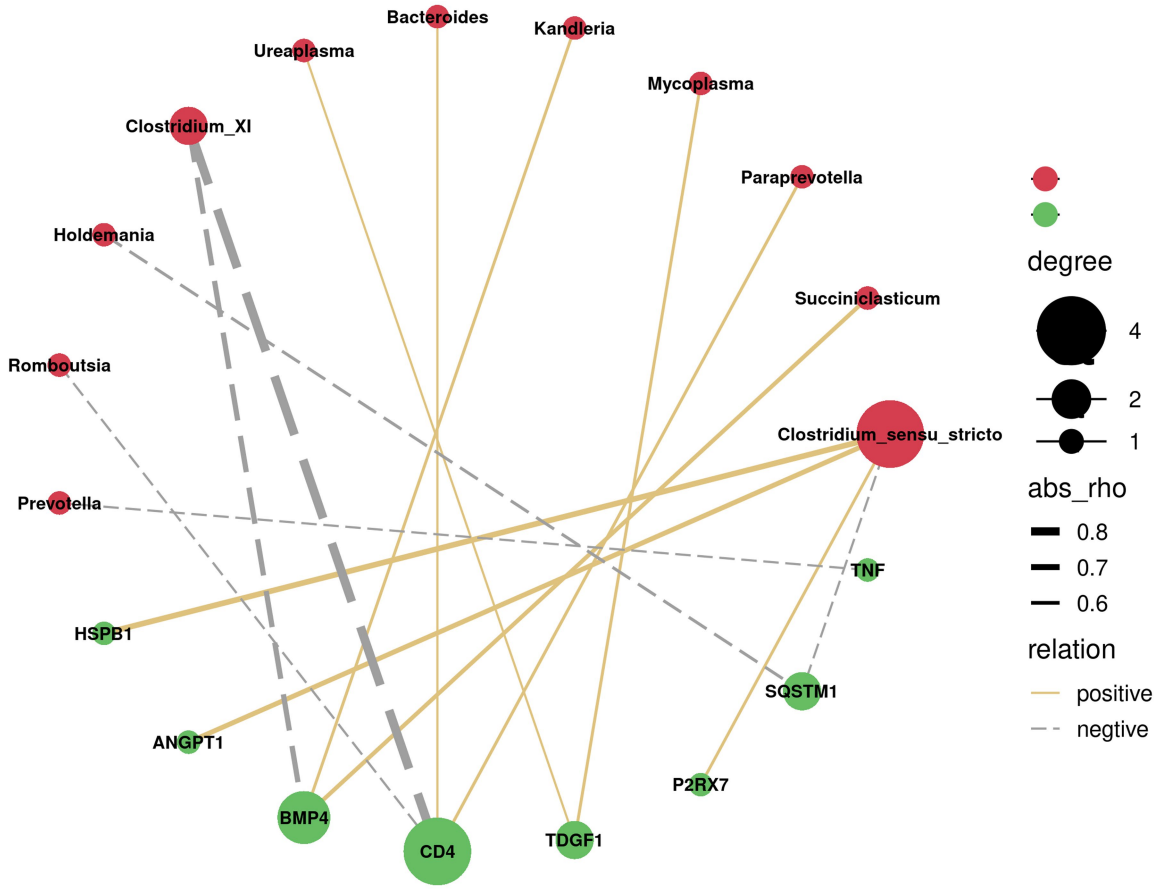


FIGURE 5
Correlation network analysis between the core genes (green circles) of positive regulation of MAPK cascade and regulation of I-kappaB kinase/NF-kappaB signalling and the predominant bacteria at genus level (red circles) in the ileum. Silver lines, negative correlation ($p$<0.05); Golden lines, positive correlation ($p$<0.05).

reports for the gut of healthy lambs (Gebeyew et al., 2021). However, the relative abundance of *Sharpea* belongs to *Firmicutes* was decreased in the SIL group, which was reported to be enriched in the low-methane producing sheep (Kamke et al., 2016). Studies have found a positive association of *Proteobacteria* with rumen biohydrogenation in ruminants (Salami et al., 2021). The lower relative abundance of *Proteobacteria* in the SIL group suggested that replacing soybean meal with silymarin marianum meal may alter the ruminal biohydrogenation. The phylum *Synergistetes* is generally found in the gut as a minor component, where it co-metabolizes secondary phytocompounds (Jumas-Bilak et al., 2009). In addition, Kang et al. (2020) found that *Pyramidobacter* from *Synergistetes* phylum can degrade natural toxic substances in plants. Interestingly, the relative abundance of *Synergistetes* phylum and *Pyramidobacter* genera in the SIL group was higher in the present study, which may be associated with the presence of some secondary compound in the silybum marianum meal. *Saccharofermentans*, as sugar fermenters, are able to ferment glucose, D-fructose, esculin, sucrose, starch, galactitol, mannitol, myo inositol and adonitol (Chen et al., 2010). In our study, the LEfSe analysis showed that *Bacteroidetes*, *Firmicutes*, *Synergistetes*, *Verrucomicrobia*, *Pyramidobacter*, and *Saccharofermentans* were enriched in the rumen of the SIL group compared with the CON group, whereas the *Proteobacteria* and *Sharpea* were depleted. These differential microorganisms further suggested that silybum marianum meal can maintain the nutrient and growth needs for growing lambs.

The ileum is an important site for the digestion and absorption of nutrients such as lipids, bile salts, and vitamins (Wang et al., 2020). In addition, the ileum also gathers a relatively large number of immune-related cells and tissues, such as Paneth cells (producing antimicrobial peptides, such as lysozyme, defensins and other antibacterial substances) and Peyer's patches (gut-associated lymphoid tissue) (Mowat and Agace, 2014), making it as an important part of the body's resistance to pathogens. The innate and acquired immune function of the ileum have been reported to be significantly affected by gut microorganisms (Martinez-Guryn et al., 2019). It has been reported that the addition of 3% silybum marianum meal was able to reduce the count of *E. coli* in the ileum of laying hens (Hashemi Jabali et al., 2018). In our study, the abundance of *Clostridium_XI* was lower in the ileum of lambs from the SIL group, which was probably due to the higher fiber content in the silybum marianum meal. In this regard, Zheng et al. (2018) have indicated that feeding fermentable fiber reduced the presence of *Clostridium_XI* in the intestine of mice. In addition, our enriched abundances of *Bifidobacterium* and *Ruminococcus* genera in SIL group implied a healthier ileum

environment because of the positive effect of *Bifidobacterium* and *Ruminococcus* on intestine health (Belkaid and Hand, 2014; Bi et al., 2019; Ding et al., 2020).

Integrated transcriptome and microbial community analysis in the ileum was further conduced to explore the potential mechanisms for increased blood cytokines in SIL group. Initially, it was speculated that silybum marianum meal resulted in stress in lambs, as evidenced by the enrichment of genes related to MAPK cascade and NF-kappaB signaling identified using GSEA and the production of cytokines. The enriched core genes related to MAPK cascade and NF-kappaB signaling in the ileum, including *BMP4* (Hogg et al., 2010), *P2RX7*, *CD4* (Blanc et al., 2018), *HSPB1* (Lee et al., 2012), were expected to be involved in cytokine production. Moreover, it was observed that *Clostridium_XI* was as the dominant genus in the ileum in the present study. It has been reported that *Clostridium_XI* produces toxins that could induce intestinal damage (Wan et al., 2022). In the present study, the abundance of *Clostridium_XI* was depleted in the SIL group compared to the CON group. It would be explainable that the increases in cytokines levels may be associated with the colonization of *Clostridium_XI*. It has been reported that toxins produced by *Clostridium_XI* triggered circulating immunity to increase the expression of some interleukins, such as IL-1β, prompting immune cells to secrete antimicrobial peptides in an attempt to clear *Clostridium_XI* from the gut (Hernández Del Pino et al., 2021; Nibbering et al., 2021). This statement coincided with our negative correlation between *Clostridium_XI* and blood cytokines. It was speculated that feeding silybum marianum meal to lambs may drive MAPK cascade and NF-kappaB signaling-related gene enrichment in the ileum, which may in turn produce cytokines that mediate a decrease in the abundance of the harmful bacterium, such as *Clostridium_XI*.

## 5. Conclusion

Replacement of soybean meal with silybum marianum meal in the diet of lambs resulted in increased cytokines production without affecting growth performance, feed intakes or blood biochemical parameters in growing lambs. Feeding silybum marianum meal as a replacement for soybean meal differently altered the transcriptome profiles and microbial community in the rumen and ileum. Further integrated transcriptome and microbial community analysis in the ileum revealed that silybum marianum meal promoted the enrichment of immune-related genes (such as *BMP4* and *CD4*), which were supposed to trigger the production of cytokines and suppress the abundance of harmful bacteria, such as *Clostridium_XI*.

## Data availability statement

The data presented in the study are deposited in the NCBI Sequence Read Archive (SRA) database repository, accession number PRJNA919487 (ileal tissue transcriptome), accession number PRJNA918960 (rumen tissue transcriptome), accession number PRJNA917096 (ileal digesta 16s rDNA), accession number PRJNA917525 (rumen digesta 16s rDNA).

## Ethics statement

The experimental protocols were reviewed and approved by the Animal Care and Use Committee of the Institute of Subtropical Agriculture, Chinese Academy of Sciences (ISA-202020) and all applicable international, national, and/or institutional guidelines for the care and use of animals were followed.

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## Author contributions

TZ: conceptualization, methodology, data acquisition, data analysis, and writing–original draft. YR: and TZ: animal experiments and data acquisition. CY, KG, and MG: review and editing. ZH: conceptualization, methodology, project administration, supervision, and review and editing. ZT: conceptualization, methodology, and project administration. 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/fmicb.2023.1093129/full#supplementary-material

# References


Arviv, A., Muklada, H., Kigel, J., Voet, H., Glasser, T., Dvash, L., et al. (2016). Targeted grazing of milk thistle (Silybum marianum) and Syrian thistle (Notobasis syriaca) by goats: preference following preconditioning, generational transfer, and toxicity. *Appl. Anim. Behav. Sci.* 179, 53–59. doi: 10.1016/j.applanim.2016.03.008

Belkaid, Y., and Hand, T. W. (2014). Role of the microbiota in immunity and inflammation. *Cells* 157, 121–141. doi: 10.1016/j.cell.2014.03.011

Bi, Y., Cox, M. S., Zhang, F., Suen, G., Zhang, N., Tu, Y., et al. (2019). Feeding modes shape the acquisition and structure of the initial gut microbiota in newborn lambs. *Environ. Microbiol.* 21, 2333–2346. doi: 10.1111/1462-2920.14614

Blanc, F., Créchet, F., Bruneau, N., Piton, G., Leplat, J.-J., Andréoletti, F., et al. (2018). Impact of a CD4 gene haplotype on the immune response in minipigs. *Immunogenetics* 70, 209–222. doi: 10.1007/s00251-017-1037-z

Caporaso, J. G., Kuczynski, J., Stombaugh, J., Bittinger, K., Bushman, F. D., Costello, E. K., et al. (2010). QIIME allows analysis of high-throughput community sequencing data. *Nat. Methods* 7, 335–336. doi: 10.1038/nmeth.f.303

Chen, S., Niu, L., and Zhang, Y. (2010). *Saccharofermentans* acetigenes gen. Nov., sp. nov., an anaerobic bacterium isolated from sludge treating brewery wastewater. *Int. J. Syst. Evol. Microbiol.* 60, 2735–2738. doi: 10.1099/ijs.0.017590-0

Cholewińska, P., Wołoszyńska, M., Michalak, M., Czyż, K., Rant, W., Smoliński, J., et al. (2021). Influence of selected factors on the *Firmicutes*, *Bacteroidetes* phyla and the *Lactobacillaceae* family in the digestive tract of sheep. *Sci. Rep.* 11:23801. doi: 10.1038/s41598-021-03207-w

Dehghan, A., Rowshan Ghasrodashti, A., Esfandiari, A., Mohebbi-Fani, M., Hoshyar, M. B., and Nayeri, K. (2011). Hepatoprotective effect of silymarin during negative energy balance in sheep. *Comp. Clin. Pathol.* 20, 233–238. doi: 10.1007/s00580-010-0984-7

Ding, S., Hu, C., Fang, J., and Liu, G. (2020). The protective role of probiotics against colorectal cancer. *Oxidative Med. Cell. Longev.* 2020, 1–10. doi: 10.1155/2020/8884583

Dockalova, H., Zeman, L., Baholet, D., Batik, A., Skalickova, S., and Horky, P. (2021). Dose effect of milk thistle (Silybum marianum) seed cakes on the digestibility of nutrients, flavonolignans and the individual components of the silymarin complex in horses. *Animals* 11:1687. doi: 10.3390/ani11061687

Edgar, R. C. (2010). Search and clustering orders of magnitude faster than BLAST. *Bioinforma. Oxf. Engl.* 26, 2460–2461. doi: 10.1093/bioinformatics/btq461

Edgar, R. C. (2013). UPARSE: highly accurate OTU sequences from microbial amplicon reads. *Nat. Methods* 10, 996–998. doi: 10.1038/nmeth.2604

Edgar, R. C., Haas, B. J., Clemente, J. C., Quince, C., and Knight, R. (2011). UCHIME improves sensitivity and speed of chimera detection. *Bioinforma. Oxf. Engl.* 27, 2194–2200. doi: 10.1093/bioinformatics/btr381

Fan, H., Wang, J., Meng, Q., and Jin, Z. (2019). Extraction optimization, preliminary characterization, and bioactivities of polysaccharides from silybum marianum meal. *J. Food Meas. Charact.* 13, 1031–1039. doi: 10.1007/s11694-018-0018-8

Gebeyew, K., Yang, C., He, Z., and Tan, Z. (2021). Low-protein diets supplemented with methionine and lysine alter the gut microbiota composition and improve the immune status of growing lambs. *Appl. Microbiol. Biotechnol.* 105, 8393–8410. doi: 10.1007/s00253-021-11620-4

Grela, E. R., Świątkiewicz, M., Florek, M., and Wojtaszewska, I. (2020). Impact of milk thistle (Silybum marianum L.) seeds in fattener diets on pig performance and carcass traits and fatty acid profile and cholesterol of meat, backfat and liver. *Livest. Sci.* 239:104180. doi: 10.1016/j.livsci.2020.104180

Hadolin, M., Škerget, M., Knez, Z., and Bauman, D. (2001). High pressure extraction of vitamin E-rich oil from Silybum marianum. *Food Chem.* 74, 355–364. doi: 10.1016/S0308-8146(01)00152-2

Hashemi Jabali, N. S., Mahdavi, A. H., Ansari Mahyari, S., Sedghi, M., and Kakhki, A. M. (2018). Effects of milk thistle meal on performance, ileal bacterial enumeration, jejunal morphology and blood lipid peroxidation in laying hens fed diets with different levels of metabolizable energy. *J. Anim. Physiol. Anim. Nutr.* 102, 410–420. doi: 10.1111/jpn.12747

Hernández Del Pino, R. E., Barbero, A. M., Español, L. Á., Morro, L. S., and Pasquinelli, V. (2021). The adaptive immune response to *Clostridioides difficile*: a tricky balance between immunoprotection and immunopathogenesis. *J. Leukoc. Biol.* 109, 195–210. doi: 10.1002/JLB.4VMR0720-201R

Hogg, K., Etherington, S. L., Young, J. M., McNeilly, A. S., and Duncan, W. C. (2010). Inhibitor of differentiation (id) genes are expressed in the steroidogenic cells of the ovine ovary and are differentially regulated by members of the transforming growth factor-β family. *Endocrinology* 151, 1247–1256. doi: 10.1210/en.2009-0914

Jumas-Bilak, E., Roudiere, L., and Marchandin, H. (2009). Description of "*Synergistetes*" phyl. Nov. and emended description of the phylum "*Deferribacteres*" and of the family *Syntrophomonadaceae*, phylum "*Firmicutes*.". *Int. J. Syst. Evol. Microbiol.* 59, 1028–1035. doi: 10.1099/ijs.0.006718-0

Kamke, J., Kittelmann, S., Soni, P., Li, Y., Tavendale, M., Ganesh, S., et al. (2016). Rumen metagenome and metatranscriptome analyses of low methane yield sheep reveals a Sharpea-enriched microbiome characterised by lactic acid formation and utilisation. *Microbiome* 4:56. doi: 10.1186/s40168-016-0201-2

Kang, S., Khan, S., Webb, R., Denman, S., and McSweeney, C. (2020). Characterization and survey in cattle of a rumen *Pyrimadobacter sp*. which degrades the plant toxin fluoroacetate. *FEMS Microbiol. Ecol.* 96:fiaa077. doi: 10.1093/femsec/fiaa077

Kim, E. J., Huws, S. A., Lee, M. R. F., and Scollan, N. D. (2009). Dietary transformation of lipid in the rumen microbial ecosystem. *Asian-Aust. J. Anim. Sci.* 22, 1341–1350. doi: 10.5713/ajas.2009.r.11

Kim, D., Langmead, B., and Salzberg, S. L. (2015). HISAT: a fast spliced aligner with low memory requirements. *Nat. Methods* 12, 357–360. doi: 10.1038/nmeth.3317

Kirk, S. G., Samavati, L., and Liu, Y. (2020). MAP kinase phosphatase-1, a gatekeeper of the acute innate immune response. *Life Sci.* 241:117157. doi: 10.1016/j.lfs.2019.117157

Koh, A., De Vadder, F., Kovatcheva-Datchary, P., and Bäckhed, F. (2016). From dietary fiber to host physiology: short-chain fatty acids as key bacterial metabolites. *Cells* 165, 1332–1345. doi: 10.1016/j.cell.2016.05.041

Langmead, B., and Salzberg, S. L. (2012). Fast gapped-read alignment with bowtie 2. *Nat. Methods* 9, 357–359. doi: 10.1038/nmeth.1923

Lee, Y.-J., Lee, H.-J., Choi, S.-H., Jin, Y. B., An, H. J., Kang, J.-H., et al. (2012). Soluble HSPB1 regulates VEGF-mediated angiogenesis through their direct interaction. *Angiogenesis* 15, 229–242. doi: 10.1007/s10456-012-9255-3

Li, B., and Dewey, C. N. (2011). RSEM: accurate transcript quantification from RNA-Seq data with or without a reference genome. *BMC Bioinform.* 12:323. doi: 10.1186/1471-2105-12-323

Li, R., Li, Y., Kristiansen, K., and Wang, J. (2008). SOAP: short oligonucleotide alignment program. *Bioinforma. Oxf. Engl.* 24, 713–714. doi: 10.1093/bioinformatics/btn025

Lieu, E. L., Kelekar, N., Bhalla, P., and Kim, J. (2021). Fructose and mannose in inborn errors of metabolism and cancer. *Meta* 11:479. doi: 10.3390/metabo11080479

Lopes, L. D., de Souza Lima, A. O., Taketani, R. G., Darias, P., da Silva, L. R. F., Romagnoli, E. M., et al. (2015). Exploring the sheep rumen microbiome for carbohydrate-active enzymes. *Antonie Van Leeuwenhoek* 108, 15–30. doi: 10.1007/s10482-015-0459-6

Magoc, T., and Salzberg, S. L. (2011). FLASH: fast length adjustment of short reads to improve genome assemblies. *Bioinformatics* 27, 2957–2963. doi: 10.1093/bioinformatics/btr507

Martinez-Guryn, K., Leone, V., and Chang, E. B. (2019). Regional diversity of the gastrointestinal microbiome. *Cell Host Microbe* 26, 314–324. doi: 10.1016/j.chom.2019.08.011

Matthews, B. G. (2002). "Cytokines, cytokine antagonists, and growth factors for treating infections" in *Immunotherapy for Infectious Diseases*. ed. J. M. Jacobson (Totowa, NJ: Humana Press)

Mowat, A. M., and Agace, W. W. (2014). Regional specialization within the intestinal immune system. *Nat. Rev. Immunol.* 14, 667–685. doi: 10.1038/nri3738

Na, S. W., and Guan, L. L. (2022). Understanding the role of rumen epithelial host-microbe interactions in cattle feed efficiency. *Anim. Nutr.* 10, 41–53. doi: 10.1016/j.aninu.2022.04.002

Netea, M., Simon, A., Van De Veerdonk, F. L., Kullberg, B., Van Der Meer, J. V. D., and Joosten, L. (2010). IL-1β processing in host defense: beyond the inflammasomes. *PLoS Pathog.* 6:e1000661. doi: 10.1371/journal.ppat.1000661

Nibbering, B., Gerding, D. N., Kuijper, E. J., Zwittink, R. D., and Smits, W. K. (2021). Host immune responses to clostridioides difficile: toxins and beyond. *Front. Microbiol.* 12:804949. doi: 10.3389/fmicb.2021.804949

Nikzad, Z., Chaji, M., Mirzadeh, K., Mohammadabadi, T., and Sari, M. (2017). Effect of different levels of milk thistle (Silybum Marianum) in diets containing cereal grains with different ruminal degradation rate on rumen bacteria of Khuzestan buffalo. *Iran. J. Appl. Anim. Sci.* 7, 401–409.

Niyas, E., Simon, S., Banglavan, S. J., Mathew, J. J., Das, A. S., John, R., et al. (2019). Successful management of nitrite poisoning in crossbred dairy calves. *Indian J. Anim. Nutr.* 36:419. doi: 10.5958/2231-6744.2019.00068.9

Okunishi, K., Dohi, M., Nakagome, K., Tanaka, R., Mizuno, S., Matsumoto, K., et al. (2005). A novel role of hepatocyte growth factor as an immune regulator through suppressing dendritic cell function. *J. Immunol.* 175, 4745–4753. doi: 10.4049/jimmunol.175.7.4745

Pourgholaminejad, A., Aghdami, N., Baharvand, H., and Moazzeni, S. M. (2016). Is TGFβ as an anti-inflammatory cytokine required for differentiation of inflammatory T $_{H}$ 17 cells? *J. Immunotoxicol.* 13, 775–783. doi: 10.1080/1547691X.2016.1193574

Ramos, S. C., Jeong, C. D., Mamuad, L. L., Kim, S. H., Kang, S. H., Kim, E. T., et al. (2021). Diet transition from high-forage to high-concentrate alters rumen bacterial community composition, epithelial transcriptomes and ruminal fermentation parameters in dairy cows. *Animals* 11:838. doi: 10.3390/ani11030838

Salami, S. A., Valenti, B., Luciano, G., Lanza, M., Umezurike-Amahah, N. M., Kerry, J. P., et al. (2021). Dietary cardoon meal modulates rumen biohydrogenation and bacterial community in lambs. *Sci. Rep.* 11:16180. doi: 10.1038/s41598-021-95691-3

Šťastník, O., Mrkvicová, E., Pavlata, L., Roztočilová, A., Umlášková, B., and Anzenbacherová, E. (2019). Performance, biochemical profile and antioxidant activity of hens supplemented with addition of milk thistle (Silybum marianum) seed cakes in diet. *Acta Univ. Agric. Silvic. Mendel. Brun.* 67, 993–1003. doi: 10.11118/actaun201967040993

Stastnik, O., Pavlata, L., and Mrkvicova, E. (2020). The milk thistle seed cakes and hempseed cakes are potential feed for poultry. *Animals* 10:1384. doi: 10.3390/ani10081384

Suchý, P., Straková, E., Kummer, V., Herzig, I., Písaříková, V., Blechová, R., et al. (2008). Hepatoprotective effects of milk thistle (Silybum marianum) seed cakes during the chicken broiler fattening. *Acta Vet. Brno* 77, 31–38. doi: 10.2754/avb200877010031

Sun, S.-C. (2017). The non-canonical NF-κB pathway in immunity and inflammation. *Nat. Rev. Immunol.* 17, 545–558. doi: 10.1038/nri.2017.52

Sun, Y., Liu, W.-Z., Liu, T., Feng, X., Yang, N., and Zhou, H.-F. (2015). Signaling pathway of MAPK/ERK in cell proliferation, differentiation, migration, senescence and apoptosis. *J. Recept. Signal Transduct.* 35, 600–604. doi: 10.3109/10799893.2015.1030412

Tajmohammadi, A., Razavi, B. M., and Hosseinzadeh, H. (2018). *Silybum marianum* (milk thistle) and its main constituent, silymarin, as a potential therapeutic plant in metabolic syndrome: a review: *silybum marianum* and metabolic syndrome. *Phytother. Res.* 32, 1933–1949. doi: 10.1002/ptr.6153

Vahabzadeh, M., Amiri, N., and Karimi, G. (2018). Effects of silymarin on metabolic syndrome: a review: silymarin and metabolic syndrome. *J. Sci. Food Agric.* 98, 4816–4823. doi: 10.1002/jsfa.9115

Wan, J., Zhang, Y., He, W., Tian, Z., Lin, J., Liu, Z., et al. (2022). Gut microbiota and metabolite changes in patients with ulcerative colitis and *Clostridioides* difficile infection. *Front. Microbiol.* 13:802823. doi: 10.3389/fmicb.2022.802823

Wang, B., and Shen, J. (2022). NF-κB inducing kinase regulates intestinal immunity and homeostasis. *Front. Immunol.* 13:895636. doi: 10.3389/fimmu.2022.895636

Wang, Y., Song, W., Wang, J., Wang, T., Xiong, X., Qi, Z., et al. (2020). Single-cell transcriptome analysis reveals differential nutrient absorption functions in human intestine. *J. Exp. Med.* 217:e20191130. doi: 10.1084/jem.20191130

Zheng, W., Wang, K., Sun, Y., and Kuo, S.-M. (2018). Dietary or supplemental fermentable fiber intake reduces the presence of clostridium XI in mouse intestinal microbiota: the importance of higher fecal bacterial load and density. *PLoS One* 13:e0205055. doi: 10.1371/journal.pone.0205055

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# Exploring the growing forest musk deer (*Moschus berezovskii*) dietary protein requirement based on gut microbiome

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It is necessary to assess the appropriate dietary protein level of the forest musk deer (FMD), as nutritional needs are unclear. The microbiome in gastrointestinal tracts plays an important role in regulating nutrient utilization, absorption and host growth or development. Thus, we aimed to evaluate growth performance, nutrient digestibility and fecal microbiome of growing FMD supplied with different protein levels of diets. Eighteen 6-month-old male FMD with an initial weight 5.0±0.2kg were used in a 62-day trial. The animals were randomly distributed to three groups, the dietary crude protein (CP) level was 11.51% (L), 13.37% (M), and 15.48% (H). The results showed that the CP digestibility decreased as dietary CP level increased ($p$<0.01). Compared with group L and H, FMD in M group has higher average daily gain, feed efficiency and neutral detergent fiber digestibility. For the fecal bacterial community, the percentage of Firmicutes was increased, Bacteroidetes was decreased and the diversity of microbiota significantly reduced ($p$<0.05) with the increasing of dietary protein. The proportion of *Ruminococcaceae_005*, *Ruminococcaceae_UCG-014* and *uncultured_bacterium_f_Lachnospiraceae* were significantly increased wtih rising CP, the proportions of *Bacteroides* and *Rikenellaceae_RC9_gut_group* were significantly decrease nevertheless at the genus level. The higher abundance of *f_Prevotellaceae* and *g_Prevotellaceae_UCG_004* were found at M group by LEfSe analysis. The relative abundance of *uncultured_bacterium_f_Ruminococcaceae* was positively correlated with the average daily gain and feed conversion ratio ($p$<0.05), whereas *Family_XIII_AD3011_group* was negatively correlated with feed conversion ratio ($p$<0.05). The UPGMA tree showed L and M groups were closer in clustering relationship, while H group was clustered separately into a branch, which indicated that the bacterial structure had changed greatly with protein level increased from 13.37 to 15.48%. Overall, our results indicated that the optimum dietary CP for the growing FMD was 13.37%.

KEYWORDS

***Moschus berezovskii*, crude protein, 16S rRNA, gut microbiota, growth performance**

# 1. Introduction

Forest musk deer (*Moschus berezovskii*), one of the small ruminants, belongs to a special economic animal in China. Musk, which secreted by male forest musk deer (FMD), is a valuable resource in the traditional Asian medicine and the international perfume industry (Wang et al., 2022). The number of wild FMDs was sharply declined as the overhunting for the musk. In 2008, wild FMD was listed as an endangered species by the International Union for Conservation of Nature (Wang and Harris, 2008). In China, captive breeding the FMD has become an important way for obtaining the musk (Meng et al., 2006). In this way, artificial breeding not only meets the demand of the musk for drug synthesis and perfume making, but also protects the population of wild FMD from the further decline. However, the captive FMDs are still fed in a relatively primitive way and the food items and composition are vary greatly from farms (Wang et al., 2015). So, limited feeding standard and incomplete nutritional requirements limit the growth performance and health status of the FMD. Better body condition of FMD during the growing phase plays an important role in a series of physiological processes, such as rut, breeding and musk secretion. In local feeding farm, besides leaves, the feedstuffs for FMD are simple mixture of the chopped pumpkin, carrot and concentrate, and the concentrate mainly consisted of corn meal, soybean meal and bran, with different proportions in different farms. But the moldy leaves or rotten vegetables constantly lead to the intestinal diseases (Wang et al., 2013), which further induced the defective of the FMD normal growth in breeding industry (Zhu et al., 2012; Li et al., 2017; Yang et al., 2021).

Dietary protein levels are essential factors that affect the growth, development and health conditions of animals (Schroeder et al., 2006). Therefore, exploring the dietary protein requirement of FMD is first. Chinese management policy for the FMD breeding industry is very strict, slaughter or injury the FMD are strictly forbidden, so obtaining the samples for dietary protein demand research is restricted, even for the rumen fermentation sampling. Considering the pivotal role of the intestinal microbiota in the growth and development of the host (Schwarzer et al., 2016), studying the changes of fecal flora structure is an important way to monitor the health status of the forest musk deer (Hu et al., 2018; Liu et al., 2019). Changes in diet can alter the fecal microbiome of FMD. As the diet of FMD changes from consuming milk to eating plants, the richness and diversity of intestinal microbiota of young FMD increased significantly (Li et al., 2021). What's more, previous found that dietary protein levels not only affect the growth performance of ruminants, but also play an important role in regulating the microbial structure of the digestive tract (Liu et al., 2022; Zhang et al., 2022).

The present study was carried out to explore the effects of different crude protein (CP) levels of TMR pellet feed on the growth performance, nutrient digestibility and fecal microorganisms of FMD during the growing phase, and provide reference data for the standardization of the nutritional needs for the captive FMD.

# 2. Materials and methods

## 2.1. Animals and diets

Eighteen 6-month-old FMD with a similar initial weight (5.0 ± 0.2 kg) were randomly divided into 3 groups consisting of 3 replicates with 3 FMD per replicate. The FMDs were fed with low CP diet (CP 11.51%, L), middle CP diet (CP 13.37%, M) and high CP diet (CP 15.48%, H), respectively. The experiment last for 62 days and all FMDs were fed at 9: 00 a.m. and 16: 00 p.m. All animals were housed individually and provided feed and water *ad libitum*. The health condition of each animal was closely monitored. The composition of the diets is presented in Table 1. The air-dried feed materials were crushed, then the pellet with the length of 8.0 mm-15.0 mm was made through the ring die granulator (compression ratio 1: 8, aperture 4.0 mm).

## 2.2. Sampling and measurement

At 1 and 62 d of the trial period, body length gain (BLG), chest girth gain (CGG), body oblique length gain (BOLG) and weight gain were measured individually after overnight fasting (12 h), meanwhile, average daily feed intake (ADFI), average daily gain (ADG) and feed to gain (F: G) were calculated. Fresh fecal samples were collected on days 56–62 of the trial. Feces for apparent nutrient digestibility analysis were collected into valve bags, and 10 ml of 10% $H_2SO_4$ solution was evenly added to 0.1 kg of feces to fix fecal nitrogen. Other samples were snap-frozen in liquid nitrogen, and then stored at −80°C for further analysis.

$$\text{BLG} = \text{Final body length} - \text{Initial body length}$$

TABLE 1 Composition of the diets (%, as-fed basis).

| Item | L | M | H |
|---|---|---|---|
| Ingredients | | | |
| Corn | 29.14 | 24.86 | 21.88 |
| Soybean meal | 1.33 | 4.93 | 10.91 |
| Wheat bran | 7.15 | 4.95 | 6.02 |
| Alfalfa hay | 38.23 | 41.51 | 46.10 |
| Mulberry branch bark powder | 7.02 | 6.41 | 4.88 |
| *Broussonetia papyrifera* silage | 7.31 | 6.82 | 4.71 |
| Beet pulp | 3.82 | 5.02 | 0.00 |
| Soybean oil | 1.00 | 0.50 | 0.50 |
| Permix[1] | 5.00 | 5.00 | 5.00 |
| Dry matter | | | |
| Digestible energy, MJ/kg | 10.65 | 10.68 | 10.63 |
| Crude protein | 11.51 | 13.37 | 15.48 |
| Crude fiber | 12.74 | 12.39 | 11.99 |
| Neutral detergent fiber | 25.79 | 25.75 | 24.75 |
| Lysine | 1.70 | 1.70 | 1.70 |
| Methionine + Cystine | 0.60 | 0.60 | 0.60 |
| Calcium | 0.90 | 0.91 | 0.90 |
| Available phosphorus | 0.24 | 0.24 | 0.24 |

[1]Provided per kilogram diet: 8,000 IU of vitamin A; 500 IU of vitamin $D_3$; 10 IU of vitamin E; 2 mg of vitamin $K_3$; 50 mg of Fe (as ferrous sulfate); 4 mg of Cu (as copper sulfate); 2 mg of Mn (as manganese sulfate); 50 mg of Zn (as zinc sulfate); 0.2 mg of I (as KI); 0.2 mg of Se (as $Na_2SeO_3$).

$$\text{CGG} = \text{Final chest girth} - \text{Initial chest girth}$$

$$\text{BOLG} = \text{Final body oblique length} - \text{Initial body oblique length}$$

$$\text{ADFI} = \text{Total food intake during the test} / \text{Days of test}$$

$$\text{ADG} = \left(\text{final body weight} - \text{initial body weight}\right) / \text{Days of test}$$

$$\text{F : G} = \text{ADFI} / \text{ADG}$$

## 2.3. Chemical analysis

All feed and fecal samples were assessed in triplicate with a 1-mm screen after finely ground. Samples were analyzed for dry matter (DM), CP, neutral detergent fiber (NDF) and acid detergent fiber (ADF), and apparent total tract digestibility of DM, CP, NDF and ADF were accumulated as previously described by Williams et al. (1962).

## 2.4. 16S rRNA gene profiling

The total genomic DNA of fecal bacteria was extracted using TIANGEN DNA Kit (TIANGEN, Beijing, China) according to the manufacturer's instructions. The genomic DNA was used as a template for PCR amplification. Full-length (V1-V9) 16S rRNA was amplified by using the 27F (5′-AGR GTT YGA TYM TGG CTC AG-3′) and 1492R (5′-RGY TAC CTT GTT ACG ACT T-3′) primers and sample-specific barcodes. 10 μl mixture containing 40 ng of template DNA, primers, buffer, Taq polymerase and dd$H_2O$. The following thermal cycling conditions was used: initial denaturation of 5 min at 95°C, 25 cycles of denaturation at 95°C for 30 s, annealing at 50°C for 30 s, extension at 72°C for 40 s, and a final extension at 72°C for 7 min. Fresh PCR products of almost-full-length 16S rRNA gene were purified by agarose gel and ligated into TA cloning kit of TOP 10 (Invitrogen, United States). Library construction was undertaken, then transformants were picked up randomly and the 16S rRNA gene was sequenced on the PacBio Sequel sequencing platform.

Circular consensus sequences (CCSs) were obtained by correcting the original subreads (SMRT link, version 8.0) according to minPasses of ≥5 and minPredictedAccuracy of ≥0.9. Lima v1.7.0 software was used to identify different samples according to the barcode. Cutadapt V2.7 (error rate 0.2) was used to identify the forward and reverse primers, and CCSs without a primer were discarded. Finally, CCS length was filtered, and sequences that did not meet the length threshold (16S: 1,200 bp to 1,650 bp; ITS: 300 bp to 1,000 bp) were discarded. Usearch v 10.0.240 (Edgar, 2013) was used to cluster CCSs at 97% similarity level, obtain operational taxonomic units (OTUs).

Venn diagram was drawn with VennDiagram version 1.6.20 (Chen and Boutros, 2011). Conduct taxonomic annotation of the OTUs based on Silva 132 (bacteria) (Quast et al., 2013) taxonomy databases. Query sequences were blasted against the reference database using the classify-consensus-blast methods, and the nonmatched sequences were further classified by classify-sklearn. Data on the community composition of each sample was obtained at various classification levels (phylum, class, order, family, genus, and species). The alpha diversity index of each sample was evaluated using QIIME2 software (Bolyen et al., 2019). Analysis of similarities (ANOSIM) was performed on unweighted UniFrac distance matrices for fecal communities and on Bray-Curtis similarities using Primer 6 software and the R package vegan. Principal coordinate analysis (PCoA) was performed using the mixOmics package in R (v3.2.1) based on the Bray-Curtis distances; non-metric multidimensional scaling was performed using the R package "Vegan." Phylogenetic trees were constructed by unweighted pair-group method with arithmetic means (UPGMA). Spearman analysis was used to calculate the correlation coefficient between the different fecal microbiota and the growth performance (Wu et al., 2021). Microbiota-based biomarker discoveries were done with LEfSe using the online server[1], and the LDA scores derived from LEfSe analysis (Segata et al., 2011) were used to show the relationship between taxon using a cladogram (circular hierarchical tree) of significantly increased or decreased bacterial taxa in the gut microbiota between groups.

## 2.5. Statistical analysis

An individual FMD was considered as an experimental unit in all statistical analysis. All data were analyzed using SPSS statistical package (IBM SPSS, Chicago, IL; 26.0 software). After checking the normality of the data distribution, the growth performance data were analyzed by one-way analysis of variance (ANOVA). All data were expressed as the mean with standard error (SEM), differences were considered as statistically significant at $p < 0.05$, and greatly significantly different at $p < 0.01$.

# 3. Results

## 3.1. Growth performance and nutrient digestibility

The effects of different dietary protein level on the growth performance and morphometric traits are shown in Table 2. The FMD receiving 13.37% CP diet had a higher ADG ($p < 0.05$) and lower F:G ($p < 0.05$) than that receiving dietary CP at 11.51%. Meanwhile, BLG significantly increased ($p < 0.05$) in M group compared with the L group.

As presented in Table 3, the digestibility of DM in H group was higher ($p < 0.05$) than other groups. With the increase of CP in diet of FMD, the digestibility of CP significantly decreased ($p < 0.01$). FMD received 13.37% CP diet have the highest NDF digestibility ($p < 0.01$), but no difference was observed among groups on ADF digestibility ($p > 0.05$).

1 https://huttenhower.sph.harvard.edu/galaxy/

TABLE 2 Effects of different dietary crude protein levels on growth performance and morphometric traits in growing forest musk deer.

| Items | *L* | *M* | *H* | SEM | *p*-value |
|---|---|---|---|---|---|
| ADFI, g/d | 186.47 | 182.45 | 183.82 | 1.350 | 0.494 |
| ADG, g/d | $8.20^{B}$ | $10.17^{A}$ | $9.44^{A}$ | 0.227 | <0.001 |
| F:G | $22.79^{A}$ | $18.03^{B}$ | $19.51^{B}$ | 0.571 | <0.001 |
| BLG, cm | $1.85^{b}$ | $2.23^{a}$ | $2.11^{ab}$ | 0.072 | 0.045 |
| BOLG, cm | 2.03 | 2.32 | 2.21 | 0.058 | 0.111 |
| CGG, cm | 1.13 | 1.18 | 1.18 | 0.027 | 0.701 |

A row with different lowercase letter superscripts were significantly different ($p < 0.05$); a row with different capital letter were highly significant different ($p < 0.01$). The same as below.

TABLE 3 Effects of different dietary crude protein levels on apparent nutrient digestibility in growing forest musk deer.

| Items | *L* | *M* | *H* | SEM | *p*-value |
|---|---|---|---|---|---|
| DM (%) | $59.91^{b}$ | $59.78^{b}$ | $60.74^{a}$ | 0.37 | 0.040 |
| CP (%) | $70.01^{A}$ | $67.43^{B}$ | $62.31^{C}$ | 0.86 | <0.001 |
| NDF (%) | $57.45^{B}$ | $61.40^{A}$ | $60.84^{A}$ | 1.13 | 0.003 |
| ADF (%) | 31.05 | 32.12 | 32.63 | 0.34 | 0.154 |

A row with different lowercase letter superscripts were significantly different ($p < 0.05$); a row with different capital letter were highly significant different ($p < 0.01$).

## 3.2. Diversity of bacteria

As shown in Figure 1A, a total of 931 OTU were shared in three groups; 34, 13 and 31 unique OTU were detected in group H, M and L, respectively. Further, the beta diversity on ANOSIM revealed that the three group' fecal microbial communities were significantly different ($p < 0.05$, Figure 1B). There were significant differences of microbiota among three groups as shown in PCoA and NMDS (Figures 1C,D). Alpha-diversity indexes (Shannon, Simpson, Chao1 and ACE) were investigated and shown in Figure 2. Highest bacterial diversity (Shannon and Simpson indexes) were observed in FMD that fed with high CP dietary ($p < 0.01$), but the species richness values (Chao1 and ACE indexes) exhibited a similar trend.

## 3.3. Microbiota composition

The composition and abundance of fecal microbiota for FMD fed with different CP diets were analyzed. As shown in Figure 3A, the percentage of Firmicutes and Bacteroides were the highest at the phyla level. And the relative abundance of Firmicutes increased, Bacteroidetes decreased with the increase of dietary protein.

With the increase of crude protein level in dietary, Ruminococcaceae and Christensenellaceae increased, but Rikenellaceae reduced at the family level (Figure 3B).

As presented in Figure 3C, at the genus level, *Ruminococcaceae_005*, *Bacteroides, Ruminococcaceae_014*, *Lachnospiraceae*, *Mollicutes_RF39 and Rikenellaceae_RC9* were the major bacterial genera. Among these, the proportion of *Ruminococcaceae_005*, *Ruminococcaceae_014* and *Lachnospiraceae* increased with the increase of dietary protein, the proportions of *Bacteroides* and *Rikenellaceae_RC9* were decreased.

Based on LEfSe analysis in Figure 4, the higher abundance of *g_uncultured_bacterium_o_Choroplast*, *f_uncultured_bacterium_o_Choroplast*, *g_Streptococcus*, *f_Streptococcaceae*, *g_Shuttleworthia*, *g_uncultured_bacterium_f_Ruminococcaceae*, *f_Ruminococcaceae* and *o_Clostridiales* were found in H group; the higher abundance of *g_Prevotellaceae_UCG_004* and *f_Prevotellaceae* were determinde in M group; and the higher abundance of *g_Alistipes*, *g_Rikenellaceae_RC9_gut_group*, *f_Rikenellaceae* and *o_Bacteroidales* were determined in group L.

As shown in Figure 5A, the Spearman correlation matrix illustrated that the relative abundance of *uncultured_bacterium_f_Ruminococcaceae* was positively correlated with the ADG and feed conversion ratio of FMD ($p < 0.05$), only *Family_XIII_AD3011_group* was positively correlated with feed conversion ratio ($p < 0.05$). In contrast, *Ruminococcaceae_UCG-010* was negatively correlated with feed conversion ratio ($p < 0.05$). The UPGMA tree showed significant differences in the structure of fecal microbiota among three dietary treatments, L and M groups were closer in clustering relationship. This indicated that the high protein played an important role in the change of fecal microbial communities (Figure 5B).

# 4. Discussion

## 4.1. Changes in growth performance and nutrient digestibility of FMD Fed diets with different CP levels

Daily intake of food is required for metabolism in animals, and the feed intake is based on energy requirement. Lu and Potchoiba (1990) observed that dry matter intake decreased curvilinearly when dietary ME density increased in growing goat. In current study, although the 3 dietary protein levels were different, the energy values are similar, which is the reason why ADFI was not significantly different among the 3 diets despite the *ad libitum* feeding mode. Whereas in the growing phase of all animals, protein was required to make up the body. From the results of ADG, F:G and BLG data in this experiment, it can be concluded that compared with the 13.37 and 15.48% groups, the 11.51% protein level did not meet the nutritional needs of the growing FMD. During growing period of animals, bones are developed first, followed by muscles, and finally fat, namely the chest girth is lagging behind (Zhang, 2018). Therefore, compared with BLG and ADG, CGG is not significantly different in the 3 treatments. Dietary nutrient balance is vital to improve the feed conversion rate. Although the difference of F:G between M and H were not significant ($p > 0.05$), there was a trend of increase in the feed conversion ratio compared with H, which indicated that 15.48% CP level may be nutritional imbalance. Excessive dietary protein would reduce the growth performance and feed conversion rate. Jennings et al. (2018) noted that in the body, excess nitrogen is converted to urea in the liver and then excreted in the urine. Huntington and Archibeque (2000) calculated that 2.5 to 5% of whole body oxygen consumption was attributable to ureagenesis in the liver. Hales et al. (2013) reported that fed steers diet containing excess CP, whereas CP levels did not significantly affect N retention. Martin and Blaxter (1965) estimated the energy cost of synthesis of urea from ammonia to be $3.80 \pm 0.57$ Kcal/g of N in sheep. They noted that heat production, as a percent of metabolizable energy intake (MEI), increased and energy retention as a percent of MEI decreased as dietary CP concentration increased, suggesting that overall efficiency of energy utilization decreased with increasing CP content. These losses in energetic efficiency may potentially have a negative impact on animal growth and production

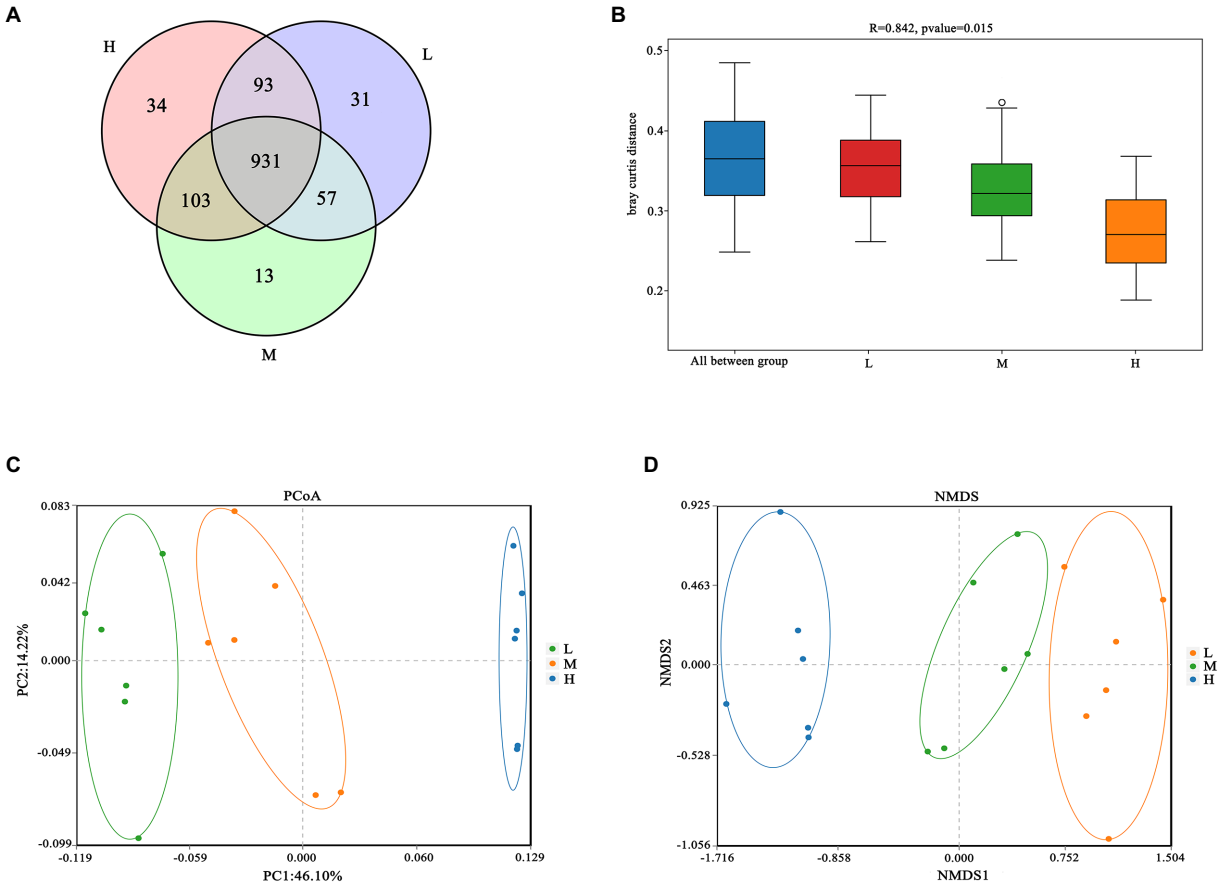


FIGURE 1
**(A)** Venn diagram; **(B)** Analysis of similarities (ANOSIM); **(C)** Principal coordinate analysis (PCoA); **(D)** Nonmetric multidimensional scaling (NMDS).

efficiency. In addition, there is accumulation of excess N in the ruminal environment as ammonia which is no longer directed to the growth of microorganisms and therefore affects microbial efficiency as well as the optimization of degradation of available fodder (Pinheiro et al., 2020). This may explained the higher dietary protein level, the lower digestibility in the results.

Rumen microbes play an important role in uptake and digestion of the feed energy and nutrients, which help to convert the food into more valuable metabolites for the host animal. The growth and reproduction of microorganisms not only need to provide sufficient nitrogen, but also a sufficient carbon, carbon mainly comes from the decomposition of glucose, starch and cellulose. NDF is one of the main sources of carbon for rumen microorganisms (Xue et al., 2022). In this study, the digestibility of NDF in group L was significantly lower than that in M and H groups ($p < 0.01$). Cappellozza et al. (2021) also observed that protein supplementation to ruminants increased NDF digestibility. This may be due to the relative shortage of N supply in group L, which makes the microbial activity was weakened in group L, and finally leads to the reduction of the digestibility of NDF (Matthews et al., 2019). NDF is mainly composed of cellulose, hemicellulose, lignin and silicate, of which cellulose and hemicellulose can be decomposed by rumen microorganisms to produce volatile fatty acids (Miller et al., 2021), while ADF is mainly composed of lignin, in addition to less cellulose and ashes (Miranda et al., 2020). Compared with cellulose and hemicellulose, lignin is difficult to be decomposed and utilized by rumen microorganisms (Khonkhaeng and Cherdthong, 2019), therefore the digestibility of the 3 groups of ADF is not significantly different ($p > 0.05$).

The rumen and its microbiota play a particularly important role in the degradation of feedstuffs for FMD. A nutritionally balanced diet is important as it provides an environment that maximizes the growth and activity of these microbes. Rumen microorganism produces end products that are either utilized directly by the host or by other microorganisms as energy (Matthews et al., 2019). Further buffering is provided by ammonia produced during fermentation, which can then be used for microbial growth in the rumen. If the degradable protein in the diet is insufficient, it would cause a deficiency of ammonia nitrogen in the rumen, which further inhibits microbial growth and fiber digestion (Allen, 2000). In this experiment, the NDF levels of the 3 diets were similar, and with the increase of CP, the Shannon index and Simpson index increased, indicating that the rumen microbial diversity increased, which further suggested that the L group had insufficient N.

## 4.2. Changes in fecal microbiome of FMD Fed diets with different CP levels

The Venn analysis showed that the L, M and H groups had 31, 13 and 34 unique OTU, respectively, and shared 931 OTU. PCoA and

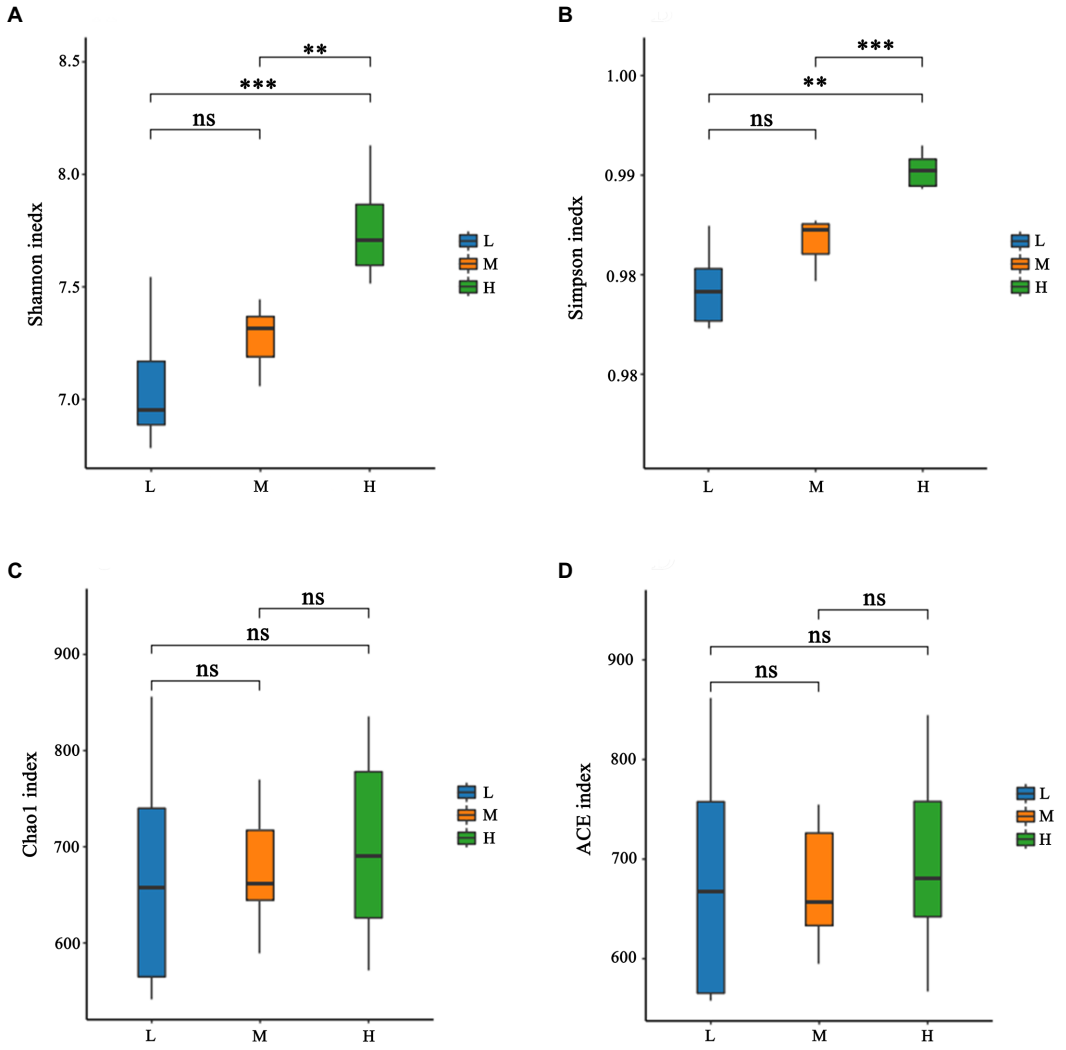


FIGURE 2
Alpha-diversity of fecal microbiota of forest musk deer fed diets with different levels of crude protein. **(A)** Shannon index; **(B)** Simpson index; **(C)** Chao1 index; **(D)** ACE index. ***, **, *, and ns were demonstrated $p<0.001$, $p<0.01$, $p<0.05$, and not significant, respectively.

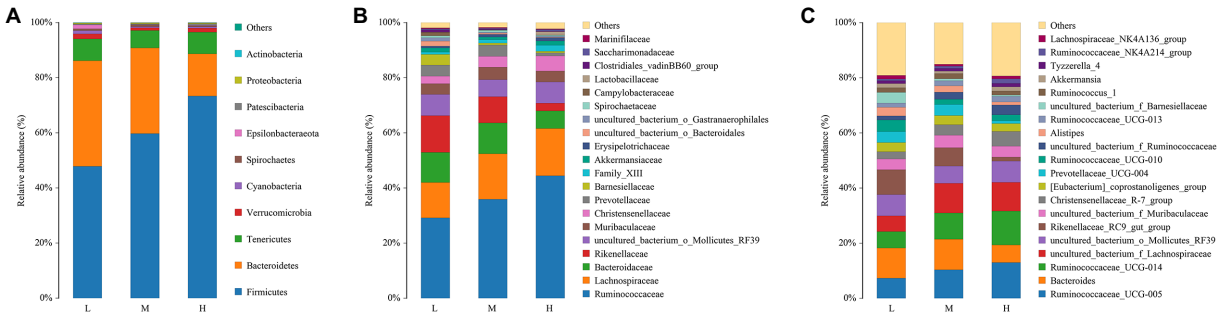


FIGURE 3
Relative abundance of bacterial composition in fecal contents at phyla **(A)**, family **(B)** and genus **(C)** levels among different dietary crude protein of growing forest musk deer. Each color represents one bacterium. The X-axis shows the different dietary treatments and the Y-axis shows the percentage of the bacteria.

NMDS showed that the structure of microflora changed regularly with the improvement of CP levels. This indicated that the microflora structure of M group is between L and H. Jiang et al. (2022) pointed out that higher α-diversity of gut microbiota probably benefits FMD adaption to different habitats. In the present research, group H has the highest α-diversity of fecal microbiota, this might indicate that the high protein of Group H has caused certain stress to the FMD, so that the intestinal bacteria have to make changes to cope with this stress.

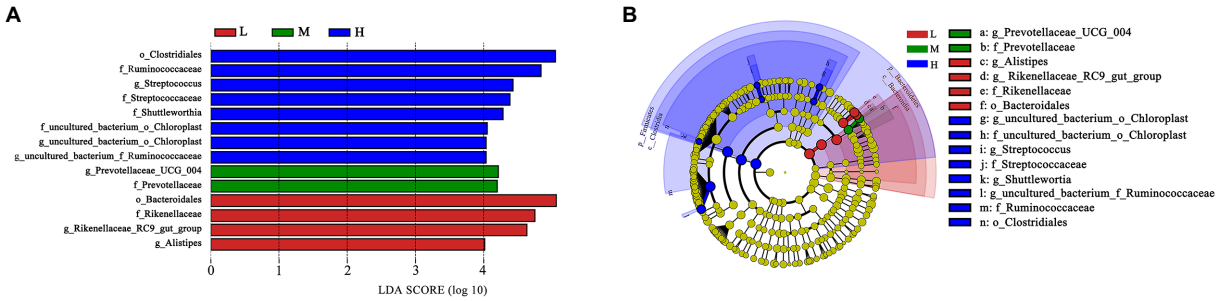


FIGURE 4
LEfSe analysis. **(A)** Different taxa microbes analysis in feces based on LEfSe method at 11.51% (L), 13.37% (M), and 15.48% (H) CP of FMD. The default parameters were LDA score>2 and $p<0.05$. Bacterium with red, green or blue colors mean that they are higher at L, M and H, respectively. **(B)** Taxonomic cladogram of forest musk deer fecal microbes at 11.51% (L), 13.37% (M), and 15.48% (H) CP levels. From inside to outside, different color circle represent different classification level (phylum, class, order, family and genus). The color of circles with letters mean that the bacterium was higher at specific crude protein levels of L, M or H groups are colored by red, green or blue, respectively.

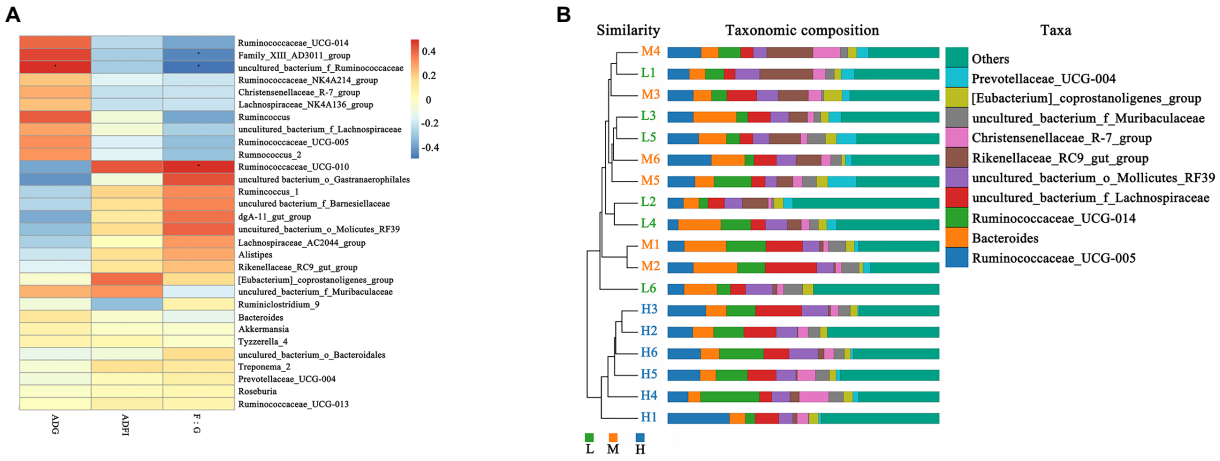


FIGURE 5
Correlation analysis and Unweighted Pair-group Method with Arithmetic Mean (UPGMA) analysis. **(A)** Spearman correlation analysis between differential genera and performance of growing forest musk deer. Significant correlations are noted by: $*$ $0.01<p\leq0.05$. **(B)** UPGMA based on OTU abundance at genus level for forest musk deer fed diets with different crude protein levels.

Firmicutes and Bacteroidetes had the hightest relative abundance in this study, which was consistent with the previous studies (Hu et al., 2017; Sun et al., 2020; Li et al., 2021; Su et al., 2022). Whereas the abundance of Firmicutes increased, and Bacteroidetes decreased with the increasing of dietary CP.

At the family level, the relative abundances of Ruminococcaceae and Christensenellaceae increased, while the relative abundances of Rikenellaceae and Barnesiellaceae decreased. In the current study, with the increase of CP levels, the relative abundance of fecal *Ruminococcaceae_UCG-005*, *Ruminococcaceae_ucg-014*, *uncultured_bacterium_f_Ruminococcaceae*, *Ruminococcaceae_NK4A214_group* and *Ruminococcaceae_UCG-013* increased at the genus level. Combined with ADFI, ADG, F:R and morphometric traits of growth FMD, it can be concluded that except for *Ruminococcaceae_UCG-013*, most genera in Ruminococcaceae were positively correlated with growth performance, and *uncultured_bacterium_f_Ruminococcaceae* being the most representative. In addition, *Family_XIII_AD3011_group* was also positively correlated with the growth performance of FMD during the growing phase ($p<0.05$). Xie et al. (2022) noted that Prevotellaceae and Ruminococcaceae some members of these families can produce propanoic acid and butanoic acid, which have the effects of regulating the proliferation and differentiation of the intestinal stem cells. Ruminococcaceae plays critical roles in degrading a variety of polysaccharides and fibers, are generally the producers of short-chain fatty acids (Zhu et al., 2017).

As the main precursor for glucose synthesis, higher propionate levels are generally play beneficial effect on ruminant production (Bergman, 1990). Poudel et al. (2019) found Prevotellaceae possess the ability to increase ruminal propionate concentrations. The higher abundance of *f_Prevotellaceae* and *g_prevotellaceae_UCG_004* were found at M group as shown in taxonomic cladogram. It indicated that Prevotellaceae might plays an important role in promoting growth performance in FMD. According to the results of this experiment, it is speculated that the bacterium needs a suitable C:N ratio.

In present experiment, the growth performance of FMD in medium protein group is better than that H and L group. The feed conversion ratio and growth performance were improved linearly with an increased level of dietary CP (Prusty et al., 2016), but the growth performance would decreases if the protein exceeds the critical value. Excessive protein reduced protein utilization, and changed the

structure of intestinal flora, promoted the proliferation of harmful intestinal bacteria (Chen, 2020). According to the fecal microbial community clustering results, at genus level, the H group was clustered separately into a branch, the clustering relationship between L and M groups is closer, indicating that the bacterial structure had changed greatly with protein level increased from 13.37% (M) to 15.48% (H), i.e., 15.48% dietary CP is excessive. Therefore, combined with growth performance and microbiome, the ideal dietary CP level was 13.37% for the growing FMD.

## 5. Conclusion

In conclusion, based on growth performance and microbiome, the relative abundance of *uncultured_bacterium_f_Ruminococcaceae*, *Family_XIII_AD3011_group*, *f_Prevotellaceae* and *g_prevotellaceae_UCG_004* could be used as a positive correlation indicators with growth performance, and *Ruminococcaceae_UCG-013*, might be a negative correlation discriminant index. Collectively, these findings also suggest optimal dietary CP levels of 13.37% for growing forest musk deer.

## 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 Northwest Agriculture & Forest University Animal Care and Use Committee.

## Author contributions

RG: conceptualization, methodology, data curation, and writing—original draft preparation. SS: investigation and data curation. YA: data curation. SW: sample collection and software. XD: visualization, investigation, and writing—review and editing. ZR: supervision and writing—reviewing, editing, project administration, and funding acquisition. HX, BJ, and LZ: resources. All authors contributed to the article and approved the submitted version.

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## Conflict of interest

LZ is employed by Shaanxi Shenglinyuan Biotechnology 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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## References

Allen, M. (2000). Effects of diet on short-term regulation of feed intake by lactating dairy cattle. *J. Dairy Sci.* 83, 1598–1624. doi: 10.3168/jds.S0022-0302(00)75030-2

Bergman, E. (1990). Energy contribution of volatile fatty acids from the gastrointestinal tract in various species. *Physiol. Rev.* 70, 567–590. doi: 10.1152/PHYSREV.1990.70.2.567

Bolyen, E., Rideout, J., Dillon, M., Bokulich, N., Abnet, C., Al-Ghalith, G., et al. (2019). Reproducible, interactive, scalable and extensible microbiome data science using QIIME 2. *Nat. Biotechnol.* 37:1091. doi: 10.1038/s41587-019-0252-6

Cappellozza, B., Bohnert, D., Reis, M., Emon, M., Schauer, C., Falck, S., et al. (2021). Influence of amount and frequency of protein supplementation to ruminants consuming low-quality cool-season forages: efficiency of nitrogen utilization in lambs and performance of gestating beef cows. *J. Anim. Sci.* 99:6. doi: 10.1093/jas/skab122

Chen, J. (2020). *Effects of different Nutrition Levels Dietary on Growth Peformance, Carcass Traits and Meat Quality of Ningxiang Pigs*. Dissertation/Doctor's Thesis. Changsha: Hunan Agricultural University.

Chen, H., and Boutros, P. C. (2011). VennDiagram: a package for the generation of highly-customizable Venn and Euler diagrams in R. *BMC Bioinformatics* 12:35. doi: 10.1186/1471-2105-12-35

Edgar, R. (2013). UPARSE: highly accurate otu sequences from microbial amplicon reads. *Nat. Methods* 10, 996–998. doi: 10.1038/nmeth.2604

Hales, K., Cole, N., and MacDonald, J. (2013). Effects of increasing concentrations of wet distillers grains with solubles in steam-flaked, corn-based diets on energy metabolism, carbon-nitrogen balance, and methane emissions of cattle. *J. Anim. Sci.* 91, 819–828. doi: 10.2527/jas.2012-5418

Hu, X., Liu, G., Li, Y., Wei, Y., Lin, S., Liu, S., et al. (2018). High-throughput analysis reveals seasonal variation of the gut microbiota composition within forest musk deer (*Moschus berezovskii*). *Front. Microbiol.* 9:1674. doi: 10.3389/fmicb.2018.01674

Hu, X., Liu, G., Shafer, A., Wei, Y., Zhou, J., Lin, S., et al. (2017). Comparative analysis of the gut microbial communities in forest and alpine musk deer using high-throughput sequencing. *Front. Microbiol.* 8:572. doi: 10.3389/fmicb.2017.00572

Huntington, G., and Archibeque, S. (2000). Practical aspects of urea and ammonia metabolism in ruminants. *J. Anim. Sci.* 77, 1–11. doi: 10.2527/JAS2000.77E-SUPPL1Y

Jennings, J., Meyer, B., Guiroy, P., and Cole, N. (2018). Energy costs of feeding excess protein from corn-based by-products to finishing cattle. *J. Anim. Sci.* 96, 653–669. doi: 10.1093/jas/sky021

Jiang, F., Song, P., Wang, H., Zhang, J., Liu, D., Cai, Z., et al. (2022). Comparative analysis of gut microbial composition and potential functions in captive forest and alpine musk deer. *Appl. Microbiol. Biotechnol.* 106, 1325–1339. doi: 10.1007/s00253-022-11775-8

Khonkhaeng, B., and Cherdthong, A. (2019). *Pleurotus ostreatus* and *Volvariella volvacea* can enhance the quality of purple field corn Stover and modulate ruminal fermentation and feed utilization in tropical beef cattle. *Animals* 9:1084. doi: 10.3390/ani9121084

Li, Y., Hu, X., Yang, S., Zhou, J., Zhang, T., Qi, L., et al. (2017). Comparative analysis of the gut microbiota composition between captive and wild forest musk deer. *Front. Microbiol.* 8:1750. doi: 10.3389/fmicb.2017.01705

Li, Y., Zhang, T., Shi, M., Zhang, B., Hu, X., Xu, S., et al. (2021). Characterization of intestinal microbiota and fecal cortisol, T3, and IgA in forest musk deer (*Moschus berezovskii*) from birth to weaning. *Integr. Zool.* 16, 300–312. doi: 10.1111/1749-4877.12522

Liu, H., Ran, T., Zhang, C., Yang, W., Wu, X., Degen, A., et al. (2022). Comparison of rumen bacterial communities between yaks (*Bos grunniens*) and Qaidam cattle (*Bos taurus*) fed a low protein diet with different energy levels. *Front. Microbiol.* 13:982338. doi: 10.3389/fmicb.2022.982338

Liu, X., Zhao, W., Yu, D., Cheng, J. G., Luo, Y., Wang, Y., et al. (2019). Effects of compound probiotics on the weight, immunity performance and fecal microbiota of forest musk deer. *Sci. Rep.* 9:19146. doi: 10.1038/s41598-019-55731-5

Lu, C., and Potchoiba, M. (1990). Feed intake and weight gain of growing goats fed diets of various energy and protein levels. *J. Anim. Sci.* 68, 1751–1759. doi: 10.2527/1990.6861751X

Martin, A., and Blaxter, K.. (1965). *The Energy Cost of Urea Synthesis in Sheep*. Proceedings of the 3rd Symposium on Energy Metabolism. UK: Academic Press.

Matthews, C., Crispie, F., Lewis, E., Reid, M., O'Toole, P., and Cotter, P. (2019). The rumen microbiome: a crucial consideration when optimising milk and meat production and nitrogen utilisation efficiency. *Gut Microbes* 10, 115–132. doi: 10.1080/19490976.2018.1505176

Meng, X., Zhou, C., Hu, J., Li, C., Meng, Z., Feng, J., et al. (2006). Musk deer farming in China. *Anim. Sci.* 82, 1–6. doi: 10.1079/ASC200516

Miller, M., Kokko, C., Ballard, C., Dann, H., Fustini, M., Palmonari, A., et al. (2021). Influence of fiber degradability of corn silage in diets with lower and higher fiber content on lactational performance, nutrient digestibility, and ruminal characteristics in lactating Holstein cows. *J. Dairy Sci.* 104, 1728–1743. doi: 10.3168/jds.2020-19088

Miranda, R. L., Tirado, G. D., Tirado, E. G., Améndola, M. R., Sandoval, G. L., Ramírez, V. R., et al. (2020). Quantifying non-fibrous carbohydrates, acid detergent fiber and cellulose of forage through an in vitro gas production technique. *J. Sci. Food Agric.* 100, 3099–3110. doi: 10.1002/jsfa.10342

Pinheiro, J., Ferreira, R., Neto, S., Signoretti, R., Henriques, L., and Oliveira, J. (2020). Dynamics of degradation of high-quality tropical forage according to levels of nitrogen supplementation. *Trop. Anim. Health Prod.* 52, 1309–1314. doi: 10.1007/s11250-019-02136-1

Poudel, P., Froehlich, K., Casper, D., and St-Pierre, B. (2019). Feeding essential oils to neonatal Holstein dairy calves results in increased ruminal prevotellaceae abundance and propionate concentrations. *Microorganisms* 7:120. doi: 10.3390/microorganisms7050120

Prusty, S., Kundu, S., Mondal, G., Sontakke, U., and Sharma, V. K. (2016). Effect of energy and protein levels on nutrient utilization and their requirements in growing Murrah buffaloes. *Trop. Anim. Health Prod.* 48, 807–815. doi: 10.1007/s11250-016-1033-6

Quast, C., Pruesse, E., Yilmaz, P., Gerken, J., Schweer, T., Yarza, P., et al. (2013). The SILVA ribosomal RNA gene database project: improved data processing and web-based tools. *Nucleic Acids Res.* 41, D590–D596. doi: 10.1093/nar/gks1219

Schroeder, G., Titgemeyer, E., Awawdeh, M., Smith, J., and Gnad, D. (2006). Effects of energy level on methionine utilization by growing steers. *J. Anim. Sci.* 84, 1497–1504. doi: 10.2527/2006.8461497x

Schwarzer, M., Makki, K., Storelli, G., Machuca-Gayet, I., Srutkova, D., Hermanova, P., et al. (2016). *Lactobacillus plantarum* strain maintains growth of infant mice during chronic undernutrition. *Science* 351, 854–857. doi: 10.1126/science.aad8588

Segata, N., Izard, J., Waldron, L., Gevers, D., Miropolsky, L., Garrett, W., et al. (2011). Metagenomic biomarker discovery and explanation. *Genome Biol.* 12:R60. doi: 10.1186/gb-2011-12-6-r60

Su, R., Dalai, M., Luvsantseren, B., Chimedtseren, C., and Hasi, S. (2022). Comparative study of the function and structure of the gut microbiota in siberian musk deer and forest musk deer. *Appl. Microbiol. Biotechnol.* 106, 6799–6817. doi: 10.1007/s00253-022-12158-9

Sun, Y., Sun, Y., Shi, Z., Liu, Z., Zhao, C., Lu, T., et al. (2020). Gut microbiota of wild and captive alpine musk deer (*Moschus chrysogaster*). *Front. Microbiol.* 10:3156. doi: 10.3389/fmicb.2019.03156

Wang, Y., and Harris, R. (2008). *Moschus berezovskii The IUCN Red List of Threatened Species*. Version 2010.3.

Wang, W., He, L., Liu, B., Li, L., Wei, N., Zhou, R., et al. (2015). Feeding performance and preferences of captive forest musk deer while on a cafeteria diet. *Folia Zool.* 64, 151–160. doi: 10.25225/fozo.v64.i2.a8.2015

Wang, Y., Sun, M., Chang, F., Wang, J., Wang, Y., Tang, J., et al. (2022). The essential differences in microbial and chemical components of musk of different qualities secreted by captive male forest musk deer (*Moschus berezovskii*). *Microb. Biotechnol.* 15, 1783–1794. doi: 10.1111/1751-7915.14002

Wang, J., Wang, C., Jie, W., Dai, X., and Xie, X. (2013). Study on domestic musk deer fed with pellet feed. *J. Econ. Anim.* 17, 23–26. doi: 10.13326/j.jea.2013.01.012

Williams, C., David, D., and Lismaa, O. (1962). The determination of chromic oxide in faeces samples by atomic absorption spectrophotometry. *J. Agric. Sci.* 59, 381–385. doi: 10.1017/S002185960001546XCorpus

Wu, X., Huang, S., Huang, J., Peng, P., Liu, Y., Han, B., et al. (2021). Identification of the potential role of the rumen microbiome in milk protein and fat synthesis in dairy cows using metagenomic sequencing. *Animals* 11:1247. doi: 10.3390/ani11051247

Xie, J., Li, L., Dai, T., Qi, X., Wang, Y., Zheng, T., et al. (2022). Short-chain fatty acids produced by ruminococcaceae mediate α-linolenic acid promote intestinal stem cells proliferation. *Mol. Nutr. Food Res.* 66:e2100408. doi: 10.1002/mnfr.202100408

Xue, B., Wu, M., Yue, S., Hu, A., Li, X., Hong, Q., et al. (2022). Changes in rumen bacterial community induced by the dietary physically effective neutral detergent fiber levels in goat diets. *Front. Microbiol.* 13:820509. doi: 10.3389/fmicb.2022.820509

Yang, C., Huang, W., Sun, Y., You, L., Jin, H., and Sun, Z. (2021). Effect of probiotics on diversity and function of gut microbiota in *Moschus berezovskii*. *Arch. Microbiol.* 203, 3305–3315. doi: 10.1007/s00203-021-02315-5

Zhang, Y. (2018). *Animal Breeding. 2nd*. Beijing: China Agriculture Press.

Zhang, Z., Shahzad, K., Shen, S., Dai, R., Lu, Y., Lu, Z., et al. (2022). Altering dietary soluble protein levels with decreasing crude protein may be a potential strategy to improve nitrogen efficiency in hu sheep based on rumen microbiome and metabolomics. *Front. Nutr.* 8:815358. doi: 10.3389/fnut.2021.815358

Zhu, C., Wang, F., Tang, Q., and Li, P. (2012). Research on the diagnosis, treatment, and prevention of common diseases of domestic musk deer. *Chin. J. Wildl.* 33, 3–4. doi: 10.3969/j.issn.1000-0127.2012.01.002

Zhu, X., Zhou, Y., Wang, Y., Wu, T., Li, X., Li, D., et al. (2017). Production of high-concentration n-caproic acid from lactate through fermentation using a newly isolated ruminococcaceae bacterium CPB6. *Biotechnol. Biofuels* 10, 102–114. doi: 10.1186/s13068-017-0788-y

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# Different microbial genera drive methane emissions in beef cattle fed with two extreme diets

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The ratio of forage to concentrate in cattle feeding has a major influence on the composition of the microbiota in the rumen and on the mass of methane produced. Using methane measurements and microbiota data from 26 cattle we aimed to investigate the relationships between microbial relative abundances and methane emissions, and identify potential biomarkers, in animals fed two extreme diets - a poor quality fresh cut grass diet (GRASS) or a high concentrate total mixed ration (TMR). Direct comparisons of the effects of such extreme diets on the composition of rumen microbiota have rarely been studied. Data were analyzed considering their multivariate and compositional nature. Diet had a relevant effect on methane yield of +10.6 g of methane/kg of dry matter intake for GRASS with respect to TMR, and on the centered log-ratio transformed abundance of 22 microbial genera. When predicting methane yield based on the abundance of 28 and 25 selected microbial genera in GRASS and TMR, respectively, we achieved cross-validation prediction accuracies of 66.5 ± 9% and 85 ± 8%. Only the abundance of *Fibrobacter* had a consistent negative association with methane yield in both diets, whereas most microbial genera were associated with methane yield in only one of the two diets. This study highlights the stark contrast in the microbiota controlling methane yield between animals fed a high concentrate diet, such as that found on intensive finishing units, and a low-quality grass forage that is often found in extensive grazing systems. This contrast must be taken into consideration when developing strategies to reduce methane emissions by manipulation of the rumen microbial composition.

KEYWORDS

**enteric methane emissions, beef cattle, concentrate-based diets, zero-grazed grass diet, microbiota by diet interaction**

## Introduction

In 2019, methane levels in the atmosphere reached record levels, about 2.5 times higher than in the pre-industrial era. Ruminants account for 16% of global methane ($CH_4$) emissions, of which 35% and 30% correspond to beef and dairy industries (Tseten et al., 2022). Methane is an unnecessary by-product of microbial fermentation of mainly complex carbohydrates in the rumen, synthesized by methanogenic archaea and released into the environment through the animal's mouth and nose. Eructated methane from ruminal microbial fermentation not only contributes to global warming, but also results

in unnecessary loss of energy, compromising feed efficiency (Manzanilla-Pech et al., 2022). Because the microbiome plays a key role in the synthesis of methane, most methane mitigation strategies in ruminants rely on altering the rumen conditions and microbial ecology. Desired changes include promoting the growth of microorganisms able to reduce hydrogen ($H_2$) production through propionate fermentation, re-partition $H_2$ to other products by acetogenesis, inhibiting methanogens or promoting methane oxidation (Cottle et al., 2011). To this end, both short-term, e.g., use of feed-additives (Dijkstra et al., 2018; Tseten et al., 2022), and long-term strategies, e.g., microbiome-driven breeding (Martínez-Álvaro et al., 2022), have been proposed. A combination of these strategies adapted to specific production systems could be the most cost-effective solution.

In addition, implementation of strategies to reduce enteric methane emissions depends on the collection of quality methane emission data on a large-scale. Respiration chambers are the gold-standard method for measuring methane emissions from ruminants but has low throughput. Alternatives include the use of tracer gases (sulfur hexafluoride) which require boluses and attachment of equipment to the animal, and collection of multiple short-term point measurements over longer time periods (van Breukelen et al., 2022). Over the past decade, genetic sequencing costs have decreased and efficiencies have increased, resulting in rising attention to the rumen microbiome as a potential proxy measurement (Shi et al., 2014; Wallace et al., 2015; Auffret et al., 2018; Martínez-Álvaro et al., 2020). Targeted amplification of specific regions of 16S rRNA loci (16S) is a robust and cost-effective method for characterizing bacterial and archaeal community composition. Compared to whole-metagenome sequencing, it is less costly and does not require the same depth of sequencing, although it has limitations in identifying taxa at high resolution and does not provide functional information (Rubiola et al., 2022). The main statistical challenges in analyzing microbial composition data are sparsity (especially when using 16S) and their compositional nature (Gloor et al., 2017), which can lead to misleading conclusions if not addressed properly (Martínez-Álvaro et al., 2021). To circumvent these problems, appropriate techniques to exclude sparse non-informative taxa (Roesch et al., 2020) and the use of log-ratio transformations, e.g., centered log-ratio (*clr*), (Greenacre et al., 2021) have been proposed. However, these methods have only recently been utilized in microbiome studies.

Finishing diets for beef production in the UK and around the world vary in their composition, from high in concentrate (e.g., barley beef systems), to forage and grass-based diets, depending on variables such as breeding system, forage stocks, grass availability and quality, breed type, or market requirements (Agriculture and Horticulture Development Board, 2020). Both rumen microbiome composition and methane yield are highly sensitive to the diet of the animal, which determines the inputs available for microbial fermentation and the corresponding $H_2$ production. Numerous studies report that the microbiome structure and methane traits are significantly altered by the forage to concentrate ratio, and by type of forage and forage quality (Janssen, 2010; Troy et al., 2015; Duthie et al., 2017; Gruninger et al., 2019; Li et al., 2019). Diet could not only cause changes in the magnitude of microbial abundances, but also alter their microbial interactions and their effects on methane emissions. Under this hypothesis, mitigation strategies or predictive equations developed for one specific diet might not be suitable for other diets.

The objectives of this study were (i) to evaluate the effects of two extreme contrasting diets (either high concentrate or low quality fresh cut grass) on methane emissions and rumen microbiota composition; and (ii) to explore the relationships between microbial abundances and methane yield and identify potential biomarkers in these two extreme feeding systems.

# Materials and methods

## Experimental design, animals, and diets

A total of 36 beef steers, 18 Limousin cross (LIMx) and 18 Aberdeen Angus cross (AAx), were selected for this trial. The cattle were paired based on breed, sire, and body weight, with one of each pair being randomly allocated to either a low quality fresh cut grass diet (GRASS) or a high concentrate total mixed ration (TMR). The 36 cattle were housed in two pens (one per treatment) and bedded on sawdust. The GRASS group had an average body weight of 499 $\pm$ 7.9 kg and the TMR group 510 $\pm$ 7.8 kg at the beginning of the trial.

All cattle had *ad libitum* access to feed and water throughout the trial. For the GRASS group, a mixed ryegrass and clover sward was mowed each morning and fed directly to the cattle top dressed with Downland Intensive Beef minerals (Downland Marketing, Carlisle, UK; 0.8% of diet dry matter). The TMR diet was mixed in a feeder wagon and had a forage to concentrate ratio of 136:864, the ingredients and nutritional composition of the diets are given in **Table 1**. The animals were adapted to these diets over a 2-week period, over which the proportion of concentrate or grass in the diet was gradually increased.

To measure methane emissions, animals were allocated to six indirect open-circuit respiration chambers over a 6-week period so that three of each treatment and three from each breed were assigned to each chamber, and paired animals were measured in the same week. Before entering the respiration chambers, animals were individually housed in training pens, which are identical in construct to the ones inside the chambers, for 6 days. After this training period, animals were isolated in the respiration chambers for 3 days, during which time they were fed once daily. Dry matter intake was recorded daily and averaged over the 3 days.

## Respiration chamber measurements

Methane measurements were undertaken in six indirect open-circuit respiration chambers and concentrations of $CH_4$ in air samples exhausted from the respiration chambers were measured by infra-red absorption spectroscopy (MGA3000; Analytical Development Company Limited, Amersham, UK). The method of measurement in the respiration chambers is described in detail in Troy et al. (2015). Methane production (ppm) was determined by calculating the concentration difference between inlet and exhaust air multiplied by volumetric dry air flow and corrected to standard temperature and pressure (25°C and 101,300 Pa). Daily gas production was calculated as the average of all recorded values

TABLE 1 Ingredients and chemical composition of two extreme offered diets fresh cut grass (Grass) and high concentrate (TMR) based.

| | TMR | Grass |
|---|---|---|
| Ingredient composition (g/kg DM)[1] | | |
| Barley | 851 | – |
| Maize dark grains | 890 | – |
| Barley Straw | 865 | – |
| Molasses | nd | – |
| Minerals[2] | 8.1 | 8.0 |
| Grass | – | 210 |
| Chemical composition (g/kg DM)[3] | | |
| Dry matter (g/kg) | 833 | 211.7 |
| Ash | 43 | 76 |
| Crude protein | 83.7 | 84.9 |
| Neutral detergent fiber | 339 | 557 |
| Starch | 356 | <5 |
| Metabolizable energy (MJ/kg DM) | 11.7 | 9.0 |
| Gross energy (MJ/kg DM) | 17.9 | 18.1 |

[1]Ingredient composition is the mean of the daily diets received by the animals across the experimental period.
[2]Contained (mg/kg): Fe, 500; Mn, 3,000; Zn, 2,000; Iodine, 100; Co, 30; Cu, 800; Se, 35. Vitamins (μg/kg): vitamin A, 400,000: vitamin D3, 100,000; vitamin E, 1,500.
[3]Chemical composition is the mean of 3 analyses per treatment.

per chamber per day and expressed on a mass basis (g/day). The values reported in this paper are from the final 48 h of the 72-h period. Due to a technical failure of one of the respiration chambers, only 26 out of the 36 animals could be used to investigate the effects of diet type on methane yield. The remaining 26 animals were still balanced for diet treatment and breed.

## Collection of rumen samples and 16S rRNA gene analysis

There were only 26 animals with adequate rumen samples available to investigate the impact of diet on the rumen microbiome, of which 24 had methane emissions measurement, these were also still balanced for breed and diet. Rumen fluid samples were collected from each animal within 2 h of exiting the respiration chambers using a naso-gastric tube (16- by 2,700-mm Equivet stomach tube; JørgenKruuse A/S, Langeskov, Denmark) and aspirating manually. For each animal approximately 50 ml of rumen fluid was filtered through two layers of muslin and samples were stored in a −80°C freezer until sent for 16S rRNA gene analysis.

DNA was extracted from the rumen samples following the protocol of Yu and Morrison (2004) combining chemical lysis and bead beating, followed by purification on column using the QIAamp DNA Mini Kit (Qiagen, Manchester, UK). Genomic DNA was quantified using Nanodrop. Illumina TruSeq DNA libraries (Illumina Inc., San Diego, CA, USA) were prepared from genomic DNA following Illumina protocol. The V4 region of the 16S rRNA gene was amplified by PCR using Q5® High-Fidelity DNA Polymerase (NEB, Hitchin, UK) and the primers 16SMiFwd and 16SMiRev, recommended by Illumina. The full sequence of the primers used were 515F = 5′-TCG TCG GCA GCG TCA GAT GTG TAT AAG AGA CAG GTG YCA GCM GCC GCG GTA A-3′ for 16SMiFwd and 806R = 5′-GTC TCG TGG GCT CGG AGA TGT GTA TAA GAG ACA GGG ACT CAN VGG GTW TCT AAT-3′ for 16SMiRev. Amplicons were purified using the ProNex® Size-Selective Purification System kit (Promega, Madison, WI, USA), quantified using Qubit® dsDNA HS Assay Kits (Life Technologies, Paisley, UK) prior to be pooled and sequenced on a Miseq Illumina instrument (Illumina Inc., San Diego, CA, USA) by Edinburgh Genomics (Edinburgh, UK). Sequencing provided a yield of average of 153.5 ± 47.4 Mb per sample and $5.06 \pm 1.6 \times 10^5$ reads per sample. The 16S rRNA amplicon sequences obtained were analyzed with the pipeline MGRAST to generate comprehensive taxonomic profiles using the standard reference database, excluding host reference genome. One table of hit counts was created for each taxonomic level, resulting in 203 genera, 115 families and 21 phyla identified.

The analysis of the taxonomic composition of the rumen microbiome was focused on (i) the natural log ratio between archaea and bacteria (A:B) and (ii) microbial composition at the genus level. The abundance of each microbial genus was normalized by the total sum of counts per sample. The microbial composition at the genus level contained a large proportion of 0 counts per sample, with an average percentage of 0 counts per sample of 78 ± 8.7% (**Supplementary Figure 1**). To maximize microbial information per sample, we discarded microbial genera with a high count of 0 across all samples only if they did not contribute to the discrimination of samples within diets. To achieve this, we used the Prevalence Interval for Microbiome Evaluation (PIME) workflow from the R package PIME (Roesch et al., 2020) to establish the minimum % of samples that a microbial genera should contain in order to be kept, i.e., minimum prevalence. Briefly, PIME uses a machine learning algorithm to find which is the optimal prevalence threshold to maximize the discrimination ability between GRASS/TMR diets. In our analysis, a minimum prevalence threshold of 20% maximized discriminatory ability between diets. After removing microbial genera present in less than 20% of samples, we retained 103 microbial genera for further analysis. The remaining 0 genera were imputed using a Bayesian multivariate composition approach implemented in the R package *zcompositions* (Palarea-Albaladejo and Martín-Fernández, 2015). The descriptive analysis of the rumen microbiome composition was presented in relative abundances to help their interpretability (**Supplementary Figure 2**). For remaining statistical analysis, the compositional nature of microbiome data was taken into account (Gloor et al., 2017) by applying a centered log-ratio (*clr*) transformation to the genera abundances, as described in Greenacre (2019) using the *zcompositions* R package.

## Data analysis

### Effect of diet on dry matter intake, methane intensity, methane yield, and archaea: bacteria ratio (A:B)

To investigate the diet effect on DMI (kg/day), methane production (g/day), methane yield (g/kg DMI) and A:B, we fitted

three different linear models including these traits as dependent variables; diet, breed, chamber, and week as fixed effects and the body weight of the animal when entering the chamber (ranging from 438 to 656 kg) as a covariate. Bayesian inference was used with bounded flat priors for all unknowns. Analyses were run with the R function *runRabbit* developed by the Institute for Animal Science and Technology from Universitat Politècnica de València.[1] After some exploratory analyses, results were based on marginal posterior distributions of 60,000 iterations, with a burning period of 10,000 and only 1 of every 10 samples were saved for inferences. In all analyses, convergence was tested using the Z criterion of Geweke and Monte Carlo sampling errors were computed using time-series procedures (Sorensen and Gianola, 2002). The parameters obtained from the marginal posterior distributions of the differences between diets were: the mean; the highest posterior density region at 95% probability ($HPD_{95\%}$); the probability of the difference being greater than 0 when the mean is positive or lower than 0 when the mean is negative ($P_0$); and the probability of the difference being greater than $r$ or lower than $-r$ if the mean is positive or negative ($P_r$), being $r$ a relevant amount having economical or biological significance. In our case, we took as $r$ one-third of the phenotypic standard deviation (Blasco, 2017).

The linear relationship between methane production or methane yield and A:B within each diet was also computed. After some exploratory analysis, methane traits were analyzed within diet separately, with a model including the fixed effect of breed and the covariates body weight and A:B. In this case, we computed the mean, $HPD_{95\%}$, and $P_0$ of the marginal posterior distribution of the regression coefficient between the *clr*-transformed abundance of the microbial genera and methane yield.

## Effect of diet on the ruminal microbiome composition

A multivariate approach was used to identify which microbial genera showed different abundances due of different diets offered, using 26 samples (13 per diet group). A discriminant analysis based on projection to latent structures (DA-PLS) computed using R package *mixOmics* was fitted, considering GRASS/TMR diet treatments as dependent variables, and the 103 *clr* transformed microbial genera abundances as explanatory variables. The number of components in the model was selected based on a cross-validation procedure with 4 cross validation groups repeated 20 times, following the procedure described in Lê Cao and Welham (2022). First, we computed the Residual Sum of Squares (RSS) in the complete dataset for a given dimension $h$, using all the samples ($n = 26$):

$$RSS_h = \sum_{i=1}^{n} (y_i^{(h)} - \hat{y}_i)^2$$

where $y_i^{(h)}$ is the predicted value of sample $i$ according to dimension $h$ and $\hat{y}_i$ is its "reconstructed" value, i.e., the product between the latent component and the vector of regression coefficients for a given dimension $h$. Under each repetition, we computed the Predicted Sum of Squares (PRESS) as the sum of squares of the residuals of the testing set in each cross-validation fold:

$$PRESS_h = \sum_{i \in test} (y_i^{(h)} - \hat{y}_i)^2$$

And averaged $PRESS_h$ across repetitions. The $Q^2$ criterion was computed to assess the gain in prediction accuracy when one more dimension was added to the model. For each dimension:

$$Q_h^2 = 1 - \frac{PRESS_h}{RSS_{h-1}}$$

We stop adding dimensions when $Q_h^2 \leq 0.0975$ as proposed by Lê Cao and Welham (2022). The most influential variables discriminating between the two diets were selected based on their variable importance for projection (VIP) criterion >0.8 and based on their Jack Knife interval of regression coefficients not containing zero, as described in Martínez-Álvaro et al. (2021). An iterative process was followed and stopped when the removal of one more microbial genus did not improve the discrimination ability of the model. Authors are aware of the high propensity of DA-PLS models in overfitting (Westerhuis et al., 2008). We attempted to overcome this statistical issue by validating the discrimination ability of the final DA-PLS model with two different strategies. First, the final DA-PLS model was tested under a new fourfold cross validation repeated 20 times. In each fold, a DA-PLS model was re-fitted for the given set of selected variables and number of components with 3/4 of the data (training) and then used to predict to the diet of the samples in the remaining testing set (1/4 of the data). The final misclassification rate (%) was obtained by averaging the misclassification rate (%) obtained across the fourfolds in each of the 20 replicates. Second, we integrated a permutation test (randomization of diet labels in the testing set) in the currently described validation procedure as recommended by Westerhuis et al. (2008) and computed the misclassification rate (%) after permutation. It has to be realized that the same individuals in the validation set were also used to optimize the final PLS-DA model parameters (e.g., number of selected components and selected variables), and thus they are not completely independent as is requested for a proper cross validation. However, the short sample size of our study did not allowed to properly performed an external validation.

Additionally, we aimed to quantify the magnitude of the divergences between diets on the abundance of the DA-PLS selected microbial genera. To this aim, we fitted one univariate linear model for each selected microbial genera, including the fixed effects of diet and breed and body weight as a covariate. Analyses were run with the R function *runRabbit* as already described. As previously, we computed the mean, $HPD_{95\%}$, $P_0$ and $P_r$ the marginal posterior distributions of the differences between diets. To help interpretability, their magnitude of the differences was expressed not only as units of *clr*-transformed abundances, but also as effect size, defined as the median of the ratio of the between the difference and the variance of the traits after correction for breed, diet and body weight.

## Associations between rumen microbiota composition and methane yield among different diets

To investigate the relationship between rumen microbiota composition and methane yield in different diets, we fitted

1 https://github.com/VLabUPV/runRabbit

TABLE 2 Means and differences between diets [fresh cut grass (GRASS) and high concentrate (TMR)] in daily dry matter intake (kg/day), methane traits and natural log archaea to bacteria ratio.

| | [1]Mean TMR | [1]Mean GRASS | [2]TMR-GRASS | [3]$P_0$ | [4]$r$ | [5]$P_r$ |
|---|---|---|---|---|---|---|
| Daily dry matter intake (kg/day) | 9.16 [8.18, 10.2] | 5.95 [4.69, 7.48] | 3.25 [1.04, 5.19] | 1.00 | 0.49 | 0.99 |
| Methane production (g/day) | 160 [141, 181] | 146 [128, 165] | 14.5 [17.8, 53.4] | 0.80 | 4.9 | 0.71 |
| Methane yield (g/kg DMI) | 14.4 [9.70, 19.5] | 25.0 [20.7, 28.9] | −10.6 [−18.2, −1.27] | 0.99 | 0.98 | 0.98 |
| Ln (Archaea: Bacteria) | −4.72 [−5.69, −3.75] | −0.43 [−1.59, 0.58] | −4.30 [−6.17, −2.49] | 1.00 | 0.3 | 1.00 |

[1]Means and highest posterior density intervals at 95% probability of the marginal posterior distributions of the means of TMR and GRASS.
[2]Median of the marginal posterior distribution of the difference between TMR and GRASS andighest posterior density interval at 95% probability.
[3]Probability of the marginal posterior distribution of the differences of being greater than 0 when the mean is positive or lower than 0 when the mean is negative.
[4]Relevant value considered as the minimum value with economic importance.
[5]Probability of the marginal posterior distribution of the differences of being greater than $r$ when the mean is positive or lower than $r$ when the mean is negative.

two (one per diet group) linear projections to latent structures regression (PLS) analysis to determine the abundances of microbial genera that best explained methane yield (g/kg DMI) within each diet ($n$ = 12 animals per group). Methane yield was considered as the dependent variable and the 103 *clr*-transformed abundances of microbial genera as explanatory variables. The number of components and the selection of explanatory variables in the model were chosen as explained at PLS-DA. After the final PLS model was built, its predictive ability was tested by threefold cross-validation, which was repeated 20 times. The final predictive ability of the model was calculated as the square of the correlation between the predicted and observed values in the training set, averaged over the threefolds and then over the replicates. As before, we integrated a permutation test (randomization of diet labels in the testing set) in the validation procedure and computed the predictive ability after permutation.

In addition, we aimed to study the linear regression between each PLS-selected *clr*-transformed microbial genera in each diet group and methane yield in a univariate context, to show whether a linear relationship existed between specific microbial genera and the environmental trait. To this aim, we used the data divided within diet group to fit one univariate linear model for each selected microbial genera, including methane yield as dependent variable and the fixed effect of breed and covariates of *clr*-transformed abundance of the microbial genera and body weight as covariates. Analyses were run with the R function *runRabbit* as already described. We computed the mean, $HPD_{95\%}$, and $P_0$ of the marginal posterior distribution of the regression coefficient between the *clr*-transformed abundance of the microbial genera and methane yield. We additionally computed the Pearson correlation between each selected microbial genera and methane yield after correcting the data by breed and body weight.

# Results

## Effect of the diet on dry matter intake, methane traits, and A:B traits

Table 2 shows the descriptive statistics and the differences in DMI, methane traits, and A:B ratio among the diets. On average, individual animal DMI varied by ± 0.4 kg between days for GRASS animals (ranging from 0.1 to 1.3), and ± 0.7 kg for TMR animals (ranging from 0.0 to 1.5 kg). Repeatability of methane measurements between the second and third day was high, with ± 1.9 ppm average variation between days (ranging from 0.1 to 6.4 ppm). Daily dry matter intake was higher in the TMR group than in the GRASS group by 3.25 [1.04, 5.19] kg/day ($P_0$ = 1.00). However, the TMR group had lower methane yield (−10.6 [−18.2, −1.27] g $CH_4$/kg DMI) and also lower A:B (−4.30 [−6.17, −2.49]) than GRASS with $P_0 \geq 0.99$, and, in all cases, differences were relevant ($P_r \geq 0.98$). Diet groups did not show relevant different in methane production (g/day). Breed effect did not present strong evidence of being different from zero ($P_0 \leq 0.88$) or relevant ($P_r \leq 0.72$) for any trait.

## Effect of diet type on the composition of the rumen microbiota at the genera level

To further examine the effects of the two extreme diets on the taxonomic composition of the rumen microbiota, we used a multivariate DA-PLS to determine which microbial genera abundances were discriminatory between the two diet groups (Supplementary Table 1; Figure 1A). We identified 28 microbial genera that, combined in a single DA-PLS component, were able to discriminate between the GRASS and TMR diets with a misclassification rate of 0% after fourfold cross-validation and 20 replicates. When the model was used to predict a randomized vector of the diet labels, it the misclassification rate increased up to 46%. Once we identified the 28 microbial genera with discrimination ability between the diets, we fitted a linear model for each microbial genus to quantify differences between diets in the *clr*-transformed abundances. Of the 28 microbial genera tested, 22 microbial genera had different abundances between the two diets ($P_0 \geq 0.95$, Supplementary Table 1; Figure 1B) and the differences were relevant, i.e., larger than 1/3 their standard deviation ($P_r \geq 0.90$). Whilst we do not have enough power to detect differences between diets in the most ubiquitous methanogenic genera *Methanobrevibacter* and *Methanobacterium* (i.e., the $HPD_{95\%}$ were very wide [−1.93, 2.32] and [−3.19, 5.74], both including the relevant values 0.48 and 1.00), unclassified *Archaea* were much more abundant in the GRASS animals, with difference being 9.42 [6.27, 12.7] (relevant value was 0.74 and $P_r$ = 1.00), having an effect size of 4.25. The major fiber degraders *Fibrobacter* and *Bacillus* were also more abundant in the GRASS group, as were unclassified *Actinobacteria, Microbacterium*, *Chryseobacterium*, *Lactococcus*, and three microbial genera from the Proteobacteria phylum (*Pseudomonas, Stenotrophomonas,* and *Rhizobium*), with effect sizes ranging from 1.44 to 2.63 ($P_r \geq 0.93$). In contrast, *Desulfovibrio, Lactobacillus,* and unclassified Bacteria were much more abundant in the TMR group with effect

sizes of −5.31, −3.64, and −3.93 ($P_r$ = 1.00). *Bifidobacterium, Succinimonas, Aerococcus*, and *Dialister,* and *Rothia* amongst others, were also relevantly more abundant in TMR ($P_r \geq 0.93$), with effect sizes ranging from −1.40 to −2.78 (see **Supplementary Table 1**).

## Multivariate prediction of methane yield based on abundance of microbial genera and investigation of linear microbiota-methane relationships in different diets

A strong positive linear relationship between methane yield and A:B was observed in the GRASS animals, with a positive regression coefficient of +3.91 [0.28, 7.38] g $CH_4$/kg DMI gain per unit A:B ($P_0$ = 0.98). This linear relationship was also positive in animals fed TMR, although the regression coefficient was lower (0.93 [−0.45, 2.43], $P_0$ = 0.91). When methane emission is expressed in g/day, we did not had enough evidence to stablish the sign of relationship with A:B ($P_0$ = 0.69 in GRASS and 0.83 in TMR). **Figure 2** shows the distribution of methane production (A) and methane yield (B) with A:B, after correcting the data for breed and body weight. As expected, the greatest $R^2$ was obtained for methane yield and A:B in GRASS ($R^2$ = 0.34) and TMR ($R^2$ = 0.27), with Pearson correlations of 0.58 ± 0.21 and 0.52 ± 0.23. Based on this result, we concentrated on relationships between methane yield and rumen microbiota composition at lower taxonomic levels.

We fitted two multivariate PLS models (one with the GRASS animals and one with TMR-fed animals) with methane yield as the dependent variable and the 103 *clr*-transformed microbial genera as explanatory variables. In GRASS animals, a one-component PLS model built with the *clr*-transformed abundance of 28 microbial genera showed 85 ± 8% predictive ability for methane yield after 20 repetitions of 3-fold cross-validation (**Supplementary Table 2**; **Figure 3**). When the model was used to predict a randomized vector of methane yield in GRASS animals, the prediction ability decreased down to 36%. In animals fed a TMR diet, a single-component PLS model constructed with the *clr*-transformed abundance of 25 microbial genera showed 66.5 ± 9% predictive ability; and when methane yield was randomized, the prediction ability decreased down to 37%.

We next examined whether there was a linear relationship between the *clr*-transformed abundance of these microbial genera and methane yield in a univariate approach (**Supplementary Table 2**). Of the 28 and 25 *clr*-transformed microbial genera selected in the PLS, only six genera were selected by the PLS algorithm as part of the model in both diets, indicating large differences in the microbiota compositions associated with methane yield in animals fed extremely different diets. Of the 6 *clr*-transformed microbial genera showing PLS predictive ability for methane yield under both diets, only the microbial genus *Fibrobacter* had a similar (negative) association with this trait (**Figure 4A**; **Supplementary Table 2**). Its *clr*- abundance showed a negative Pearson correlation with methane yield under both GRASS (−0.41 ± 0.26) and TMR (−0.53 ± 0.22) and a negative regression coefficient of −0.25 [−0.67, 0.15] ($P_0$ = 0.89) and −0.75 [−1.63, 0.17] ($P_0$ = 0.95), respectively. Although our PLS analysis did not select *Methanobrevibacter* as an optimal predictor of methane yield, we wanted to pay particular attention to it because its role in rumen methanogenesis is well known (Evans et al., 2019). The *clr*-abundance of *Methanobrevibacter* genus was positively associated with methane yield in both diets, but the s.e. of Pearson correlations were very large (0.25 ± 0.30 in GRASS and 0.41 ± 0.26 in TMR) and the probability of the linear regression coefficients of being positive were only moderate (in TMR it was 1.10 [−0.69, 2.88] $P_0$ = 0.89 and in GRASS it was 0.65 [−1.12, 2.60] $P_0$ = 0.77). Interestingly, the other five microbial genera expressing PLS predictive ability of methane yield in both diets were bacterial genera (*Weissella, Kurthia, Raoultella, Herminiimonas*, and *Micrococcus*) that suggests a changing association with methane yield depending on the diet. Their *clr*-transformed abundance was associated with a decrease in methane yield under TMR diet, with *Raoultella, Kurthia* and *Weissella* showing the strongest correlations with methane yield of −0.60 ± 0.20, −0.52 ± 0.23 and −0.52 ± 0.23, and regression coefficients of −4.74 [−9.58, −0.17] $P_0$ = 0.98; −4.30 [−9.58, 1.18] $P_0$ = 0.95 and −1.340 [−3.06, 0.22] $P_0$ = 0.95, respectively. In contrast, their abundance was associated with an increase in methane yield within the GRASS diet, *Kurthia, and Herminiimonas* showing the strongest correlations with methane yield of 0.69 ± 0.17 and 0.49 ± 0.24 and regression coefficients of 1.71 [0.33, 3.09] $P_0$ = 0.99 and 0.85 [−0.31, 2.01] $P_0$ = 0.93, respectively (**Figure 4A**). These results suggest that the effect of abundance of a particular microorganism on methane yield may depend on diet-induced rumen environmental conditions.

The majority of the microbial genera showing predictive ability in PLS were different between diets (19/25 and 22/28 in TMR and GRASS, respectively). Of these, 12 and 5 showed a linear regression coefficient with methane yield that was different from 0 with a $P_0 \geq 0.95$ (**Figures 4B, C**). Under TMR conditions (**Figure 4B**; **Supplementary Table 2**), *clr*-transformed abundance of 12 bacterial genera, including five Proteobacteria (*Comamonas, Variovorax, Acidovorax, Corallococcus, and Methylobacterium*) and unclassified Bacteria, had a mitigation effect on methane yield (Pearson correlation of −0.58 ± 0.21 to −0.64 ± 0.19 and regression coefficient from −1.08 [−2.21, 0.10] to −5.75 [−11.4, −0.25], $P_0 \geq 0.96$), while abundance of the four bacterial genera (*Dialister, Rothia, Succinimonas,* and *Oerskovia*) was associated with an increase in emissions (Pearson correlation from 0.54 ± 0.22 to 0.67 ± 0.17 and regression coefficient from 0.46 [−0.13, 0.99] to 1.90 [−0.13, 3.65], $P_0 \geq 0.95$). Under GRASS conditions (**Figure 4C**; **Supplementary Table 2**), methane yield was positively correlated with the bacterial genus *Pseudobutyrivibrio* and the archaea Unclassified *Euryarchaeota* (Pearson correlation of 0.64 ± 0.19 and 0.57 ± 0.22 and regression coefficient of 0.89 [0.07, 1.74] and 0.34 [−0.01, 0.74] $P_0 \geq 0.97$), whereas the Proteobacterial genera *Stenotrophomonas* and *Rhizobium* and Actinobacterial genera *Kineococcus* were negatively associated (Pearson correlation from −0.54 ± 0.22 to −0.66 ± 0.18 and regression coefficient from −1.00 [−1.96, 0.01] to −1.41 [−2.66, −0.11], $P_0 \geq 0.95$).

# Discussion

Because of the large contribution of ruminants to total greenhouse gas emissions from livestock (Gerber et al., 2013),

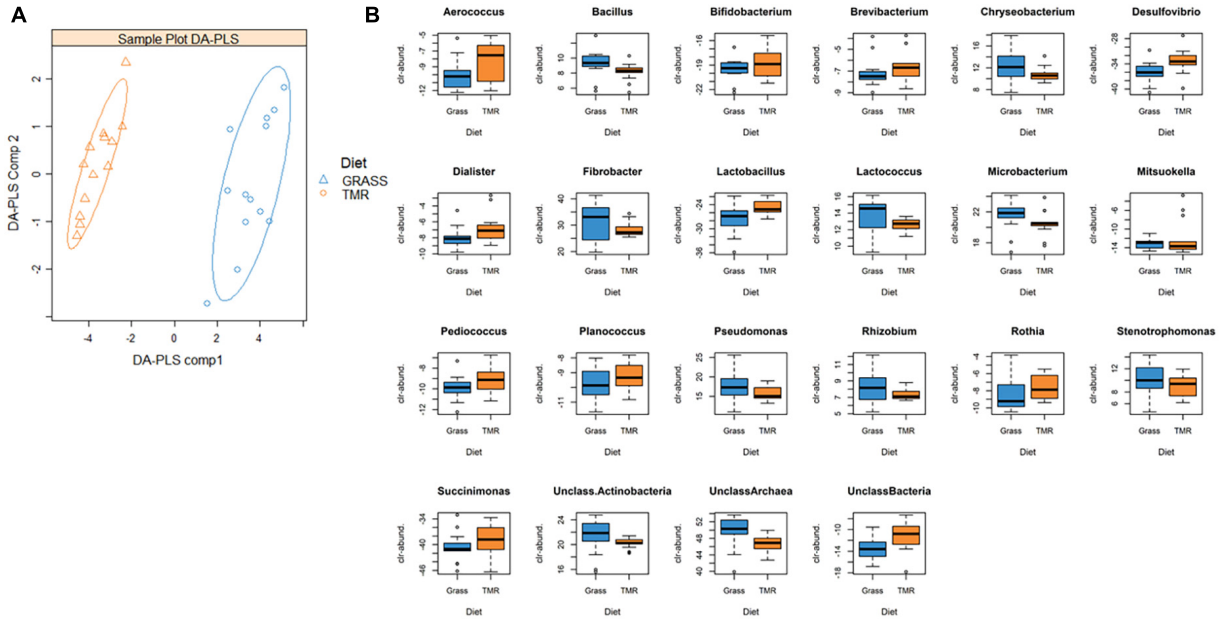


FIGURE 1
**(A)** Sample plot from a Discriminant by projection of latent structures analysis (DA-PLS) fitted to discriminate amongst the fresh cut grass (Grass) and high concentrate (TMR) fed animals based on 28 clr-transformed microbial genera abundances. The model was built with a single component and presented a miss-classification rate of 0%. For visualization purposes, sample plot is based on two components. **(B)** Microbial genera identified by DA-PLS which presented differential abundances between grass and TMR (probability of the difference of being different from 0 $\geq$ 0.95). Differences are expressed in units of clr-transformed abundances. Full details of the PLS analysis and linear models can be found in **Supplementary Table 1**.

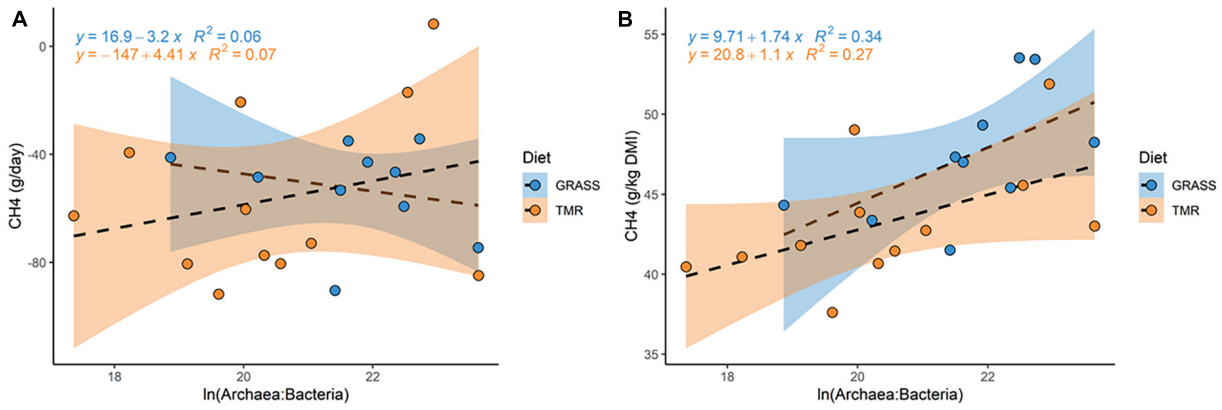


FIGURE 2
Data distribution of methane emissions [expressed as **(A)** methane production (g/day) or **(B)** methane yield (g/kg DMI)] and the natural log-ratio between archaea and bacteria abundances within animals offered two contrasting diets, high concentrate (TMR) or fresh cut grass (Grass). Methane and natural log-ratio between archaea and bacteria abundances was pre-corrected by fixed effects of breed, and body weight as a covariate.

there is urgent interest in reducing enteric methane emissions and minimizing the environmental impact of beef and dairy farming. Beef cattle are raised worldwide under a variety of different conditions and feeding systems that need to be considered when developing methane mitigation strategies, especially if they are based on microbiome measurements, in which diet has a major impact. In this study, we provide insight into how microbial genera abundances associated with methane emissions differ between extreme diets, and therefore which pose the best proxies for predicting the trait under specific diet types. The influence of these extreme diets (high concentrate and fresh cut grass) on microbial biota have rarely been directly compared (McGovern et al., 2020), and never, as far as we are aware, with a low-quality grass.

As a target for mitigation, methane emissions can be expressed as daily production (g/day) or relative to inputs (e.g., methane yield, g/kg DMI) or outputs (e.g., methane intensity, g/kg of meat), among others (de Haas et al., 2017). The obvious issue with targeting methane production is that it is correlated with feed intake. Our study showed that methane yield was more closely related to microbiota parameters (A:B) than methane production (g/day), especially on the poor quality grass diet, and therefore it might be more appropriate to consider it as a methane

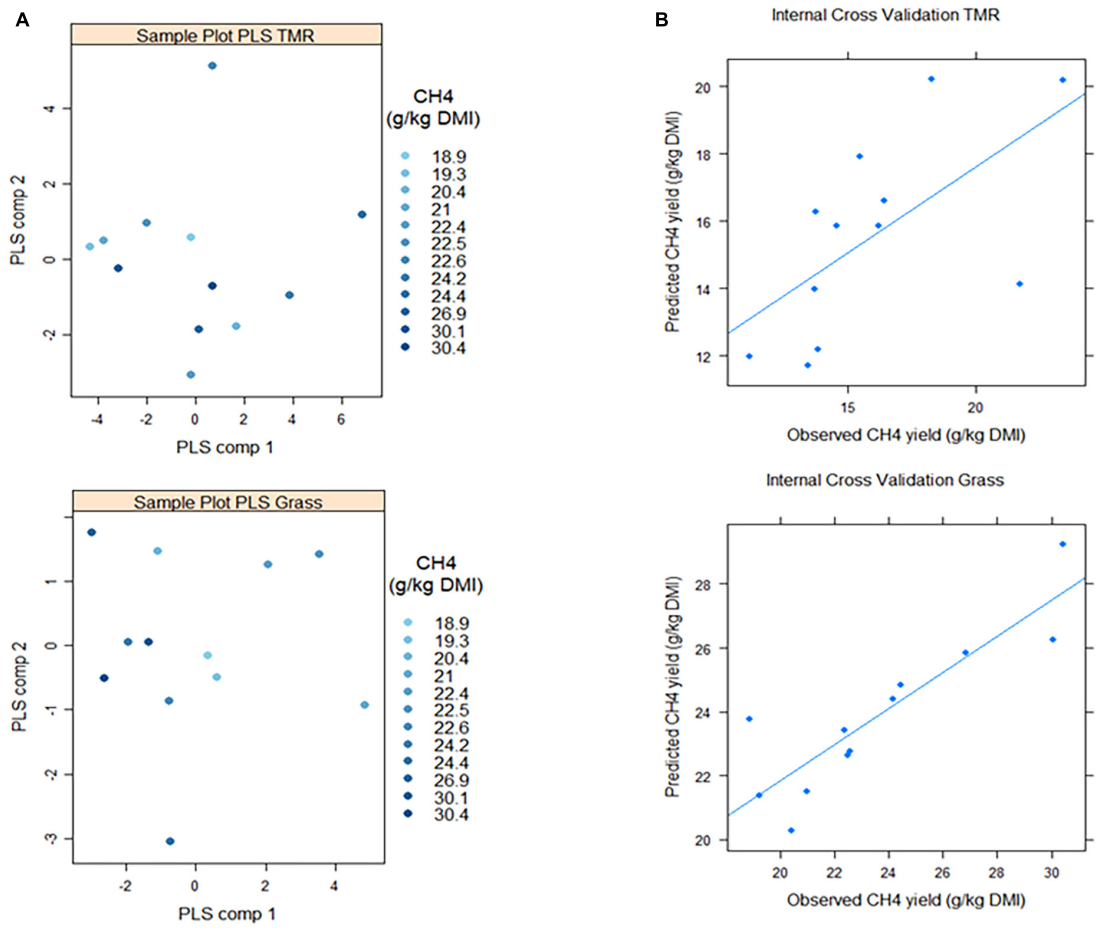


FIGURE 3
Results from linear projection of latent structures regression (PLS) models aiming to predict methane yield (g/kg DMI) based on 28 or 25 *clr*-transformed microbial genera in fresh cut grass (Grass) or high concentrate (TMR) fed animals. Full details of the PLS models can be found in **Supplementary Table 2**. **(A)** Sample plots (both PLS models were built with one single component, but two components were fitted only for visualization purposes). **(B)** After a threefold cross-validation procedure repeated 20 times, methane yield was predicted with 85 ± 8% (Grass) and 66.5 ± 9% prediction accuracy.

measurement when searching for microbiome proxies targeting methane mitigation.

As expected, diet had a strong effect on methane yield (Lovett et al., 2003; Aguerre et al., 2011; Troy et al., 2015; Olijhoek et al., 2018). Animals fed low quality fresh cut grass had 10.6 g of methane/kg of dry matter intake (73%) higher methane yield than those fed a high concentrate TMR. This is well explained in the literature by the passage rate of grain solids being faster than those of forage, and concentrate diets being richer in starch and proteins. Higher passage rate, together with fermentation of starch, are associated with fermentation pathways that lead to more propionate and less hydrogen available for archaea to reduce carbon dioxide to methane, in comparison to slower passage rates and fermentation of fiber in forage diets (Wolin, 1979; Janssen, 2010). Diet composition is also a critical factor in microbial ecology (Gruninger et al., 2014), as it determines the rumen conditions that favor the adaptation and growth of specific microbial species. Our extreme diets altered the ratio between the abundance of archaea and bacteria, with the poor quality fresh cut grass having a detrimental impact on the bacterial community.

Diet had a strong influence on the composition of the microbiota, even when analyzed at lower taxonomic levels, with 28 genera perfectly discriminating between the two extreme diets. However, the observed changes in methane yield and microbiota composition caused by the diet were not necessarily related. For example, we found that animals fed a poor quality fresh cut grass had significantly increased relative abundance of *Bacillus*, *Lactococcus, Pseudomonas, Microbacterium*, and unclassified *Actinobacteria* and a significant decrease in *Mitsuokella, Planococcus*, *Lactobacillus, Desulfovibrio,* and *Aerococcus* compared with TMR-fed animals. However, none of these abundances were selected in a PLS model to predict methane yield in each diet group. This can be explained by the fact that diet alters rumen conditions, which affects the microbial genera associated with methane emissions, but also other non-related microbial genera. It should be noted that these results may reflect an actual lack of methane-microbe associations, or a minor effect not captured in this study due to lack of power (loss of data points due to mechanical chamber failure).

The lack of a strong correlation of methane yield and *Methanobrevibacter* abundance could be due to the fact

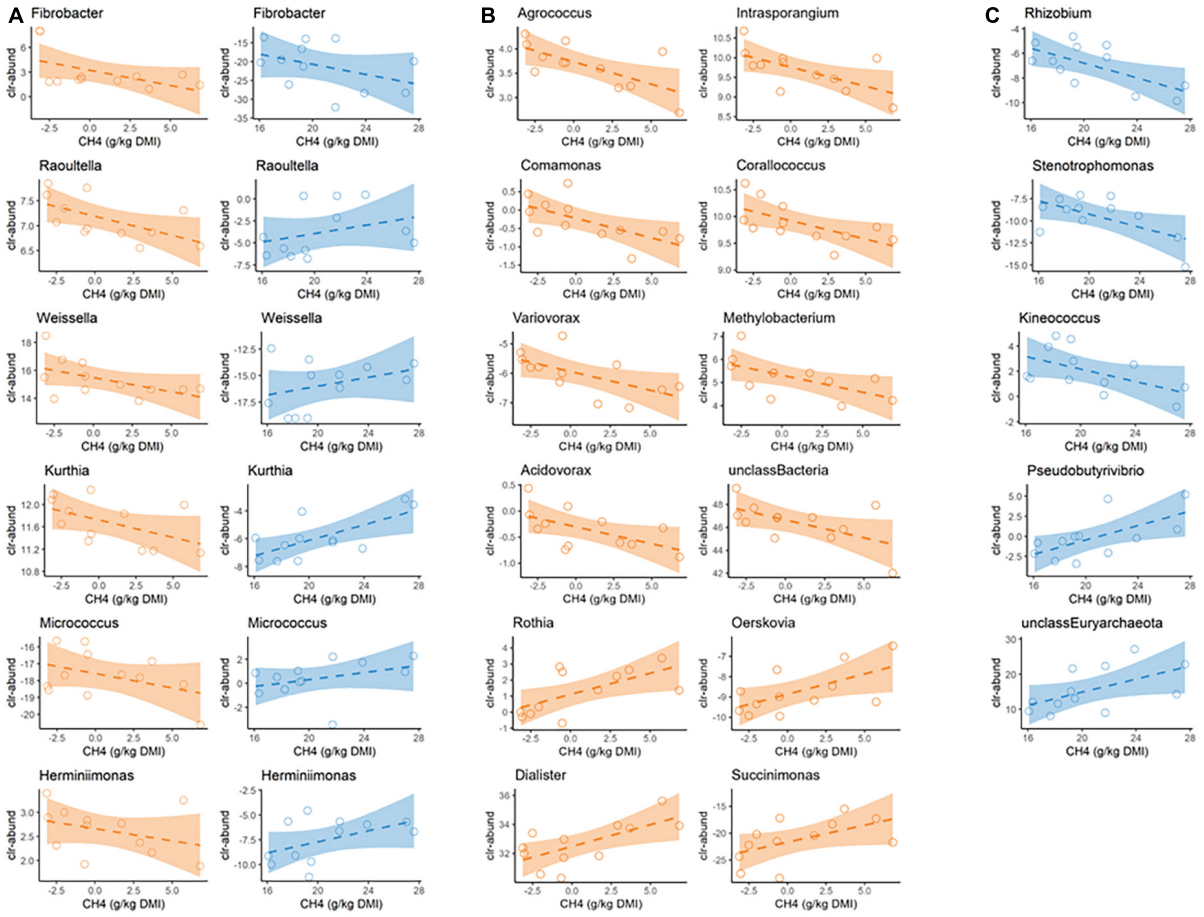


FIGURE 4
Visualization of linear associations between *clr*-transformed abundances of microbial genera identified with PLS analysis and methane yield in fresh cut grass (Grass, in blue) or high concentrate (TMR, in orange) fed animals. Full details of the analysis can be found in **Supplementary Table 2**. **(A)** Six microbial genera were selected in the PLS model in both diet groups, although only *Fibrobacter* was associated with methane yield in the same direction. **(B,C)** Microbial genera presenting a linear regression coefficient with a probability of being different from 0 ≤ 0.95 on methane yield only under TMR **(B)** or grass **(C)** diets.

that different *Methanobrevibacter* strains classified within *Methanobrevibacter* genera are functionally versatile and therefore have different effects on methane yield, as observed in Kittelmann et al. (2013) and Martínez-Álvaro et al. (2022). In other cases, diet significantly altered the abundance of certain microbial genera that were associated with methane yield (selected in PLS) and therefore could help explain the 73% difference between diets in methane yield. For example, the abundance of Unclassified Bacteria was relevantly increased in the high concentrate diet and had a significant mitigation effect on methane yield on this diet group. On the other hand, *Bifidobacterium* was increased in the high concentrate diet but had a positive effect on methane emissions under a poor quality fresh cut grass diet, although the regression coefficient was different from zero with only a moderate probability. *Bifidobacterium* species produce lactic acid and acetic acid, fermentation products usually associated with increased hydrogen production, which potentially increases the synthesis of methane (Moss et al., 2000; Danielsson et al., 2017). Another example is the abundance of the dominant cellulolytic genus *Fibrobacter*, which was increased in animals fed the low-quality grass diet and showed predictive ability and a negative correlation with methane yield in both diet groups, with stronger effect in the high concentrate diet. In their study, Kittelmann et al. (2013) inferred a co-occurrence of bacteria from the *Fibrobacteaceae* family, including the major cellulolytic bacterium *Fibrobacter succinogenes*, which produces only formate and no $H_2$ (Rychlik and May, 2000), and hydrogenotrophic- methanogenesis was reduced (Smith and Hungate, 1958; Leahy et al., 2013). In contrast, bacteria from the *Ruminococcaceae* family, some of which produce large amounts of $H_2$ (e.g., *Ruminococcus* sp.), co-occurred with *M. gottschalkii*, which is capable of producing methane from $H_2$ and $CO_2$ but not from formate (Miller and Lin, 2002). Later, the same authors associated greater abundance of *Fibrobacter* sp. with a low methane emitting ruminotype and greater abundance of *sp.* from *Ruminococcus* genus and other *Ruminococcaceae* with a high methane emitting ruminotype (Kittelmann et al., 2014). These studies may explain our negative association between *Fibrobacter* and methane yield observed in both diets; although *Ruminococcus* genus abundance was not associated with methane yield in any case (data not shown).

Except for *Fibrobacter,* the few microbial genera that were predictive of methane yield under both diets had different effects

on methane yield. In addition, most microbial genera that were predictive of methane yield differed between diets, suggesting that the main microbial drivers of methane yield depend on the products available for substrates microbial fermentation. On the high concentrate diet, several genera from the *Proteobacteria* phylum (*Acidovorax, Comamonas, Corallococcus, Variovorax*, and *Methylobacterium*) were negatively associated with methane yield along with other unclassified Bacteria. In the low-quality fresh cut grass group, some Proteobacteria were also negatively associated with methane yield, but they belonged to other genera (*Stenotrophomonas* and *Rhizobium*). The abundance of some of these genera from the Proteobacteria phylum has been negatively associated with methane in the literature (Wallace et al., 2015; Danielsson et al., 2017; Auffret et al., 2018; Martínez-Álvaro et al., 2020). The explanation might be based on the fact that these *Proteobacteria* genera are methanotrophic in the rumen (e.g., *Methylobacterium*); or capable of fixing nitrogen (e.g., *Rhizobium*), at least in the host plant (Munoz Aguilar et al., 1988). In the rumen, $N_2$ reduction to ammonia may act as an alternative $H_2$ consuming sink competing with ruminal methanogenesis (Bulen and LeComte, 1966). On a low-quality fresh cut grass diet, unclassified *Euryarchaeota*, *Pseudobutyrivibrio* and *Kurthia* had a strong effect increasing methane emissions, which we did not observe in the high concentrate diet. Instead, *Succinimonas*, *Oerskovia, Rothia* and *Dialister* had the positive and strongest effect increasing methane yield. Methanogenic archaea belong to the *Euryarchaeota* phylum, while *Pseudobutyrivibrio* plays an important role in the release of formate (Tapio et al., 2017) and other by-products such as butyrate, known to be associated with an increase in methane emissions (Kamke et al., 2016). Only in a few cases did we observed a changing effect of the abundance of a particular microbe under different diets (e.g., *Kurthia* or *Weissella,* see **Figure 4A**); but only for *Kurthia* the regression coefficients were different from 0 in both diets and allows to confirm a diet-microbiota cross-over interaction.

In this study, we found that different microbes drive emissions when hosts are fed with diets extreme in their forage to concentrate ratio; therefore, different mitigation strategies targeting specific microbial mechanisms must be adequate to each feeding system. To date, targeted sequencing of specific regions of the 16S gene is the most cost-effective method, although it may not provide high taxonomic resolution compared to metagenomic sequencing. Nevertheless, in our study, we achieved high accuracy in predicting methane yield for both the poor-quality grass and high concentrate TMR dietary scenarios, with a PLS algorithm using almost all different microbial abundances. Our results are most relevant to the development of strategies to reduce methane emissions based on changes in the microbiome, as different diets determine which microbial taxa have the greatest impact on methane yield, and diet-specific strategies should be considered. Our study is a small-scale study with the main goal of identifying the difference in microbiota compositions between two extreme diets and its use for prediction of methane emissions. This study draws attention to the need for different mitigation strategies adapted to different diet types, but further larger scale studies are required to understand diet-microbiome interactions that influence methane emissions. Also, microbiome based methane predictions need to ben locally validated the ensure factors such as local dietary factors are not skewing the results.

## Conclusion

The divergent impacts on the rumen microbial composition of extreme diets, in this case high concentrate and low-quality grass, have rarely been directly compared. These differences in microbial composition can be used to predict methane yield of individual animals. Using two groups of beef cattle fed with two extreme diets on their forage to concentrate ratio, we found a reduced set of microbial genera with a high predictive accuracy for methane yield of 85 and 66% in the forage or concentrate-based diets. Among the microbial genera that predicted methane yield, there was little overlap between diets, and most microbial genera were diet specific. This finding is critical for the development of mitigation strategies based on the microbiome, where, according to this study, diet-specific strategies should be considered at least at phenotypic level.

## 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.ebi.ac.uk/ena/browser/home, PRJEB57703.

## Ethics statement

This animal trials described below were approved by the Animal Experiment Committee of SRUC and were conducted in accordance with the requirements of the UK Animals (Scientific Procedures) Act 1986.

## Author contributions

GM: conceptualization, methodology, investigation, and writing—original draft. MA: conceptualization, methodology, review, and editing. RR: conceptualization, supervision, review, and editing. HN: methodology and investigation. MM-Á: software, formal analysis, and writing—original draft. All authors reviewed and approved the manuscript.

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## Conflict of interest

MA was employed by Agrifirm.

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/fmicb.2023.1102400/full#supplementary-material

## References


Agriculture and Horticulture Development Board, (2020). *Dairy beef production systems*. Available online at: https://ahdb.org.uk/knowledge-library/dairy-beef-production-systems (accessed August 15, 2022).

Aguerre, M. J., Wattiaux, M. A., Powell, J. M., Broderick, G. A., and Arndt, C. (2011). Effect of forage-to-concentrate ratio in dairy cow diets on emission of methane, carbon dioxide, and ammonia, lactation performance, and manure excretion. *J. Dairy Sci.* 94, 3081–3093. doi: 10.3168/jds.2010-4011

Auffret, M. D., Stewart, R., Dewhurst, R. J., Duthie, C. A., Rooke, J. A., Wallace, R. J., et al. (2018). Identification, comparison, and validation of robust rumen microbial biomarkers for methane emissions using diverse *Bos Taurus* breeds and basal diets. *Front. Microbiol.* 8:1–15. doi: 10.3389/fmicb.2017.02642

Blasco, A. (2017). *Bayesian data analysis for animal scientists*. Vol. 265. New York, NY: Springer.

Bulen, W. A., and LeComte, J. R. (1966). The nitrogenase system from azotobacter: two-enzyme requirement for N2 reduction, ATP-dependent H2 evolution, and ATP hydrolysis. *Proc. Natl. Acad. Sci. U.S.A.* 56, 979–986. doi: 10.1073/pnas.56.3.979

Cottle, D. J., Nolan, J. V., and Wiedemann, S. G. (2011). Ruminant enteric methane mitigation: a review. *Animal Produ. Sci.* 51, 491–514. doi: 10.1071/AN10163

Danielsson, R., Dicksved, J., Sun, L., Gonda, H., Mller, B., Schnrer, A., et al. (2017). Methane production in dairy cows correlates with rumen methanogenic and bacterial community structure. *Front. Microbiol.* 8:226. doi: 10.3389/fmicb.2017.00226

de Haas, Y., Pszczola, M., Soyeurt, H., Wall, E., and Lassen, J. (2017). Invited review: phenotypes to genetically reduce greenhouse gas emissions in dairying. *J. Dairy Sci.* 100, 855–870. doi: 10.3168/jds.2016-11246

Dijkstra, J., Bannink, A., France, J., Kebreab, E., and van Gastelen, S. (2018). Short communication: antimethanogenic effects of 3-nitrooxypropanol depend on supplementation dose, dietary fiber content, and cattle type. *J. Dairy Sci.* 101, 9041–9047. doi: 10.3168/jds.2018-14456

Duthie, C.-A., Haskell, M., Hyslop, J. J., Waterhouse, A., Wallace, R. J., Roehe, R., et al. (2017). The impact of divergent breed types and diets on methane emissions, rumen characteristics and performance of finishing beef cattle. *Animal* 2017, 1–10. doi: 10.1017/S1751731117000301

Evans, P. N., Boyd, J. A., Leu, A. O., Woodcroft, B. J., Parks, D. H., Hugenholtz, P., et al. (2019). An evolving view of methane metabolism in the Archaea. *Nat. Rev. Microbiol.* 17, 219–232. doi: 10.1038/s41579-018-0136-7

Gerber, P. J., Steinfeld, H., Henderson, B., Mottet, A., Opio, C., Dijkman, J., et al. (2013). *Tackling climate change through livestock – a global assessment of emissions and mitigation opportunities*. Rome: Food and Agriculture Organization of the United Nations.

Gloor, G. B., Macklaim, J. M., Pawlowsky-Glahn, V., and Egozcue, J. J. (2017). Microbiome datasets are compositional: and this is not optional. *Front. Microbiol.* 8:2224. doi: 10.1080/01904168209363016

Greenacre, M. (2019). "Compositional data analysis in practise," in *Chapman & hall/crc interdisciplinary statistics series*, eds P. van der, H. N. Keiding, B. J. T. Morgan, and C. K. Wikle (Boca Raton FL: CRC Press Taylor & Francis Group).

Greenacre, M., Martínez-Álvaro, M., and Blasco, A. (2021). Compositional data analysis of microbiome and any-omics datasets: a validation of the additive logratio transformation. *Front. Microbiol.* 12:727398. doi: 10.3389/fmicb.2021.727398

Gruninger, R. J., Puniya, A. K., Callaghan, T. M., Edwards, J. E., Youssef, N., Dagar, S. S., et al. (2014). Anaerobic fungi (phylum neocallimastigomycota): advances in understanding their taxonomy, life cycle, ecology, role and biotechnological potential. *FEMS Microbiol. Ecol.* 90, 1–17. doi: 10.1111/1574-6941.12383

Gruninger, R. J., Ribeiro, G. O., Cameron, A., and Mcallister, T. A. (2019). Invited review?: application of meta-omics to understand the dynamic nature of the rumen microbiome and how it responds to diet in ruminants. *Animal Int. J. Animal Biosci.* 13, 1843–1854. doi: 10.1017/S1751731119000752

Janssen, P. H. (2010). Influence of hydrogen on rumen methane formation and fermentation balances through microbial growth kinetics and fermentation thermodynamics. *Animal Feed Sci. Technol.* 160, 1–22. doi: 10.1016/j.anifeedsci.2010.07.002

Kamke, J., Kittelmann, S., Soni, P., Li, Y., Tavendale, M., Ganesh, S., et al. (2016). Rumen metagenome and metatranscriptome analyses of low methane yield sheep reveals a Sharpea-enriched microbiome characterised by lactic acid formation and utilisation. *Microbiome* 4, 1–16. doi: 10.1186/s40168-016-0201-2

Kittelmann, S., Pinares-Patiño, C. S., Seedorf, H., Kirk, M. R., Ganesh, S., McEwan, J. C., et al. (2014). Two different bacterial community types are linked with the low-methane emission trait in sheep. *PLoS One* 9:1–9. doi: 10.1371/journal.pone.0103171

Kittelmann, S., Seedorf, H., Walters, W. A., Clemente, J. C., Knight, R., Gordon, J. I., et al. (2013). Simultaneous amplicon sequencing to explore co- occurrence patterns of bacterial, archaeal and eukaryotic microorganisms in rumen microbial communities. *PLoS One* 8:47879. doi: 10.1371/journal.pone.0047879

Lê Cao, K.-A., and Welham, Z. M. (2022). *Multivariate data integration using r methods and applications with the mixomics package*. Boca Raton FL: CRC Press, Taylor & Francis group.

Leahy, S. C., Kelly, W. J., Ronimus, R. S., Wedlock, N., Altermann, E., and Attwood, G. T. (2013). Genome sequencing of rumen bacteria and archaea and its application to methane mitigation strategies. *Animal Int. J. Animal Biosci.* 7, 235–243. doi: 10.1017/S1751731113000700

Li, R., Teng, Z., Lang, C., Zhou, H., Zhong, W., Ban, Z., et al. (2019). Effect of different forage-to-concentrate ratios on ruminal bacterial structure and real-time methane production in sheep. *PLoS One* 14:e0214777. journal.pone.0214777 doi: 10.1371/

Lovett, D., Lovell, S., Stack, L., Callan, J., Finlay, M., Conolly, J., et al. (2003). Effect of forage/concentrate ratio and dietary coconut oil level on methane output and performance of finishing beef heifers. *Livestock Produ. Sci.* 84, 135–146. doi: 10.1016/j.livprodsci.2003.09.010

Manzanilla-Pech, C. I. V., Stephansen, R. B., Difford, G. F., Løvendahl, P., and Lassen, J. (2022). Selecting for feed efficient cows will help to reduce methane gas emissions. *Front. Genet.* 13:1–10. doi: 10.3389/fgene.2022.885932

Martínez-Álvaro, M., Auffret, M. D., Stewart, R. D., Dewhurst, R. J., Duthie, C.-A., Rooke, J. A., et al. (2020). Identification of complex rumen microbiome interaction within diverse functional niches as mechanisms affecting the variation of methane emissions in bovine. *Front. Microbiol.* 11:1–13. doi: 10.3389/fmicb.2020.00659

Martínez-Álvaro, M., Auffret, M., Duthie, C., Dewhurst, R., Cleveland, M., Watson, M., et al. (2022). Bovine host genome acts on rumen microbiome function linked to methane emissions. *Commun. Biol.* 5:350. doi: 10.1038/s42003-022-03293-0

Martínez-Álvaro, M., Zubiri-Gaitán, A., Ferrer, A., Blasco, A., and Greenacre, M. (2021). Comprehensive functional core microbiome comparison in genetically obese and lean hosts under the same environment. *Commun. Biol.* 4:1246. doi: 10.1038/s42003-021-02784-w

McGovern, E., McGee, M., Byrne, C. J., Kenny, D. A., Kelly, A. K., and Waters, S. M. (2020). Investigation into the effect of divergent feed efficiency phenotype on the bovine rumen microbiota across diet and breed. *Sci. Rep.* 10, 1–11. doi: 10.1038/s41598-020-71458-0

Miller, T. L., and Lin, C. (2002). Description of methanobrevibacter gottschalkii sp. nov., methanobrevibacter thaueri sp. nov. *Int. J. Syst. Evolut. Microbiol.* 52, 819–822. doi: 10.1099/ijs.0.02022-0.02022

Moss, A., Jouany, J., Newbold, J., Moss, A., Jouany, J., and Newbold, J. (2000). Methane production by ruminants?: its contribution to global warming. *Ann. Zoot.* 49, 231–253. doi: 10.1051/animres:2000119

Munoz Aguilar, J. M., Ashby, A. M., Richards, A. J. M., Loake, G. J., Watson, M. D., and Shaw, C. H. (1988). Chemotaxis of rhizobium leguminosarum biovar phaseoli towards flavonoid inducers of the symbiotic nodulation genes. *Microbiology* 134, 2741–2746. doi: 10.1099/00221287-134-10-2741

Olijhoek, D. W., Løvendahl, P., Lassen, J., Hellwing, A. L. F., Höglund, J. K., Weisbjerg, M. R., et al. (2018). Methane production, rumen fermentation, and diet digestibility of holstein and jersey dairy cows being divergent in residual feed intake and fed at 2 forage-to-concentrate ratios. *J. Dairy Sci.* 101, 9926–9940. doi: 10.3168/jds.2017-14278

Palarea-Albaladejo, J., and Martín-Fernández, J. A. (2015). ZCompositions - r package for multivariate imputation of left-censored data under a compositional approach. *Chemometr. Intelli. Laborat. Syst.* 143, 85–96. doi: 10.1016/j.chemolab.2015.02.019

Roesch, L. F. W., Dobbler, P. T., Pylro, V. S., Kolaczkowski, B., Drew, J. C., and Triplett, E. W. (2020). pime: a package for discovery of novel differences among microbial communities. *Mol. Ecol. Res.* 20, 415–428. doi: 10.1111/1755-0998.13116

Rubiola, S., Macori, G., Civera, T., Fanning, S., Mitchell, M., and Chiesa, F. (2022). Comparison between full-length 16s RRNA metabarcoding and whole metagenome sequencing suggests the use of either is suitable for large-scale microbiome studies. *Foodborne Pathogens Dis.* 19, 495–504. doi: 10.1089/fpd.2022.0027

Rychlik, J. L., and May, T. (2000). The effect of a methanogen, methanobrevibacter smithii, on the growth rate, organic acid production, and specific ATP activity of three predominant ruminal cellulolytic bacteria. *Curr. Microbiol.* 40, 176–180. doi: 10.1007/s002849910035

Shi, W., Moon, C. D., Leahy, S. C., Kang, D., Froula, J., Kittelmann, S., et al. (2014). Methane yield phenotypes linked to differential gene expression in the sheep rumen microbiome. *Gen. Res.* 24, 1517–1525. doi: 10.1101/gr.168245.113

Smith, P. H., and Hungate, R. E. (1958). Isolation and charcaterization of methanobacterium ruminatum n sp. *J. Bacteriol.* 75, 713–718.

Sorensen, D., and Gianola, D. (2002). *Likelihood, bayesian, and MCMC methods in quantitative genetics*. New York: Springer.

Tapio, I., Snelling, T. J., Strozzi, F., and Wallace, R. J. (2017). The ruminal microbiome associated with methane emissions from ruminant livestock. *J. Animal Sci. Biotechnol.* 8:7. doi: 10.1186/s40104-017-0141-0

Troy, S. M., Duthie, C., Hyslop, J. J., Roehe, R., Ross, D. W., Wallace, R. J., et al. (2015). Effectiveness of nitrate addition and increased oil content as methane mitigation strategies for beef cattle fed two contrasting basal diets 1. *J. Animal Sci.* 93, 1815–1823. doi: 10.2527/jas2014-8688

Tseten, T., Sanjorjo, R. A., Kwon, M., and Kim, S. (2022). Strategies to mitigate enteric methane emissions from ruminant animals. *J. Microbiol. Biotechnl.* 32, 269–277. doi: 10.4014/jmb.2202.02019

van Breukelen, A. E., Aldridge, M. N., Veerkamp, R. F., Koning, L., Sebek, L. B., and de Haas, Y. (2022). "Combining measurements of enteric CH4 emissions from two non-invasive sensors for genetic evaluations," in *Proceeding of the WIAS annual conference*, (Wageningen), 60.

Wallace, R. J., Rooke, J. A., McKain, N., Duthie, C.-A., Hyslop, J. J., Ross, D. W., et al. (2015). The rumen microbial metagenome associated with high methane production in cattle. *BMC Geno.* 16:839. doi: 10.1186/s12864-015-2032-0

Westerhuis, J. A., Hoefsloot, H. C. J., Smit, S., Vis, D. J., Smilde, A. K., Velzen, E. J. J., et al. (2008). Assessment of PLSDA cross validation. *Metabolomics* 4, 81–89. doi: 10.1007/s11306-007-0099-6

Wolin, M. J. (1979). The rumen fermentation?: a model for microbial interactions in anaerobic ecosystems. *Adv. Microbial Ecol.* 3, 49–77.

Yu, Z., and Morrison, M. (2004). Improved extraction of PCR-quality community DNA from digesta and fecal samples. *BioTechniques* 36, 808–812. doi: 10.2144/04365st04

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# Effects of dietary crude protein levels in the concentrate supplement after grazing on rumen microbiota and metabolites by using metagenomics and metabolomics in Jersey-yak

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**Introduction:** The crude protein level in the diet will affect the fermentation parameters, microflora, and metabolites in the rumen of ruminants. It is of great significance to study the effect of crude protein levels in supplementary diet on microbial community and metabolites for improving animal growth performance. At present, the effects of crude protein level in supplementary diet on rumen fermentation parameters, microbial community, and metabolites of Jersey-Yak (JY) are still unclear.

**Methods:** The purpose of this experiment was to study the appropriate crude protein level in the diet of JY. The rumen fermentation indexes (volatile fatty acids and pH) were determined by supplementary diets with crude protein levels of 15.16 and 17.90%, respectively, and the microbial community and metabolites of JYs were analyzed by non-target metabonomics and metagenome sequencing technology, and the changes of rumen fermentation parameters, microbial flora, and metabolites in the three groups and their interactions were studied.

**Results and Discussion:** The crude protein level in the supplementary diet had significant effects on pH, valeric acid, and the ratio of acetic acid to propionic acid ($p < 0.05$). The protein level had no significant effect on the dominant microflora at the phylum level ($p > 0.05$), and all three groups were Bacteroides and Firmicutes. The results of metabolite analysis showed that the crude protein level of supplementary diet significantly affected the metabolic pathways such as Bile secretion and styrene degradation ($p < 0.05$), and there were different metabolites between the LP group and HP group, and these different metabolites were related to the dominant microbial to some extent. To sum up, in this experiment, the effects of crude protein level in supplementary diet on rumen microorganisms and metabolites of JY and their relationship were studied, which provided the theoretical basis for formulating a more scientific and reasonable supplementary diet in the future.

KEYWORDS

**protein level, compensatory feeding, rumen microbial, metagenome, metabolome, Jersey-Yak**

# 1. Introduction

Yak, as a unique animal species living on the Qinghai-Tibet Plateau, provides basic means of production and living for local herdsmen (Qiu et al., 2012). In order to improve the production performance of yak, artificial insemination was utilized to improve yak's hybridization with high-quality frozen semen of Jersey and Angus cattle etc. However, the Qinghai-Tibet Plateau is in the grass-withering period from November to May of the following year. Climate change will affect the quality (mainly the content level of crude protein) and yield of forage grass. The nutrition in forage grass is not enough to meet the nutritional needs of yak and its hybrid offspring. Both yak and its hybrid offspring will suffer from a decline in productivity due to insufficient nutrition intake, and most importantly, they will reduce their weight, thus reducing the economic benefits of herders (Zhao and Zhou, 1999; Xue et al., 2005; Xu et al., 2017). Therefore, supplementary feeding during the dry season can appropriately reduce the loss of herders, and a reasonable protein level in the diet is one of the crucial measures to improve the growth performance and weight of animals.

As one of the main digestive organs for ruminants to degrade and digest the feed, the rumen is rich in microbial communities such as archaea, bacteria, fungi, and protozoa to ferment the feed (Zhao et al., 2022; Wang et al., 2022a; Xue et al., 2022a). The rich microbial community is closely related to the digestion and metabolism of the host feed, which can degrade and ferment organic substances such as plant fibers and polysaccharides into volatile fatty acids (VFAs), which are the principal energy sources of the host (Liu et al., 2021; Chen et al., 2022a; Xue et al., 2022a). Among them, the structure and function of the rumen microbial community are affected by many factors, such as variety, age, feeding mode, feeding level, etc. (Zhang et al., 2019; Li et al., 2020; Huang et al., 2021). 16S rRNA and metabonomics were used to study the bacterial composition in the rumen, and it was found that the nutritional level of diet could significantly change the relative abundance of dominant bacteria and metabolites in the rumen (Granja-Salcedo et al., 2016; Wang et al., 2020a, 2022b; Yi et al., 2022a). The level of protein in the diet will not only affect the composition of microorganisms and metabolites in the rumen but also affect the rumen fermentation indexes and serum biochemical indexes of the host (Wang et al., 2020b, 2022b).

Although the effects of dietary nutrient levels on rumen microbials and metabolites in ruminants have been extensively studied, most of them are limited to the level of rumen bacteria using techniques such as 16S rRNA. Moreover, due to the differences in digestive and metabolic characteristics and environmental adaptability between Jersey cattle, yak, and other cattle species, the effects on microbial communities and metabolites may also be different (Latham et al., 2018; Li et al., 2022; Pang et al., 2022a). With the deepening of microbial research, people gradually combine metagenomics and metabonomics to explore the effects of dietary nutrition level on host traits and rumen microbial community, (Xue et al., 2020, 2022a). Compared with 16S rRNA technology, the metagenomic can not only study the composition and diversity of bacteria in the host microbial but also conduct in-depth research on gene and function (Ghurye et al., 2016). However, when studying the effect of dietary nutrition level on Jersey-Yak (JY, hybrid offspring of female yaks by artificial insemination with high-quality frozen semen of Jersey), metagenomics and metabolomics have not been used for multi-omics research.

Therefore, in this study, in order to verify the influence of protein level in supplementary diet on rumen microbial community structure and metabolites of JYs, and the data of metagenomics, rumen metabolomics and serum metabolomics were integrated for joint analysis, the influence of protein level in supplementary diet on growth index and rumen fermentation parameters of JY was discussed. The correlation between different metabolites in serum and rumen and dominant microflora in rumen was analyzed, and the microflora and metabolism were discussed. Provide more scientific guidance for fattening Jersey, and help herders acquired more economic benefits.

# 2. Materials and methods

The animal experiments involved in this experiment were approved by the Lanzhou Institute of Husbandry and Pharmaceutical Sciences of the Chinese Academy of Agricultural Sciences (CAAS; approval number: 1610322020018). All sampling procedures are strictly in accordance with the Guidelines for Ethical Treatment of Experimental Animals in China.

## 2.1. Animal and experimental design

The experiment was conducted in Xiahe County, Gannan Tibetan Autonomous Prefecture. In this experiment, 18 healthy 6-month-old male JYs were selected, and their weight before the preliminary experiment was 62.20 ± 2.64 kg. According to the weighing data before the pre-experiment, 18 JYs were randomly divided into three treatment groups by R software (Version 4.1.2). Feeding management is as follows: each group grazes at 9:00 a.m. and 6:00 p.m. every day, all grazing in the same pasture. After grazing every day, each group was raised in a separate column, and then the treatments of the three groups were: (1) No supplementary feeding, grazing (as control group, Control); (2) Supplementing low-protein diet (protein content: 15.16%; LP); (3) Supplementing high-protein diet (protein content: 17.90%; HP). See Supplementary material S1 for the composition and content of supplementary diet. Among them, the supplementary feeding amount is adjusted according to the monthly weighing data of Juangu cattle, and the monthly supplementary feeding amount is calculated according to 1.2% of the weight of JY in the HP and LP group.

This experiment includes two stages: pre-experiment (15 days) and formal experiment (120 days). The purpose of the experiment is to make the experimental animals adapt to the supplementary diet in advance. After the end of the pre-experiment and the beginning of the formal experiment, the weight of JY was measured regularly every month.

## 2.2. Sample collection

After the formal experiment, JYs in each group fasted for 1 day. The blood of each JY was collected by jugular vein puncture with a blood collection tube (5 ml) without additives, then centrifuged at 4500 × *g* for

15 min with a portable medical centrifuge to separate the serum. The separated serum samples were stored in foam boxes with ice bags, transported back to the laboratory and stored in a refrigerator at −20°C for biochemical index analysis. After blood samples were collected, 150 ml rumen fluid samples of each JY were collected by oral stomach tube. After filtration, it was distributed into a 50 ml centrifuge tube, then immediately placed in a liquid nitrogen tank. After the sample was sent to the laboratory, it was stored in a refrigerator at −80°C for subsequent metagenome sequencing analysis and determination of VFAs.

## 2.3. Determination of rumen fermentation parameters

After the rumen fluid was collected, it was filtered with four layers of gauze, and then the pH of the filtered rumen fluid was measured with a portable pH meter. Each sample was repeated three times. Rumen liquid transported back to the laboratory was thawed on ice, and centrifuged for 15 min at 4°C and 10000 × *g* by low-temperature freezing centrifuge. The supernatant was transferred to a solution containing an internal standard (2-Ethylbutyric acid, 2 EB) and a concentration of 25% metaphosphoric acid. Then the concentration of VFAs was determined by gas chromatography using the method introduced by Erwin et al. (1961).

## 2.4. Determination and analysis of serum and rumen fluid non-target metabolome

The rumen fluid sample (100 μl) was sucked into a 1.5 ml centrifuge tube, and 400 ml of extractive solution (acetonitrile: methanol = 1:1) was added, respectively, for low-temperature ultrasonic extraction (5°C, 40 kHz); Standing at < 20°C for 30 min; Centrifuge at 4°C, 13000 × *g* for 15 min, remove the supernatant, add 100 μl complex solution (acetonitrile: water = 1:1) for redissolving, and perform low-temperature ultrasonic extraction; Centrifuge at 4°C and 13000 × *g* for 5 min, and suck the supernatant into the sample bottle for computer analysis.

The obtained raw data were filtered by the software Progenesis QI (Waters Corporation, Milford, United States) for baseline, peak identification, integration, and retention time correction, etc., and the data matrix of retention time, mass-to-charge ratio, and peak intensity was obtained. Then, the data matrix was compared with HMDB[1], and the information on metabolites was obtained. Data pre-processing: pre-processing the matrix data, mainly including missing value filtering, filling in vacant values, data normalization, quality control sample verification, and data conversion. During the verification of quality control samples, the data with relative standard deviation (RSD) > 30% are mainly deleted and not used for subsequent analysis.

According to the expression of metabolites in different groups of samples, partial least squares discriminant analysis (PLS-DA) was performed on the samples by using the "Vegan" package of R software (Version 1.6.2) to judge the degree of separation between the samples. According to the variable importance in the projection (VIP) value obtained by PLS-DA analysis, the Kruskal–Wallis H test was used to screen the different metabolites among the three groups. The difference metabolites between the LP group and HP were judged by the Wilcoxon rank sum test, and they were all realized by the "stats" package of R software, in which $p < 0.05$ was regarded as statistically significant. The annotation of metabolites was obtained by using the Kyoto Encyclopedia of Genes and Genomes (KEGG) and HMDB, and then the "scipy" in Python (Version 1.0.0) was used to analyze the metabolic pathway and enrichment of target metabolites.

## 2.5. DNA extraction, metagenome sequencing, and data processing

Total genomic DNA from rumen fluid was extracted by repeated bead beating plus column (RBB + C) method introduced by Yu and Morrison (2004). Then the quality of the extracted DNA was detected by 1% agarose gel electrophoresis, and the DNA concentration was measured by ultraviolet spectrophotometer. The sequencing of macro genome was completed in Shanghai Meiji Biomedical Technology Co., Ltd. Covaris M220 was used for fragmentation (about 400 bp in length), PE library was constructed according to the instructions of Next Flex Rapid DNA-SeqKit (Bioo Scientific, United States), Nova Seq Reagent Kits/His EQ X Reagent Kits were used for bridge PCR and Illumina Novaseq6000 instrument was used for sequencing. All the raw data were submitted to the NCBI Sequence Read Archive (SRA) database (Accession number: PRJNA904725).

Make statistics and quality control on the original reads obtained by sequencing, and obtain high-quality reads to ensure the accuracy of subsequent analysis. It mainly uses FASTP (Version 0.23.0, https://github.com/OpenGene/fastp) software to cut the adapter sequences at the 3′ and 5′ ends of the sequences and remove the low-quality reads (the length is less than 50 bp, the average mass value is less than 20, and it contains N bases). After quality control, the high-quality data were assembled by MEGAHIT (Version 1.1.2; contig ≥ 300 bp), and the open reading frame (ORF) in the splicing result was predicted by Prodigal (Version 2.6.3; the sequence length was > 100 bp), and it was translated into the amino acid sequence (Hyatt et al., 2010; Li et al., 2015). Using CD-HIT software (Version 4.6.1) to cluster the predicted gene sequences (identity > 90%, coverage > 90%) to obtain gene sets (Fu et al., 2012). Using SOAPaligal software (Version: 2.21), the gene abundance (RPKM) was calculated by comparing each sample with the gene set by formula (Li et al., 2009; Lawson et al., 2017).

Use Diamond software (Version 2.0.13) to compare the gene set with the RefSeq database (parameter: *E*-value ≤ 1*e*−5) to make a taxonomic annotation on rumen microorganisms at the phylogenetic level, and compare with KEGG to obtain the corresponding functional annotation information of the samples (Buchfink et al., 2015, 2021). The annotation of carbohydrate-active enzyme (CAZyme) genes was obtained by using HMMER (Version 3.1b2) software against carbohydrate-active enzyme database (hmmscan; *E*-value ≤ 1*e*−05).

According to the gene abundance calculated by the RPKM method, the microbial composition at the phylum and genus level was analyzed and mapped by using the vegan package in R (Version: 4.1.2). Column diagram was used to visualize the microbial composition of the three treatment groups at phylum and genus level. Among the three treatment groups, microbials with significant differences between the LP group and the HP group were subjected to the Kruskal–Wallis H test by using the stats package of R software (Version: 4.1.2).

1 http://www.hmdb.ca/

## 2.6. Correlation analysis

According to VIP > 1, $p < 0.05$, the correlation between different metabolites selected from serum and rumen fluid and rumen fermentation parameters (acetic acid, propionic acid, *iso*-butyric acid, butyric acid, *iso*-valeric acid, pentanoic acid, and hexyl-propyl ratio) was analyzed. SciPy of Python (Version 1.0.0) was used to analyze the Spearman correlation between different metabolites and rumen fermentation parameters based on the Bray_Curtis distance, and the cluster heat map was used for visualization.

Metabolites in serum and rumen with VIP > 1, $p < 0.05$ were interactively analyzed with the top 50 dominant microflora in phylum and genus taxonomy. Based on Bray_Curtis distance, the Spearman correlation between differential metabolites and dominant microbial phylum and dominant microbial genus was analyzed by using "scipy" of Python (Version 1.0.0), and these correlations were visualized by cluster heat map.

## 2.7. Statistical analysis

SAS software (Version 9.4) was used to analyze the monthly weight data and weight changes of each group of JY designed by random block with one-way analysis of variance (ANOVA). The statistical results were expressed by mean and standard deviation. Then use GraphPad (Version 9.0) for visualization. Blood biochemical indexes and VFAs were tested by SPSS software (Version 26) for normality and homogeneity of variance, and ANOVA was used to analyze the differences among the three groups, among which $p < 0.05$ was regarded as statistically significant. Finally, the results were expressed by the standard deviation (SD) of the mean value.

# 3. Results

## 3.1. Changes in growth index

The effects of different protein levels of supplementary diet on the body weight and monthly body weight change of JY during the formal trial period are shown in Figure 1. According to SAS, ANOVA (Supplementary material S2) was carried out on the body weight and weight changes of the three groups. The results showed that there were significant differences in the body weight each month among the three groups ($p < 0.05$), and the body weight of the HP group was higher than that of the LP group and Control group. In terms of the monthly weight gain rate, JY of the LP group and HP group with supplementary feeding were in a state of weight gain in the formal experiment, but the weight of the control group without supplementary feeding showed a significant downward trend in the last month of the formal experiment.

## 3.2. Changes of rumen fermentation parameters

The effects of three treatments on rumen fermentation parameters of JYs are shown in Table 1. The valeric acid content and pH of the LP and HP groups were significantly higher than those of the Control group ($p < 0.05$). The isopropyl ratio of the Control group was significantly higher than the other two groups ($p < 0.05$). There was no significant difference in the content of acetic acid, valeric acid, *iso*-butyric acid, butyric acid, and *iso*-valeric acid among the three groups ($p > 0.05$).

## 3.3. Statistics of metagenomic data

A total of 864,673,934 raw reads were obtained from 18 samples in three treatment groups, with an average of 48,037,441 ± 4,449,071 raw reads per sample. After quality control, 851,239,334 clean reads were retained, with an average of 47,291,074.11 ± 4,397 clean reads per sample (Supplementary material S3). A total of 11,800,805 contigs were generated by *de novo* assembly of the retained data, with an average of 655,600.28 ± 81,518.10 contigs per sample, of which the length of N50 per sample was 772.00 ± 99.47 bp. Gene prediction showed that there were 15,548,998.00 open reading frame ORFs in all samples (Supplementary material S3). Bacteria accounted for 96.67%, eukaryotes accounted for 1.35%, archaea accounted for 1.07%, and viruses accounted for 0.59% (Supplementary material: S4).

## 3.4. Microbial maps of three treatment groups

The microbial composition of 18 samples (at Phylum and Genus levels) was analyzed by non-metric multidimensional scale analysis (NMDS), which was analyzed and plotted by the vegan package of R software (Figures 2A,B). The results showed that the three treatment groups had a good clustering effect, which could be followed up. According to the visualization of the microbial composition of the three treatment groups at the phylum and genus level (Figures 2C,D), the results showed that at the phylum level, the dominant phylum of the three treatment groups was *Bacteroidetes*, *Firmicutes*, *Kiritimatiellaeota*, and *Proteobacteria*, etc. At the genus level, the dominant microbial genera in the three treatment groups mainly include *Prevotella*, *unclassified_o_bacteroidales*, *unclassified_f_lachnospiraceae*, *unclassified_c_kiritimatiellae*, *unclassified_f_paludibateraceae*, *Ruminococcus*, and *Methanobrevibacter*.

In this study, based on the difference matrix of Bray_Curtis, the difference of microbial composition in three treatment groups was estimated (Figure 3A). The content of *unclassified_c_alphabetobacter* in HP group was significantly higher than that in the other two groups. Besides, the contents of *Streptomyces*, *Butyricimonas*, *Mucilaginibacter*, *Candidatus_Cryptobacteroides*, *Massilioclostridium*, and other microbials were also significantly different among the three treatment groups. In the comparison of microbials between LP and HP groups (Figure 3B), the contents of *Oribacterium*, *Slackia*, *unclassified_o_burkholderiales*, *Duncaniella*, and *epulopsis* in HP group were significantly higher than those in LP group.

## 3.5. Enzyme map of carbohydrate activity in three treatment groups

The function of microbial is partly determined by genes encoding carbohydrate-active enzymes (CAZymes). The genes encoding CAZymes were analyzed by NMDS (Figure 4A), and the results showed that the genes encoding CAZymes in three treatment groups

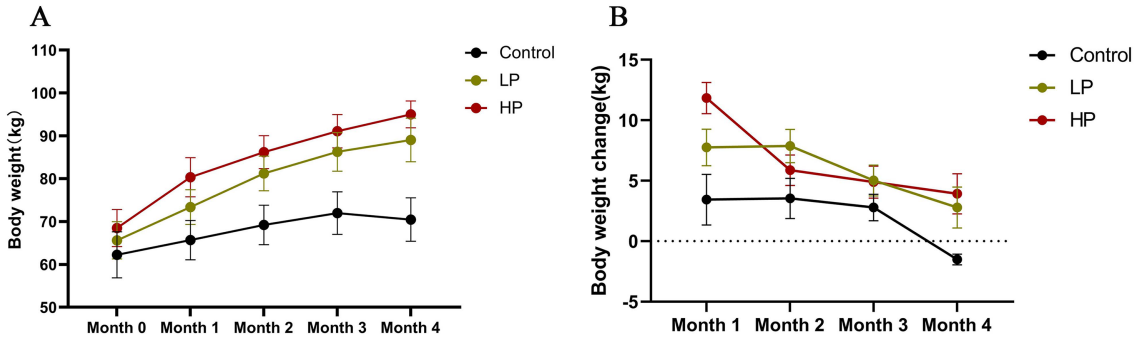


FIGURE 1
Effects of dietary protein level on body weight **(A)** and body weight change **(B)**. The mean value and standard deviation (SD) of each group were used to represent. Control: control group, no supplementary feeding; LP: low protein group, supplementary feeding with low protein diet; HP: high protein group, supplementary feeding with high protein diet.

**TABLE 1** Effects of supplemental diets with different protein levels on rumen fermentation parameters of Jersey-Yak.

| Item | Treatment group (Mean±SD) | | | *p*-Value |
|---|---|---|---|---|
| | Control | LP | HP | |
| Acetate | 33.02 ± 5.52 | 35.75 ± 10.33 | 34.40 ± 3.82 | 0.628 |
| Propionate | 6.68 ± 1.18 | 8.40 ± 2.62 | 7.91 ± 1.91 | 0.112 |
| *Iso*-butyrate | 0.31 ± 0.04 | 0.37 ± 0.08 | 0.34 ± 0.08 | 0.105 |
| Butyrate | 4.16 ± 1.08 | 5.84 ± 2.79 | 4.96 ± 1.16 | 0.086 |
| *Iso*-valerate | 0.32 ± 0.07 | 0.41 ± 0.10 | 0.37 ± 0.13 | 0.095 |
| Valerate | $0.26 \pm 0.05^{b}$ | $0.47 \pm 0.24^{a}$ | $0.36 \pm 0.16^{ab}$ | 0.021 |
| Acetate: Propionate | $4.96 \pm 0.44^{a}$ | $4.35 \pm 0.63^{b}$ | $4.49 \pm 0.67^{b}$ | 0.040 |
| pH | $6.57 \pm 0.17^{B}$ | $6.92 \pm 0.18^{A}$ | $7.02 \pm 0.16^{A}$ | 0.006 |

The value of each treatment group is expressed by the Mean ± standard deviation (SD). In the same line, superscript with different lowercase letters shows significant differences among groups, while uppercase letters show significant differences among groups.

had a good clustering effect. A total of 527 CAZyme genes were identified (Figure 4B), including 260 Glycoside Hydrolases (GHs), 87 Glycosyl Transferases (GTs), and 77 Polysaccharide Lyases (PLs). There are 6 Carbohydrate Esterases (CEs), 70 Carbohydrate-Binding Modules (CBMs), and 17 and auxiliary oxidoreductases (AAs). Among them, the genes encoding glycosidase GHs and GTs are the most.

The differences of CAZyme coding genes among three treatment groups were compared (Figure 4C). The results showed that the relative abundance of GT28, GT13, GH149, and GH103 in HP group was significantly higher than that in other two groups, and the relative abundance of GH31, GH95, GH43_29, and PL11_2 in LP group was significantly higher than that in other two groups. Compared with HP group (Figure 4D), the results showed that the contents of GH31, GH95, GH43_29, GH67, and GH98 in LP group are significantly higher than those in HP group, and the relative abundance of GT28, CE11, GT27, and GH149 in HP group is significantly higher than that in LP group.

## 3.6. Serum and rumen fluid metabolomics

Metabolites were detected in the serum of three treatment groups, and 884 metabolites were obtained. According to the PLS-DA scores of metabolites in the serum of three treatment groups in cation mode and anion mode (Figures 5A,B), the results show that the clustering effect of samples in the group is good, which can be used for subsequent analysis. Among them, after the Kruskal–Wallis H test, 125 metabolites were screened out among the three treatment groups, and there were significant differences among 48 metabolites between LP and HP groups after Wilcox'test ($p < 0.05$ VIP > 1).

The products with the top 20 abundance were selected and represented by cluster heat map (HCA; Figure 6A). The results showed that the differential metabolites could be clustered into five clusters. The first cluster mainly included two metabolites, LysoPC [22:5(4Z, 7Z, 10Z, 13Z, 16Z)] and PC [20:5(5Z, 8Z, 11Z, 14Z, 17Z)/0:0]. The second cluster mainly includes 10 metabolites including Notoginsenoside T1, 6-Hydroxyhexanoic acid, N-acetyl-L-glutamate5-semi aldehyde, and L-Carnitine. The third cluster mainly includes three metabolites of Dihydrocaffeic acid 3-sulfate, Isoglobotriaose, and 2,4-Dimethylpyridine, the fourth cluster mainly includes PSOROMIC ACID, Cyclodopa glucoside, and N, N-dimethyl-Safingol, and the fifth cluster mainly includes 3-Methyl-L-histidine and lysopc. The different metabolic pathways among the three treatment groups (Figure 6B) mainly include Sphingolipid metabolism, Bile secretion, Linoleic acid metabolism, Histidine metabolism, and so on. According to the VI*p* value and *P* value obtained by PLS-DA analysis, the differential metabolites between LP and HP groups were screened (Figure 6C). The results showed that there were mainly 2-amino-14,16-dimethyloctodecan-3-ol, Isoglobotriaos, Tragopogonsaponin A, and 2-hydroxyhexadecanoic acid between the two treatment groups.

Metabolites in rumen fluid of three treatment groups were detected, among which 1,667 metabolites were identified. According to the PLS-DA scores of metabolites in the rumen of three treatment groups in cation mode and anion mode (Figures 5C,D), the results showed that the clustering effect of samples in the group is good, which can be used for subsequent analysis. As with the analysis method of metabolites in serum, 204 metabolites were significantly different among the three treatment groups, and 45 metabolites were significantly different between LP and HP groups.

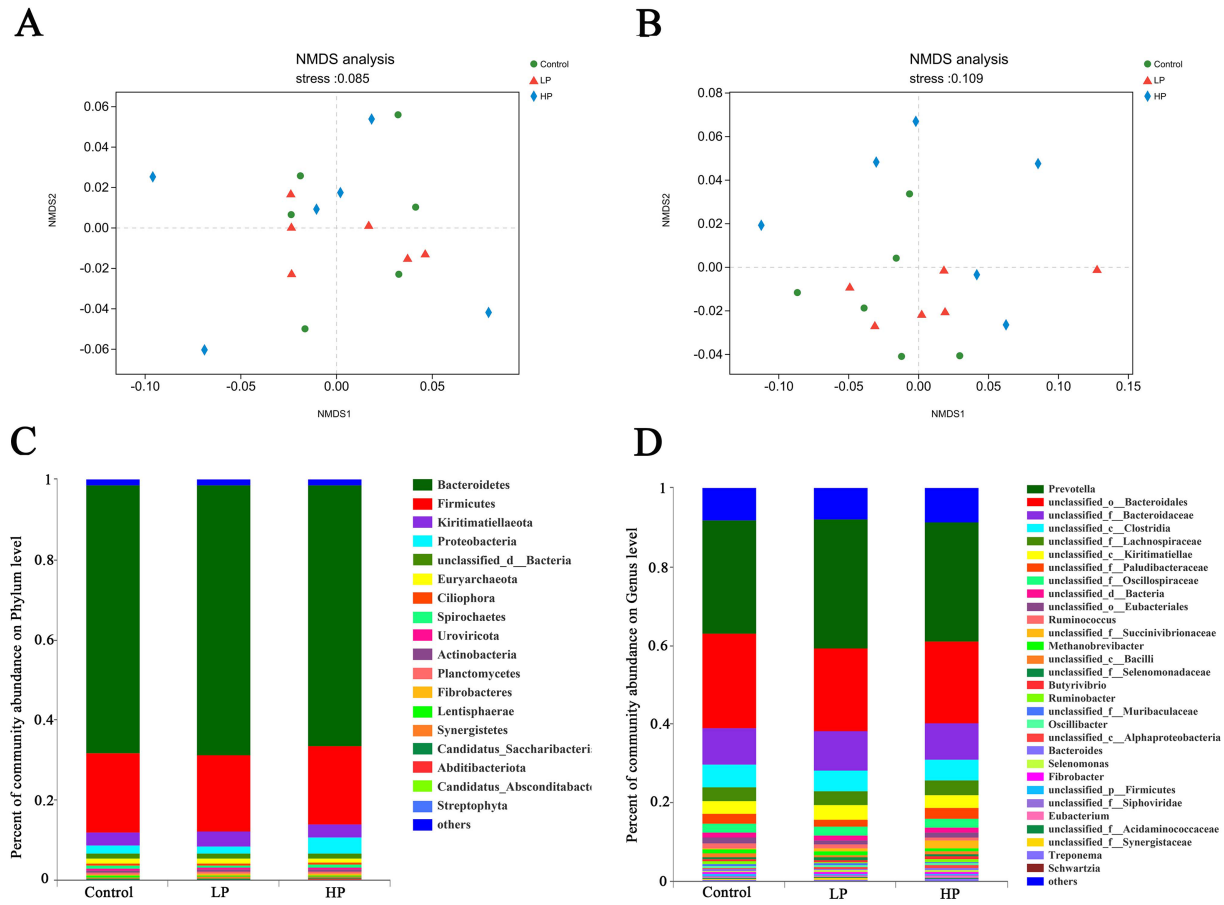


FIGURE 2
Non-metric multi-dimensional analysis (NMDS) of metagenome sequencing and microbial composition at genus and phylum level in three treatment groups. NMDS of metagenome sequencing of all samples at phylum **(A)** and genus **(B)** level. Microbial composition profiles of three treatment groups at phylum **(C)** and genus **(D)** levels.

The top 20 metabolites in abundance were selected and visualized by cluster thermogram (Figure 7A). The results show that the different metabolites in rumen fluid can be clustered into five clusters, the first cluster contains methyl (r)-9-hydroxy-10-undecene-5, 7-diynoate glucoside and Alpha-Cyano-4-hydroxycinnamic acid are two metabolites, and the second cluster contains three differential metabolites of PE (14.0/0:0), Tangeraxanthin and Girinimbine. The third cluster contains 11 metabolites including Prehumulinic acid, 9-Decenoic acid, and 2-deoxycastastrone, the fourth cluster contains a metabolite of Dehydrocyanaropicrin, and the fifth cluster contains 5′-Hydroxy-3′,4′, 7-Trimethoxylavan, Phenethylamine glucuronide, and N-Acetyl-DL-Methionine, three metabolites. According to the analysis of the different metabolic pathways among the three treatment groups (Figure 7B), the results showed that there were mainly different metabolic pathways such as Th17 cell differentiation, Styrene degradation, and Bile secretion. According to the VI*p* value and *P* value obtained by PLS-DA analysis, the different metabolites in the rumen fluid of LP and HP groups were screened (Figure 7C). The results showed that there were significant differences in metabolites such as Acetaminophen cysteine, Nicotine glucuronide, Phenethylamine glucuronide, Hydroxygaleon, and N-Acetyl-DL-Methionine between the two groups (LP and HP).

## 3.7. Relationship between different metabolites in serum and rumen fluid and rumen fermentation parameters

To determine the difference metabolites in serum and rumen fluid and rumen fermentation parameters (acetate, propionate, *iso*-butyrate, butyrate, *iso*-valerate, valerate, and acetic/propionic ratio), Spearman rank correlation analysis was carried out on the difference metabolites, and these parameters. According to the correlation analysis of differential metabolites in the serum and VFA parameters (Figure 8A), results showed that Acetate, Propionate, Butyrate, and L-Carnitine, PC (18:1/0:0), 2-amino-4-({2-[(2-carbon-2-hydroxy-1-phenyl ethyl) sulfinyl]-1-(carbo) Metabolites such as 3-Buten-1-amine and Glucosylsphingosine were negatively correlated with N,N-dimethyl-Safingol, 16-Hydroxy hexadecanoic acid, Polyoxyethylene 40 monostearate and PC [16:0/18:2(9Z, 2z)] and other metabolites showed positive correlation, among which Butyrate and L-Carnitine had extremely significant negative correlation ($p < 0.05$), Isovalerate and PC (18:1/0:0) and PC [20:5(5Z,8Z,11Z,14Z,17Z)/0:0]. Isopropyl ratio is positively correlated with most of the differential metabolites.

In the analysis of the correlation between different metabolites in rumen fluid and VFAs (Figure 8B), the results show that acetate, propionate, *iso*-butyrate, butyrate, *iso*-valerate, and valerate are

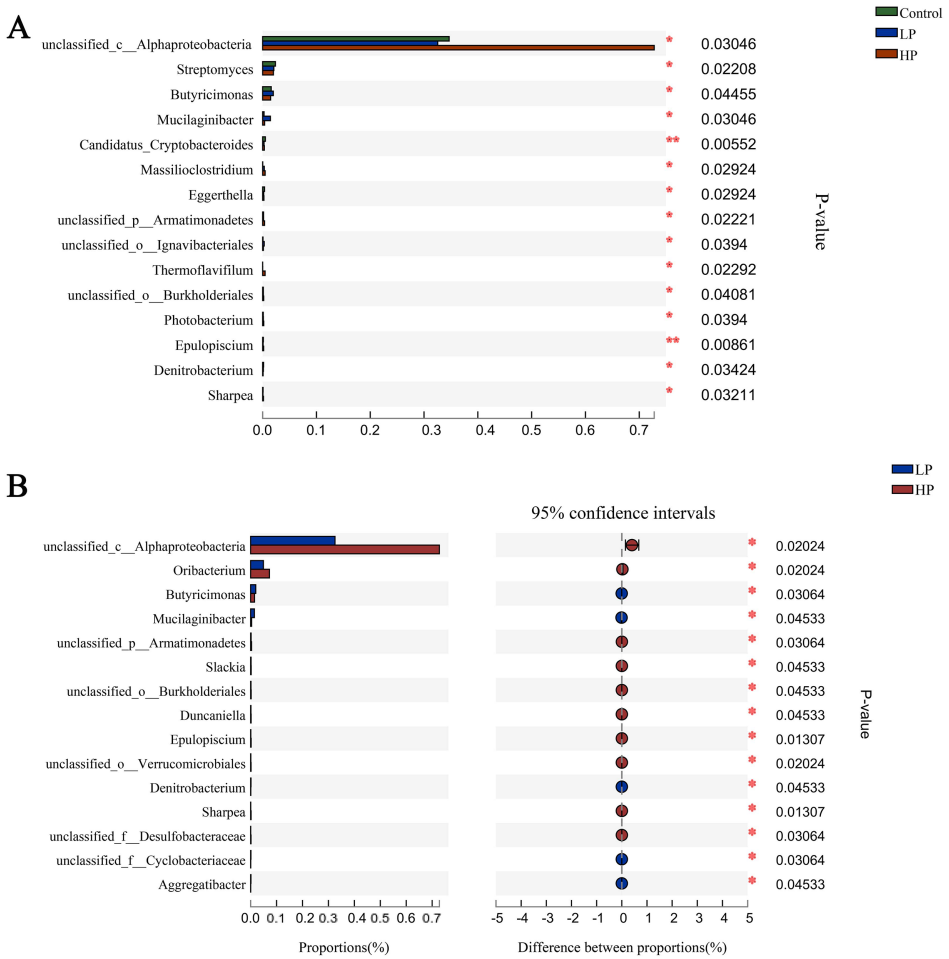


FIGURE 3
Composition differences of microbials in three treatment groups and LP_HP groups at genus level. **(A)** The difference of microbial composition in three treatment groups at genus level. **(B)** Difference of microbial composition between LP and HP groups at genus level. "*" indicates that metabolites are significantly different among groups. "**" indicates that there is a significant difference in metabolites between groups.

negatively correlated with most of the different metabolites, in particular, butyrate and valerate are negatively correlated with metabolites such as Thromboxane B2, Costunolide and Erinacine D ($p < 0.05$), but the indexes of propionate, butyrate and valerate are correlated with metabolites DG [20: 2 (11z, 14z)/18: 3 (9z, 12z, 15z]/. *Iso*-propyl ratio showed a negative correlation with DG [20: 2 (11Z, 14Z)/18: 3 (9Z, 12Z, 15Z)/0: 0] and other metabolites, but showed a positive correlation with most metabolites.

## 3.8. Relationship between rumen dominant microflora and rumen fermentation parameters and metabolites

To determine the potential interaction between rumen microflora and host metabolism, the dominant microflora with the top 20 phylum and genus levels were selected to correlate with Spearman rank between different metabolites in serum and rumen fluid, respectively. According to the correlation analysis between serum differential metabolites and dominant microflora (Figures 9A,B), the results show that at the phylum level, *Bacteroidetes*, *Firmicutes*, *Actinobacteria*, and *Fibrobacteres* are negatively correlated with 10,20-Dihydroxyeicosanoic acid and 6-hydroxytadecanoic acid, while other dominant microflora are positively correlated with these two metabolites. *Actinobacteria* showed an obvious positive correlation with metabolites such as 3-Buten-1-amine, Deoxycholic acid, MEDICA 16, 10,20-Dihydroxyeicosanoic acid, and 6-hydroxytadecanoic acid, There was a significant negative correlation with metabolites such as 16-Hydroxy hexadecanoic acid, Polyoxyethylene 40 monostearate and N,N-dimethyl-Safingol, and there was a significant positive correlation with metabolites such as 3-Buten-1-amine and PC (18:1/0:0; $p < 0.05$). Metabolites such as PC (18:1/0:0) and PC (17:0/0:0) have significant negative correlations with dominant phylum such as Streptophyta and Chytridiomycota ($p < 0.05$). At the genus level, *Prevotella* has a negative correlation with most of the differential metabolites, and a very significant positive correlation with 3-o 3-O-Acetylepisamarcandin ($p < 0.01$). The dominant microbial phylum *Ruminococcus* and *Methanobrevibacter* were positively correlated with the metabolites of 16-Hydroxy hexadecanoic acid and Polyoxyethylene 40 monostearate, among which *Methanobrevibacter* was positively correlated with Deoxycholic acid ($p < 0.05$).

According to the correlation analysis between the differential metabolites in rumen fluid and the dominant microflora

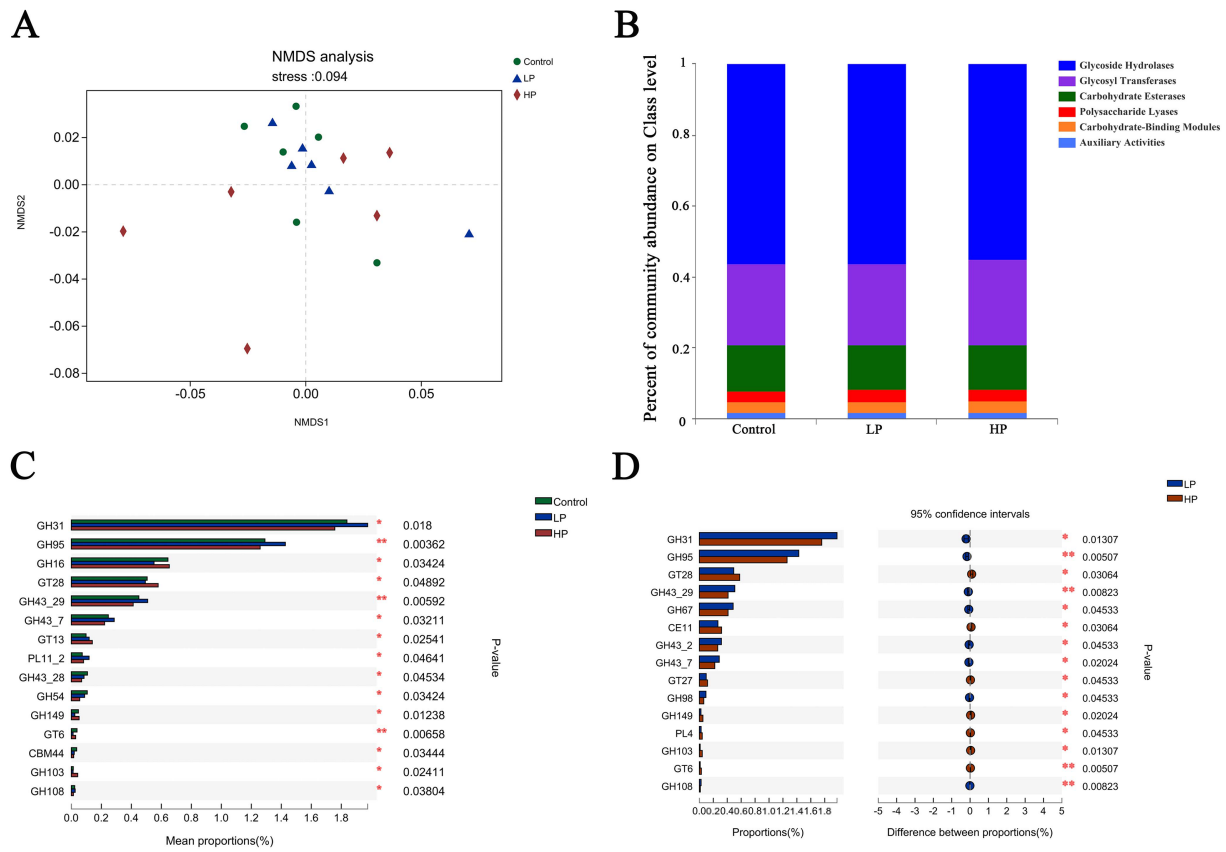


FIGURE 4
Non-metric multi-dimensional analysis of carbohydrate-active enzymes **(A)** and composition of three treatment groups **(B)**. There are significant differences in CAZymes between the three treatment groups **(C)** and LP_HP two treatment groups **(D)**.

(Figures 9C,D), the results show that: at the phylum level, *Bacteroidetes* showed an obvious negative correlation with most metabolites except Tangeraxan, Xi-1-Butoxy-1-methoxyethane, Butylisopropylamine, Decyl acetate, and Hexylamine, there is a significant positive correlation between *Bacteroidetes* and Xi-1-Butoxy-1-methoxyethane, Butylisopropylamine, and Decyl acetate ($p < 0.05$). *Synergistetes* are negatively correlated with most of the differential metabolites, among which there is a significant negative correlation with Decyl acetate, Hexylamine, and DG (18:1(11Z)/18:1(9Z)/0:0; $p < 0.05$). At the genus level, *Prevotella* showed a significant negative correlation with most metabolites except Butylisopropylamine, DG (20: 2 (11Z, 14Z)/18: 3 (9Z, 12Z, 15Z)/0: 0), and Decyl acetate, among which DG (20: 2) 11Z Unclassified_o_bacteria showed an extremely significant positive correlation with most of the differential metabolites, including Artemin, Walleminone, Thromboxane B2, and Costunolide.

## 4. Discussion

When ruminants on Qinghai-Tibet Plateau are supplemented during the dry season, the proper nutrition level in the supplementary diet can not only significantly improve the rumen microbial community and metabolites composition of ruminants but also regulate the rumen fermentation index of ruminants, which is the key to improve the growth performance of the host (Yáñez-Ruiz et al., 2015; Xu et al., 2017; Newbold and Ramos-Morales, 2020). Therefore, metagenomics and metabonomics were used to study the effects of different protein levels on the rumen microbial community and metabolites of JY, and the effects on rumen fermentation parameters and growth performance were also discussed.

In recent years, researches on the effect of dietary nutrition level on the growth performance of ruminants showed that the type and nutrition level of diet will have a significant impact on the daily gain and weight of ruminants (Xu et al., 2017; Estrada-Angulo et al., 2018; Wang et al., 2022b). Protein is one of the most important nutrients in ruminants' diets. Ruminants need to get protein from various feeds in order to maintain their lives and engage in various production. Therefore, the differential supply of protein in supplementary diets may lead to the different growth performances of JY. In this study, the weight of JYs was significantly increased by supplementing diets with different crude protein levels, and the weight of the LP group and HP group was significantly higher than that of the control group. In addition, the weight of the control group showed an obvious downward trend in the last month of the formal experiment, indicating that with the increase in the age of JY, the food intake increased, and the nutrients obtained from pasture during grazing could not meet its growth and development needs, which led to the decline of the weight of JY. Consistent with previous research results, the increase of crude protein levels in the diet can increase the body weight and daily gain of animals (Estrada-Angulo et al., 2018; Qiu et al., 2018; Wang et al., 2020b). Moreover, when feeding lambs with different crude protein levels, the results showed that the daily gain

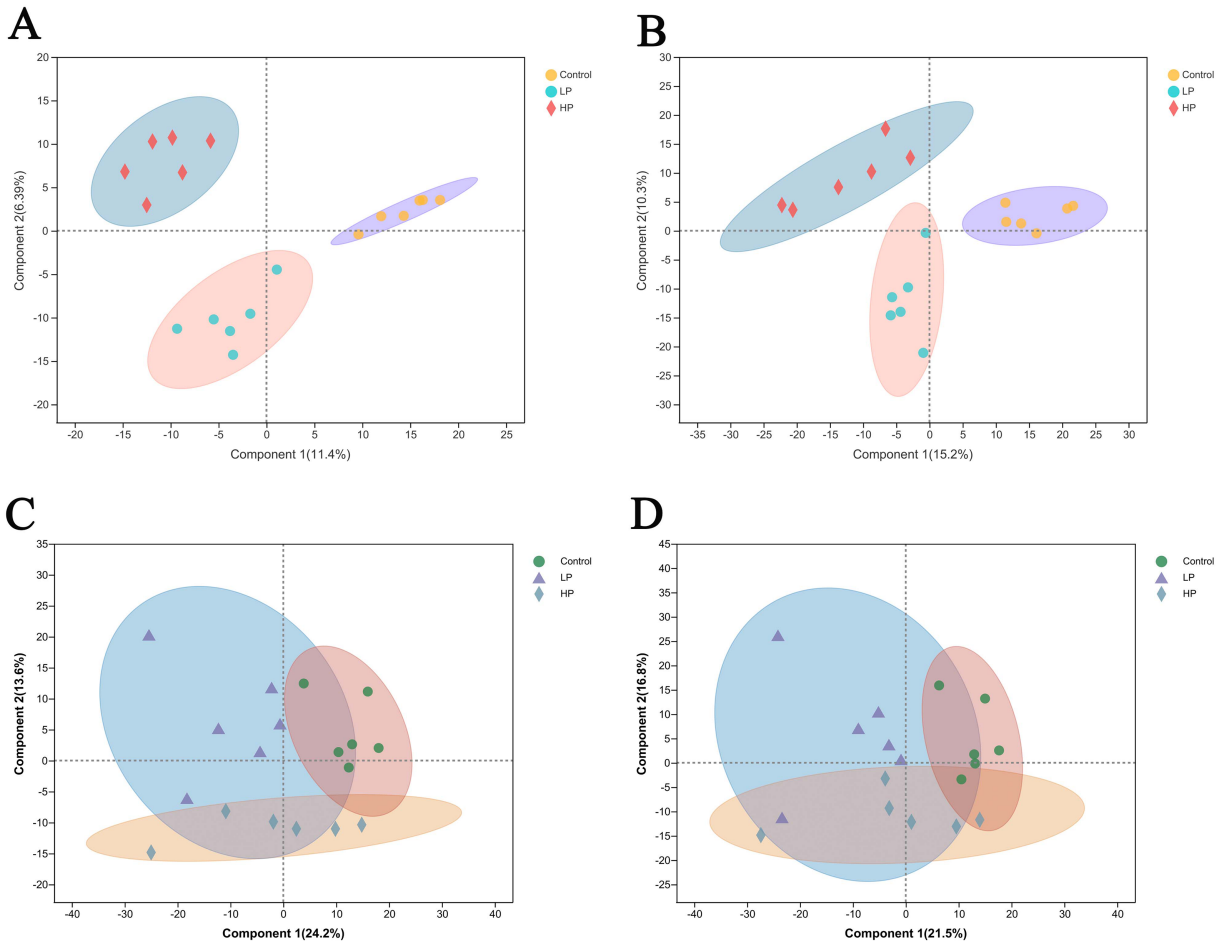


FIGURE 5
Partial least squares discriminant analysis (PLS-DA) scores of serum metabolism group and rumen metabolism group. PLS-DA analysis of serum metabolites in three treatment groups under cation mode **(A)** and anion mode **(B)**. PLS-DA analysis of rumen metabolites in three treatment groups under cation mode **(C)** and anion mode **(D)**.

and feed conversion efficiency of lambs were significantly improved by using high protein levels (Karlsson and Martinsson, 2011). In this experiment, the body weight and daily gain of JYs in the HP group were higher than those in the LP group. Therefore, according to the results of this experiment and previous research report (Bihuniak and Insogna, 2015), we speculate that increasing the crude protein level in the diet within a reasonable range can improve the growth performance of animals.

Rumen is a unique structure of ruminants, and it is a living fermentor for ruminants. The stable rumen environment is the basic guarantee for ruminants to digest feed, while pH and VFAs are the main indicators to measure it, and VFAs are the final products of ruminants' degradation and digestion of protein in diet (Weller et al., 1969; Wang et al., 2012; Melo et al., 2013a). Therefore, in this study, the indexes of VFAs and pH in the rumen were measured. Rumen pH represents the buffering capacity of the rumen, which is used to reflect the stability of the internal environment in the rumen. It is an important index to evaluate rumen fermentation. It is in a dynamic change, with the normal range of 5.5 ~ 7.5, at this time, protozoa and cellulolytic bacteria in the rumen grow rapidly and produce a large number of VFAs to meet the energy needed by animals (Beijer, 1952; Palmonari et al., 2010; Melo et al., 2013a; Mizrahi et al., 2021). In this experiment, the rumen pH in the control group was lower than that in the LP group and the HP group, indicating that the supplementary feeding diet had a certain impact on the rumen internal environment, but the pH among the three groups were within the normal range, indicating that the body itself had certain self-regulation ability. VFAs are the final products of rumen microbials in ruminants when they ferment and degrade diets, and are the main sources of energy metabolism for ruminants' growth and development (Warner, 1964; Harirchi et al., 2022; Weimer, 2022). In this experiment, the concentrations of Acetate, Propionate, *Iso*-Butyrate, Butyrate, *Iso*-Valerate, and valerate are the lowest in the Control group, which is consistent with other research results (Karimi et al., 2021; Mirzakhani et al., 2021; Yi et al., 2022a). The crude protein level in the supplementary diet has a significant impact on Valerate in JY. The concentration in the LP is higher than that in the HP, which may be related to the internal environment in the rumen of JY. According to previous studies, either too high or too low pH in the rumen can affect rumen absorption of VFAs in ruminants (Calsamiglia et al., 2008). Therefore, we speculate that the difference in acetate concentration among the three groups may be partly related to rumen

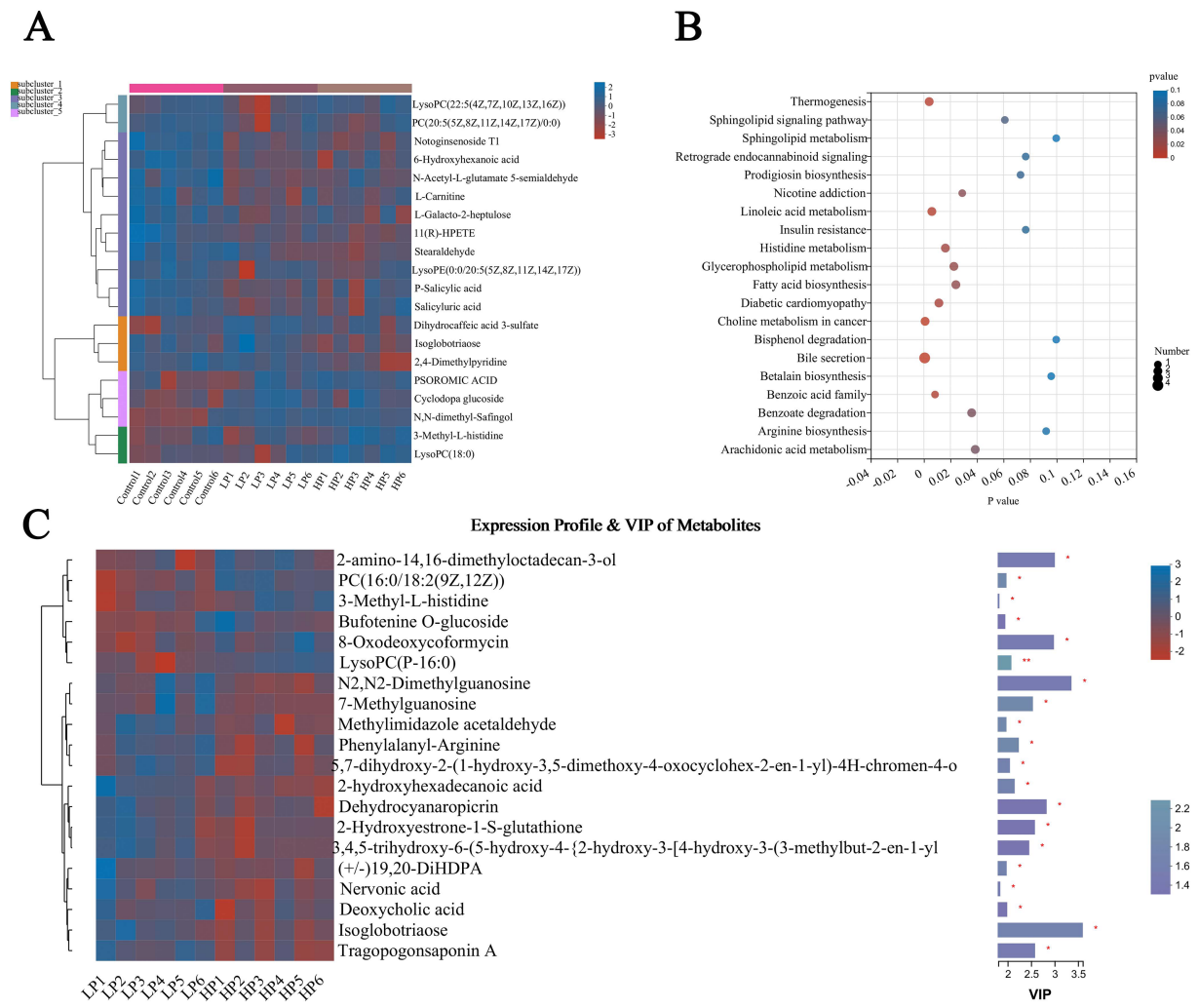


FIGURE 6
Differential metabolites and metabolic pathways in serum of three treatment groups. Differential metabolites in serum of three treatment groups **(A)** were visualized by cluster thermogram. Bubble chart was used to show the different metabolic pathways in the serum of the three treatment groups **(B)**. Differential metabolites in serum of LP and HP **(C)** were visualized by thermogram and VIP value. The tree diagram of metabolites cluster on the left side shows that each column shows a sample, the lower side shows the name of the sample, and each row shows a metabolite. Bar chart showing VIP value on the right. "*" indicates that metabolites are significantly different among groups. "**" indicates that there is a significant difference in metabolites between groups. The same below.

pH, and the pH among the three groups is different. The ratio of Acetate/Propionate can affect microbial diversity. The ratio of Acetate/Propionate in the rumen is different with different feed types or microbial community structures. The higher the proportion of concentrate in the ruminant diet, the lower the ratio of Acetate/Propionate (Pang et al., 2022a; Yi et al., 2022a). In this experiment, the ratio of Acetate/Propionate in the control group was higher than that in the LP and the HP group, which was consistent with the results of Dai et al. (2022). Therefore, we speculated that the ratio of concentrate in the diet was changed when JYs were supplemented with different crude protein levels, thus reducing the ratio of Acetate/Propionate in the HP and the LP group.

Genomics can study the composition and functional characteristics of rumen microbials, by annotating the genes encoding CAZymes, we can quickly and comprehensively understand the effects of different protein levels of supplementary diets on rumen microflora in JY (Ghurye et al., 2016; Ngara and Zhang, 2018; Taş et al., 2021). In this study, as in previous studies, the dominant microbial compositions of the three treatment groups at phylum level are *Bacteroides*, *Sclerotinia*, *Kiritimatiellaeota*, and *Proteus*. *Bacteroidetes* and *Firmicutes* contain a large number of fiber-degrading bacteria, which can degrade cellulose in feed to produce VFAs, and play an important role in carbohydrate degradation in rumen, thus promoting the growth and development of ruminants (Chen et al., 2022a; Xue et al., 2022b). At the genus level, the dominant microbial genera in the three treatment groups are *Prevotella* and *Ruminococcus*, etc. *Prevotella* can make use of starch and plant cell walls in rumen, such as xylan and pectin. *Ruminococcus* can produce a large amount of cellulase and hemicellulase. More importantly, *Ruminococcus* is an important process involved in the degradation of oligopeptides into amino acids, which is regarded as a restrictive step in protein hydrolysis in rumen (Calsamiglia et al., 2010; Kim et al., 2017; Gaffney et al., 2021; Sharma et al., 2022). There was no significant difference in the relative abundance of these dominant microbials at the phylum and genus

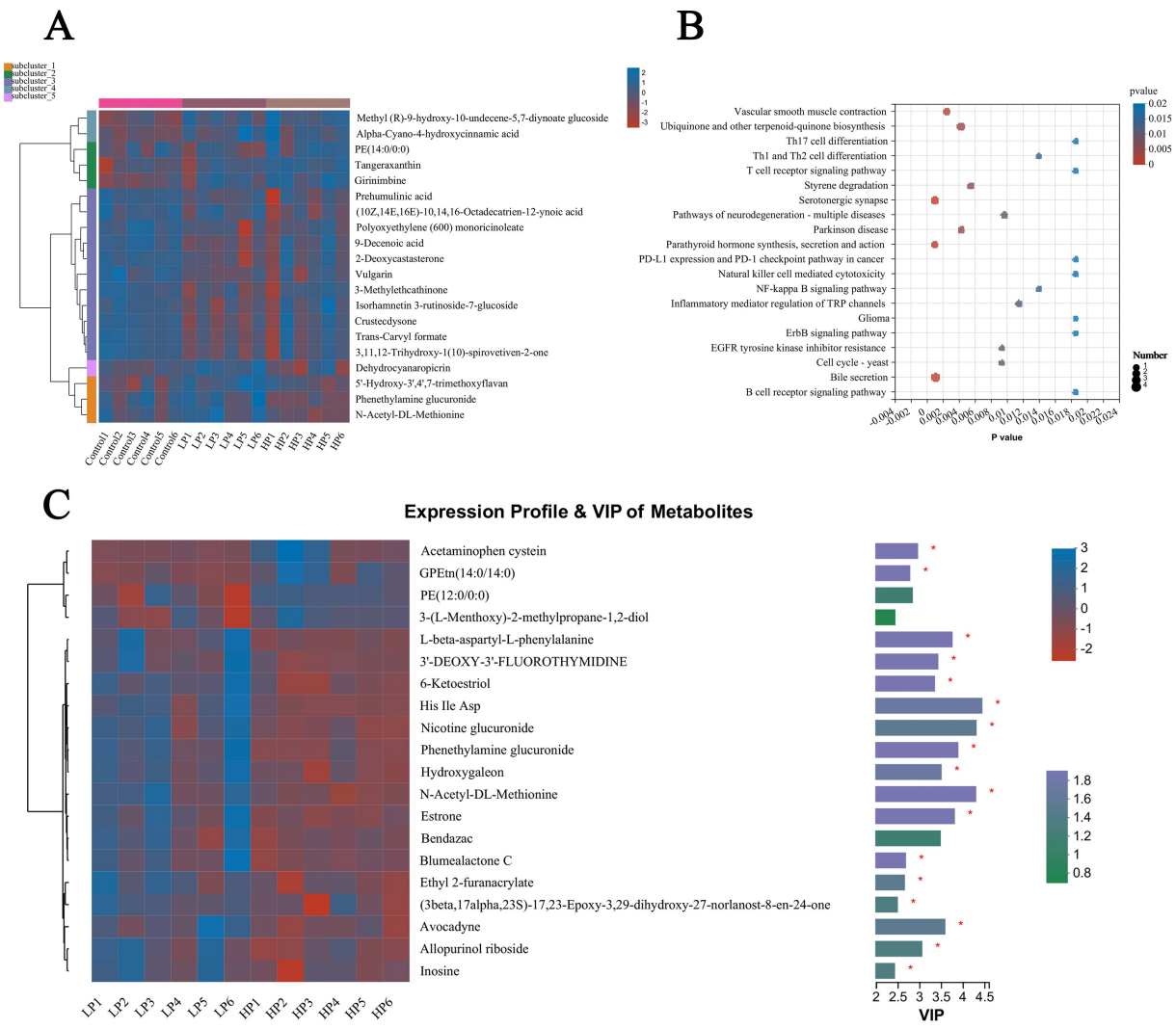


FIGURE 7
Differential metabolites and metabolic pathways in rumen fluid of three treatment groups. Differential metabolites in rumen fluid of three treatment groups **(A)** were visualized by cluster thermogram. Bubble diagram was used to show the different metabolic pathways in rumen fluid of three treatment groups **(B)**. Differential metabolites in rumen fluid of LP group and HP group **(C)** were visualized by heat map and VIP value. The tree diagram of metabolites cluster on the left side shows that each column shows a sample, the lower side shows the name of the sample, and each row shows a metabolite. Bar chart showing VIP value on the right. "*" indicates that metabolites are significantly different among groups. "**" indicates that there is a significant difference in metabolites between groups.

levels among the three treatment groups, which may be due to their adaptation to different supplementary diets through the ruminant rumen self-regulation system. It is worth noting that although the relative abundance of *Streptomyces*, *Butyricimonas*, and *unclassified_c_Alphaproteobacteria* is relatively low, these microbials have significant differences between the three treatment groups, which may be due to these differences. The microbial community leads to different degradation capabilities of the supplementary diet between the three treatment groups.

Metagenomics can be used to annotate the genes encoding CAZymes (Kunath et al., 2017); therefore, in this study, the genes encoding CAZymes in rumen microbials were annotated, and it was found that the three treatment groups were GHs and GTs with the largest number. Carbohydrate in the ruminant diet provides fermentation substrate for rumen microorganisms, which can account for more than 60% of the diet. Complex carbohydrates are decomposed into monosaccharides and oligosaccharides under the catalysis of various hydrolases, and then these oligosaccharides are quickly decomposed into pyruvate by microorganisms, which is fermented through metabolic pathways and finally produce acetic acid and propionic acid. Among them, GHs can destroy the glycosidic bond of carbohydrates through hydrolysis, and GTs can catalyze the formation of glycosidic bonds by using sugar donors containing nucleoside phosphate or lipid phosphate leaving groups (Bourne and Henrissat, 2001; Lairson et al., 2008; Ardèvol and Rovira, 2015). CE mainly removes the ester group of polysaccharides and participates in the degradation of the carbohydrate side chain, which can promote GHs and PLs to break the glycosidic bond of polysaccharides (Biely, 2012). PLs can break the glycosidic bond through the mechanism in the body, and the CBMs itself has no catalytic activity, but it can assist GHs and PLs by fixing CAZymes on the substrate surface to increase the contact time and area (Cantarel et al., 2009; Lombard et al., 2010;

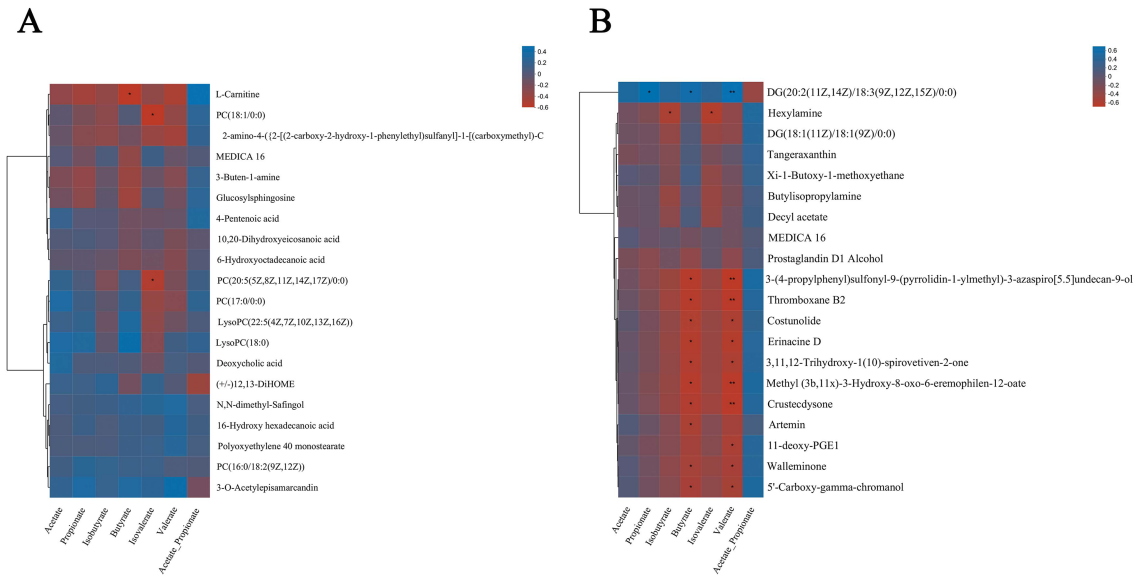


FIGURE 8
Spearman relationship between different metabolites in serum and rumen fluid and VFAs. Spearman correlation between different metabolites screened from serum **(A)** and rumen fluid **(B)** and VFAs in three treatment groups.

Jones et al., 2018). There are significant differences among GT28, GT13, GH149, and GH103 among the three treatment groups. In the comparison of CAZymes between LP and HP groups, the number of GH31, GH95, GH67, and GH98 in LP group is significantly higher than that in HP group. Previous studies have shown that GH149 and GH98 play an important role in the degradation of complex carbohydrates (Rigden, 2005; Kuhaudomlarp et al., 2018). Therefore, we speculate that the differences between these CAZymes may affect the degradation process of carbohydrates in the diet of JY, resulting in the difference in valerate concentration in the three groups. Valic acid is the substrate of rumen fermentation, which can be further processed into ruminants as a carbon source, moreover, valeric acid also participates in cellular immunity and is an effective immunomodulator (Nadeau et al., 2003; Feng et al., 2022). Therefore, the difference in valeric acid concentration in the rumen will affect the nutrient absorption capacity of ruminants, which will lead to the difference in growth performance, but the specific mechanism between them needs further study.

In order to further explore the potential relationship between rumen dominant microflora and rumen fermentation parameters and metabolites, Spearman correlation analysis was conducted based on Bray_Curtis distance. According to previous studies, more and more evidences showed that the concentration of metabolites will affect the function of rumen microflora (Zhang et al., 2017; Xue et al., 2020). The results of this experiment are consistent with those of yak and Holstein cattle, indicating that the changes between microbial flora and metabolites are closely related to the health and growth of the body (Zhang et al., 2017, 2021). In this experiment, the results showed that there is a significant positive or negative correlation between dominant microorganisms and differential metabolites. The metabolic pathways of these differential metabolites annotated by the KEGG database mainly include sphingomyelin metabolism, linoleic acid metabolism, histidine metabolism, and bile secretion et al. Deoxycholic acid is a differential metabolite among the three groups, which is significantly related to Proteobacteria. Deoxycholic acid is a steroid and one of the main components of circulating bile acid, which plays an important role in bile secretion (Funabashi et al., 2020). 4-Pentenoic acid is a differential metabolite among the three groups, and it is significantly related to the dominant microorganisms at the phylum level. The metabolite is a lipid and lipid molecule, which has been proved that inhibiting the combination of alanine and glucose in pyruvate carboxylase and glyceraldehyde 3-phosphate dehydrogenase can inhibit the oxidation reaction of fatty acids, thus affecting the growth and development of animals (Gorin and Zendowski, 1975; Zhong et al., 1985). Therefore, we speculate that the dominant microorganisms in JY can change the metabolic pathway in the body by regulating the concentration of these metabolites, and then affect the growth and development of the host.

## 5. Conclusion

In this study, the effects of crude protein level in supplementary diet on growth performance and rumen fermentation parameters of JYs were compared, and the effects on microbial community composition and metabolites were explored by metagenomics and metabolomics. The crude protein level in the supplementary diet significantly increased the daily gain of JY. The pH and concentration of valerate in the LP and HP group were significantly higher than those in the Control group, and the A:P ratio in the Control group was significantly higher than that in the other two groups. The crude protein level in the supplementary diet has no significant effect on the dominant microbial at the taxonomic level of phylum and genus. However, the dominant microorganisms are closely related to the different metabolites in

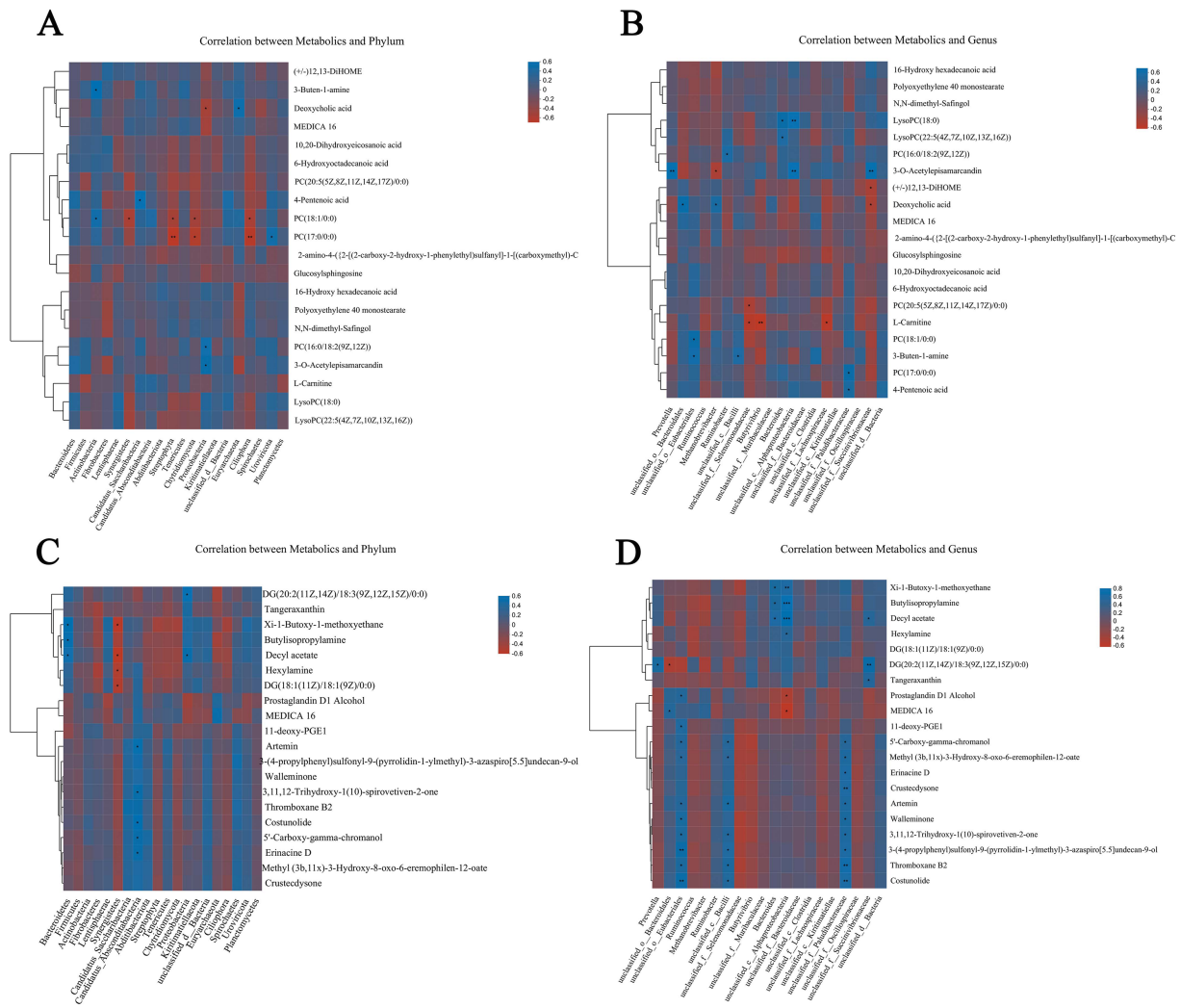


FIGURE 9
Correlation among rumen metagenome, serum and rumen metabolomics. Spearman level correlation between rumen microflora and metabolites. Correlation analysis between dominant microbials and serum differential metabolites at taxonomic level of phylum **(A)** and genus **(B)**. Correlation between dominant microflora at taxonomic level of phylum **(C)** and genus **(D)** and differential metabolites in rumen fluid.

serum and rumen, which are related to bile secretion and histidine metabolism, and are closely related to rumen fermentation parameters. To sum up, it is necessary to supplement the diet with suitable crude protein levels for JY during the dry season. This study is helpful to further explore the microbial and metabolic functions of JY fed with different crude protein levels, and then understand the protein requirements of JY in the dry season, so as to provide basic data for formulating more scientific supplementary diets in the future.

## 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: NCBI: PRJNA904725.

## Ethics statement

The animal study was reviewed and approved by Lanzhou Institute of Husbandry and Pharmaceutical Sciences of the Chinese Academy of Agricultural Sciences. Written informed consent was obtained from the owners for the participation of their animals in this study.

## Author contributions

CL, PY, and RFD contributed to conception and design of this study. RFD, RQD, XYM, and CH conducted relevant experiments. RFD drafted the manuscript. RFD, XYM, XMM, and YL organized the database. RFD and CH participated in the editing of the manuscript. CL and PY provided the funding supports. All authors contributed to the article 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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## Supplementary material

The Supplementary material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2023.1124917/full#supplementary-material

## References

Ardèvol, A., and Rovira, C. (2015). Reaction mechanisms in carbohydrate-active enzymes: glycoside hydrolases and glycosyltransferases. Insights from ab initio quantum mechanics/molecular mechanics dynamic simulations. *J. Am. Chem. Soc.* 137, 7528–7547. doi: 10.1021/jacs.5b01156

Beijer, W. H. (1952). Methane fermentation in the rumen of cattle. *Nature* 170, 576–577. doi: 10.1038/170576a0

Biely, P. (2012). Microbial carbohydrate esterases deacetylating plant polysaccharides. *Biotechnol. Adv.* 30, 1575–1588. doi: 10.1016/j.biotechadv.2012.04.010

Bihuniak, J. D., and Insogna, K. L. (2015). The effects of dietary protein and amino acids on skeletal metabolism. *Mol. Cell. Endocrinol.* 410, 78–86. doi: 10.1016/j.mce.2015.03.024

Bourne, Y., and Henrissat, B. (2001). Glycoside hydrolases and glycosyltransferases: families and functional modules. *Curr. Opin. Struct. Biol.* 11, 593–600. doi: 10.1016/s0959-440x(00)00253-0

Buchfink, B., Reuter, K., and Drost, H.-G. (2021). Sensitive protein alignments at tree-of-life scale using DIAMOND. *Nat. Methods* 18, 366–368. doi: 10.1038/s41592-021-01101-x

Buchfink, B., Xie, C., and Huson, D. H. (2015). Fast and sensitive protein alignment using DIAMOND. *Nat. Methods* 12, 59–60. doi: 10.1038/nmeth.3176

Calsamiglia, S., Cardozo, P. W., Ferret, A., and Bach, A. (2008). Changes in rumen microbial fermentation are due to a combined effect of type of diet and pH. *J. Anim. Sci.* 86, 702–711. doi: 10.2527/jas.2007-0146

Calsamiglia, S., Ferret, A., Reynolds, C. K., Kristensen, N. B., and van Vuuren, A. M. (2010). Strategies for optimizing nitrogen use by ruminants. *Animal* 4, 1184–1196. doi: 10.1017/s1751731110000911

Cantarel, B. L., Coutinho, P. M., Rancurel, C., Bernard, T., Lombard, V., and Henrissat, B. (2009). The carbohydrate-active EnZymes database (CAZy): an expert resource for Glycogenomics. *Nucleic Acids Res.* 37, D233–D238. doi: 10.1093/nar/gkn663

Chen, X., Yan, F., Liu, T., Zhang, Y., Li, X., Wang, M., et al. (2022a). Ruminal microbiota determines the high-fiber utilization of ruminants: evidence from the ruminal microbiota transplant. *Microbiol. Spectr.* 10:e0044622. doi: 10.1128/spectrum.00446-22

Dai, D., Pang, K., Liu, S., Wang, X., Yang, Y., Chai, S., et al. (2022). Effects of concentrate supplementation on growth performance, rumen fermentation, and bacterial community composition in grazing yaks during the warm season. *Animals (Basel)* 12:1398. doi: 10.3390/ani12111398

Erwin, E. S., Marco, G. J., and Emery, E. M. (1961). Volatile fatty acid analyses of blood and rumen fluid by gas chromatography. *J. Dairy Sci.* 44, 1768–1771. doi: 10.3168/jds.S0022-0302(61)89956-6

Estrada-Angulo, A., Castro-Pérez, B. I., Urías-Estrada, J. D., Ríos-Rincón, F. G., Arteaga-Wences, Y. J., Barreras, A., et al. (2018). Influence of protein level on growth performance, dietary energetics and carcass characteristics of Pelibuey×Katahdin lambs finished with isocaloric diets. *Small Rumin. Res.* 160, 59–64. doi: 10.1016/j.smallrumres.2018.01.012

Feng, S., Ngo, H. H., Guo, W., Chang, S. W., Nguyen, D. D., Liu, Y., et al. (2022). Volatile fatty acids production from waste streams by anaerobic digestion: a critical review of the roles and application of enzymes. *Bioresour. Technol.* 359:127420. doi: 10.1016/j.biortech.2022.127420

Fu, L., Niu, B., Zhu, Z., Wu, S., and Li, W. (2012). CD-HIT: accelerated for clustering the next-generation sequencing data. *Bioinformatics* 28, 3150–3152. doi: 10.1093/bioinformatics/bts565

Funabashi, M., Grove, T. L., Wang, M., Varma, Y., McFadden, M. E., Brown, L. C., et al. (2020). A metabolic pathway for bile acid dehydroxylation by the gut microbiome. *Nature* 582, 566–570. doi: 10.1038/s41586-020-2396-4

Gaffney, J., Embree, J., Gilmore, S., and Embree, M. (2021). *Ruminococcus bovis* sp. nov., a novel species of amylolytic Ruminococcus isolated from the rumen of a dairy cow. *Int. J. Syst. Evol. Microbiol.* 71:004924. doi: 10.1099/ijsem.0.004924

Ghurye, J. S., Cepeda-Espinoza, V., and Pop, M. (2016). Metagenomic assembly: overview, challenges and applications. *Yale J. Biol. Med.* 89, 353–362.

Gorin, E., and Zendowski, S. (1975). Effect of methoxyindole 2-carboxylic acid and 4-pentenoic acid on adipose tissue metabolism. *Biochim. Biophys. Acta* 388, 268–276. doi: 10.1016/0005-2760(75)90132-0

Granja-Salcedo, Y. T., Ribeiro Junior, C. S., de Jesus, R. B., Gomez-Insuasti, A. S., Rivera, A. R., Messana, J. D., et al. (2016). Effect of different levels of concentrate on ruminal microorganisms and rumen fermentation in Nellore steers. *Arch. Anim. Nutr.* 70, 17–32. doi: 10.1080/1745039x.2015.1117562

Harirchi, S., Wainaina, S., Sar, T., Nojoumi, S. A., Parchami, M., Parchami, M., et al. (2022). Microbiological insights into anaerobic digestion for biogas, hydrogen or volatile fatty acids (VFAs): a review. *Bioengineered* 13, 6521–6557. doi: 10.1080/21655979.2022.2035986

Huang, C., Ge, F., Yao, X., Guo, X., Bao, P., Ma, X., et al. (2021). Microbiome and metabolomics reveal the effects of different feeding systems on the growth and ruminal development of yaks. *Front. Microbiol.* 12:682989. doi: 10.3389/fmicb.2021.682989

Hyatt, D., Chen, G.-L., LoCascio, P. F., Land, M. L., Larimer, F. W., and Hauser, L. J. (2010). Prodigal: prokaryotic gene recognition and translation initiation site identification. *BMC Bioinform.* 11, 1–11. doi: 10.1186/1471-2105-11-119

Jones, D. R., Thomas, D., Alger, N., Ghavidel, A., Inglis, G. D., and Abbott, D. W. (2018). SACCHARIS: an automated pipeline to streamline discovery of carbohydrate active enzyme activities within polyspecific families and de novo sequence datasets. *Biotechnol. Biofuels* 11:27. doi: 10.1186/s13068-018-1027-x

Karimi, A., Alijoo, Y. A., Kazemi-Bonchenari, M., Mirzaei, M., and Sadri, H. (2021). Effects of supplemental fat sources and forage feeding levels on growth performance, nutrient digestibility, ruminal fermentation, and nitrogen utilization in dairy calves. *Animal* 15:100179. doi: 10.1016/j.animal.2021.100179

Karlsson, L., and Martinsson, K. (2011). Growth performance of lambs fed different protein supplements in barley-based diets. *Livest. Sci.* 138, 125–131. doi: 10.1016/j.livsci.2010.12.010

Kim, J. N., Méndez–García, C., Geier, R. R., Iakiviak, M., Chang, J., Cann, I., et al. (2017). Metabolic networks for nitrogen utilization in Prevotella ruminicola 23. *Sci. Rep.* 7:7851. doi: 10.1038/s41598-017-08463-3

Kuhaudomlarp, S., Patron, N. J., Henrissat, B., Rejzek, M., Saalbach, G., and Field, R. A. (2018). Identification of Euglena gracilis β-1,3-glucan phosphorylase and establishment of a new glycoside hydrolase (GH) family GH149. *J. Biol. Chem.* 293, 2865–2876. doi: 10.1074/jbc.RA117.000936

Kunath, B. J., Bremges, A., Weimann, A., McHardy, A. C., and Pope, P. B. (2017). Metagenomics and CAZyme discovery. *Methods Mol. Biol.* 1588, 255–277. doi: 10.1007/978-1-4939-6899-2_20

Lairson, L. L., Henrissat, B., Davies, G. J., and Withers, S. G. (2008). Glycosyltransferases: structures, functions, and mechanisms. *Annu. Rev. Biochem.* 77, 521–555. doi: 10.1146/annurev.biochem.76.061005.092322

Latham, E. A., Weldon, K. K., Wickersham, T. A., Coverdale, J. A., and Pinchak, W. E. (2018). Responses in the rumen microbiome of Bos taurus and indicus steers fed a low-quality rice straw diet and supplemented protein. *J. Anim. Sci.* 96, 1032–1044. doi: 10.1093/jas/sky023

Lawson, C. E., Wu, S., Bhattacharjee, A. S., Hamilton, J. J., McMahon, K. D., Goel, R., et al. (2017). Metabolic network analysis reveals microbial community interactions in anammox granules. *Nat. Commun.* 8:15416. doi: 10.1038/ncomms15416

Li, S., Du, M., Zhang, C., Wang, Y., Lee, Y., and Zhang, G. (2022). Diet type impacts production performance of fattening lambs by manipulating the ruminal microbiota and metabolome. *Front. Microbiol.* 13:824001. doi: 10.3389/fmicb.2022.824001

Li, D., Liu, C.-M., Luo, R., Sadakane, K., and Lam, T.-W. (2015). MEGAHIT: an ultra-fast single-node solution for large and complex metagenomics assembly via succinct de Bruijn graph. *Bioinformatics* 31, 1674–1676. doi: 10.1093/bioinformatics/btv033

Li, R., Yu, C., Li, Y., Lam, T.-W., Yiu, S.-M., Kristiansen, K., et al. (2009). SOAP2: an improved ultrafast tool for short read alignment. *Bioinformatics* 25, 1966–1967. doi: 10.1093/bioinformatics/btp336

Li, H., Yu, Q., Li, T., Shao, L., Su, M., Zhou, H., et al. (2020). Rumen microbiome and metabolome of Tibetan sheep (Ovis aries) reflect animal age and nutritional requirement. *Front. Vet. Sci.* 7:609. doi: 10.3389/fvets.2020.00609

Liu, K., Zhang, Y., Yu, Z., Xu, Q., Zheng, N., Zhao, S., et al. (2021). Ruminal microbiota-host interaction and its effect on nutrient metabolism. *Anim. Nutr.* 7, 49–55. doi: 10.1016/j.aninu.2020.12.001

Lombard, V., Bernard, T., Rancurel, C., Brumer, H., Coutinho, P. M., and Henrissat, B. (2010). A hierarchical classification of polysaccharide lyases for glycogenomics. *Biochem. J.* 432, 437–444. doi: 10.1042/bj20101185

Melo, L. Q., Costa, S. F., Lopes, F., Guerreiro, M. C., Armentano, L. E., and Pereira, M. N. (2013a). Rumen morphometrics and the effect of digesta pH and volume on volatile fatty acid absorption. *J. Anim. Sci.* 91, 1775–1783. doi: 10.2527/jas.2011-4999

Mirzakhani, A., Ghorbani, G. R., Sadeghi-Sefidmazgi, A., Rafiee, H., and Drackley, J. K. (2021). Effects of starter protein content and alkali processing of wheat straw on growth, ruminal fermentation, and behavior in Holstein calves. *J. Dairy Sci.* 104, 3098–3108. doi: 10.3168/jds.2020-19247

Mizrahi, I., Wallace, R. J., and Morais, S. (2021). The rumen microbiome: balancing food security and environmental impacts. *Nat. Rev. Microbiol.* 19, 553–566. doi: 10.1038/s41579-021-00543-6

Nadeau, J. A., Andrews, F. M., Patton, C. S., Argenzio, R. A., Mathew, A. G., and Saxton, A. M. (2003). Effects of hydrochloric, valeric, and other volatile fatty acids on pathogenesis of ulcers in the nonglandular portion of the stomach of horses. *Am. J. Vet. Res.* 64, 413–417. doi: 10.2460/ajvr.2003.64.413

Newbold, C. J., and Ramos-Morales, E. (2020). Review: ruminal microbiome and microbial metabolome: effects of diet and ruminant host. *Animal* 14, s78–s86. doi: 10.1017/s1751731119003252

Ngara, T. R., and Zhang, H. (2018). Recent advances in function-based metagenomic screening. *Genom. Proteom. Bioinform.* 16, 405–415. doi: 10.1016/j.gpb.2018.01.002

Palmonari, A., Stevenson, D. M., Mertens, D. R., Cruywagen, C. W., and Weimer, P. J. (2010). pH dynamics and bacterial community composition in the rumen of lactating dairy cows. *J. Dairy Sci.* 93, 279–287. doi: 10.3168/jds.2009-2207

Pang, K., Chai, S., Yang, Y., Wang, X., Liu, S., and Wang, S. (2022a). Dietary forage to concentrate ratios impact on yak ruminal microbiota and metabolites. *Front. Microbiol.* 13:964564. doi: 10.3389/fmicb.2022.964564

Qiu, K., Zhang, X., Jiao, N., Xu, D., Huang, C., Wang, Y., et al. (2018). Dietary protein level affects nutrient digestibility and ileal microbiota structure in growing pigs. *Anim. Sci. J.* 89, 537–546. doi: 10.1111/asj.12946

Qiu, Q., Zhang, G., Ma, T., Qian, W., Wang, J., Ye, Z., et al. (2012). The yak genome and adaptation to life at high altitude. *Nat. Genet.* 44, 946–949. doi: 10.1038/ng.2343

Rigden, D. J. (2005). Analysis of glycoside hydrolase family 98: catalytic machinery, mechanism and a novel putative carbohydrate binding module. *FEBS Lett.* 579, 5466–5472. doi: 10.1016/j.febslet.2005.09.011

Sharma, G., Garg, N., Hasan, S., and Shirodkar, S. (2022). Prevotella: an insight into its characteristics and associated virulence factors. *Microb. Pathog.* 169:105673. doi: 10.1016/j.micpath.2022.105673

Taş, N., de Jong, A. E., Li, Y., Trubl, G., Xue, Y., and Dove, N. C. (2021). Metagenomic tools in microbial ecology research. *Curr. Opin. Biotechnol.* 67, 184–191. doi: 10.1016/j.copbio.2021.01.019

Wang, L., Li, Y., Zhang, Y., and Wang, L. (2020a). The effects of different concentrate-to-forage ratio diets on rumen bacterial microbiota and the structures of Holstein cows during the feeding cycle. *Animals* 10:957. doi: 10.3390/ani10060957

Wang, X., Li, X., Zhao, C., Hu, P., Chen, H., Liu, Z., et al. (2012). Correlation between composition of the bacterial community and concentration of volatile fatty acids in the rumen during the transition period and ketosis in dairy cows. *Appl. Environ. Microbiol.* 78, 2386–2392. doi: 10.1128/aem.07545-11

Wang, X., Xu, T., Zhang, X., Geng, Y., Kang, S., and Xu, S. (2020b). Effects of dietary protein levels on growth performance, carcass traits, serum metabolites, and meat composition of Tibetan sheep during the cold season on the Qinghai-Tibetan plateau. *Animals* 10:801. doi: 10.3390/ani10050801

Wang, X., Xu, T., Zhang, X., Zhao, N., Hu, L., Liu, H., et al. (2022b). The response of ruminal microbiota and metabolites to different dietary protein levels in Tibetan sheep on the Qinghai-Tibetan plateau. *Front. Vet. Sci.* 9:922817. doi: 10.3389/fvets.2022.922817

Wang, K., Zhang, H., Hu, L., Zhang, G., Lu, H., Luo, H., et al. (2022a). Characterization of the microbial communities along the gastrointestinal tract in crossbred cattle. *Animals* 12:825. doi: 10.3390/ani12070825

Warner, A. C. (1964). Production of volatile fatty acids in the rumen: methods of measurement. *Nutr. Abstr. Rev.* 34, 339–352.

Weimer, P. J. (2022). Degradation of cellulose and hemicellulose by ruminal microorganisms. *Microorganisms* 10:2345. doi: 10.3390/microorganisms10122345

Weller, R. A., Pilgrim, A. F., and Gray, F. V. (1969). Volatile fatty acid production in the rumen of the grazing sheep: its use as an indicator of pasture value. *Br. J. Nutr.* 23, 97–111. doi: 10.1079/bjn19690013

Xu, T., Xu, S., Hu, L., Zhao, N., Liu, Z., Ma, L., et al. (2017). Effect of dietary types on feed intakes, growth performance and economic benefit in Tibetan sheep and yaks on the Qinghai-Tibet plateau during cold season. *PLoS One* 12:e0169187. doi: 10.1371/journal.pone.0169187

Xue, M.-Y., Sun, H.-Z., Wu, X.-H., Liu, J.-X., and Guan, L. L. (2020). Multi-omics reveals that the rumen microbiome and its metabolome together with the host metabolome contribute to individualized dairy cow performance. *Microbiome* 8:64. doi: 10.1186/s40168-020-00819-8

Xue, M. Y., Wu, J. J., Xie, Y. Y., Zhu, S. L., Zhong, Y. F., Liu, J. X., et al. (2022b). Investigation of fiber utilization in the rumen of dairy cows based on metagenome-assembled genomes and single-cell RNA sequencing. *Microbiome* 10:11. doi: 10.1186/s40168-021-01211-w

Xue, M.-Y., Xie, Y.-Y., Zhong, Y., Ma, X.-J., Sun, H.-Z., and Liu, J.-X. (2022a). Integrated meta-omics reveals new ruminal microbial features associated with feed efficiency in dairy cattle. *Microbiome* 10:32. doi: 10.1186/s40168-022-01228-9

Xue, B., Zhao, X. Q., and Zhang, Y. S. (2005). Seasonal changes in weight and body composition of yak grazing on alpine-meadow grassland in the Qinghai-Tibetan plateau of China. *J. Anim. Sci.* 83, 1908–1913. doi: 10.2527/2005.8381908x

Yáñez-Ruiz, D. R., Abecia, L., and Newbold, C. J. (2015). Manipulating rumen microbiome and fermentation through interventions during early life: a review. *Front. Microbiol.* 6:1133. doi: 10.3389/fmicb.2015.01133

Yi, S., Dai, D., Wu, H., Chai, S., Liu, S., Meng, Q., et al. (2022a). Dietary concentrate-to-forage ratio affects rumen bacterial community composition and metabolome of yaks. *Front. Nutr.* 9:927206. doi: 10.3389/fnut.2022.927206

Yu, Z. T., and Morrison, M. (2004). Improved extraction of PCR-quality community DNA from digesta and fecal samples. *BioTechniques* 36:808. doi: 10.2144/04365st04

Zhang, T., Mu, Y., Zhang, D., Lin, X., Wang, Z., Hou, Q., et al. (2019). Determination of microbiological characteristics in the digestive tract of different ruminant species. *Microbiology* 8:e00769. doi: 10.1002/mbo3.769

Zhang, J., Shi, H., Wang, Y., Li, S., Cao, Z., Ji, S., et al. (2017). Effect of dietary forage to concentrate ratios on dynamic profile changes and interactions of ruminal microbiota and metabolites in Holstein heifers. *Front. Microbiol.* 8:2206. doi: 10.3389/fmicb.2017.02206

Zhang, X., Xu, T., Wang, X., Geng, Y., Zhao, N., Hu, L., et al. (2021). Effect of dietary protein levels on dynamic changes and interactions of ruminal microbiota and metabolites in yaks on the Qinghai-Tibetan plateau. *Front. Microbiol.* 12:684340. doi: 10.3389/fmicb.2021.684340

Zhao, C., Wang, L., Ke, S., Chen, X., Kenez, A., Xu, W., et al. (2022). Yak rumen microbiome elevates fiber degradation ability and alters rumen fermentation pattern to increase feed efficiency. *Anim. Nutr. (Zhongguo xu mu shou yi xue hui)* 11, 201–214. doi: 10.1016/j.aninu.2022.07.014

Zhao, X. Q., and Zhou, X. M. (1999). Ecological basis of alpine meadow ecosystem management in Tibet: Haibei alpine meadow ecosystem Research Station. *Ambio* 28, 642–647.

Zhong, J., Fong, J. C., and Schulz, H. (1985). Inhibition of carnitine acetyltransferase by metabolites of 4-pentenoic acid. *Arch. Biochem. Biophys.* 240, 524–529. doi: 10.1016/0003-9861(85)90058-x

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# Effects of dietary N-carbamylglutamate on rumen fermentation parameters, and bacterial community diversity of Holstein dairy cows in Tibet

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**Introduction:** The Tibetan Plateau is characterized by low temperature and hypoxia. N-carbamylglutamic acid (NCG) can increase blood oxygen saturation, and have the potential to be used to prevent the high-altitude hypoxia stress state of cows. However, its beneficial effect on the rumen microbiota of Holstein dairy cows remains unclear.

**Methods:** Hence, the experiments 12 multiparous (parity ranged from 2 to 7) Holstein dairy cows (413.0±42 kg) were randomly assigned to 2 treatments with 6 replicates in each treatment: basal diet (CON, control group) and basal diet plus 20g/d/cow of NCG (NCG, experiment group), respectively. To study the effects of dietary NCG supplementation on rumen microbiota of Holstein dairy cows in Tibet. The experiment lasted for 45 days, with 15 days of pre-feeding and 30days of formal trail period.

**Results:** The results showed that ruminal $NH_3$-N concentration in NCG group was lower ($p<0.05$) than that in the CON group, while molar proportion of acetic acid and total volatile fatty acid (VFA) concentration were increased ($p<0.05$) with the addition of NCG. Microbial diversity increased ($p<0.05$) in NCG group, with *Bacteroidetes*, *Firmicutes,* and *Patescibacteria* as the most abundant phyla. The KEGG pathway analysis showed that the potential function of ruminal bacteria was mainly enriched in metabolism (carbohydrates, amino acids, lipids, energy, and nucleotides) and genetic information processing (replication, repair, and translation).

**Conclusion:** In conclusion, NCG can improve rumen nitrogen utilization, total VFA and acetic acid production, and increase rumen microbial diversity, all of which could make the introduced Holstein dairy cows to better adapt to the harsh environment in Tibet and improve their production performance.

KEYWORDS

**N-carbamylglutamate, rumen bacteria, rumen fermentation, Tibet, Holstein dairy cow**

# 1. Introduction

Due to the special geographical and environmental conditions, the Qinghai-Tibet Plateau has a long cold climate, strong ultraviolet radiation, strong wind and low oxygen content in the air (Liu H. et al., 2022; Liu Z. et al., 2022), leading to an extremely harsh living environment for livestock. As the hinterland of plateau, the living environment is even worse in Tibet, making it a major restricting factor for the development of Tibet's animal husbandry. Local animals like Yaks (*Bos grunniens*) and Tibetan sheep are well-adapted to the harsh living environment in Tibet; however, introduced animals like Holstein dairy cows and Jersey cows could not stand with the harsh environment there. For example, introduced Jersey cows in this area have to live and produce under the challenge of low temperature and hypoxia, which is the major reason of hypoxemia and tissue hypoxia (Kong et al., 2019). Moreover, it is prone to a series of pathophysiological changes that will greatly affect the body's health and production performance, such as pulmonary hypertension, decreased milk production, and other metabolic disorders and clinical symptoms (Neary and Garry, 2014).

Offering nutritional feed additives to animals that have the risk of suffering hypoxic have been proved efficient (Wenk, 2000). Although Arg (Arginine) could attenuate inflammatory stress and significantly improve lactating performance of dairy cows (Wang, 2016; Zhao et al., 2018; Ding et al., 2019), and improve the altitude reaction in high altitude animals by increasing blood oxygen saturation (Schneider et al., 2001), the rapid degradation in the rumen (Chacher et al., 2012) and the high price hindered its utilization in ruminant production. In the meanwhile, N-carbamoyl glutamate (NCG), a structural analog of N-acetylglutamate acts as an allosteric activator of essential cofactor aminophosphate synthase in endogenous arginine synthesis (Gessler et al., 2010), is much more stable than Arg in the rumen (Ma et al., 2019). The NCG is non-toxic to livestock (Guffon et al., 1995), and previous studies consistently proven that supplementation of NCG to ruminant diets could improve milk yield and production performance for dairy cows (Chacher et al., 2014; Ma et al., 2020; Gu et al., 2021), and improve reproduction of gestation Hu sheep (Sun et al., 2017) and improved the health and growth of suckling Hu lambs (Zhang et al., 2018). Therefore, it is widely used as a viable and cost-effective alternative to Arg. Our parallel study indicated that dietary supplementation of NCG by 20 g/d/head prevented hypoxic stress and tended to improve milk quality of Jersey dairy cows in Tibet (Liu H. et al., 2022; Liu Z. et al., 2022). This suggests that NCG also has beneficial effects on the physiological regulation of introduced animals at high altitude plateau.

This led to the hypothesis that supplementation of NCG would alter rumen metabolism via regulating ruminal microbial community of Holstein dairy cows that introduced to Tibet. Hence, this study was designed to assess the effect of NCG supplementation on rumen metabolism and microbiota of Holstein dairy cows that have been introduced to Tibet for a long time.

# 2. Materials and methods

## 2.1. Ethics statement

The animals and experimental procedures used in this study were approved by the Institutional Animal Care and the Use Committee, Institute of Subtropical Agriculture, Chinese Academy of Sciences, Changsha, PR China (Approval number: ISA-2019-0115).

## 2.2. Animals, experimental design, and diets

This study was conducted during the warn season (from June to July 2021) in Changzhu dairy farm (Latitude N: 28°57′17″, Longitude E: 91°39′4″, Altitude: 3700 m), Shannan City, Tibet Autonomous Region, China. The hybrid Holstein breed (Holstein × yellow cattle) was selected, Average milk yield 9.6 kg/d, dry matter intake 16.91 kg/d. Twelve multiparous (parity ranged from 2 to 7) Holstein dairy cows (413.0 ± 42 kg) in pre-lactation with no clinical signs of disease were used as experimental animals in a completely random designed experiment.

All Holstein dairy cows randomly assigned into two groups (6 cows/group). Both groups of cows were randomly assigned to one of two treatments, with one group fed a basal diet (CON group) and another group fed the basal diet plus 20 g NCG per day for each cow (NCG group). The basal diet was offered in the form of total mixed ration (TMR), while the NCG was offered by top-dressing on the basal TMR at morning feeding to make sure been fully consumed. The NCG, with an ≥80%, used in the current study was supplied by Nenghe Biotechnology Co., Ltd., Zhongshan, China. The ingredients and chemical compositions of the basal diet are illustrated in Table 1. The experiment lasted 45 days, with 15 days of pre-feeding and 30 days of formal experimentation. All cows were fed two equal meals at 06:00 and 18:00 h every day. All animals were allowed free access to feed and fresh water throughout the experimental period.

## 2.3. Sample collection and measurements

Diet offered and refused for each cow were recorded, and samples of diet and orts were collected daily throughout the formal experimental period. Feed and orts samples were dried at 65°C for 72 h, and ground to pass a 1 mm screen. Then the samples were analyzed for analytical dry matter (DM; method 934.01), ether extract (EE; method 920.39), crude protein (CP; method 976.06), acid detergent fiber (ADF, method 985.29), calcium (Ca; method 978.02), and total phosphorus (TP; method 946.06) according to the procedures of the Association of Official Analytical Chemist (AOAC, 2012). The neutral detergent fiber (NDF) contents were determined using a Fibretherm Fiber Analyzer with F57 filter bags (ANKOM A200, ANKOM Technology Corp., Fairport, NY, United States) according to Van Soest et al. (1991).

Rumen fluid samples (100 mL) were collected from each cow using an oral stomach tube at 2 h after morning feeding on the final day of the experiment as reported previously (Shen et al., 2012). The initial 50 mL was discarded to minimize contamination of oral saliva. The pH value was measured immediately using a pH meter (PHS-100 portable acidity meter, Tianqi Mdt InfoTech Ltd., Shanghai, China). Thereafter, rumen fluid samples were filtered through a 4-layer cheesecloth and stored at −80°C until analysis. During sample analysis, 10 mL of rumen fluid was used for microbial crude protein (MCP) determination, and 5 mL of rumen fluid was centrifuged (10,000 g, 4°C, 15 min) to obtain supernatants, which was used for

TABLE 1 Ingredients and nutrient compositions of the basal diet (DM basis).

| Items | Content (%) |
|---|---|
| **Ingredients** | |
| Corn silage | 62.50 |
| Wheat grass | 20.83 |
| Alfalfa hay | 8.33 |
| Oat | 0.92 |
| Wheat bran | 0.75 |
| DDGS | 0.67 |
| Corn | 4.37 |
| Soybean meal | 0.50 |
| Cottonseed meal | 0.42 |
| Premix[1] | 0.63 |
| NaCl | 0.08 |
| Total | 100 |
| **Nutrient levels[2]** | |
| CP | 9.23 |
| EE | 4.84 |
| NDF | 51.99 |
| ADF | 24.35 |
| $NE_L$/(MJ/kg) | 5.15 |
| Calcium | 0.55 |
| Phosphorus | 0.26 |

[1]Per kilogram of premix contained the following: Ferric, 4,500 mg; Copper, 1,600 mg; Manganese, 3,000 mg; Zinc, 5,500 mg; Selenium, 30 mg; Cobalt, 20 mg; Iodine, 30 mg; vitamin A 600,000 IU; vitamin D 200,000 IU; vitamin E 200 IU.
[2]CP, crude protein; EE, ether extract; NDF, neutral detergent fiber; ADF, acid detergent fiber, $NE_L$, milk production and net energy.

volatile fatty acid (VFA) and ammonia nitrogen ($NH_3$-N) determination. The VFA concentration was measured using high-performance liquid chromatography (Agilent 1,100, Santa Clara, CA), referring to the method of Wang et al. (2020). The treated sample was automatically injected into the Agilent DBFFAP gas capillary column (30 m × 0.25 mm × 0.25 μm). Injector temperature was set to 250°C and detector temperature was set to 280°C. The split ratio of all samples was set at 50:1. The column temperature was programmed to heat from 60 to 220°C at a rate of 20°C/min, and then held for 5 min. A colorimetric method was used to detect $NH_3$-N and MCP as described by Wang et al. (2019).

## 2.4. DNA extraction, PCR amplification, and 16S rRNA sequencing

Total microbial DNA was extracted using the QIAamp® DNA Stool Mini Kit (Qiagen, Hilden, Germany), and the quality and quantity of extracted DNA were measured by a NanoDrop 2000 (Thermo Scientific, Waltham, United States). The quantitative real-time PCR analysis of total bacteria was carried out with an ABI 7900 sequence detection system (Applied Biological System, Foster, CA, United States), using SYBR green premix Pro Taq HS qPCR kit AG11701 (Magigene Bioinformatics Technology, Guangzhou, China). The specific test procedures were in accordance with Jiao et al. (2015). The V3-V4 hypervariable region of 16S rRNA genes of bacteria was amplified with primer 338F (5′-ACT CCT ACG GGA GGC AGC A-3′) and 806R (5′-GGA CTA CHV GGG TWT CTA AT-3′) as reported by Mumbi et al. (2015). The PCR procedures and conditions were performed according to the method described by Jiao et al. (2016) The PCR products were purified using the QIAquick Gel Extraction Kit (QIAGEN, Germany), quantified using real-time PCR, and sequenced on the MiSeq platform at Magigene Bioinformatics Technology (Guangzhou, China). The raw paired-end reads analysis and the quality control were referenced to the methods as reported by Huang et al. (2020). Qualified reads were separated using sample-specific barcode sequences and trimmed using the Illumina Analysis Pipeline version 2.6. The dataset was then analyzed using QIIME 2. The sequences with a similarity level of more than 97% were clustered into the same operational taxonomic units (OTUs), using the method of Uparse Software (7.1 version).[1] Analyses of alpha diversities were performed using the ggplot2 and vegan packages of R 1.7 version (Oksanen et al., 2013). All computed distance matrix was visualized in a non-metric multidimensional scaling (NMDS) graph. The R software was used to analyze the differences of beta diversity index between groups.

## 2.5. Correlation analysis of ruminal fermentation and rumen bacteria

Spearman correlation analysis was carried out to analyze the relationship between the relative abundances of rumen bacteria and ruminal fermentation parameters. The absolute value of correlation coefficient was greater than 0.6 and the $p < 0.05$, the correlation was considered significant. The R Pretty Heatmaps package was applied to obtain heatmaps to visualize the robustness of correlations.

## 2.6. Functional prediction of microbial pathway abundances by PICRUSt

The functional potential of rumen microbes was inferred from the ASVs table using PICRUSt (Phylogenetic Investigation of Communities by Reconstruction of Unobserved States) (Douglas et al., 2020). First, execute the normalize_by_copy_number.py script to normalize the ASVs. Second, the standardized ASVs table was entered into the predict_metagnomes.py script to generate the Kyoto Encyclopedia of Genes and Genomes (KEGG)-based prediction of microbial pathway abundance.

## 2.7. Statistical analysis

The Shapiro–Wilk test was used for normal distribution of ruminal fermentation parameters and alpha diversity index data of the

1 http://drive5.com/uparse/

two groups, followed by Student's *t*-test for comparison of means. The Wilcoxon rank-sum test was used to compare the relative abundance of bacterial taxa between the two groups. All the data were analyzed using the SPSS Statistics 22 (IBM, Chicago, United States) software. The data were expressed as means and standard error of means (SEM). The treatment effects was significant at $p < 0.05$, and the trends was discussed at $0.05 \leq p < 0.10$.

# 3. Results

## 3.1. Rumen fermentation parameters

The ruminal pH and MCP production did not differ between CON and NCG treatments, but the ruminal $NH_3$-N concentration in CON was significantly greater ($p < 0.01$) than that in NCG (Table 2). The concentration of total VFA tended ($p = 0.09$) to be greater in NCG than that of CON (Table 2). Greater ($p = 0.03$) molar proportion of acetate was observed in NCG than that in CON group, thus lead to a tendency ($p = 0.07$) of greater Acetate/Propionate ratio in CNG group; meanwhile, the molar proportion of butyric acid tended ($p = 0.09$) to be higher in CON than that in NCG (Table 2). Whereas, the molar proportion of the rest individual VFA were not different between CON and NCG treatments.

TABLE 2 Effects of NCG supplementation on ruminal fermentation and microbial crude protein of Holstein dairy cows in Tibet.

| Items | Treatments[1] | | SEM[2] | *p*-value |
|---|---|---|---|---|
| | CON | NCG | | |
| pH | 6.90 | 6.89 | 0.103 | 0.89 |
| $NH_3$-N, mg/dL | 9.84[a] | 6.09[b] | 1.161 | < 0.01 |
| MCP[3], mg/mL | 1.38 | 1.49 | 0.154 | 0.49 |
| Total VFA[4], mmol/L | 55.31 | 66.43 | 5.735 | 0.09 |
| Acetic acid, mmol/100 mmol | 58.65[b] | 61.70[a] | 1.320 | 0.03 |
| Propionic acid, mmol/100 mmol | 21.25 | 19.93 | 0.767 | 0.11 |
| Acetic/Propionic | 2.80 | 3.12 | 0.174 | 0.07 |
| Butyric acid, mmol/100 mmol | 15.54 | 14.17 | 0.767 | 0.09 |
| Isobutyric acid, mmol/100 mmol | 1.11 | 1.07 | 0.091 | 0.46 |
| Valeric acid, mmol/100 mmol | 1.25 | 1.20 | 0.124 | 0.70 |
| Isovaleric acid, mmol/100 mmol | 2.19 | 1.86 | 0.311 | 0.30 |

Values within a row with different superscripts differ at $p < 0.05$.
[1]CON, basal diet, without additive; NCG, basal diet + 20 g NCG/d.
[2]SEM, standard error of means.
[3]MCP, microbial crude protein.
[4]VFA, volatile fatty acid.

## 3.2. Rumen microbial richness and diversity

A total of 1,050,460 high-quality reads were obtained for all 12 rumen fluid samples, with an average of 87,538 ± 4,713 reads per sample (Supplementary Figure 1). Rarefaction curves showed that the sequencing depth covered essentially all species in all samples and accurately reflected microbial communities (Figure 1A). A total of 3,077 OTUs were obtained, with more than 95% annotated at phylum, class, and order level, approximately 80% annotated at family level, and only 56.87 and 31.04% annotated at genus and species level (Supplementary Table 1), respectively. Among which, 1839 were shared OTUs in the con and NCG cows, and 524 and 713 were unique OTUs in the con and NCG cows, respectively (Supplementary Figure 2). Alpha diversity indices including Chao 1 and Shannon diversity were analyzed, and the Chao 1 did not differ ($p > 0.05$) between treatments, while, the Shannon index was greater ($p < 0.05$) in NCG than that in CON (Figure 1B), suggesting that offering NCG to cows resulted in a greater ruminal bacteria diversity. The β-diversity was done by NMDS analysis, and the result showed that the ruminal bacteria community structure was greatly ($p = 0.026$) different between the CON and NCG groups (Figure 2; stress = 0.0994). As compared to the CON cows, the ruminal bacteria of cows received NCG supplementation had a clear cluster, indicating that they have a more similar microbial community structure.

## 3.3. Taxonomic analysis of ruminal microbial communities

Except some unclassified phyla, a total number of 19 phyla were identified in the ruminal microbiota of Holstein dairy cows in Tibetan plateau, with those greater than 1% in at least one group shown in Table 3. The five most abundant phyla were *Firmicutes* (averaged 54%), *Bacteroidetes* (averaged 17%), *Patescibacteria* (averaged 16%), *Proteobacteria* (averaged 6%), and *Tenericutes* (averaged 2%), accounting more than 95% of the entire bacteria identified. Notably, *Patescibacteria* was found to be a dominant phylum in Holstein dairy cow for the first time. At the family level, *Ruminococcaceae* (averaged 34%), *Saccharimonadaceae* (averaged 15%), *Lachnospiraceae* (averaged 11%), and *Prevotellaceae* (averaged 6%) were the four most abundant family (Table 4). Meanwhile, at the genus level, a total number of 77 genera were identified, and only the top 10 genera (including unassigned and uncultured) are listed in Table 5, with the *Candidatus_Saccharimonas* (averaged 15%), *Ruminococcus_1* (averaged 11%), *Papillibacter* (averaged 4%), *Ruminococcus_2* (averaged 4%), and *Lachnospiraceae_XPB1014_group* (averaged 4%) determined as the five most abundant genus. However, dietary NCG supplementation had no significant ($p > 0.05$) effect on relative abundances of ruminal bacterial at either phyla, family, or genus level.

## 3.4. Correlation of rumen bacterial with fermentation parameters

The Spearman's rank correlation analysis was used to investigate the correlations between ruminal fermentation parameters and rumen bacteria relative abundance at family level for dairy cows (Figure 3 and Supplementary Tables 2, 3). The production of ruminal MCP positively

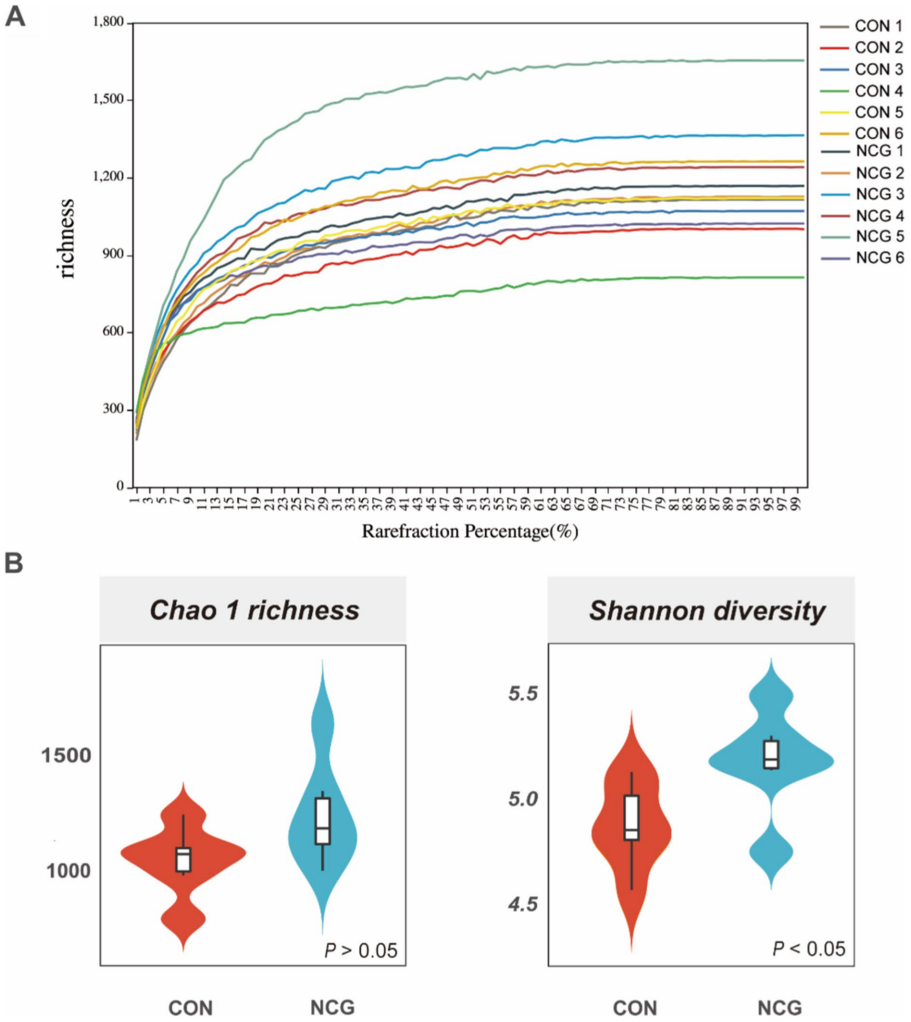


FIGURE 1
Rarefaction curve **(A)** and alpha diversity indices **(B)**; Chao 1 and Shannon indexes of ruminal bacteria of lactating cows fed CON and NCG diets. CON, basal diet; NCG, basal diet +20g NCG/cow/d.

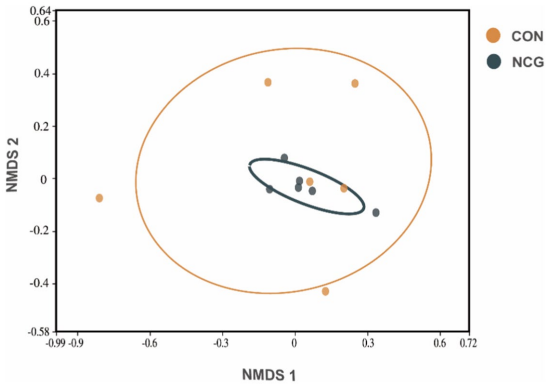


FIGURE 2
Non-metric Multidimensional Scaling (NMDS) analysis based on Bray Curtis (stress=0.0994) distance of ruminal fluid samples of lactating cows fed diets with or without N-carbamoyl glutamate (NCG). The centroid of each ellipse represents the group mean, and the shape was defined by the covariance within each group. CON, basal diet; NCG, asal diet +20g NCG/cow/d.

correlated with the relative abundance of family *F082* ($r=0.832$, $p=0.001$); the Acetic/Propionic positively ($r=0.713$, $p=0.012$) correlated with the relative abundance of family *Ruminococcaceae*, whereas it negatively ($r=0.832$, $p=0.019$) correlated with family *Lachnospiraceae*. In contrast, the molar proportion of propionic acid positively correlated with the relative abundance of family *Lachnospiraceae* ($r=0.699$, $p=0.015$), but negatively correlated with the family *Ruminococcaceae* ($r=0.678$, $p=0.019$). Attention should also be paid that the relative abundances of unassigned bacteria positively correlated with the molar proportion of propionic acid and valeric acid, as well as the uncultured bacteria negatively correlated with the molar proportion of acetic acid. This suggests that unassigned and uncultured bacteria are also played an important role in ruminal fermentation.

## 3.5. Rumen microbial function prediction

The functions of ruminal microbes were predicted using KEGG pathway analysis, with more than 77% of the bacteria of both treatments allocated to the function of "Metabolism" at level 1 of KEGG

TABLE 3 Relative abundances (%) of ruminal bacteria at phylum level in dairy cows received or not received NCG.

| Phylum | Treatments[1] | | SEM[2] | *p*-value |
|---|---|---|---|---|
| | CON | NCG | | |
| *Firmicutes* | 0.53 | 0.55 | 0.033 | 0.90 |
| *Bacteroidetes* | 0.18 | 0.15 | 0.026 | 0.91 |
| *Patescibacteria* | 0.15 | 0.17 | 0.032 | 0.89 |
| *Proteobacteria* | 0.07 | 0.05 | 0.013 | 0.91 |
| *Tenericutes* | 0.02 | 0.03 | 0.006 | 0.90 |
| *Spirochaetes* | 0.01 | 0.01 | 0.004 | 0.89 |
| *Cyanobacteria* | <0.01 | 0.01 | 0.004 | 0.89 |
| *Kiritimatiellaeota* | 0.01 | 0.01 | 0.003 | 0.88 |
| *Fibrobacteres* | <0.01 | 0.01 | 0.002 | 0.89 |
| *Lentisphaerae* | 0.01 | <0.01 | 0.004 | 0.91 |

[1]CON, basal diet; NCG, control diet + 20 g NCG/cow/d.
[2]SEM, standard error of means.

TABLE 4 Relative abundances (%) of ruminal bacteria at family level in dairy cows received or not received NCG.

| Family | Treatments[1] | | SEM[2] | *p*-value |
|---|---|---|---|---|
| | CON | NCG | | |
| *Ruminococcaceae* | 0.34 | 0.35 | 0.037 | 0.89 |
| *Saccharimonadaceae* | 0.15 | 0.16 | 0.032 | 0.89 |
| *Lachnospiraceae* | 0.10 | 0.12 | 0.029 | 0.88 |
| *Prevotellaceae* | 0.07 | 0.05 | 0.018 | 0.99 |
| *Bacteroidales_RF16_group* | 0.04 | 0.05 | 0.009 | 0.88 |
| *F082* | 0.04 | 0.03 | 0.010 | 0.90 |
| *Christensenellaceae* | 0.03 | 0.04 | 0.006 | 0.88 |
| *Clostridiales_vadinBB60_group* | 0.03 | 0.02 | 0.014 | 0.89 |
| (Unassigned) | 0.04 | 0.06 | 0.009 | 0.88 |
| Uncultured | 0.05 | 0.05 | 0.012 | 0.91 |

[1]CON, basal diet; NCG, basal diet + 20 g NCG/cow/d.
[2]SEM, standard error of means.

(Supplementary Figure 3). A total of 33 functional pathways were identified at the level 2 of KEGG orthologs (KO), with the top 20 pathways shown in Figure 4. In this potential pathway functional profile, the functional structure of the ruminal bacteria community identified in rumen fluid samples collected from dairy cows in Tibet was found to be dominated by pathways involved in metabolism (carbohydrates, amino acids, lipid, energy, and nucleotides), and genetic information processing (replication, and repair, translation). Moreover, the pathways responsible for cell growth and death, and cell motility were found to be dominant in pathways concerning cellular processes.

## 4. Discussion

Dietary supplementation of NCG altered ruminal fermentation, namely enhanced concentration of total VFA and acetic acid but lower

TABLE 5 Relative abundances (%) of ruminal bacteria at genus level in dairy cows received or not received NCG.

| Genus | Treatments[1] | | SEM[2] | *p*-value |
|---|---|---|---|---|
| | CON | NCG | | |
| *Candidatus_saccharimonas* | 0.15 | 0.16 | 0.032 | 0.80 |
| *Ruminococcus_1* | 0.10 | 0.11 | 0.026 | 0.81 |
| *Papillibacter* | 0.04 | 0.05 | 0.011 | 0.80 |
| *Ruminococcus_2* | 0.05 | 0.04 | 0.023 | 0.80 |
| *Lachnospiraceae_XPB1014_group* | 0.05 | 0.04 | 0.012 | 0.86 |
| *Prevotella_1* | 0.04 | 0.03 | 0.008 | 0.87 |
| *Christensenellaceae_R-7_group* | 0.03 | 0.04 | 0.006 | 0.74 |
| *Ruminococcaceae_UCG-010* | 0.03 | 0.03 | 0.008 | 0.8 |
| Unassigned | 0.24 | 0.24 | 0.028 | 0.81 |
| Uncultured | 0.03 | 0.03 | 0.006 | 0.80 |

[1]CON, basal diet, without additive; NCG, basal diet + 20 g NCG/cow/d.
[2]SEM, standard error of means.

$NH_3$-N, which indicate enhanced ruminal N utilization. VFA can provide 75% of the energy for ruminants (Cheng et al., 2021), and also play an important role in immunity and growth of ruminants (Fan et al., 2020). The enhanced total VFA in NCG treatment was mainly because of increased molar proportion of acetic acid, suggesting a possibility increased fiber digestibility even though the nutrient digestibility were not determined. It was previously reported that NCG can promote the production of citrulline, which is further converted into fumaric acid and Arg under the action of enzymes (Hu et al., 2019). Fumaric acid is primary converted into acetic acid instead of lactic acid, which is beneficial for ruminal fermentation (Zhou et al., 2012). This might be the reason for the greater acetic acid production in cows fed NCG. Moreover, greater milk fat contents were determined in Jersey cows offered same dose of NCG like the current study (Liu H. et al., 2022; Liu Z. et al., 2022), suggesting that enhanced acetic acid are beneficial for milk fat syntheses. Dietary supplementation of NCG did not affect the molar proportion of propionate acid, thus lead to greater acetate to propionate ratio. Additionally, supplementation of NCG can promote urea cycle (Chacher et al., 2014), improve ruminal $NH_3$-N utilization (Oba et al., 2005), and thus provide more N sources for MCP synthesis. It was proved in *in vitro* and *in vivo* studies that NCG can decrease ruminal $NH_3$-N concentration (Chacher et al., 2013, 2014), enable rumen microbes to synthesize more MCP, thus reducing nitrogen excretion and improving nitrogen utilization (Chacher et al., 2014). The lowered ruminal $NH_3$-N concentration in NCG cows was in consistence with previous reports by Chacher et al. (2013, 2014); however, only numerically greater contents of ruminal MCP were observed in NCG feeding cows. Taken together, supplementation of NCG to diet of Holstein dairy cows in Tibet do have benefits in improving ruminal fermentation and nutrient utilization, which will help the introduced animals to better adapt to the harsh environment of plateau hypoxia.

Biodiversity is essential for promoting the sustainability and productivity of numerous ecosystems. Similarly, the diversity of the rumen microbiota is closely related to metabolic ability, ruminal

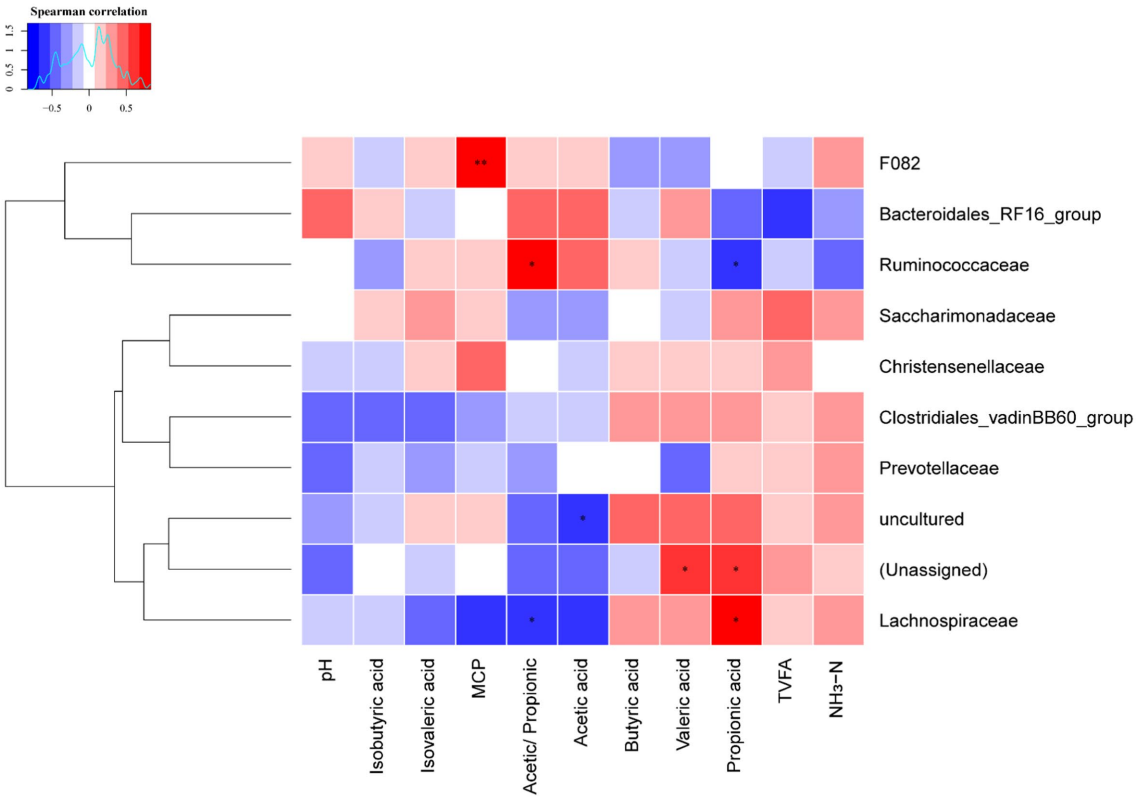


FIGURE 3
Heatmap of the correlations between the relative abundance of ruminal bacteria at family level and ruminal fermentation parameters. Blue color indicates negative correlations, and red color represents positive correlations. * Represents $p<0.05$ and ** represent $p<0.01$. MCP, microbial crude protein; TVFA, total volatile fatty acid; $NH_3$-N, ammonia nitrogen.

stability of ruminants, and health status. The current experiment studied the effects of diet add of NCG on rumen fermentation and rumen microbiota of Holstein dairy cows in Qinghai-Tibet Plateau. The results of 16 s rRNA gene sequencing indicated that dietary NCG supplementation resulted in an increased rumen microbial diversity of Tibetan Plateau dairy cows. We speculated that dairy cows of the NCG group would benefit from the increased microbial diversity, which will increase the stability of rumen environment and increase body energy to meet their survival requirements in the high-altitude and hypoxic environment in the Tibetan plateau. The more clustered ruminal bacteria community in NCG supports this speculation to some extends. Human studies have also shown that the diversity of intestinal microbiota improves the fermentation efficiency of dietary fiber and contributes to the stability of intestinal ecosystems (Tap et al., 2015). Moreover, the increased diversity of human gut microbes also reflects stronger metabolic capacity and improved health (Clarke et al., 2014).

The phyla *Firmicutes* and *Bacteroidetes* were dominant in the rumen of Tibetan Plateau dairy cows. *Firmicutes* and *Bacteroidetes* are consistently detected as the predominant phylum in rumen (Cui et al., 2019), suggesting that these bacteria play a significant role in rumen function and ecology of ruminants living in both low and high altitude. Furthermore, it should be noted that a high relative abundance of phyla *Patescibacteria* was detected in the rumen of Tibetan plateau dairy cows, which was not reported previously. Patescibacteria, also known as CPR, bacteria with special cell forms that are an important subset of the smallest known family of bacteria on Earth (He et al., 2015), it can be detected in wide range natural habitats (Rinke et al., 2013; Probst et al., 2018; Bokhari et al., 2020). Phyla *Patescibacteria* are commonly found in a variety of anaerobic environments such as wastewater treatment systems, soil, and human oral cavity, but are not a permanent member of the rumen environment (Wrighton et al., 2014). The plentiful *Patescibacteria* was not positive correlation with the nutrient index of water, indicating that the bacteria could adapt to oligotrophic environment (Herrmann et al., 2019). Cabello et al. (2019) were also able to assemble and obtain CPR MAGs in the bottom water of Belga Lake, again verified that CPR bacteria can live in an oligotrophic environment. Hence, we speculated that in high-altitude and low-oxygen regions, there may be some adaptive microbial community in the rumen microbes of Holstein cows. The enhanced microbes in the body may participate in important physiological functions of the body and maintain the health of the host, thus adapting to the harsh environment of high altitude and low oxygen. Nevertheless, their functional roles in Tibetan Plateau dairy cows still remain unclear and deserve further investigation.

Correlation analysis refers to the analysis of two or more variable elements with correlation, and is commonly applied to reveal the relationship between microbes and metabolites or fermentation parameters in rumen. In the current study, the relative abundances of *F082*, *Ruminococcaceae* and *Lachnospiraceae* family were found closely associated with ruminal fermentation and MCP production. The *Ruminococcaceae* and *Lachnospiraceae* belong to the *Firmicutes* phylum, was involved in the metabolism of a variety of carbohydrates which play

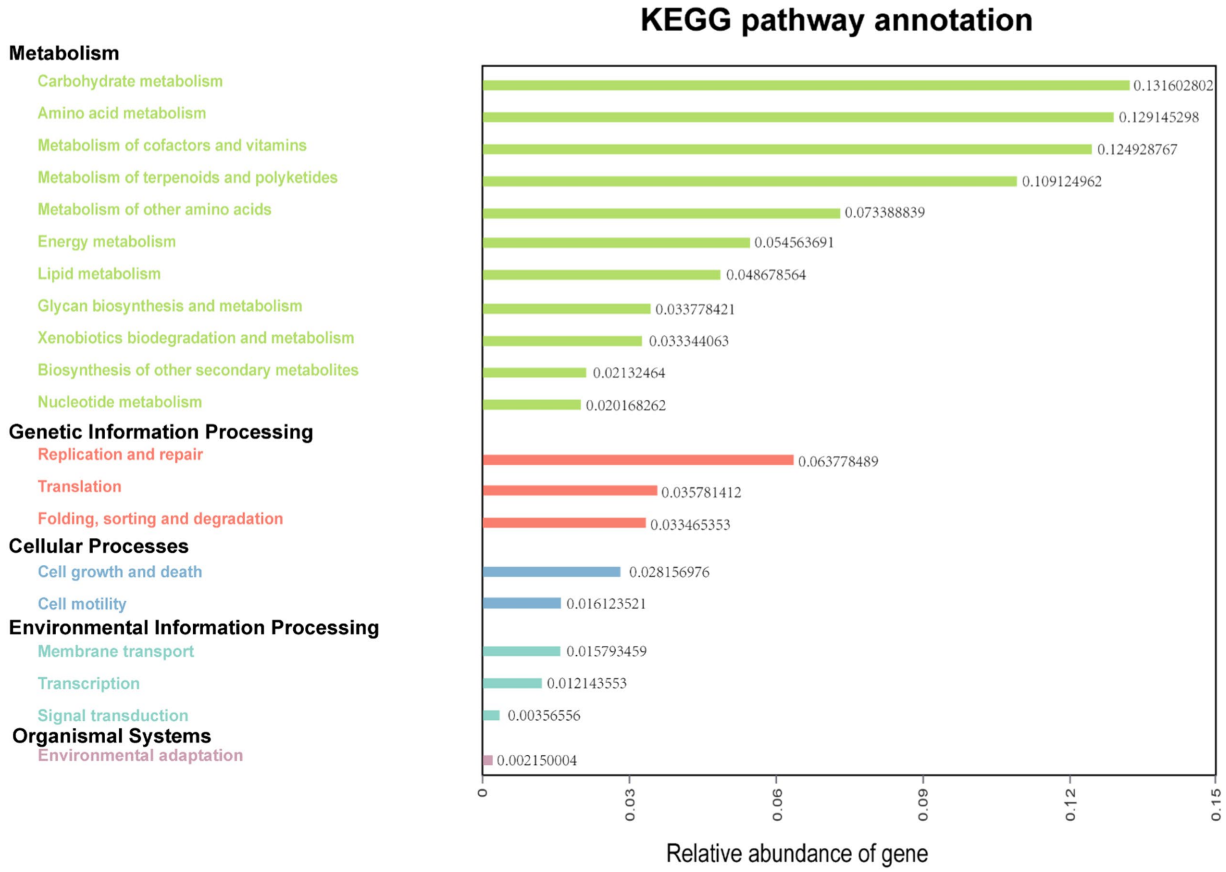


FIGURE 4
Predictive functional analysis showing relative abundance of assigned KEGG identity to the pathways of metabolisms at subsystem levels ($KO_2$).

a vital role in energy utilization (Lopes et al., 2021). The positive correlation between microbes and rumen VFA ensured the energy source of the host. Most of the VFAs formed in the rumen are absorbed across by the host ruminal epithelium (Zhang et al., 2016). Thus, dietary supplementation of NCG might possess the ability to more efficiently transport and absorb VFAs. The main VFA (acetic acid) in the rumen may cut down the abundance of *Escherichia coli* and maintain rumen health (Kong et al., 2014). This study showed that Acetic/propionic acid was positively correlated with *Ruminococcaceae*, and the response of VFA to rumen microbes indicated that dietary NCG supplementation could promote the interaction between microorganism and organism and adapt to the harsh growing conditions in Tibetan Plateau.

Rumen is a huge biological resource bank, and ruminal microbes play vital roles on the body's immunity, nutrient absorption and degradation, and even enzyme metabolism. It is of great importance to excavate functional genes of rumen bacteria that are closely related to important nutritional physiological functions (Martin et al., 2010). In the current study, potential function prediction of ruminal bacteria of Tibetan Plateau dairy cows were carried out using KEGG pathway analysis. The results indicate that the most of ruminal bacteria were enriched in carbohydrate metabolism and amino acid metabolism at level 2 of KEGG pathways. This suggests that most ruminal bacteria are engaged in energy and amino acid metabolism so that to let the cows better adapt to the high-altitude hypoxic environment. Dietary NCG supplementation can increase endogenous arginine synthesis, promote amino acid metabolism, and increase amino acid utilization (Hu et al., 2019), which is identical with the results of functional prediction. In addition, genes related to replication and repair were the most abundant among genetic information processing. Studies have suggested that low oxygen and high ultraviolet radiation may cause DNA and protein damage in a plateau environment, while genes associated with replication and repair may help reduce damage to biomolecules (Fan et al., 2020). However, our results are based only on functional predictions of rumen bacteria. Further direct sequencing of the rumen metagenome of plateau cattle is needed to more accurately explore the role of these genes in NCG addition and environmental adaptation.

In conclusion, dietary NCG supplementation (20 g/d/head) reduced ruminal $NH_3$-N concentration and enhanced ruminal total VFA production, especially acetic acid, suggesting enhanced N utilization and energy acquisition in rumen of Holstein dairy cows introduced to Tibet. The enhanced ruminal fermentation and N utilization is likely due to the enhanced ruminal microbial diversity, which needs to be further studied.

## 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/search/all/?term=PRJNA900013.

## Ethics statement

The animals and experimental procedures used in this study were approved by the Institutional Animal Care and the Use Committee, Institute of Subtropical Agriculture, Chinese Academy of Sciences, Changsha, PR China (Approval number: ISA-2019-0115).

## Author contributions

CZ and DX were involved in the methodology and conceptualization. YW and AJ conducted the experiment. JZ and YW analyzed the sequencing data, interpreted the prepared the tables and figures, and wrote the manuscript. TR, CZ, DX, and JZ reviewed and edited the manuscript. CZ, BL, and ZT were involved in project administration. All authors have read and agreed to the published version of the 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.The reviewer HN declared a shared affiliation with the authors JZ, YW, AJ, and CZ to the handling editor at the time of review.

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## Supplementary material

The Supplementary material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2023.1101620/full#supplementary-material

## References

AOAC (2012). *Official methods of analysis. 17th edn*. Association of Official Analytical Chemists, Arlington, VA.

Bokhari, R. H., Amirjan, N., Jeong, H., Kim, K. M., and Caetano-Anollés, N. A. (2020). Bacterial origin and reductive evolution of the cpr group. *Genome Biol. Evol.* 12, 103–121. doi: 10.1093/gbe/evaa024

Cabello, P. J., Zemskaya, T. I., Zakharenko, A. S., Sakirko, M. V., Ivanov, V. G., Ghai, R., et al. (2019). Microbiome of the deep lake baikal, a unique oxic bathypelagic habitat. *Limnol. Oceanogr.* 65, 1471–1488. doi: 10.1002/lno.11401

Chacher, B., Liu, H., Wang, D., and Liu, J. (2013). Potential role of N-carbamoyl glutamate in biosynthesis of arginine and its significance in production of ruminant animals. *J Anim Husbandry Biotech.* 4:6. doi: 10.1186/2049-1891-4-16

Chacher, B., Wang, D. M., Liu, H. Y., and Liu, J. X. (2012). Degradation of l-arginine and n-carbamoyl glutamate and their effect on rumen fermentation *in vitro*. *Ital. J. Anim. Sci.* 11, 374–377. doi: 10.4081/ijas.2012.e68

Chacher, B., Zhu, W., Ye, J. A., Wang, D. M., and Liu, J. X. (2014). Effect of dietary Ncarbamoylglutamate on milk production and nitrogen utilization in highyielding dairy cows. *J. Dairy Sci.* 97, 2338–2345. doi: 10.3168/jds.2013-7330

Cheng, L., Cantalapiedra-Hijar, G., Meale, S., Rugoho, I., Jonker, A., Khan, M., et al. (2021). Review: markers and proxies to monitor ruminal function and feed efficiency in young ruminants. *Animal* 15:100337. doi: 10.1016/j.animal.2021.100337

Clarke, S. F., Murphy, E. F., Osullivan, O., Lucey, A. J., Humphreys, M., Hogan, A., et al. (2014). Exercise and associated dietary extremes impact on gut microbial diversity. *Gut* 63, 1913–1920. doi: 10.1136/gutjnl-2013-306541

Cui, X. X., Wang, Z. F., Yan, T. H., Chang, S. H., Wang, H., and Hou, F. J. (2019). Rumen bacterial diversity of Tibetan sheep (*Ovis aries*) associated with different forage types on the Qinghai-Tibetan plateau. *Can. J. Microbiol.* 65, 859–869. doi: 10.1139/cjm-2019-0154

Ding, L., Shen, Y., Wang, Y., Zhou, G., Zhang, X., Wang, M., et al. (2019). Jugular arginine supplementation increases lactation performance and nitrogen utilization efficiency in lactating dairy cows. *J. Anim. Sci. Biotechnol.* 10, 3–10. doi: 10.1186/s40104-018-0311-8

Douglas, G. M., Maffei, V. J., Zaneveld, J. R., Yurgel, S. N., Brown, J. R., Taylor, C. M., et al. (2020). PICRUSt2 for prediction of metagenome functions. *Nat. Biotechnol.* 38, 685–688. doi: 10.1038/s41587-020-0548-6

Fan, Q., Wanapat, M., Yan, T., and Hou, F. (2020). Altitude influences microbial diversity and herbage fermentation in the rumen of yaks. *BMC Microbiol.* 20:370. doi: 10.1186/s12866-020-02054-5

Gessler, P., Peter, B., Hans, U., and Schwenk, B. W. (2020). Favourable long-term outcome after immediate treatment of neonatal hyperammonemia due toN-acetylglutamate synthase deficiency. *European Journal of Pediatrics*. 169, 197–199. doi: 10.1007/s00431-009-1006-0

Gu, F. F., Miao, C., Jiang, L., Wang, D. N., Liu, H. Y., and Liu, J. X. (2021). Dietary supplementation with N-carbamoylglutamate initiated from the prepartum stage improves lactation performance of postpartum dairy cows. *Anim. Nutr.* 7, 232–238. doi: 10.1016/j.aninu.2020.07.002

Guffon, N., Vianey-Saban, C., Bourgeois, J., Rabier, D., Colombo, J., and Guibaud, P. A. (1995). New neonatal case of N-acetylglutamate synthase deficiency treated by carbamylglutamate. *J. Inherit. Metab. Dis.* 18, 61–65. doi: 10.1007/BF00711374

He, X., McLean, J. S., Edlund, A., Yooseph, S., Hall, A. P., Liu, S. Y., et al. (2015). Cultivationof a human-associated TM7 phylotype reveals a reduced genome and epibiotic parasitic lifestyle. *Proc. Natl. Acad. Sci. U. S. A.* 112, 244–249. doi: 10.1073/pnas.1419038112

Herrmann, M., Wegner, C. E., Taubert, M., Geesink, P., and Küsel, K. (2019). Predominance of cand. *Patescibacteria* in groundwater is caused by their preferential mobilization from soils and flourishing under oligotrophic conditions. *Front. Microbiol.* 10:1407. doi: 10.3389/fmicb.2019.01407

Hu, Y., Shao, D., Wang, Q., Xiao, Y., Zhao, X., Shen, Y., et al. (2019). Effects of dietary N-carbamylglutamate supplementation on growth performance, tissue development and blood parameters of yellow-feather broilers. *Poult. Sci.* 98, 2241–2249. doi: 10.3382/ps/pey591

Huang, T. T., Wu, Z. Y., and Zhang, W. X. (2020). Effects of garlic addition on bacterial communities and the conversions of nitrate and nitrite in a simulated pickle fermentation system. *Food Control* 113:107215. doi: 10.1016/j.foodcont.2020.107215

Jiao, J., Li, X., Beauchemin, K. A., Tan, Z., Tang, S., and Zhou, C. (2015). Rumen development process in goats as affected by supplemental feeding v. grazing: age-related anatomic development, functional achievement and microbial colonisation. *Br. J. Nutr.* 113, 888–900. doi: 10.1017/S0007114514004413

Jiao, J., Lu, Q., Forster, R. J., Zhou, C., Wang, M., Kang, J., et al. (2016). Age and feeding system (supplemental feeding versus grazing) modulates colonic bacterial succession and host mucosal immune maturation in goats. *J. Anim. Sci.* 94, 2506–2518. doi: 10.2527/jas.2015-0081

Kong, Z. W., Zhou, C. S., Bin, L., Jiao, J. Z., Chen, L., Ren, A., et al. (2019). Integrative plasma proteomic and microRNA analysis of Jersey cattle in response to high-altitude hypoxia. *J. Dairy Sci.* 102, 4606–4618. doi: 10.3168/jds.2018-15515

Kong, X. F., Zhou, X. L., Lian, G. Q., Blachier, F., Liu, G., Tan, B., et al. (2014). Dietary supplementation with chitooligosaccharides alters gut microbiota and modifies intestinal luminal metabolites in weaned Huanjiang minipiglets. *Livest. Sci.* 160, 97–101. doi: 10.1016/j.livsci.2013.11.023

Liu, H., Ran, T., Zhang, C., Yang, W., Wu, X., Degen, A., et al. (2022). Comparison of rumen bacterial communities between yaks (*Bos grunniens*) and Qaidam cattle (*Bos taurus*) fed a low protein diet with different energy levels. *Front. Microbiol.* 13:982338. doi: 10.3389/fmicb.2022.982338

Liu, Z., Yan, F., and Mi, H. (2022). N-carbamoylglutamate supplementation on the digestibility, rumen fermentation, milk quality, antioxidant parameters, and metabolites of Jersey cattle in high-altitude areas. *Front. Vet. Sci.* 9:4. doi: 10.3389/fvets.2022.848912

Lopes, D., Duarte, M., Reau, A. J. L., Chaves, I. Z., and Hilário, C. M. (2021). Assessing the relationship between the rumen microbiota and feed efficiency in Nellore steers. *J. Anim. Sci. Biotechnol.* 12:79. doi: 10.1186/s40104-021-00599-7

Ma, N., Li, Y., Ren, L. Y., Hu, L. Q., Xu, R. T., Shen, Y. Z., et al. (2020). Effects of dietary N-carbamylglutamate supplementation on milk production performance, nutrient digestibility and blood metabolomics of lactating Holstein cows under heat stress. *Anim. Feed Sci. Technol.* 273:114797. doi: 10.21203/rs.3.rs-30617/v1

Ma, Y., Zeng, Z., Kong, L., Chen, Y., and He, P. (2019, 2019). Determination of N-carbamylglutamate in feeds and animal products by high performance liquid chromatography tandem mass spectrometry. *Molecules* 24:3172. doi: 10.3390/molecules24173172

Martin, R., Nauta, A. J., Amor, K. B., Knippels, L. M. J., Knol, J., and Garssen, J. (2010). Early life: gut microbiota and immune development in infancy. *Benef Microbes.* 1, 367–382. doi: 10.3920/BM2010.0027

Mumbi, M. P., Eissa, N., Noah, B. C., Ehsan, K., Jean-Eric, G., and Heimesaat, M. M. (2015). Antepartum antibiotic treatment increases offspring susceptibility to experimental colitis: a role of the gut microbiota. *PLoS One* 10:e0142536. doi: 10.1371/journal.pone.0142536

Neary, J. M., and Garry, F. B. (2014). Impaired alveolar-arterial oxygen transfer is associated with reduced milk yield in primiparous post-partum dairy heifers at moderate altitude. *J. Dairy Res.* 81, 434–439. doi: 10.1017/S0022029914000430

Oba, M., Baldwin, L. R., Owens, L. S., and Bequette, J. B. (2005). Metabolic fates of ammonia–N in ruminal epithelial and duodenal mucosal cells isolated from growing sheep. *J. Dairy Sci.* 88, 3963, 3963–3970. doi: 10.3168/jds.S0022-0302(05)73082-4

Oksanen, J., Blanchet, F. G., Kindt, R., Legendre, P., and Wagner, H. (2013). Multivariate analysis of ecological communities in R: vegan tutorial. R package version 1.7.2013.

Probst, A. J., Ladd, B., Jarett, J. K., Geller-Mcgrath, D. E., Sieber, C. M. K., Emerson, J. B., et al. (2018). Differential depth distribution of microbial function and putative symbionts through sediment-hosted aquifers in the deep terrestrial subsurface. *Nat. Microbiol.* 3, 328–336. doi: 10.1038/s41564-017-0098-y

Rinke, C., Schwientek, P., Sczyrba, A., Ivanova, N. N., Anderson, I. J., Cheng, J. F., et al. (2013). Insights into the phylogeny and coding potential of microbial dark matter. *Nature* 499, 431–437. doi: 10.1038/nature12352

Schneider, J. C., Blazy, I., Déchaux, M., Rabier, D., Mason, N. P., and Richalet, J. P. (2001). Response of nitric oxide pathway to l-arginine infusion at the altitude of 4, 350 m. *Eur. Respir. J.* 18, 286–292. doi: 10.1183/09031936.01.00073401

Shen, J. S., Chai, Z., Song, L. J., Liu, J. X., and Wu, Y. M. (2012). Insertion depth of oral stomach tubes may affect the fermentation parameters of ruminal fluid collected in dairy cows. *J. Dairy Sci.* 95, 5978–5984. doi: 10.3168/jds.2012-5499

Sun, L. W., Zhang, H., Fan, Y. X., Wang, R. C., Zhang, G. M., and Wang, F. (2017). Metabolomic profiling in umbilical venous plasma reveals effects of dietary rumen-protected arginine or N-carbamylglutamate supplementation in nutrient-restricted Hu sheep during pregnancy. *Reprod. Domest. Anim.* 52, 376–388. doi: 10.1111/rda.12919

Tap, J., Furet, J. P., Bensaada, M., Philippe, C., Roth, H., Rabot, S., et al. (2015). Gut microbiota richness promotes its stability upon increased dietary fibre intake in healthy adults. *Environ. Microbiol.* 17, 4954–4964. doi: 10.1111/1462-2920.13006

Van Soest, P. J., Robertson, J. B., and Lewis, B. A. (1991). Methods for dietary fiber, neutral detergent fiber, and nonstarch polysaccharides in relation to animal nutrition. *J. Dairy Sci.* 74, 3583–3597. doi: 10.3168/jds.S0022-0302(91)78551-2

Wang, M. Z. (2016). 1500 effects of arginine infusion through jugular vein on the milk performance and casein synthesis in midlactation cows. *J. Anim. Sci.* 94:728. doi: 10.2527/jam2016-1500

Wang, L., Liu, K., Wang, Z., Bai, X., Peng, Q., and Jin, L. (2019). Bacterial community diversity associated with different utilization efficiencies of nitrogen in the gastrointestinal tract of goats. *Front. Microbiol.* 10:239. doi: 10.3389/fmicb.2019.00239

Wang, Q., Wang, Y., Wang, X., Dai, C., Tang, W., Li, J., et al. (2020). Effects of dietary energy levels on rumen fermentation, microbiota, and gastrointestinal morphology in growing ewes. *Food Sci. Nutr.* 8, 6621–6632. doi: 10.1002/fsn3.1955

Wenk, C. (2000). Recent advances in animal feed additives such as metabolic modifiers, antimicrobial agents, probiotics, enzymes and highly available minerals-review. *Asian Australasian J. Anim. Sci.* 13, 86–95. doi: 10.5713/ajas.2000.86

Wrighton, K. C., Castelle, C. J., Wilkins, M. J., Hug, L. A., Sharon, I., Thomas, B. C., et al. (2014). Metabolic interdependencies between phylogenetically novel fermenters and respiratory organisms in an unconfined aquifer. *ISME J.* 8, 1452–1463. doi: 10.1038/ismej.2013.249

Zhang, Z., Xu, D., Wang, L., Hao, J., Wang, J., Zhou, X., et al. (2016). Convergent evolution of rumen microbiomes in high-altitude mammals. *Curr. Biol.* 26, 1873–1879. doi: 10.1016/j.cub.2016.05.012

Zhang, H., Zhao, F. F., Peng, A., Dong, L., Wang, M. Z., Yu, L. H., et al. (2018). Effects of dietary L-arginine and N-carbamylglutamate supplementation on intestinal integrity, immune function and oxidative status in intrauterine growth retarded suckling lambs. *J. Agric. Food Chem.* 66, 4145–4154. doi: 10.1021/acs.jafc.8b00726

Zhao, F. F., Wu, T. Y., Wang, H. R., Ding, L. Y., Ahmed, G., Li, H. W., et al. (2018). Jugular arginine infusion relieves lipopolysaccharide-triggered inflammatory stress and improves immunity status of lactating dairy cows. *J. Dairy Sci.* 101, 5961–5970. doi: 10.1093/jas/sky250

Zhou, Y. W., Mcsweeney, C. S., Wang, J. K., and Liu, J. X. (2012). Effects of disodium fumarate on ruminal fermentation and microbial communities in sheep fed on high-forage diets. *Animal* 6, 815–823. doi: 10.1017/S1751731111002102

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# Effects of urbanization and lifestyle habits on the intestinal microbiota of adolescents in eastern China

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**Introduction:** Owing to urbanization, living habits have changed widely, leading to alterations in the intestinal microbiota of urban residents. However, there are few studies on the characteristics of intestinal microbiota of adolescents living in different urbanized areas in China.

**Methods:** A total of 302 fecal samples collected from adolescent students in eastern China were examined. 16S rRNA high-throughput sequencing was used to identify the fecal microbiota. These data were combined with questionnaire survey results to investigate the effect of urbanization on the intestinal microbiota of adolescents in eastern China. Moreover, the role of lifestyle habits in this relationship was also evaluated.

**Results:** The results revealed significant differences in the structure of the intestinal microbiota among adolescents living in regions with different levels of urbanization. Adolescents living in urban regions had a significantly higher proportion of *Bacteroides* ($p < 0.001$, FDR=0.004), whereas those living in towns and rural regions had higher proportions of *Bifidobacterium* ($p < 0.001$, FDR<0.001) and *Prevotella* ($p < 0.05$, FDR=0.019). The diversity of the intestinal microbiota was higher in urban residents than in adolescents living in towns and rural regions ($p < 0.05$). In addition, the differences in intestinal microbiota across individuals living in cities, towns, and rural regions were related to dietary preferences, flavor preferences, and sleep and exercise durations. Adolescents who ate more meat had more *Dorea* (LDA=3.622, $p$ =0.04), while the abundance of *Escherichia–Shigella* is higher among adolescents who ate more condiments (LDA=4.285, $p$ =0.02). The abundance of *Dialister* was significantly increased in adolescents with longer sleep durations (LDA=4.066, $p$= 0.03). Adolescents who exercised for a long duration had more *Faecalibacterium* than those who exercised for a shorter duration (LDA=4.303, $p$ =0.04).

**Discussion:** Our research has preliminarily demonstrated that there were differences in the composition of Gut microbiome in stool samples of adolescents living in different urbanized areas, and provide a scientific basis for the maintenance of a healthy intentional microbota in adolescences.

KEYWORDS

**adolescents, urbanization, intentional microbiota, lifestyle habits, China**

## 1. Introduction

The human gastrointestinal tract contains 200–300 square meters of mucous membrane, which hosts more than 1,000 types of microorganisms with a total weight of 1–1.5 kg. These microorganisms, dispersed across 50 bacterial phyla, constitute the human intestinal microbiota (Qin et al., 2010; Backhed et al., 2015).

The intestinal microbiota plays an important role in health. Studies have shown that the intestinal microbiota influences the body's metabolism, immune system, and brain function (Turnbaugh et al., 2007; Cho and Blaser, 2012; Shreiner et al., 2015). The gastrointestinal microbiota promotes the digestion of dietary fibers and improves lipid metabolism by inhibiting lipase activity in adipocytes (Hooper et al., 2001). In addition, the intestinal mucosa plays an important role in immunity, protecting the intestinal epithelium from the direct attachment of microorganisms. The intestinal microbiota can promote the maturation of the mucosal layer and improve immunity *via* FoxP3 + CD4 + cells (Nagano et al., 2012). There also exists a bidirectional network of communication between the intestinal microbiota and the brain called the gut–brain axis. Short-chain fatty acids, which are bacterial metabolites, affect the integrity of the blood–brain barrier by increasing the production of tight junction proteins. This, in turn, activates neurons and alters neurological function, leading to emotional and behavioral changes (Gomaa, 2020; Morais et al., 2021).

Previous studies have demonstrated a strong connection between the intestinal microbiota and gut disorders. Irritable bowel syndrome (IBS) and inflammatory bowel disease (IBD) are known to be caused by imbalances in the intestinal microbiota, leading to the growth of pathogenic microorganisms and subsequent damage to the intestinal mucosa (Ghoshal et al., 2012; Parekh et al., 2015). In addition, the intestinal microbiota plays an important role in host energy metabolism. Previous studies have revealed significant differences in the intestinal microbiota between healthy individuals and patients with obesity, fatty liver disease, diabetes, cardiovascular disease, and other diseases (Gomaa, 2020). Obese individuals show a higher proportion of *Porphyromonas*, *Campylobacter*, *Bacteroides*, *Staphylococcus*, and *Parabacteroides* and a decrease in the proportion of probiotic bacteria (Le Chatelier et al., 2013; Crovesy et al., 2020).

The intestinal microbiota is also related to immune and nervous system diseases. The intestinal microbiota of patients with allergy and asthma is significantly different from that of healthy individuals (Frati et al., 2018). Dysfunction of the brain–gut axis is associated with many neurological and psychiatric disorders, including stress-related disorders such as anxiety and depression, neurodevelopmental disorders such as autism, and neurodegenerative diseases such as Alzheimer's disease (Clarke et al., 2013; Kelly et al., 2016; Sherwin et al., 2016; Strati et al., 2017). Studies have also shown that probiotic regimens can have a significant therapeutic effect on these psychiatric disorders (McKean et al., 2017). Taken together, this evidence suggests that more attention should be paid to gut health and the maintenance of a healthy intestinal microbiota, which is important for overall health.

In humans, the intestinal microbiota changes with age. Previously, it was believed that the structure of the intestinal microbiota in 3-year-old children was similar to that in adults (Chana Palmer et al., 2007). However, accumulating evidence shows that the development of the human intestinal microbiota is not as rapid as previously described. Instead, the intestinal microbiota gradually matures with age and even during adolescence (Kim et al., 2018; Vijayakumar et al., 2018). Adolescence is a critical period of growth and development. Physical development during adolescence lays the foundation for health in adulthood. Adolescents experience rapid physical changes, including changes in their body shape, sexual development and maturation, brain and nerve development, and increased energy metabolism (Golden et al., 2014; Vijayakumar et al., 2018). Thus, maintaining a healthy intestinal microbiota could promote the absorption of nutrients, prevent diseases, and improve physical development in adolescents, laying the foundation for good overall health in adulthood.

Urbanization is closely related to a decrease in the diversity of the human microbiome. Thus, the intestinal microbiota of adolescents living in regions with different levels of urbanization may also differ, affecting their growth and development at this stage. In previous studies, it was found that *Bacteroides* was more abundant in the intestines of adolescents living in cities, while *Prevotella* was more abundant in adolescents living in rural areas (Nakayama et al., 2015). *Bacteroides* can maintain intestinal stability by producing short-chain fatty acids (Tian et al., 2017). The decrease of *Prevotella* has been proved to be related to many diseases (Moon et al., 2017). Maintaining the balance of intestinal microecology is of great significance to health. Therefore, analyzing the differences in the intestinal microbiota in adolescents from areas with different levels of urbanization is of great significance for helping them maintain a healthy intestinal microenvironment.

Further, economic gaps contribute to variations in the environments and living habits of urban residents, thus influencing their intestinal microbiota. According to previous studies, differences in economic status and dietary habits have resulted in large differences in the intestinal microbiota of individuals from the East and West (Yatsunenko et al., 2012). The fecal samples of westerners show a high proportion of *Bacteroides* owing to the consumption of more high-fat meat. However, individuals in the East show a higher proportion of *Prevotella* because they consume more high-fiber carbohydrates (Nakayama et al., 2015). In addition, behavioral habits such as exercise and sleep durations can also affect the intestinal microbiota. Studies have reported that exercise has a significant impact on the intestinal microbiota in adolescents. Further, after exercise interventions, the intestinal microbiota of children with diabetes appears similar to that of healthy children (Quiroga et al., 2020). In breast cancer survivors, sleep disturbances are linked to the abundance of *Paracoccus*, *Rikenellaceae*, and *Clostridium* groups (Paulsen et al., 2017). Moreover, evidence suggests that metabolic disorders associated with sleep deprivation could be mediated by the overgrowth of specific intestinal bacteria (Matenchuk et al., 2020).

However, few previous studies have found links between living habits and urbanization or explored the reasons for variations in the intestinal microbiota among residents of areas with different urbanization levels.

This study investigated the characteristics of the intestinal microbiota in adolescents from regions in Hangzhou with different levels of urbanization. We analyzed the effects of diet, exercise and sleep on these characteristics to provide a scientific basis for the maintenance of intestinal health in adolescents from regions with different levels of urbanization.

# 2. Materials and methods

## 2.1. Study population and collection of fecal samples

Using stratified cluster sampling, five districts/counties in Hangzhou were selected: Shangcheng District, Gongshu District, Xihu District, Fuyang District, and Chun'an County. Four schools—including two junior high schools and two primary schools—were selected from each district. Subsequently, one class with more than 16 students was selected from each school.

According to the urbanization level of different districts/counties, the regions were divided into three groups: urban, town, and rural areas. The urban group was composed of students from Shangcheng District and Gongshu District; the town group was composed of students from four schools in Xihu District and the adolescents from the town in Fuyang District; and the rural group was composed of the students from Chun'an County and the adolescents from the rural region in Fuyang District. Adolescents aged 7–15 years (contained the younger age) were included in this study and referred to the national health industry standard WS/T 610-2018. Exclusion criteria: Antibiotics have been used in the past 15 days (Qin et al., 2010; Backhed et al., 2015). Gastrointestinal dysfunction, previous gastrointestinal disease history (Shreiner et al., 2015). Diarrhea, abdominal distension, abdominal pain or constipation within the past 15 days. Finally, 302 adolescents were included in the study.

Fecal samples were obtained from the participants and stored at 4°C immediately following collection and sent to the laboratory within 4 h and frozen for DNA sample extraction. This research was approved by the Ethics Committee of Hangzhou Center for Disease Control and Prevention (No. 20047), and written informed consent was obtained from the parents or guardians of each adolescent.

## 2.2. Questionnaire design

The questionnaire contained questions on basic demographic information such as sex, age, and grade. Lifestyle habits of nearly 7 days, such as dietary preferences, flavor preferences, sleep durations, and exercise durations were also surveyed. The questionnaire was self-evaluated. In order to prevent adolescents from providing inaccurate responses while filling out their questionnaire owing to a lack of question comprehension or recall bias, they were accompanied by their parents while filling out the questionnaire. Thus, parents and adolescents completed the questionnaire together. Dietary preferences were divided into three categories: 1. Preference for vegetables (Vegetable intake is higher than meat), 2. Preference for meat (Meat intake is higher than vegetables), and 3. Balanced proportion of vegetables and meat. Flavor preferences were divided into three categories: 1. Light flavor, 2. Heavy seasoning (spicy, salty, sweet, sour, etc.), and 3. Balanced flavor. Sleep duration was divided into 1. Less than 7 h, 2. 7–9 h, and 3. More than 9 h. Exercise duration was divided into four groups: 1. Less than 0.5 h, 2. 0.5–1 h, 3. 1–2 h, and 4. More than 2 h.

This questionnaire was designed by project team members based to the opinions of experts, strictly following the principles of probability and statistics. This questionnaire was designed according to "Zhejiang Adult Behavior Risk Factors Monitoring Questionnaire." It has been pre-investigated, expertly demonstrated, and conducted face-to-face investigations by trained and qualified investigators.

## 2.3. DNA extraction and PCR amplification

Total microbial genomic DNA was extracted from fecal samples using the E.Z.N.A.® soil DNA Kit according to manufacturer's instructions. The quality and concentration of DNA were determined by 1.0% agarose gel electrophoresis and a NanoDrop® ND-2000 spectrophotometer (Thermo Scientific Inc., United States) and kept at −80°C prior to further use. The hypervariable region V3–V4 of the bacterial 16S rRNA gene were amplified with primer pairs 806R(5′-GGACTACHVGGGTWTCTAAT-3′; Liu et al., 2016) by an ABI GeneAmp® 9,700 PCR thermocycler (ABI, CA, United States). The PCR reaction mixture including 4 μL 5 × Fast Pfu buffer, 2 μL 2.5 mM dNTPs, 0.8 μL each primer (5 μM), 0.4 μL Fast Pfu polymerase, 10 ng of template DNA, and ddH2O to a final volume of 20 μL. PCR amplification cycling conditions were as follows: initial denaturation at 95°C for 3 min, followed by 27 cycles of denaturing at 95°C for 30 s, annealing at 55°C for 30 s and extension at 72°C for 45 s, and single extension at 72°C for 10 min, and end at 4°C. All samples were amplified in triplicate. The PCR product was extracted from 2% agarose gel and purified using the AxyPrep DNA Gel Extraction Kit (Axygen Biosciences, Union City, CA, United States) according to manufacturer's instructions and quantified using Quantus™ Fluorometer (Promega, United States).

## 2.4. Illumina MiSeq sequencing

Purified amplicons were pooled in equimolar amounts and paired-end sequenced on an Illumina NovaSeq PE250 platform (Illumina, San Diego, United States) according to the standard protocols by Majorbio Bio-Pharm Technology Co. Ltd. (Shanghai, China). The raw sequencing reads were deposited into the NCBI Sequence Read Archive (SRA) database (Accession Number: PRJNA856408).

## 2.5. Amplicon sequence processing and analysis

After demultiplexing, the resulting sequences were quality filtered with fastp (0.19.6; Chen et al., 2018) and merged with FLASH (v1.2.11; Magoč and Salzberg, 2011). Then the high-quality sequences were denoised using DADA2 (Callahan et al., 2016) plugin in the Qiime2 (Bolyen et al., 2019; version 2020.2) pipeline with recommended parameters, which obtains single nucleotide resolution based on error profiles within samples. DADA2 denoised sequences are usually called amplicon sequence variants (ASVs). To minimize the effects of sequencing depth on alpha and beta diversity measure, the number of sequences from each sample was rarefied to 24,635, which still yielded an average Good' s coverage of 99.10%. Chao1 and Shannon index were calculated with Mothur (v1.30.1; Schloss et al., 2009). Taxonomic assignment of ASVs was performed using the Naive bayes consensus taxonomy classifier implemented in Qiime2 and the SILVA 16S rRNA database (v138).

TABLE 1 Characteristics of the adolescent population in different regions.

| | | City | Town | Rural area | *p* |
|---|---|---|---|---|---|
| Age (years) ($x \pm s$) | | 11.11 ± 2.746 | 10.61 ± 2.760 | 10.21 ± 2.959 | 0.078 |
| Gender | Male | 52(51%) | 47(48.5%) | 55(53.4%) | 0.746 |
| | Female | 50(49%) | 50(51.5%) | 48(46.6%) | |
| Grade | Primary school | 49(48%) | 52(53.6%) | 55(53.4%) | 0.668 |
| | Junior school | 53(52%) | 45(46.4%) | 48(46.6%) | |
| Dietary preference | Vegetables | 6(5.9%) b | 13(13.4%) a, b | 17(16.5%) a | 0.048 |
| | Meat | 23(22.5%) | 31(32.0%) | 24(23.3%) | |
| | Balanced | 73(71.6%) a | 53(54.6%) b | 62(60.2%) a, b | |
| Flavor preference | Light | 12(11.8%) a | 9(9.3%) b | 24(23.3%) a, b | 0.04 |
| | Heavy | 22(21.6%) | 27(27.8%) | 24(23.3%) | |
| | Balanced | 68(66.7%) | 61(62.9%) | 55(53.4%) | |
| Sleep duration | <7 h | 7(6.9%) | 8(8.2%) | 2(1.9%) | 0.008 |
| | 7–9 h | 68(66.7%) a | 80(82.5%) b | 81(78.6%) a, b | |
| | >9 h | 27(26.5%) a | 9(9.3%) b | 20(19.4%) a, b | |
| Exercise duration | <0.5 h | 16(15.7%) b | 15(15.5%) b | 33(32.0%) a | 0.001 |
| | 0.5–1 h | 52(51.0%) a, b | 61(62.9%) b | 36(35.0%) a | |
| | 1–2 h | 27(26.5%) a | 12(12.4%) b | 22(21.4%) a, b | |
| | >2 h | 7(6.9%) | 9(9.3%) | 12(11.7%) | |

*a and b: There are significant differences between the two groups; a, b: There is no significant difference between this group and the other two groups.

## 2.6. Statistical analysis

The differences of Alpha diversity index (Chao index and Shannon index) between groups were determined by Kruskal–Wallis rank-sum test. Beta diversity principal coordinate analysis (PCoA) is determined based on ASV table and Bray–Curtis distance algorithm. The abundance of each microbiota at the level of phylum and genus was counted and displayed as Veen diagram and Bar diagram. The comparative analysis of genus intestinal microflora was determined by Kruskal-Wallis rank sum test and Tukey–Kramer back testing, False discovery rates (FDRs) were calculated to account for multiple testing, FDR < 0.05 was considered statistically significant. LEfSe (Linear discriminant analysis Effect Size) online software[1] was used for linear discriminant analysis (LDA ≥ 2) to determine the bacteria with significant differences in different groups (Segata et al., 2011). Canonical correlation analysis (CCA) combines correspondence analysis with multiple regression analysis, and each step is calculated by regression with environmental factors, usually used to determine the correlation between sample distribution, intestinal microbiota and environmental factors (Mccune, 1997). The above data analysis and mapping are all carried out on the online platform of Majorbio Cloud Platform[2]. IBM SPSS version 21 software was used for statistical analysis, and Chi-square analysis was used to compare demographic characteristics among adolescents. Bonferroni adjustment was used for *post-hoc* testing. Significant difference was defined as $p < 0.05$.

1 http://huttenhower.sph.harvard.edu/galaxy/root?Tool_id=lefse_upload

2 www.majorbio.com

# 3. Results

## 3.1. Basic characteristics of the study population

In total, 302 adolescents were included in the study: 102 from cities areas (age, 11.11 ± 2.746 years), 97 from towns (age, 10.61 ± 2.760 years), and 103 from rural areas (age, 10.21 ± 2.959 years). There were no significant differences in age, grade, and gender among the three regions (Table 1).

The dietary and flavor preferences and exercise and sleep durations of adolescents from different regions are shown in Table 1. There were significant differences in these lifestyle habits among the three regions. The proportion of adolescents with a balanced dietary intake was highest in cities, and that of those who consumed more vegetables was the highest in rural areas. Regarding flavor preferences, the proportion of adolescents with a balanced flavor preference was the highest in cities, and adolescents with light flavor in cities are significantly more than those in town. Cities also had the highest proportion of adolescents who slept for more than 9 h. However, adolescents who exercised for more than 2 h a day were the most common in rural areas. These differences in lifestyle habits may have created differences in the intestinal microbiota among adolescents living in different areas of Hangzhou.

## 3.2. Characteristics of the intestinal microbiota based on the level of urbanization

16S rRNA sequencing technology was used to analyze the fecal microbiota in adolescents from different regions. First, the Alpha diversity of the intestinal microbiota in adolescents was calculated

using the Wilcoxon rank-sum test. The Chao index reflects the richness of communities in the samples, and the Shannon index reflects their diversity. Our results showed no significant difference in the Chao index among adolescents from different regions. However, the Shannon index was significantly different ($p < 0.001$). The Shannon index was the highest in adolescents from cities, followed by those from towns and rural areas. Hence, the intestinal microbiota of city adolescents was more diverse than that of adolescents from other regions (Figures 1A,B).

Next, we examined the relationship between microbial communities in different regions using PCoA based on Bray-Curtis distances. The results showed that the composition of the intestinal microbiota was significantly different among adolescents from different regions (PERMANOVA, $p = 0.001$; Figure 1C), except between adolescents from town and rural area ($p = 0.37$; Supplementary Figure S1). As shown in the Venn diagram, there were 282 bacterial species shared among residents of cities, towns, and rural areas. However, 41, 40, and 47 bacterial species were exclusive to those from cities, towns, and rural areas, respectively (Figure 1D). Combined with the results of Alpha diversity, the results showed that adolescents from regions of higher urbanization had a more diverse microbiota.

The distribution of the intestinal microbiota in adolescents from different regions was analyzed at the phylum and genus level using a community bar diagram. The relative abundances of *Firmicutes*, *Actinobacteria*, *Bacteroidota*, and *Proteobacteria* were different among the different regions. In cities, *Firmicutes* accounted for 65.63% of the standardized readings, followed by *Bacteroidota* (16.92%), *Actinobacteria* (11.90%), and *Proteobacteria* (4.41%). In towns, *Firmicutes* accounted for 64.42% of standardized readings, followed by *Actinobacteria* (18.82%), *Bacteroidota* (10.79%), and *Proteobacteria* (4.2%). In rural areas, *Firmicutes* accounted for 64.61% of standardized readings, followed by *Actinobacteria* (19.06%), *Bacteroidota* (10.35%), and *Proteobacteria* (4.89%). Compared with towns and rural areas, the relative abundance of *Bacteroidota* in cities was higher (16.92 vs. 10.79 vs. 10.35%, $p = 0.003$, FDR = 0.027), while the proportion of *Actinobacteria* was lower (11.90 vs. 18.82 vs. 19.06%, $p < 0.001$, FDR < 0.001; Figure 2). The ratios of *Firmicutes* to *Bacteroidota* in cities, towns, and rural areas were 3.88, 5.97, and 6.25, respectively.

The Wilcoxon rank-sum test was used to assess differences in the distribution of gut microbes at the genus level among adolescents from different regions. Among the 15 bacterial genera with the highest relative abundance, 10 genera showed statistical differences in the gut of adolescents from different regions (Figure 3). *Bifidobacterium* was more abundant in the intestinal microbiota of adolescents from rural areas, and the least abundant in those from cities ($p < 0.001$). *Bacteroides* was most commonly found in cities and less commonly in towns and rural areas ($p < 0.001$). *Blautia* and the *Eubacterium hallii* group were more frequent in towns and rural areas than in cities ($p < 0.001$). The abundance of *Collinsella*,

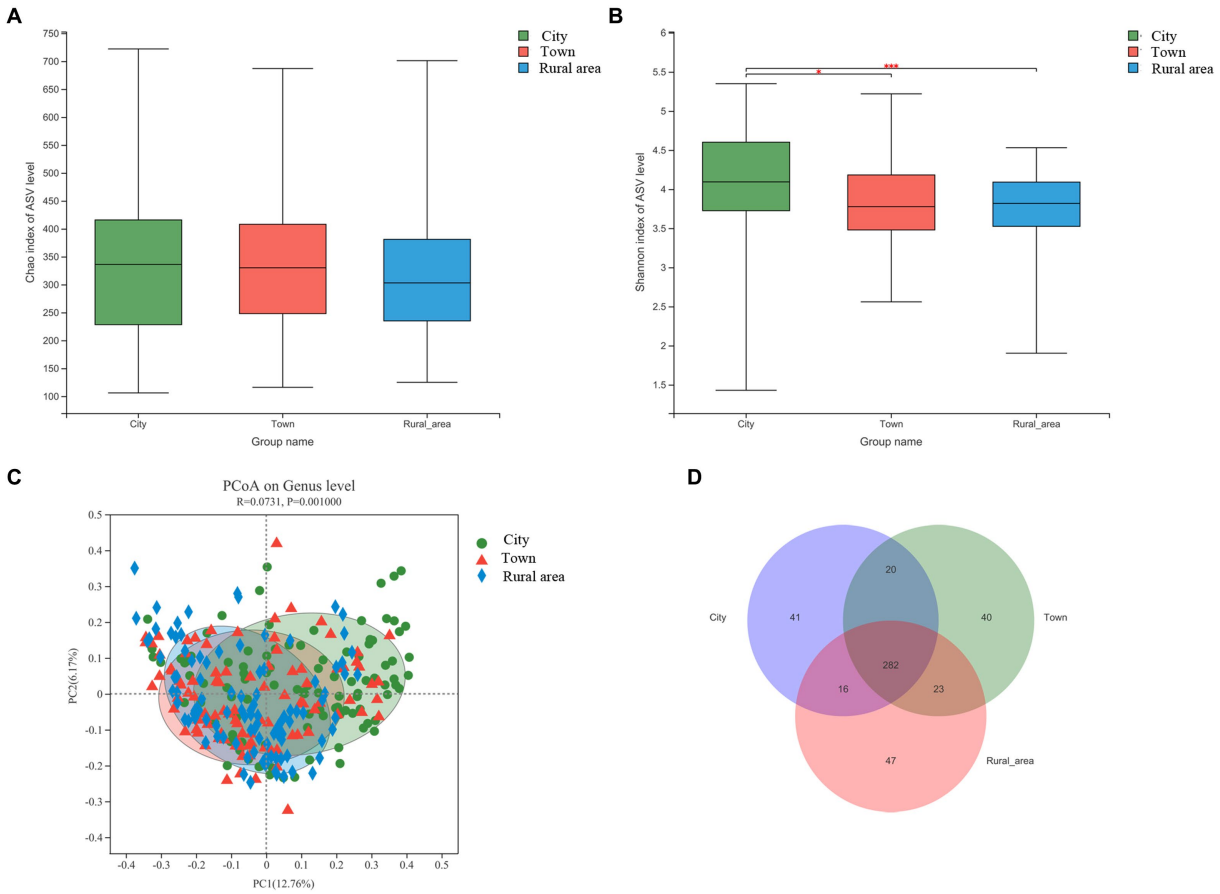


FIGURE 1
Alpha and beta diversity of the intestinal microbiota from adolescents in different regions, **(A)** Chao index; **(B)** Shannon index; **(C)** Beta diversity; **(D)** Venn diagram. Statistical significance thresholds were: *$0.01 < p \leq 0.05$; **$0.001 < p \leq 0.01$; ***$p \leq 0.001$.

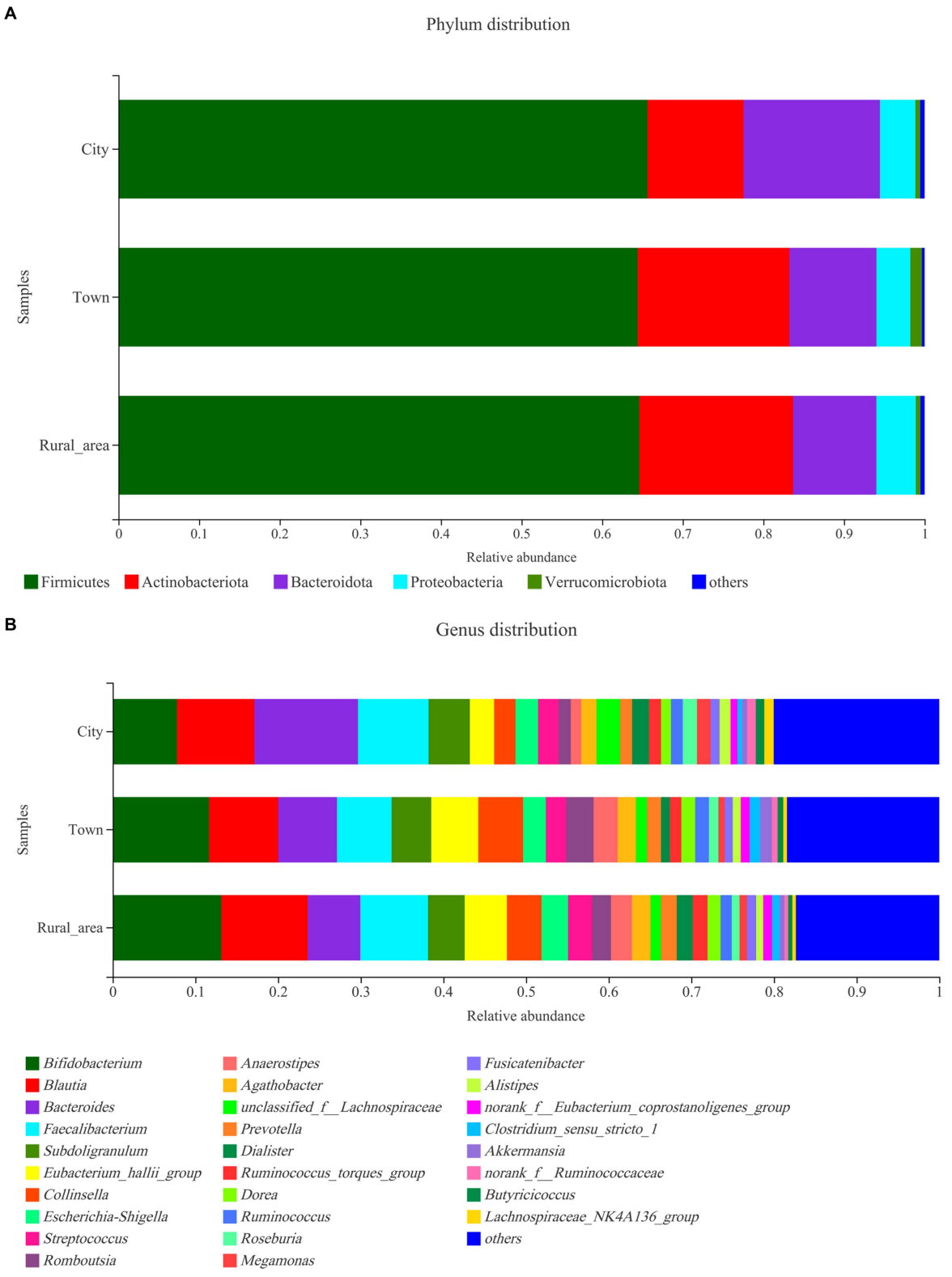


FIGURE 2
Community distribution of the intestinal microbiota in adolescents from different regions at the phylum and genus levels. **(A)** phylum level; **(B)** genus level.

*Romboutsia*, *Anaerostipes*, *unclassified_f_Lachnospiraceae*, *Prevotella*, *Dialister* are also significantly different among adolescents in different areas. However, after FDR correction, the statistical significance of *Dialister* disappeared. There was no significant difference in *Escherichia–Shigella* among the three regions (Table 2).

LEfSe was used to analyze the key intestinal bacteria contributing to community structure differences among adolescents from different regions. The results showed that the intestinal microbiota of adolescents in cities was rich in *Bacteroidales* (LDA = 4.480, $p = 0.001$) order, *Bacteroidia* (LDA = 4.480, $p = 0.001$) class, *Bacteroidota* (LDA = 4.480,

$p = 0.001$) phylum, *Bacteroides* (LDA = 4.475, $p < 0.001$) genus, and *Bacteroidaceae* (LDA = 4.475, $p < 0.001$) family. In towns, the abundance of *Coriobacteriales* (LDA = 4.198, $p < 0.001$) order, *Coriobacteriia* (LDA = 4.198, $p < 0.001$) class, *Collinsella* (LDA = 4.157, $p < 0.001$) genus, *Coriobacteriaceae* (LDA = 4.156, $p < 0.001$) family and the *Eubacterium hallii* group (LDA = 4.109, $p < 0.001$) genus was significantly higher than that in other areas. Adolescents from rural areas showed a higher enrichment of *Actinobacteriota* (LDA = 4.548, $p < 0.001$) phylum, *Actinobacteria* (LDA = 4.407, $p < 0.001$) class, *Bifidobacterium* (LDA = 4.405, $p < 0.001$) genus, *Bifidobacteriaceae* (LDA = 4.404, $p < 0.001$) family, *Bifidobacteriales* (LDA = 4.404, $p < 0.001$) order, *Blautia* (LDA = 3.936, $p = 0.001$) genus, *Prevotellaceae* (LDA = 3.828, $p = 0.001$) family, *Prevotella* (LDA = 3.782, $p = 0.001$) genus (Figure 4).

## 3.3. Factors influencing the intestinal microbiota in adolescents from regions with different levels of urbanization

In order to further analyze the impact of lifestyle habits on the relationship between urbanization and intestinal microbiota, CCA (canonical correlation analyses) was conducted between intestinal microbiota and environmental factors. As shown in Figure 5, urbanization and flavor preference are the two factors that have the greatest influence on the intestinal microbiota of adolescents in Hangzhou, and urbanization has a positive correlation with dietary preference and sleep duration, indicating that the differences of intestinal microbiota of adolescents in different areas may be related to their lifestyle habits. The distribution of intestinal microbiota in adolescents with different lifestyle habits was observed based on beta diversity. There were significant differences in the genus-level bacterial community structure (beta diversity) among adolescents with different sleep durations (Supplementary Figure S2). We used the Wilcoxon rank-sum test to analyze the characteristics of the adolescent intestinal microbiota under different living habits. All these factors influenced the intestinal microbiota at the genus level. Regarding dietary preference, adolescents who preferred to eat meat and had a balanced diet had more *Bacteroides* in the intestinal tract than those who preferred to eat vegetables ($p < 0.05$, FDR = 0.633; Figure 6A). With respect to flavor preference, *Escherichia–Shigella* were significantly more abundant in the intestinal tract of adolescents who preferred heavy seasoning than in that of those with a preference for light or balanced flavors ($p < 0.05$, FDR = 0.519; Figure 6B). *Dialister* is more common in the intestines of adolescents who sleep more ($p < 0.05$, FDR = 0.625; Figure 6C). However, exercise duration had an influence on the intestinal microbiota of adolescents. The abundance of *Faecalibacterium* increased with exercise duration ($p < 0.05$, FDR = 0.765; Figure 6D). However, these differences disappeared after FDR correction.

In addition, LEfSe analysis was used to identify the key intestinal bacteria contributing to community structure differences in adolescents with different lifestyle conditions. The intestinal microbiota of adolescents with a light flavor preference, and strong flavor preference was characterized by the *Peptoclostridium* (LDA = 3.180, $p = 0.003$), and *Escherichia-Shigella* (LDA = 4.285, $p = 0.02$), respectively. Adolescents with different dietary preferences also had different intestinal microbiota community structures. *Ruminococcaceae* (LDA = 3.321, $p = 0.03$) was the specific genus in the intestines of adolescents with a balanced diet. In adolescents who preferred meat, the specific bacterium was *Dorea* (LDA = 3.622, $p = 0.04$), and *UCG-003* (LDA = 3.055, $p = 0.02$) was the specific strain in those who preferred to eat vegetables. The intestinal microbiota of adolescents who slept for less than 7 h, 7–9 h, more than 9 h was characterized by the *Lachnoclostridium* (LDA = 3.523, $p = 0.002$),

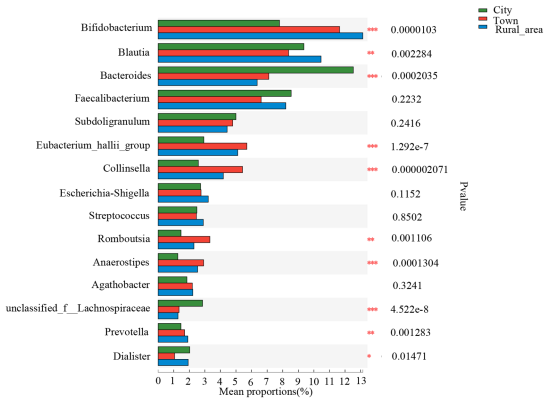


FIGURE 3
Comparison of the fecal microbiota across different regions at the genus level. Statistical significance thresholds were: *0.01<$p \leq 0.05$; **0.001<$p \leq 0.01$; ***$p \leq 0.001$.

TABLE 2 Comparison of the fecal microbiota across different regions at the genus level.

| Genus | City | Town | Rural area | *p* | *Q* |
|---|---|---|---|---|---|
| *Bifidobacterium* | 7.79 ± 10.41 | 11.65 ± 10.12 | 13.14 ± 13.5 | <0.001 | <0.001 |
| *Blautia* | 9.356 ± 10.28 | 8.376 ± 5.031 | 10.46 ± 7.82 | 0.002 | 0.031 |
| *Bacteroides* | 12.53 ± 13.86 | 7.104 ± 8.816 | 6.357 ± 8.174 | <0.001 | 0.004 |
| *Eubacterium hallii group* | 2.936 ± 3.068 | 5.695 ± 4.967 | 5.111 ± 4.776 | <0.001 | <0.001 |
| *Collinsella* | 2.58 ± 6.403 | 5.416 ± 7.41 | 4.185 ± 5.775 | <0.001 | <0.001 |
| *Romboutsia* | 1.47 ± 2.246 | 3.323 ± 4.7 | 2.3 ± 2.295 | 0.001 | 0.018 |
| *Anaerostipes* | 1.264 ± 1.305 | 2.92 ± 3.973 | 2.534 ± 2.701 | <0.001 | 0.003 |
| *unclassified_f__Lachnospiraceae* | 2.852 ± 4.719 | 1.345 ± 1.521 | 1.279 ± 1.182 | <0.001 | <0.001 |
| *Prevotella* | 1.465 ± 7.561 | 1.7 ± 5.327 | 1.91 ± 5.112 | 0.001 | 0.019 |
| *Dialister* | 1.948 ± 4.369 | 1.097 ± 2.142 | 1.927 ± 4.215 | 0.01 | 0.128 |

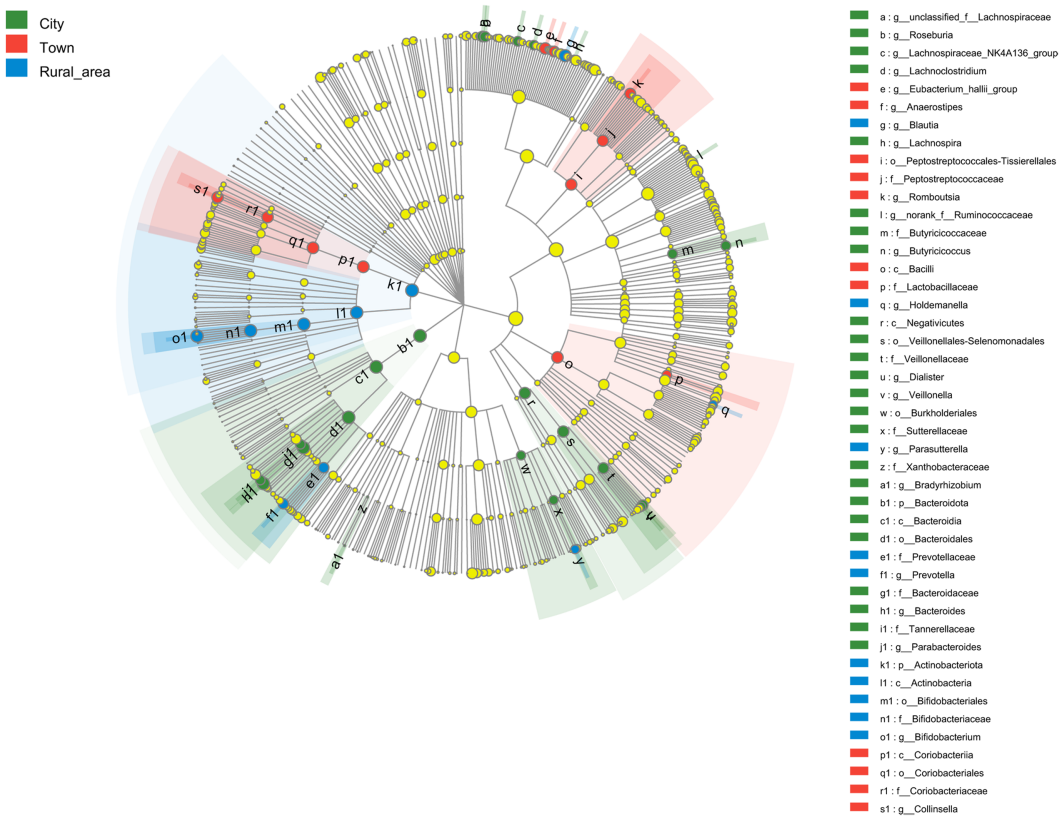


FIGURE 4
LefSe analysis of the intestinal microbiota from adolescents in different regions.

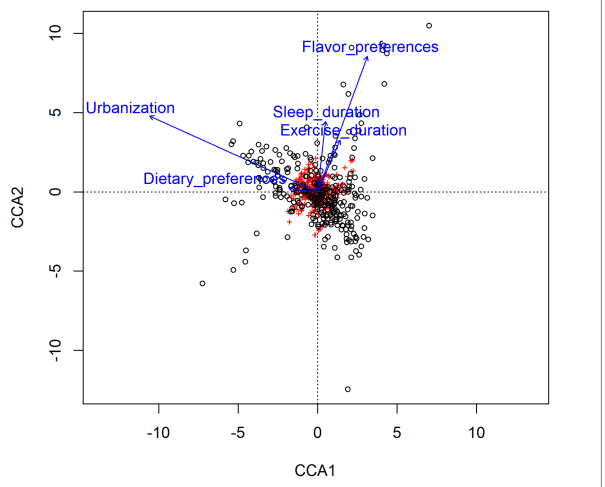


FIGURE 5
Canonical correlation analyses (CCA) of the correlation between gut microbiota and related environmental factors. Arrows represent different environmental factors, red labels represent genera of gut microbiota, and black circles represent samples. The length of the arrow represents the degree of correlation between the environmental factor and gut microbiota, and the longer the arrow represents the greater the influence on the composition of gut microbiota by related environmental factor. The Angle between the arrow lines represents the direction of the correlation, the acute angle indicates the positive correlation between the two environmental factors, and the obtuse angle is the negative correlation.

*NK4A214_group* (LDA = 3.006, $p = 0.012$), and *Dialister* (LDA = 4.066, $p = 0.03$), respectively. In the analysis of exercise durations, *Intestinimonas* (LDA = 2.146, $p < 0.001$), *Ruminococcus* (LDA = 3.622623, $p = 0.001$), *Megasphaera* (LDA = 3.223, $p = 0.01$), *Faecalibacterium* (LDA = 4.303, $p = 0.04$) are the specific bacterium in the intestines of adolescents who exercise for less than half an hour, half an hour to 1 h, 1–2 h and more than 2 h, respectively (Figure 7).

## 4. Discussion

Previous studies have found regional differences in the human intestinal microbiota. This high abundance of intestinal *Bifidobacterium* has also been observed in Japanese children and is believed to be linked to the Japanese diet (Nakayama et al., 2015). People from western countries consume more fat and protein. Studies have found that these individuals show a higher abundance of *Bacteroides* in their intestinal microbiota. Meanwhile, these individuals typically consume carbohydrates in their simplest forms *via* drinks, cakes, desserts, and white bread. Hence, western diets are often high in energy and low in nutritional value, altering the metabolism of intestinal microorganisms and increasing the risk of obesity, cardiovascular diseases, and diabetes (Yatsunenko et al., 2012; Sampson and Mazmanian, 2015). In contrast, the long-term consumption of complex carbohydrates (such as dietary fiber) has been proven to increase the abundance of *Prevotella*. Therefore, the amount of intestinal *Prevotella* is low among westerners, while

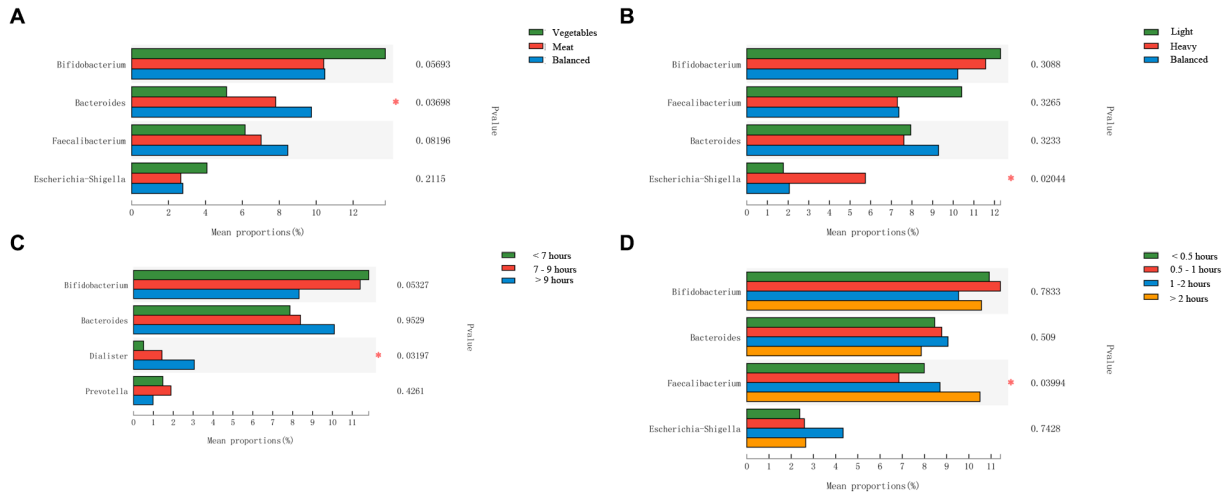


FIGURE 6
Comparison of the intestinal microbiota from adolescents with different lifestyle habits at the genus level. **(A)** Dietary preference; **(B)** Flavor preference; **(C)** Sleep duration; **(D)**: Exercise Duration. Statistical significance thresholds were: *0.01<$p$≤0.05; **0.001<$p$≤0.01; ***$p$≤0.001.

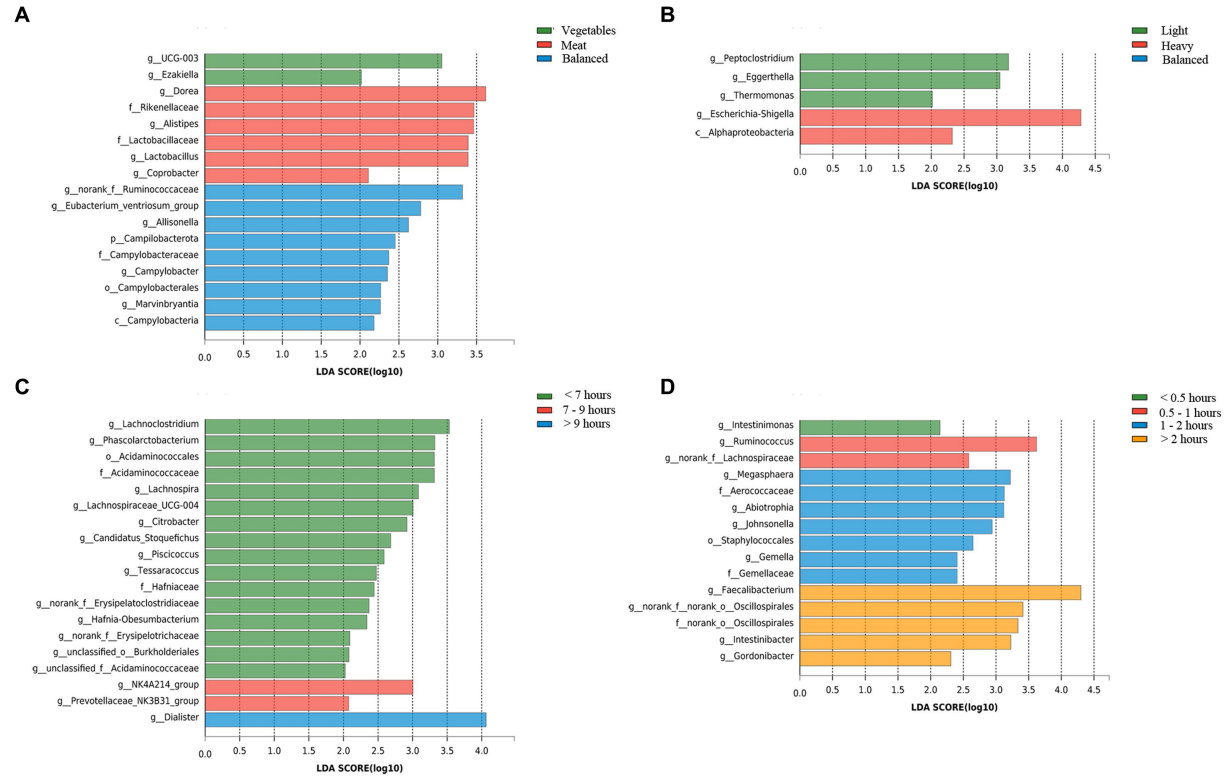


FIGURE 7
LefSe analysis of the intestinal microbiota from adolescents with different lifestyle habits. **(A)** Dietary preference; **(B)** Flavor preference; **(C)** Sleep duration; **(D)** Exercise duration.

being higher among populations that consume coarse grains, such as people from Malawi, American Indians, and people from African countries (De Filippo et al., 2010; Ayeni et al., 2018). The intestinal microbiota of people from different areas shows vast differences. In our study, it also shows consistency. The diversity of intestinal microbiota of adolescents in urban areas is higher than that in town and rural areas, and the composition of intestinal microbiota of adolescents in urban is different from that in town and rural area. Living in different geographical areas may strongly affect the diversity of the intestinal microbiota during childhood.

The ratio of *Firmicutes* to *Bacteroidetes* is thought to be related to intestinal inflammation and obesity (Stojanov et al., 2020). Higher F/B

ratios are linked to a higher risk of obesity (Ruth et al., 2005; Kasai et al., 2015; Crovesy et al., 2020) and lower risk of IBD (Manichanh et al., 2006; Walker et al., 2011; Kabeerdoss et al., 2015). Previous studies have found that the F/B ratio is higher among children living in urban Europe than in those from rural Africa (De Filippo et al., 2010). Researchers believe that this is due to the high protein and fat consumption among European children. However, the influence of the F/B ratio on diseases is still controversial. In many studies, this ratio has shown no correlation with obesity, and even the opposite conclusion has been reached (Castaner et al., 2018; Vaiserman et al., 2020). Our data showed that the abundance of *Actinobacteria* was higher than that of *Bacteroidota* in adolescents from rural regions and towns. *Bacteroidota* was significantly more abundant in adolescents from urban regions than in those from other regions. The F/B ratio was found to differ among adolescents living in cities, towns, and rural areas. Unexpectedly, it was lower in adolescents living in urban areas than in those from rural areas. However, as mentioned above, the dietary habits in the urban areas of Hangzhou were not similar to those observed in western cities. While meat consumption was prevalent in the city of Hangzhou, the high-frequency intake of vegetables and fruits was also observed. Hence, the relationship between the intestinal microbiota in adolescents from different regions of Hangzhou and obesity needs further analysis. At the genus level, many bacteria showed differences among adolescents from different types of areas. Among them, *Bifidobacterium* showed a lower abundance in adolescents from urban areas, *Bacteroides* showed a higher abundance, consistent with the intestinal microbiota observed in westerners (De Filippo et al., 2010). Hence, urban development had some influence on the intestinal microbiota of adolescents. The relative abundance of *Bifidobacterium* and *Bacteroides* was found to be higher in the intestinal tracts of children living in Bangkok, while that of *Prevotella* was higher in the intestinal tracts of children living in Khon Kaen. This was attributed to the lower frequency of vegetables and fruits consumed by children in Bangkok (Nakayama et al., 2015).Our data showed that the relative abundance of *Prevotella* is low in all three regions, but it is higher in rural areas. *Prevotella* abundance is linked to a low-fat and high-fiber diet. Traditional eating habits are maintained more frequently in rural areas, which have a relatively backward economy. Our analysis of dietary habits showed that adolescents in rural areas of Hangzhou preferred to eat more vegetables, resulting in the characteristic higher abundance of *Prevotella* and lower abundance of *Bacteroides*. In economically developed urban areas, the high protein and fat intake led to a significant increase in *Bacteroides*. The difference of intestinal microbiota among adolescents living in different areas may be related to their lifestyle habits including dietary.

Diet has long been considered an important determinant of the intestinal microbiota. Our study also found that there is a close connection between dietary habits and the intestinal microbiota in adolescents. There are more *Dorea* in the intestines of adolescents who prefer to eat meat than those in the other two groups. In previous studies, it was found that the abundance of *Dorea* in Type 2 Diabetes individuals increased significantly, and it was negatively correlated with the abundance of butyrate-producing bacteria (Li et al., 2020). The increase of *Dorea* may play a role in the development of Type 2 Diabetes. This shows that the preference for eating meat in adolescence can affect the metabolism of the body by changing the intestinal microecological environment. In other words, a balanced diet is conducive to the formation of a healthy microecological intestinal environment. Moreover, 71.6% of adolescents in urban areas had a balanced diet, which might explain the higher diversity of the intestinal microbiota in this population. So far, there has been limited research on the relationship between flavor preference and the intestinal microbiota among adolescents. Our research provides new insights into this area. Through CCA analysis, Flavor preference is the lifestyle habits that have the greatest influence on the intestinal microbiota of adolescents in Hangzhou. We also found that adolescents who consumed more seasoning-rich food had the highest abundance of *Proteobacteria*, especially *Escherichia–Shigella*. However, some probiotics were enriched in adolescents who consumed more balanced and light flavors. Previous studies have considered that prevalence of the bacterial phylum *Proteobacteria* is a marker for an unstable microbial community (dysbiosis) and a potential diagnostic criterion for disease (Shin et al., 2015). Intestinal microbiota disorders also usually involve *Escherichia–Shigella*, which are linked to various diseases, such as diarrhea, Type 2 Diabetes, and acute pancreatitis (Thingholm et al., 2019; Zhu et al., 2019; Mizutani et al., 2021). This suggests that lightly flavored food can help adolescents develop a healthy intestinal microbiota and prevent diseases.

We found that sleep duration is also related to the intestinal microbiota. The intestinal microbiota was different in adolescents who slept for less than 7 h. *Bifidobacterium* was more abundant in the group with a lower sleep duration, while *Bacteroides* was more abundant in adolescents who slept for longer, although the difference was not significant, while the abundance of *Dialister* is significantly increased in the adolescents with longer sleep time. In our study, we also found that adolescents in urban areas slept longer than those in rural areas, and the abundance of *Dialister* of adolescents in urban areas is higher. This shows that the difference of sleep duration may also have an impact on the intestinal microbiota of adolescents in different areas. Previous studies have found that vegetarianism is a risk factor for mental disorders (Johannes Michalak and Jacobi, 2012). This may be due to the low tissue concentrations of long-chain *n*–3 fatty acids and vitamin B12 in vegetarians, which may increase the risk of major depression (Kwok et al., 2002; Wolfgang Herrmann, 2002). Patients with mental disorders often have a variety of sleep problems, such as sleep interruptions and a reduced duration of sleep (Baglioni et al., 2016). Both *Dialister* related to carbohydrate metabolism (Gonçalves et al., 2016) and *Bacteroides* related to fat metabolism (Li et al., 2022) were increased in the intestinal tract of adolescents who slept longer, which might be the reason for the different distribution of intestinal microbiota in adolescents with different sleep duration. Given that adolescents are in a critical period of growth and development, a balanced diet could not only help in maintaining a stable intestinal microbiota but also promote a healthy psychological state and improve sleep quality.

Previous studies have found that exercise plays an important anti-inflammatory role in the body, and regular exercise also affects the distribution of intestinal flora. People with exercise habits had a greater diversity of gut microbiota than people without (O'Sullivan et al., 2015). This may be because exercise reduces the effects of stress-induced intestinal barrier dysfunction. Moderate exercise is also associated with a lower degree of intestinal permeability, preservation of mucus thickness, and a lower rate of bacterial translocation (Luo et al., 2014). Our study also found a correlation between exercise and the intestinal microbiota of adolescents. Individuals who exercised for a long time had more *Faecalibacterium*. In previous studies, probiotics such as *Bifidobacterium* and *Faecalibacterium* have been found to be negatively correlated with body weight (Remely et al., 2016; Barratt et al., 2022). In addition, low *Faecalibacterium* abundance has been

detected in some patients with chronic diseases (Sedighi et al., 2017; Dominika Salamon et al., 2018; Gao et al., 2018). One explanation is that the microbial microbiota promotes the biotransformation of bile acids to regulate fat digestion and metabolism, decreasing serum lipid levels (Ji et al., 2012). In our study, *Faecalibacterium* was more abundant in individuals who exercised for a long duration. This indicated that a longer exercise duration may accelerate metabolism in adolescents by improving their intestinal microbiota. Adolescents who exercised for a long duration were more common in rural areas, and individuals from rural areas also had a higher abundance of probiotics. Hence, exercise durations could have also had a significant impact on the distribution of the intestinal microbiota in adolescents from different regions.

Our findings provide new ideas for exploring the characteristics of the intestinal microbiota in adolescents living in areas with different urbanization levels and the factors that influence these characteristics. Nevertheless, our study has some limitations. First, this study explored dietary habits and sleep durations using a survey questionnaire. Hence, the results may be subject to some conformity and recall bias. Second, we did not measure some specific blood gut microbiota-related metabolites, which may help us to further explain the existing research results. Third, the differences provided in this study are nominal significance, so they should be carefully explained. Fourth, as 16S rRNA amplicon sequencing does not allow species-level resolution, more detailed analysis is needed to explore the relationship between urbanization and intestinal microbiota in the future.

## 5. Conclusion

A total of 302 adolescents participated in this study, which was the first comprehensive study examining the influence of urbanization and living habits on the intestinal microbiota of Chinese adolescents. The findings suggest that the diversity of intestinal microbiota is higher among adolescents from urban areas than in those from towns and rural areas. These differences are related to dietary preferences, flavor preferences, sleep duration, and exercise duration. Our results provide a scientific basis for the development of strategies to enable the maintenance of a healthy intestinal microbiota in adolescents living in areas with different levels of urbanization.

## 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/, PRJNA856408.

## Ethics statement

The studies involving human participants were reviewed and approved by the Ethics Committee of Hangzhou Center for Disease Control and Prevention (No. 20047). Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin.

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## Author contributions

GZ: funding acquisition, data curation, formal analysis, and writing—original draft. LX: data curation, formal analysis, and methodology. YW and BW: data curation and formal analysis. WT, ZS, QK, and WL: sample collection. XP: methodology, supervision, project administration, and review. HM: methodology, supervision, project administration, and review. All authors contributed to the article and approved the submitted version.

## 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/fmicb.2023.989303/full#supplementary-material

## References

Ayeni, F. A., Biagi, E., Rampelli, S., Fiori, J., Soverini, M., Audu, H. J., et al. (2018). Infant and adult gut microbiome and Metabolome in rural Bassa and urban settlers from Nigeria. *Cell Rep.* 23, 3056–3067. doi: 10.1016/j.celrep.2018.05.018

Backhed, F., Roswall, J., Peng, Y., Feng, Q., Jia, H., Kovatcheva-Datchary, P., et al. (2015). Dynamics and stabilization of the human gut microbiome during the first year of life. *Cell Host Microbe* 17, 690–703. doi: 10.1016/j.chom.2015.04.004

Baglioni, C., Nanovska, S., Regen, W., Spiegelhalder, K., Feige, B., Nissen, C., et al. (2016). Sleep and mental disorders: a Meta-analysis of Polysomnographic research. *Psychol. Bull.* 142, 969–990. doi: 10.1037/bul0000053

Barratt, M. J., Nuzhat, S., Ahsan, K., Frese, S. A., Arzamasov, A. A., Sarker, S. A., et al. (2022). *Bifidobacterium Infantis* treatment promotes weight gain in Bangladeshi infants with severe acute malnutrition. *Sci. Transl. Med.* 14:eabk1107. doi: 10.1126/scitranslmed.abk1107

Bolyen, E., Rideout, J. R., Dillon, M. R., Bokulich, N. A., Abnet, C. C., Al-Ghalith, G. A., et al. (2019). Reproducible, interactive, scalable and extensible microbiome data science using Qiime 2. *Nat. Biotechnol.* 37, 852–857. doi: 10.1038/s41587-019-0209-9

Callahan, B. J., McMurdie, P. J., Rosen, M. J., Han, A. W., Johnson, A. J., and Holmes, S. P. (2016). Dada2: high-resolution sample inference from Illumina amplicon data. *Nat. Methods* 13, 581–583. doi: 10.1038/nmeth.3869

Castaner, O., Goday, A., Park, Y. M., Lee, S. H., Magkos, F., Shiow, S. T. E., et al. (2018). The gut microbiome profile in obesity: a systematic review. *Int. J. Endocrinol.* 2018:4095789. doi: 10.1155/2018/4095789

Chana Palmer, E. M. B., DiGiulio, D. B., Relman, D. A., and Brown, P. O. (2007). Development of the human infant intestinal microbiota. *PLoS Biol.* 5:e177. doi: 10.1371/journal.pbio.0050177.t001

Chen, S., Zhou, Y., Chen, Y., and Gu, J. (2018). Fastp: an ultra-fast all-in-one Fastq preprocessor. *Bioinformatics* 34, i884–i890. doi: 10.1093/bioinformatics/bty560

Cho, I., and Blaser, M. J. (2012). The human microbiome: at the Interface of health and disease. *Nat. Rev. Genet.* 13, 260–270. doi: 10.1038/nrg3182

Clarke, G., Grenham, S., Scully, P., Fitzgerald, P., Moloney, R. D., Shanahan, F., et al. (2013). The microbiome-gut-brain Axis during early life regulates the hippocampal serotonergic system in a sex-dependent manner. *Mol. Psychiatry* 18, 666–673. doi: 10.1038/mp.2012.77

Crovesy, L., Masterson, D., and Rosado, E. L. (2020). Profile of the gut microbiota of adults with obesity: a systematic review. *Eur. J. Clin. Nutr.* 74, 1251–1262. doi: 10.1038/s41430-020-0607-6

De Filippo, C., Cavalieri, D., Di Paola, M., Ramazzotti, M., Poullet, J. B., Massart, S., et al. (2010). Impact of diet in shaping gut microbiota revealed by a comparative study in children from Europe and rural Africa. *Proc. Natl. Acad. Sci. U. S. A.* 107, 14691–14696. doi: 10.1073/pnas.1005963107

Dominika Salamon, A. S.-O., Kapusta, P., Szopa, M., Mrozińska, S., Ludwig-Słomczyńska, A. H., Wołkow, P. P., et al. (2018). Characteristics of gut microbiota in adult patients with type 1 and type 2 diabetes based on next-generation sequencing of the 16s Rrna gene fragment. *Pol Arch Intern Med* 128, 336–343. doi: 10.20452/pamw.4246

Frati, F., Salvatori, C., Incorvaia, C., Bellucci, A., Di Cara, G., Marcucci, F., et al. (2018). The role of the microbiome in asthma: the gut(−)lung axis. *Int. J. Mol. Sci.* 20, 123–134. doi: 10.3390/ijms20010123

Gao, R., Zhu, C., Li, H., Yin, M., Pan, C., Huang, L., et al. (2018). Dysbiosis signatures of gut microbiota along the sequence from healthy, Young patients to those with overweight and obesity. *Obesity (Silver Spring, Md)* 26, 351–361. doi: 10.1002/oby.22088

Ghoshal, U. C., Shukla, R., Ghoshal, U., Gwee, K.-A., Ng, S. C., and Quigley, E. M. M. (2012). The gut microbiota and irritable bowel syndrome: friend or foe? *Int. J. Inflamm.* 2012, 1–13. doi: 10.1155/2012/151085

Golden, N. H., and Abrams, S. A.Committee on Nutrition (2014). Optimizing bone health in children and adolescents. *Pediatrics* 134, e1229–e1243. doi: 10.1542/peds.2014-2173

Gomaa, E. Z. (2020). Human gut microbiota/microbiome in health and diseases: a review. *Antonie Van Leeuwenhoek* 113, 2019–2040. doi: 10.1007/s10482-020-01474-7

Gonçalves, C. S. G., Faveri, M., Pérez-Chaparro, P. J., Lobão, E., Figueiredo, L. C., Baccelli, G. T., et al. (2016). Association of Three Putative Periodontal Pathogens with chronic periodontitis in Brazilian subjects. *J. Appl. Oral Sci.* 24, 181–185. doi: 10.1590/1678-775720150445

Hooper, L. V. W. M., Thelin, A., Hansson, L., Falk, P. G., and Gordon, J. I. (2001). Molecular analysis of commensal host-microbial relationships in the intestine. *Science* 291, 881–884. doi: 10.1126/science.291.5505.881

Ji, Y. S., Kim, H. N., Park, H. J., Lee, J. E., Yeo, S. Y., Yang, J. S., et al. (2012). Modulation of the murine microbiome with a concomitant anti-obesity effect by *Lactobacillus Rhamnosus* Gg and *Lactobacillus Sakei* Nr28. *Benefic. Microbes* 3, 13–22. doi: 10.3920/BM2011.0046

Johannes Michalak, X. C. Z., and Jacobi, F. (2012). Vegetarian diet and mental disorders: results from a representative community survey. *Int. J. Behav. Nutr. Phys. Act.* 9, 67–76.

Kabeerdoss, J., Jayakanthan, P., Pugazhendhi, S., and Ramakrishna, B. S. (2015). Alterations of mucosal microbiota in the colon of patients with inflammatory bowel disease revealed by real time polymerase chain reaction amplification of 16s ribosomal ribonucleic acid. *Indian J. Med. Res.* 142, 23–32. doi: 10.4103/0971-5916.162091

Kasai, C., Sugimoto, K., Moritani, I., Tanaka, J., Oya, Y., Inoue, H., et al. (2015). Comparison of the gut microbiota composition between obese and non-obese individuals in a Japanese population, as analyzed by terminal restriction fragment length polymorphism and next-generation sequencing. *BMC Gastroenterol.* 15:100. doi: 10.1186/s12876-015-0330-2

Kelly, J. R., Borre, Y., Ob, C., Patterson, E., El Aidy, S., Deane, J., et al. (2016). Transferring the blues: depression-associated gut microbiota induces neurobehavioural changes in the rat. *J. Psychiatr. Res.* 82, 109–118. doi: 10.1016/j.jpsychires.2016.07.019

Kim, J. W., Lee, J. S., Kim, J. H., Jeong, J. W., Lee, D. H., and Nam, S. (2018). Comparison of microbiota variation in Korean healthy adolescents with adults suggests notable maturity differences. *OMICS* 22, 770–778. doi: 10.1089/omi.2018.0146

Kwok, T., Cheng, G., Woo, J., Lai, W. K., and Pang, C. P. (2002). Independent effect of vitamin B12 deficiency on hematological status in older Chinese vegetarian women. *Am. J. Hematol.* 70, 186–190. doi: 10.1002/ajh.10134

Le Chatelier, E., Nielsen, T., Qin, J., Prifti, E., Hildebrand, F., Falony, G., et al. (2013). Richness of human gut microbiome correlates with metabolic markers. *Nature* 500, 541–546. doi: 10.1038/nature12506

Li, Q., Chang, Y., Zhang, K., Chen, H., Tao, S., and Zhang, Z. (2020). Implication of the gut microbiome composition of type 2 diabetic patients from northern China. *Sci. Rep.* 10:5450. doi: 10.1038/s41598-020-62224-3

Li, H., Wang, S., Wang, S., Yu, H., Yu, W., Ma, X., et al. (2022). Atorvastatin inhibits high-fat diet-induced lipid metabolism disorders in rats by inhibiting Bacteroides reduction and improving metabolism. *Drug Des. Devel. Ther.* 16, 3805–3816. doi: 10.2147/dddt.S379335

Liu, C., Zhao, D., Ma, W., Guo, Y., Wang, A., Wang, Q., et al. (2016). Denitrifying sulfide removal process on high-salinity wastewaters in the presence of *Halomonas* Sp. *Appl. Microbiol. Biotechnol.* 100, 1421–1426. doi: 10.1007/s00253-015-7039-6

Luo, B., Xiang, D., Nieman, D. C., and Chen, P. (2014). The effects of moderate exercise on chronic stress-induced intestinal barrier dysfunction and antimicrobial defense. *Brain Behav Immunity* 39, 99–106. doi: 10.1016/j.bbi.2013.11.013

Magoč, T., and Salzberg, S. L. (2011). Flash: fast length adjustment of short reads to improve genome assemblies. *Bioinformatics* 27, 2957–2963. doi: 10.1093/bioinformatics/btr507

Manichanh, C., Rigottier-Gois, L., Bonnaud, E., Gloux, K., Pelletier, E., Frangeul, L., et al. (2006). Reduced diversity of faecal microbiota in Crohn's disease revealed by a metagenomic approach. *Gut* 55, 205–211. doi: 10.1136/gut.2005.073817

Matenchuk, B. A., Mandhane, P. J., and Kozyrskyj, A. L. (2020). Sleep, circadian rhythm, and gut microbiota. *Sleep Med. Rev.* 53:101340. doi: 10.1016/j.smrv.2020.101340

Mccune, B. (1997). Influence of Noisy environmental data on canonical correspondence analysis. *Ecology* 78, 2617–2623. doi: 10.1890/0012-9658(1997)078[2617:IONEDO]2.0.CO;2

McKean, J., Naug, H., Nikbakht, E., Amiet, B., and Colson, N. (2017). Probiotics and subclinical psychological symptoms in healthy participants: a systematic review and meta-analysis. *J. Altern. Complement. Med. (New York, NY)* 23, 249–258. doi: 10.1089/acm.2016.0023

Mizutani, T., Aboagye, S. Y., Ishizaka, A., Afum, T., Mensah, G. I., Asante-Poku, A., et al. (2021). Gut microbiota signature of pathogen-dependent dysbiosis in viral gastroenteritis. *Sci. Rep.* 11:13945. doi: 10.1038/s41598-021-93345-y

Moon, J. H., Kim, M., and Lee, J. H. (2017). Genome sequence of *Prevotella Intermedia* Suny Ab G8-9k-3, a biofilm forming strain with drug-resistance. *Braz. J. Microbiol.* 48, 5–6. doi: 10.1016/j.bjm.2016.04.030

Morais, L. H., Schreiber, H. L. IV, and Mazmanian, S. K. (2021). The gut microbiota-brain Axis in behaviour and brain disorders. *Nat. Rev. Microbiol.* 19, 241–255. doi: 10.1038/s41579-020-00460-0

Nagano, Y., Itoh, K., and Honda, K. (2012). The induction of Treg cells by gut-indigenous *Clostridium*. *Curr. Opin. Immunol.* 24, 392–397. doi: 10.1016/j.coi.2012.05.007

Nakayama, J., Watanabe, K., Jiang, J., Matsuda, K., Chao, S. H., Haryono, P., et al. (2015). Diversity in gut bacterial community of school-age children in Asia. *Sci. Rep.* 5:8397. doi: 10.1038/srep08397

O'Sullivan, O., Cronin, O., Clarke, S. F., Murphy, E. F., Molloy, M. G., Shanahan, F., et al. (2015). Exercise and the microbiota. *Gut Microbes* 6, 131–136. doi: 10.1080/19490976.2015.1011875

Parekh, P. J., Balart, L. A., and Johnson, D. A. (2015). The influence of the gut microbiome on obesity, metabolic syndrome and gastrointestinal disease. *Clin. Transl. Gastroenterol.* 6:e91. doi: 10.1038/ctg.2015.16

Paulsen, J. A., Ptacek, T. S., Carter, S. J., Liu, N., Kumar, R., Hyndman, L., et al. (2017). Gut microbiota composition associated with alterations in cardiorespiratory fitness and psychosocial outcomes among breast Cancer survivors. *Support Care Cancer* 25, 1563–1570. doi: 10.1007/s00520-016-3568-5

Qin, J., Li, R., Raes, J., Arumugam, M., Burgdorf, K. S., Manichanh, C., et al. (2010). A human gut microbial gene catalogue established by metagenomic sequencing. *Nature* 464, 59–65. doi: 10.1038/nature08821

Quiroga, R., Nistal, E., Estebanez, B., Porras, D., Juarez-Fernandez, M., Martinez-Florez, S., et al. (2020). Exercise training modulates the gut microbiota profile and impairs inflammatory signaling pathways in obese children. *Exp. Mol. Med.* 52, 1048–1061. doi: 10.1038/s12276-020-0459-0

Remely, M., Hippe, B., Zanner, J., Aumueller, E., Brath, H., and Haslberger, A. G. (2016). Gut microbiota of obese, type 2 diabetic individuals is enriched in *Faecalibacterium Prausnitzii*, Akkermansia Muciniphila and *Peptostreptococcus Anaerobius* after weight loss. *Endocr. Metab. Immune Disord. Drug Targets* 16, 99–106. doi: 10.2174/1871530316666160831093813

Ruth, E., Ley, F. B., Turnbaugh, P., Lozupone, C. A., Knight, R. D., and Gordon, J. I. (2005). Obesity alters gut microbial ecology. *Proc. Natl. Acad. Sci. U. S. A.* 102, 11070–11075. doi: 10.1073/pnas.0504978102

Sampson, T. R., and Mazmanian, S. K. (2015). Control of brain development, function, and behavior by the microbiome. *Cell Host Microbe* 17, 565–576. doi: 10.1016/j.chom.2015.04.011

Schloss, P., Westcott, S., Ryabin, T., Hall, J., Hartmann, M., Hollister, E., et al. (2009). Introducing Mothur: open-source, platform-independent, community-supported software for describing and comparing microbial communities. *Appl. Environ. Microbiol.* 75, 7537–7541. doi: 10.1128/AEM.01541-09

Sedighi, M., Razavi, S., Navab-Moghadam, F., Khamseh, M. E., Alaei-Shahmiri, F., Mehrtash, A., et al. (2017). Comparison of gut microbiota in adult patients with type 2 diabetes and healthy individuals. *Microb. Pathog.* 111, 362–369. doi: 10.1016/j.micpath.2017.08.038

Segata, N., Izard, J., Waldron, L., Gevers, D., Miropolsky, L., Garrett, W. S., et al. (2011). Metagenomic biomarker discovery and explanation. *Genome Biol.* 12:R60. doi: 10.1186/gb-2011-12-6-r60

Sherwin, E., Rea, K., Dinan, T. G., and Cryan, J. F. (2016). A gut (microbiome) feeling about the brain. *Curr. Opin. Gastroenterol.* 32, 96–102. doi: 10.1097/MOG.0000000000000244

Shin, N. R., Whon, T. W., and Bae, J. W. (2015). Proteobacteria: microbial signature of Dysbiosis in gut microbiota. *Trends Biotechnol.* 33, 496–503. doi: 10.1016/j.tibtech.2015.06.011

Shreiner, A. B., Kao, J. Y., and Young, V. B. (2015). The gut microbiome in health and in disease. *Curr. Opin. Gastroenterol.* 31, 69–75. doi: 10.1097/MOG.0000000000000139

Stojanov, S., Berlec, A., and Štrukelj, B. (2020). The influence of probiotics on the Firmicutes/Bacteroidetes ratio in the treatment of obesity and inflammatory bowel disease. *Microorganisms* 8, 1715–1730. doi: 10.3390/microorganisms8111715

Strati, F., Cavalieri, D., Albanese, D., De Felice, C., Donati, C., Hayek, J., et al. (2017). New evidences on the altered gut microbiota in autism Spectrum disorders. *Microbiome* 5:24. doi: 10.1186/s40168-017-0242-1

Thingholm, L. B., Ruhlemann, M. C., Koch, M., Fuqua, B., Laucke, G., Boehm, R., et al. (2019). Obese individuals with and without type 2 diabetes show different gut microbial functional capacity and composition. *Cell Host Microbe* 26, 252–264.e10. doi: 10.1016/j.chom.2019.07.004

Tian, L., Wu, A. K., Friedman, J., Waldor, M. K., Weiss, S. T., and Liu, Y. Y. (2017). Deciphering functional redundancy in the human microbiome. *Nat Commun.* 11, 6217–6227. doi: 10.1038/s41467-020-19940-1

Turnbaugh, P. J., Ley, R. E., Hamady, M., Fraser-Liggett, C. M., Knight, R., and Gordon, J. I. (2007). The human microbiome project. *Nature* 449, 804–810. doi: 10.1038/nature06244

Vaiserman, A., Romanenko, M., Piven, L., Moseiko, V., Lushchak, O., Kryzhanovska, N., et al. (2020). Differences in the gut Firmicutes to Bacteroidetes ratio across age groups in healthy Ukrainian population. *BMC Microbiol.* 20:221. doi: 10.1186/s12866-020-01903-7

Vijayakumar, N., Op de Macks, Z., Shirtcliff, E. A., and Pfeifer, J. H. (2018). Puberty and the human brain: insights into adolescent development. *Neurosci. Biobehav. Rev.* 92, 417–436. doi: 10.1016/j.neubiorev.2018.06.004

Walker, A. W., Sanderson, J. D., Churcher, C., Parkes, G. C., Hudspith, B. N., Rayment, N., et al. (2011). High-throughput clone library analysis of the mucosa-associated microbiota reveals Dysbiosis and differences between inflamed and non-inflamed regions of the intestine in inflammatory bowel disease. *BMC Microbiol.* 11:7. doi: 10.1186/1471-2180-11-7

Wolfgang Herrmann, J. G. (2002). Vegetarian lifestyle and monitoring of vitamin B-12 status. *Clin. Chim. Acta* 326, 47–59. doi: 10.1016/S0009-8981(02)00307-8

Yatsunenko, T., Rey, F. E., Manary, M. J., Trehan, I., Dominguez-Bello, M. G., Contreras, M., et al. (2012). Human gut microbiome viewed across age and geography. *Nature* 486, 222–227. doi: 10.1038/nature11053

Zhu, Y., He, C., Li, X., Cai, Y., Hu, J., Liao, Y., et al. (2019). Gut microbiota Dysbiosis worsens the severity of acute pancreatitis in patients and mice. *J. Gastroenterol.* 54, 347–358. doi: 10.1007/s00535-018-1529-0

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# *Saccharomyces cerevisiae boulardii* accelerates intestinal microbiota maturation and is correlated with increased secretory IgA production in neonatal dairy calves

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Neonatal calves have a limited capacity to initiate immune responses due to a relatively immature adaptive immune system, which renders them susceptible to many on-farm diseases. At birth, the mucosal surfaces of the intestine are rapidly colonized by microbes in a process that promotes mucosal immunity and primes the development of the adaptive immune system. In a companion study, our group demonstrated that supplementation of a live yeast probiotic, *Saccharomyces cerevisiae boulardii* (SCB) CNCM I-1079, to calves from birth to 1 week of age stimulates secretory IgA (sIgA) production in the intestine. The objective of the study was to evaluate how SCB supplementation impacts the intestinal microbiota of one-week-old male calves, and how changes in the bacterial community in the intestine relate to the increase in secretory IgA. A total of 20 calves were randomly allocated to one of two treatments at birth: Control (CON, $n = 10$) fed at 5 g/d of carrier with no live yeast; and SCB ($n = 10$) fed at 5 g of live SCB per day ($10 \times 109$ CFU/d). Our study revealed that supplementing calves with SCB from birth to 1 week of age had its most marked effects in the ileum, increasing species richness and phylogenetic diversity in addition to expediting the transition to a more interconnected bacterial community. Furthermore, LEfSe analysis revealed that there were several differentially abundant taxa between treatments and that SCB increased the relative abundance the family *Eubacteriaceae*, *Corynebacteriaceae*, *Eggerthellaceae*, *Bacillaceae*, and *Ruminococcaceae*. Furthermore, network analysis suggests that SCB promoted a more stable bacterial community and appears to reduce colonization with *Shigella*. Lastly, we observed that the probiotic-driven increase in microbial diversity was highly correlated with the enhanced secretory IgA capacity of the ileum, suggesting that the calf's gut mucosal immune system relies on the development of a stable and highly diverse microbial community to provide the necessary cues to train and promote its proper function. In summary, this data shows that supplementation of SCB promoted establishment of a diverse and interconnected microbiota, prevented colonization of *Escherichia Shigella* and indicates a possible role in stimulating humoral mucosal immunity.

KEYWORDS

intestinal immunity, microbiome, microbial colonization, calf health, immune development

# 1. Introduction

At birth, neonatal calves have a limited capacity to initiate immune responses, and the immune system gradually matures during the first months of life, concurrent with microbial colonization and development (Wopereis et al., 2014). Although less studied in calves, microbial colonization of the gastrointestinal tract (GIT) during early life has been extensively reported in other mammalian species to be critical for normal immune and intestinal development (Hooper et al., 2012). The process of colonization is highly dynamic and modulated by several environmental and maternal factors that include colostrum and milk consumption, maternal and environmental microbes, diet, antimicrobial exposure, and weaning (Wopereis et al., 2014). During establishment of the intestinal microbiota there is a suppression of adaptive immunity to promote tolerance to the increasing load of bacteria that reaches the intestine, and the amount of antigen that is encountered by immune cells (Belkaid and Hand, 2014; Yang et al., 2020). Calves are highly dependent on successful passive transfer of immunoglobulins (Ig) from colostrum since there is no transfer of Ig through placenta and their adaptive immune system is not fully developed. Failure of passive transfer results in increased morbidity and mortality (Urie et al., 2018), with diarrhea accounting for over half of reported illnesses and one-third of deaths (Urie et al., 2018; Winder et al., 2018). Traditionally, antimicrobials are used to treat and prevent diarrhea. However, variable efficacy of antimicrobials, risk of developing antimicrobial resistance, and the long-term impacts on animal health and performance make this practice unsustainable (Windeyer et al., 2014; Aghakeshmiri et al., 2017; Buss et al., 2021). As a mean to minimize antimicrobial use, preventative approaches should be emphasized and alternatives to antimicrobials need to be pursued.

As previously mentioned, the gut microbiota modulates mucosal immunity and primes the development of the adaptive immune system during early life. In addition, the gut microbiome has greater plasticity in early life and perturbations to the intestinal microbial at this stage of life can have long-term health consequences (Laforest-Lapointe and Arrieta, 2017). The same is thought to occur in calves, as pre- (Marquez, 2014) and probiotics (Abe et al., 1995) exert the greatest effects when supplemented during the first weeks of life (Cangiano et al., 2020). This could be directly related to the instability of their microbial communities, whereas later in life the microbiome is stable and more difficult to influence (Malmuthuge et al., 2015). Hence, modulating the gut microbiome during early life, by supplementing microbial-based products, could become a sustainable option to enhance mucosal immunity and ultimately improve calf health (Malmuthuge and Guan, 2017). Previously, *Saccharomyces cerevisiae boulardii* (SCB) has been shown to reduce the incidence of severe diarrhea in pre-weaned calves (Villot et al., 2019) and increase average daily gain (ADG) after weaning (Renaud et al., 2019). In a companion study, it was shown that SCB supplementation to newborn calves from birth stimulates secretory IgA (sIgA) production in the intestine at 1 week of age (Villot et al., 2020), suggesting that this yeast probiotic can prime mucosal immunity. Secretory IgA is the most abundant immunoglobulin in mucosal secretions (Pabst et al., 2016) and represents one of the main non-inflammatory defense mechanisms of the gut along with antimicrobial peptides. It generates an adaptive immune response that prevents adhesion and invasion by microorganisms, neutralizing them and forcing its clearance out of the gastrointestinal tract (Mantis et al., 2011). The enhancement of humoral immunocompetence and increased secretion of IgA by SCB supplementation has also been demonstrated in germ-free mice models (Rodrigues et al., 2000; Martins et al., 2009), in normal BALB/C mice (Qamar et al., 2001), and broiler chickens (Rajput et al., 2013). However, the exact molecular mechanisms of action and the secreted molecules that confer its protective and immunomodulatory effects remain only partially understood. It has been shown that SCB reduces dendritic cell activation in cells culture, and promotes IL-10 secretion while reducing IL-6 and TNF-α, ultimately reducing T cell proliferation (Thomas et al., 2009). In addition, SCB reduces oxygen availability in the neonatal gut, accelerating the colonization of the intestine with strict anaerobic bacteria and stimulating microbial diversity. This in turn provides the necessary signals that stimulate intestinal maturation (Pais et al., 2020). Lastly, SCB administration after antimicrobial therapy helps restore the intestinal microbiota diversity faster in mice (Barc et al., 2008; Moré and Swidsinski, 2015). Therefore, we hypothesized that supplementation of SCB to newborn calves could accelerate intestinal microbial maturation, providing the necessary signals to stimulate the intestinal naïve B cells to become activated and increase their capacity to secrete IgA. The objective of the study was to evaluate how SCB supplementation impacts the intestinal microbiota of one-week-old male calves, and how changes in the bacterial community in the GIT relate to the increase in secretory IgA secretion observed our companion study.

# 2. Materials and methods

## 2.1. Animals, housing, treatments, and experimental design

The experiment was conducted as per the guidelines of the Canadian Council of Animal Care (CCAC, 1993) at the Dairy Research and Technology Center of the University of Alberta (Edmonton, Alberta, Canada), and the methodology was previously described in our companion study (Villot et al., 2020). The animal use protocol was approved by the University of Alberta Animal Care Committee (Animal Use Protocol # 2645). A total of 20 singlet and naturally delivered Holstein bull calves born from May to August 2018 with a birth body weight (BW) between 35 and 55 kg were enrolled in this study. Calves were removed from the maternity pen

at birth to avoid contact with the dam and moved to disinfected indoor pens (150 × 125 cm) bedded with a consistent amount of fresh straw to facilitate nesting. Animals included in this study presented healthy at birth based on general appearance, rectal temperature, umbilical appearance, and nasal discharge; none were vaccinated or treated with therapeutic fluids during the trial. Calves received two meals of standardized colostrum replacer (HeadStart, Saskatoon Colostrum Co., Ltd., Saskatoon, SK, Canada). Briefly, calves received two feedings of colostrum at 2 h after birth fed at 7% of birth BW, and at 12 h after birth fed at 3% of birth BW to receive an average amount of 180, and 120 g of IgG, respectively (Villot et al., 2020). Colostrum was bottle fed, and the remainder of the meal volume was tube-fed if the calves did not consume all of the meal within 30 min. The concentration of IgG and IgA delivered to the calves was measured in a pooled colostrum batch composed of subsamples of all the colostrum fed to the calves, which confirmed that all calves received the same amount of IgG and IgA from colostrum (Villot et al., 2019). Milk replacer was bottle-fed, and it was composed of 260 g/kg of crude protein, 160 g/kg of crude fat, and 4.58 Mcal/kg of metabolizable energy on a dry matter basis (Grober Animal Nutrition, Cambridge, Ontario, Canada). The initial volume of individual meals offered to each calf was 7.5% of their birth BW during the 2 first days of the experiment and increased to 8.5% of BW from d 3 to d 7.

A generalized randomized block design was used to evaluate the impact of SCB supplementation on bacterial colonization and its impact on the development of humoral immunity of Holstein bull calves. Calves were randomly assigned to one of two treatments according to their date of birth and initial BW: Control (CON, $n = 10$) fed at 5 g/d of carrier with no live yeast; and *Saccharomyces cerevisiae boulardii* (SCB, $n = 10$) fed at 5 g of live SCB per day ($10 \times 10^9$ CFU) from birth to 1 week of life. Both the CON and SCB treatments were fed once daily starting with the first colostrum feeding and subsequently in the morning milk replacer feeding until 1 week of age when calves were euthanized.

## 2.2. Intestinal tissue and digesta sampling

At 1 week of life, 3 h after the morning meal, calves were euthanized to collect tissue and digesta samples from the gastrointestinal tract. Calves were euthanized with a pentobarbital sodium injection (Euthanyl, Vetoquinol, Lavaltrie, QC, Canada) at 0.125 mL/kg of BW administered through a jugular catheter. Once the calf reached a surgical plane of anesthesia, exsanguination was performed. The esophagus and rectum were first ligated to prevent loss of digesta and the entire gut digesta were then removed with caution to avoid transfer of digesta between the different segments. Segments of the proximal jejunum, ileum, and colon, along with their digesta were collected according to Villot et al. (2020). Briefly, the proximal jejunum sample was taken 100 cm distal to the pylorus sphincter, the ileum segment was defined as 30 cm proximal to the ileocecal junction, and the colon segment was defined as 30 cm distal to the ileocecal junction. Zip-ties were placed on each end of the segments so that a sample of digesta could be collected. Intestinal digesta were removed from the sample using tweezers and placed in a 50-mL Falcon tube. The tissue was then washed in sterile PBS (pH 7.4) until clean (~3–4 washes) and placed in a sterile bag. Both gut digesta and tissue samples were immediately snap-frozen in liquid nitrogen and then stored at −80°C until further processing.

## 2.3. Quantification of SCB, secretory IgA and SCFA in intestinal digesta

A primer set that targets the 26S rRNA gene of *S. cerevisiae* was used to estimate the copy numbers of SCB using real-time quantitative PCR (qPCR), according to Chang et al. (2007) and Villot et al. (2020). Briefly, the qPCR was performed in the high throughput Viia 7 Real-Time PCR System (Thermo Fisher Scientific, Waltham, MA) using SYBR green chemistry (Fast SYBR Green Master Mix, Applied Biosystems, Foster City, CA) with specific primers (Forward: 5′- AGGAGTGCGGTTCTTTG −3′, and Reverse: 5′- TACTTACCGAGGCAAGCTACA −3′). Genomic DNA of SCB was extracted from the commercial probiotic yeast used in the study (ProTernative Milk; CNCM I-1079 strain) to generate a standard curve to estimate the gene copy number of *S. cerevisiae* in calf digesta from proximal jejunum, ileum, and colon. All qPCR reactions were performed using the optimized conditions described previously by Villot et al. (2020), and the quantity of *S. cerevisiae* was calculated from the standard curve.

Data on sIgA concentration in intestinal digesta, as well as the analysis procedures, are presented in Villot et al. (2019). Briefly, sIgA was measured in intestinal digesta from jejunum, ileum, and colon on d 7. The concentration of sIgA was analyzed using a sandwich ELISA technique with a commercial kit (Bethyl Laboratories Inc., TX), as per the manufacturer's recommendations and as described by Villot et al. (2020). All samples were tested in duplicate, and samples with an intra and inter-assay CV greater than 10% were repeated.

The concentrations of short-chain fatty acids (SCFAs; acetic, propionic, butyric, and caproic) were determined via gas chromatography, as described by Schlau et al. (2012). Briefly, digesta samples were thawed and centrifuged at 13,000 rpm for 5 min at 4°C and the supernatant mixed with 25% meta-phosphoric acid (4:1; vol/vol). The samples were then centrifuged again at 13,000 rpm for 5 min at 4°C, and the supernatant was mixed with internal standard solution (5:1; vol/vol) before incubation at −20°C overnight. After incubation, samples were centrifuged at 19,000 × *g* at 4°C for 5 min and the supernatant transferred to vials. Samples were analyzed by gas chromatography (Varian, Palo Alto, CA) using a flame ionization detector and a capillary column (CP-WAX 58 FFAP 25 m 0.53 mm, Varian CP7767, Varian Analytical Instruments, Walnut Creek, CA).

## 2.4. DNA extraction, library preparation, 16S amplicon sequencing

Extraction of microbial genomic DNA was performed similarly to that previously reported by Yu and Morrison (2004). Briefly, the digesta and tissue samples (~0.3 g ± 0.1 g) were washed twice with Tris-EDTA buffer. After the addition of cell-lysis buffer containing 4% SDS, samples were subjected to physical disruption at 5,000 rpm for 3 min using Biospec Mini Beads Beater 8 (BioSpec, Bartlesville, OK), followed by incubation at 70°C for 15 min and centrifugation for 5 min at 16,000 × *g* at 10°C. The bead beating, incubation, and centrifugation

steps were repeated, and impurities were removed from the supernatant using 10 M ammonium acetate, followed by DNA precipitation using isopropanol. After precipitation, DNA was further purified using QIAmp fast DNA stool mini kit (Qiagen Inc., Germantown, MD). The quantity and quality of DNA were evaluated using the NanoDrop 1000 spectrophotometer, and DNA was stored at −20°C until further use.

For library preparation and sequencing, a fragment of the 16S rRNA gene spanning the V1–V3 hypervariable region was amplified by PCR using dual indexed {universal bacterial primers [9F (5′-GAGTTTGATCMTGGCTCAG-3′); 515R (5′- CCGCGGCKG CTGGCAC- 3′); Kroes et al., 1999]}. The amplicons with targeted size (~480 bp) were purified from 1% agarose gel using a QIAEX II gel extraction kit (Qiagen Inc.). The quality and quantity of purified PCR products were checked using a NanoDrop 1000 (NanoDrop Technologies, Wilmington, DE) to ensure that the concentration of DNA from all samples was higher than 25 ng/μL. Sequencing libraries were prepared using the Nextera XT Index (Illumina) and sequenced on an Illumina MiSeq platform (2 × 300 bp) at Genome Quebec (McGill University, Montreal, QC, Canada).

## 2.5. Bioinformatics and statistical analysis

The adapter sequences were trimmed from the raw fastq files, and the trimmed reads were demultiplexed according to the samples using the bcl2fastq2 conversion software version 2.20.0. (Illumina). For bioinformatic analysis, the sorted reads were imported and processed using the Quantitative Insight into Microbial Ecology (QIIME2) package version 2021.2 (Bolyen et al., 2019). First, low-quality reads (Phred score < 20) and short (<100 bp) reads were filtered out. This was followed by denoising and merging using the plugin DADA2 to generate an amplicon sequence variant (ASV) feature table. Sequences shorter than 400 bp as well as chimeric sequences and singleton ASVs were excluded from further analyses. Taxonomic classification was performed in QIIME2 using a scikit-learn naïve Bayes classifier trained for the V1-V3 region. Next, taxonomic assignment was performed using the trained classifier using the SILVA database (SILVA release 132) with 99% identity for representative bacterial sequences. Alpha and beta diversity were performed using QIIME2 with a sampling depth of 15,000 for the digesta samples, and 7,000 for the tissue samples. Alpha-diversity analysis was conducted with standard diversity metrics accessed via QIIME2, including Chao1, Shannon index, and Faith index. A nonparametric analysis of variance (Kruskal-Wallis test) was used to test differences in α-diversity among treatment groups and to calculate $p$-values within QIIME2. Beta-diversity was measured by calculating the weighted and unweighted UniFrac distances using QIIME2. Non-metric multidimensional scaling (NMDS) was applied on the resulting distance matrices, and analysis of similarity (ANOSIM) were used to calculate $p$-values and to test differences in β-diversity among treatment groups for significance. Furthermore, the linear discriminant analysis of effect size (LEfSe; Segata et al., 2011) was used to evaluate ASVs differences between treatments. The data were displayed using linear discriminant analysis score plots for significant ASVs after Kruskal–Wallis and Wilcoxon tests. A size-effect threshold of 3.5 on the logarithmic LDA score was used to identify discriminating taxa. Finally, the relative abundance of discriminant taxa from the LEfSe analysis were compared between groups using among the groups, and Mann–Whitney $U$ test was used to identify taxa that were significantly different.

Microbiota network analysis was performed at the phylum and at the genus level and separately for each treatment group. Genera with less than 0.1% mean relative abundance and less than 25% prevalence among the different samples were excluded. The ASV relative abundance table obtained from QIIME was imported into R package Phyloseq and converted into an adjacency matrix through the use of R package SPIEC-EASI (Kurtz et al., 2015). Next, the Meinshausen-Bühlman neighborhood selection method was used to calculate the conditional dependence between the different ASV pairs. This approach was developed to perform network analysis for compositional and sparse data, such as relative abundance of ASV data, which prevents indirect and spurious associations from being generated (Kurtz et al., 2015). The obtained matrices were visualized as networks at both Phylum and Genus levels. The networks model the relationships between different ASVs, where nodes represent ASVs, and edges represent their co-occurrence across different samples. In order to evaluate ASV centrality in the networks, their hub scores were calculated and extracted from the networks using the SPIEC-EASI hub_score() function, and ranked. The 10 species with the highest hub scores were defined as the keystone species in each group.

Lastly, non-parametric Spearman rank correlation coefficient analysis was used to test the relationship between secretory IgA in ileum digesta (sIgA), short chain fatty acids profile (acetate, propionate, caproate, and total SCFAs), alpha diversity measures (species richness, evenness, and phylogenetic diversity), with the bacterial communities differentially abundant between treatments and *Saccharomyces cerevisiae* gene copy numbers present in ileum digesta. Physiological data were published previously by Villot et al. (2020) in a companion paper. The resulting correlation matrix was visualized in a heatmap format generated by the corrplot package of R [Corrplot: visualization of a correlation matrix; R package version 4.1.2. 2021].

# 3. Results

## 3.1. Alpha and beta diversity measures

Supplementation of newborn calves with SCB increased species richness at 1 week of age in the ileum digesta ($p = 0.01$; Figure 1A) and tended to increase species richness in colon digesta ($p = 0.08$; Figure 1A), as denoted by the Chao1 index. No differences in evenness (Shannon index) of the bacterial microbiota of calves were observed between treatments in any of the locations in the digesta samples ($p > 0.23$; Figure 1A). Additionally, SCB supplementation increased the phylogenetic diversity (PD) in the ileum digesta ($p < 0.01$; Figure 1A), as denoted by the PD faith index. In the tissue samples, no difference between groups were observed in species richness ($p > 0.34$; Figure 1B), evenness ($p > 0.21$; Figure 1B), or PD ($p > 0.17$; Figure 1B). To compare bacterial communities between groups, we used unweighted UniFrac distance matrixes in both digesta and tissue samples of the different segments of the intestine and were visualized by NMDS (Figure 2). These results revealed that the bacterial profiles generated from ileum digesta-associated communities tended to separate into distinct clusters according to treatment (ANOSIM $R = 0.20$, $p = 0.02$; Figure 2B), with no differences observed in the other regions of the intestine both for mucosa and digesta.

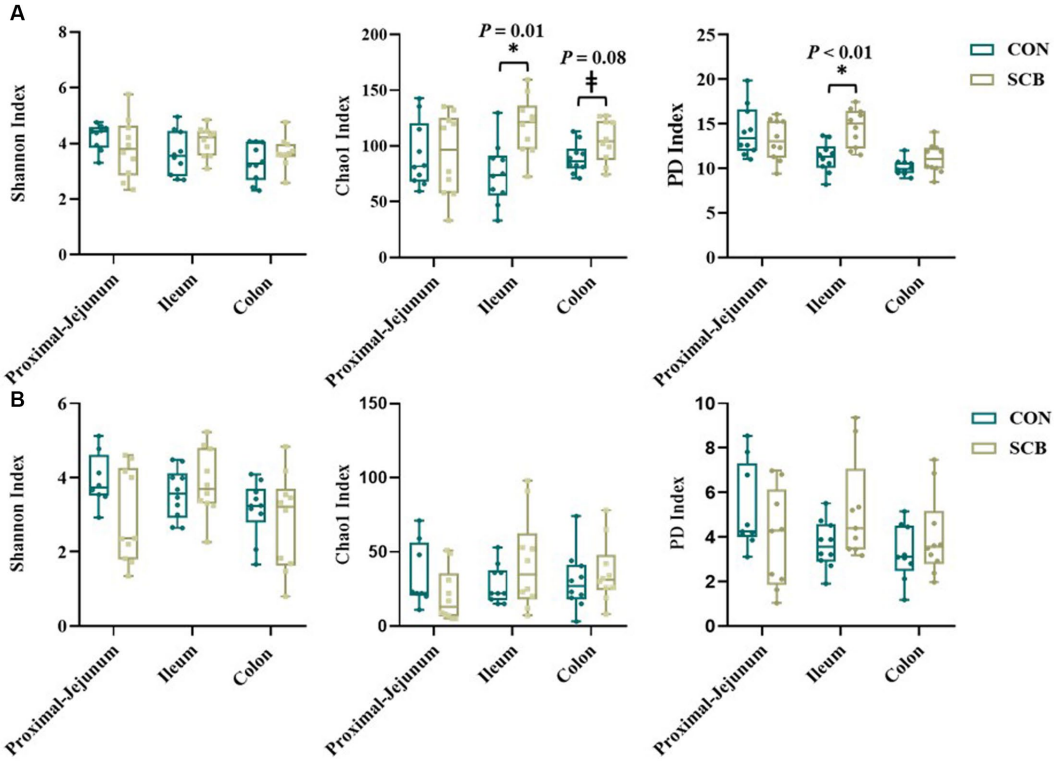


FIGURE 1
Alpha diversity index in digesta **(A)** and mucosa **(B)** at proximal jejunum, ileum, and colon of calves supplemented (SCB) or not (CON) from birth until 1 week of age with *Saccharomyces cerevisiae boulardii* CNCM I-1079 at $10 \times 10^9$ cfu/d. Species richness and diversity index were measured with three different matrices: Chao1, Shannon index, and Faith phylogenetic diversity (PD index). * Indicates a significant difference between CON and SCB groups ($p < 0.05$), and ‡ denotes a tendency for a significant difference between CON and SCB groups ($p < 0.10$). The lines, boxes, and whiskers in the box plots represent the median, and 25th, and 75th percentiles, and the min-to-max distribution of replicate values, respectively.

## 3.2. *Saccharomyces cerevisiae* concentrations, LEfSe, and relative abundance of discriminant taxa

Copy numbers of 26S rRNA *S. cerevisiae* gene were detected in the digesta of all intestinal compartments measured. Supplementation of SCB in milk significantly increased the density of *S. cerevisiae* per g of digesta in jejunum, ileum, and colon compared with non-supplemented calves ($p < 0.001$; Table 1).

We performed a linear discriminant analysis coupled with effect size measurements (LEfSe) in ileal digesta samples given that it was the only location and sample type where differences in species richness, phylogenetic diversity, and clustering of the bacterial community according to treatment were observed (Figure 1A). Results from LEfSe analysis were used to generate a circular cladogram for the ileal digesta microbiota and to identify the differentially abundant taxa between treatments (Figure 3). The LEfSe analysis in ileum digesta samples indicated that the phyla *Actinobacteriota*, including the families *Corynebacteriaceae*, *Eggerthellaceae*, as well as the families *Bacillaceae*, *Erysipelotrichaceae*, *Aerococcaceae*, *Eubacteriaceae*, and *Ruminococcaceae* were among the discriminatory taxa between CON and SCB groups. Next, the relative abundances of the discriminatory taxa between treatments were compared. The relative abundance of the families *Ruminococcaceae*, *Bacillaceae*, *Eubacteriaceae*, *Corynebacteriaceae*, *Eggerthellaceae*, *Erysipelotrichaceae*, *Aerococcaceae*, and *Eubacteriaceae* ($p < 0.04$; Figure 4A), as well as members of the genus *Eggerthella*, *Geobacillus*, and *Anoxybacillus* were significantly increased in the SCB group ($p < 0.05$; Figure 4B) compared with CON.

## 3.3. Network analysis

To further assess community ecological parameters, we performed a co-occurrence network analysis using SPIEC-EASI to infer microbial ecological networks differences between the CON and SCB groups in ileal digesta. The resulting network revealed that probiotic supplementation increased the microbial interconnectivity between nodes in the network as denoted by the increase in total edges within the SCB network (417 edges) compared to CON (176 edges), and the number of total nodes (SCB = 198 vs. CON = 135) present in the network (Figure 5). In addition, the keystone species (composed of the 10 taxa most central to the microbiota networks according to the hub scores) were different between groups and show that SCB increased the number of different taxa at the genus level that are central to the microbiota network (Figure 6). For instance, the keystone species in CON group consisted primarily of bacteria from the genus *Lactobacillus*, *Escherichia Shigella*, and *Veillonella* (Figure 6A, Hub Score > 0.6), whereas the SCB group consisted of *Streptococcus*, *Lactococcus*, *Lactobacillus*, *Pelomonas*, *Actinomyces*, *Eubacterium*, *Lachnoclostridium* (Figure 6B, Hub Score > 0.6). Furthermore, the average hub scores were higher for those keystone species in SCB

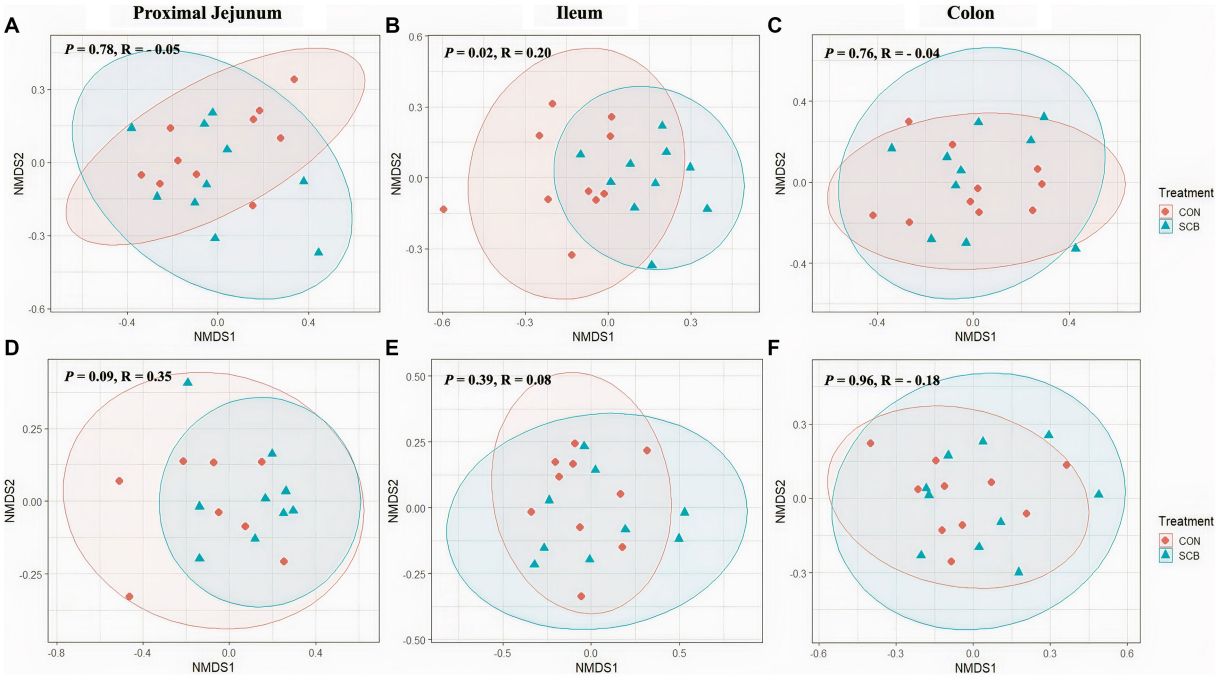


FIGURE 2
Non-Metric Multidimensional Scaling Plots (NMDS) based on unweighted unifrac distances in digesta **(A–C)** and mucosa **(D–F)** at proximal jejunum, ileum, and colon, respectively, of calves supplemented (SCB) or not (CON) from birth until 1 week of age with *Saccharomyces cerevisiae boulardii* CNCM I-1079 at $10 \times 10^9$ cfu/d. Analysis of similarity ($R$ = ANOSIM) were used to calculate $p$-values and to test differences in β-diversity among treatment groups.

TABLE 1 Concentration of *Saccharomyces cerevisiae* in the gut digesta of 1-wk-old calves in response to the supplementation of *Saccharomyces cerevisiae boulardii* CNCM I-1079.

| | Treatment[2] | | | $p$-value[3] | | |
|---|---|---|---|---|---|---|
| Concentration in digesta, $Log^{10}$ copy /g of sample | CON | SCB | SEM | Location | TRT | TRT*Location |
| *Saccharomyces cerevisiae*[1] | | | | <0.001 | <0.001 | <0.001 |
| Proximal-Jejunum | 4.97 | 5.75 | 0.16 | | <0.001 | |
| Ileum | 5.03 | 5.93 | 0.23 | | <0.001 | |
| Colon | 3.58 | 5.85 | 0.26 | | <0.001 | |

[1]Copy number of 26S rRNA *Saccharomyces cerevisiae* gene per gram of digesta.
[2]CON = nonsupplemented calves; SCB = calves supplemented with *Saccharomyces cerevisiae boulardii* CNCM I-1079 at $10 \times 10^9$ cfu/d from birth until 1 week of age. Values are least square means.
[3]TRT = Treatment.

compared with CON groups (0.75 vs. 0.60), suggesting that these keystone species were not only different between treatment groups, but were also more interconnected within their ecological networks.

## 3.4. Secretory IgA concentrations, and short-chain fatty acid profile

The concentrations of sIgA was measured in the digesta of distal jejunum, Ileum, Colon, and on feces. Supplementation of SCB significantly increased sIgA concentrations in the ileum, and colon digesta ($p < 0.001$; Table 2). Concentrations of total SCFAs, acetate, propionate, and caproic acid in proximal jejunum, ileum, and colon were not affected by treatment ($p \geq 0.25$; Figure 7; Supplementary Table S3), nor the acetate to propionate ratio ($p = 0.80$; Supplementary Table 3). However, the acetate concentration was higher in the colon ($p < 0.01$; Figure 7A; Supplementary Table S3) compared to the ileum and proximal jejunum. Furthermore, when doing pairwise comparison of treatment effects by gut location and adjusted by Tukey for multiple comparisons, the concentration of caproic acid was higher in the ileum for SCB compared to CON ($p < 0.03$; Figure 7C; Supplementary Table S3).

## 3.5. Correlation between physiological variables and intestinal digesta microbiota

The relative abundance of the discriminatory taxa obtained in LEfSe in addition to the *Saccharomyces cerevisiae* gene copy numbers

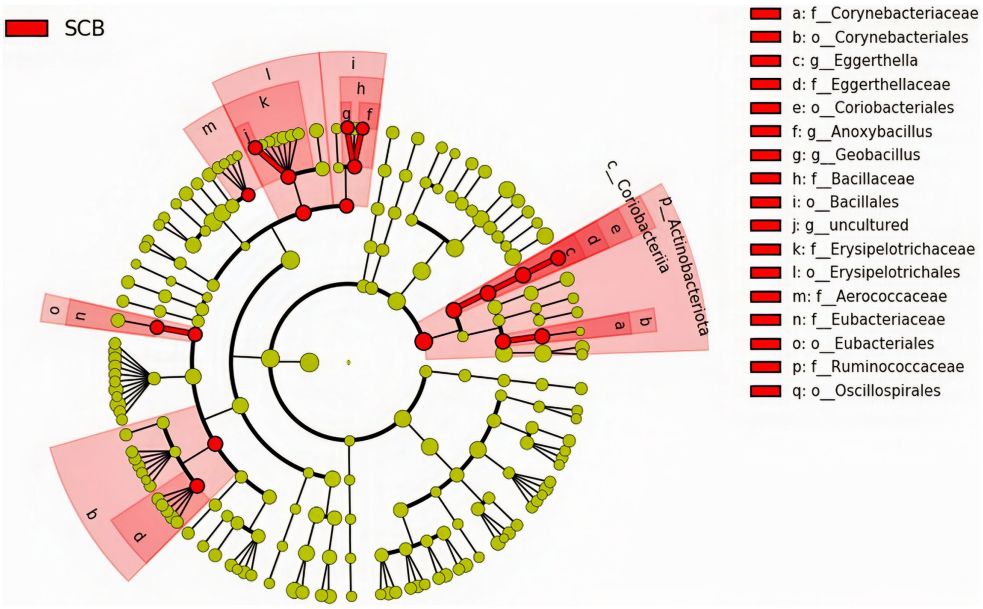


FIGURE 3
The linear discriminant analysis effect size (LEfSe) circular cladogram generated from the ileum digesta of ileum bacterial communities from phylum to genus level identified as discriminating taxa for calves supplemented with *Saccharomyces cerevisiae boulardii* CNCM I-1079 at $10 \times 10^9$ cfu/d from birth to 1 week of age (SCB), and non-supplemented calves (CON).

in ileum digesta was then correlated to sIgA, as well as short chain fatty acids profile, and alpha diversity indices in the same segment and sample type using spearman rank correlations (Figure 8). The gene copy numbers of SCB were positively correlated with the concentration of sIgA in ileum digesta ($p < 0.01$; Spearman' $\rho = 0.58$). In addition, the relative abundance of the family *Corynebacteriaceae* ($p < 0.01$; Spearman' $\rho = 0.73$), and *Eubacteriaceae* ($p < 0.01$; Spearman' $\rho = 0.65$) were positively correlated with the concentrations of sIgA in the ileum digesta. Additionally, the relative abundance of bacterial families *Ruminococcaceae* ($p < 0.01$; Spearman' $\rho = 0.62$), *Aerococcaceae* ($p < 0.01$; Spearman' $\rho = 0.59$), and *Corynebacteriaceae* ($p = 0.03$; Spearman' $\rho = 0.48$), and genus *Eggerthella* ($p = 0.01$; Spearman' $\rho = 0.55$) were positively correlated with species richness (Chao1 index). Total SCFAs, acetate, propionate, and their ratio were not correlated with SCB gene copy numbers nor with any of the differentially abundant taxa between groups (Figure 8).

## 4. Discussion

Our study revealed that supplementing calves with the strain of *Saccharomyces cerevisiae boulardii* CNCM I-1079 from birth to 1 week of age, increased species richness and phylogenetic diversity in addition to expediting the transition to a more interconnected bacterial community in the ileum, two key features of later stages of primary ecological succession (Connell and Slatyer, 1977). Because the major differences in alpha and beta diversity were observed in the ileum digesta, which also coincides with the intestinal region where the differences in sIgA production were observed in our companion paper (Villot et al., 2020), we decided to focus the rest of our exploratory analysis on this region of the intestine. We performed a LEfSe analysis to determine which discriminatory bacterial taxa were different between the treatments. This analysis revealed that there were several differentially abundant taxa between treatments and that SCB increased the relative abundance of members of the phylum *Actinobacteria*, family *Eubacteriaceae*, *Corynebacteriaceae*, *Eggerthellaceae*, *Bacillaceae*, and *Ruminococcaceae*. Members of the *Actinobacteria* phylum have been previously reported to increase in cecum and colon of piglets supplemented with SCB (Brousseau et al., 2015; Kiros et al., 2019). In addition, bacterial members of this group are major utilizers of lactose in the neonatal human gut and can modulate the pathogenicity of some bacterial groups (Bag et al., 2017). In addition, members of the *Eubacteriaceae* and *Ruminococcaceae* family are important producers of SCFAs and have been shown to be increased by supplementation with the live yeast *Saccharomyces cerevisiae* to neonatal piglets (Kiros et al., 2019), and with SCB to weaned piglets (Zhang et al., 2020). Furthermore, members of the *Ruminococcaceae* family can utilize yeast cell wall components, such as mannose, chitin, 1,3-β-glucan and 1,6-β-glucan (Czerucka et al., 2007), as fermentation substrates to produce SCFAs. As key bacterial metabolites produced by gut bacteria, SCFAs play a crucial role in linking microbiota composition and various biological effects at the mucosal level. In our study, although we observed changes in the relative abundance of major SCFAs producing bacteria, we did not observe variation in the production of SCFAs, other than a small increase in caproate. As expected, the highest concentrations of SCFA were observed in the colon, where most of the fermentation occurs in the intestine. However, the diet of the calves was composed strictly of milk replacer, that offers virtually no fiber to stimulate production of SCFAs in the lower gut, and even though SCFA producing bacteria were present in the intestine and lower gut, there was very minimal substrate available to ferment, as reflected in the low concentrations of SCFAs found in this study. It has been previously reported that in the rumen of neonatal calves of less than 1 week of age, functionally

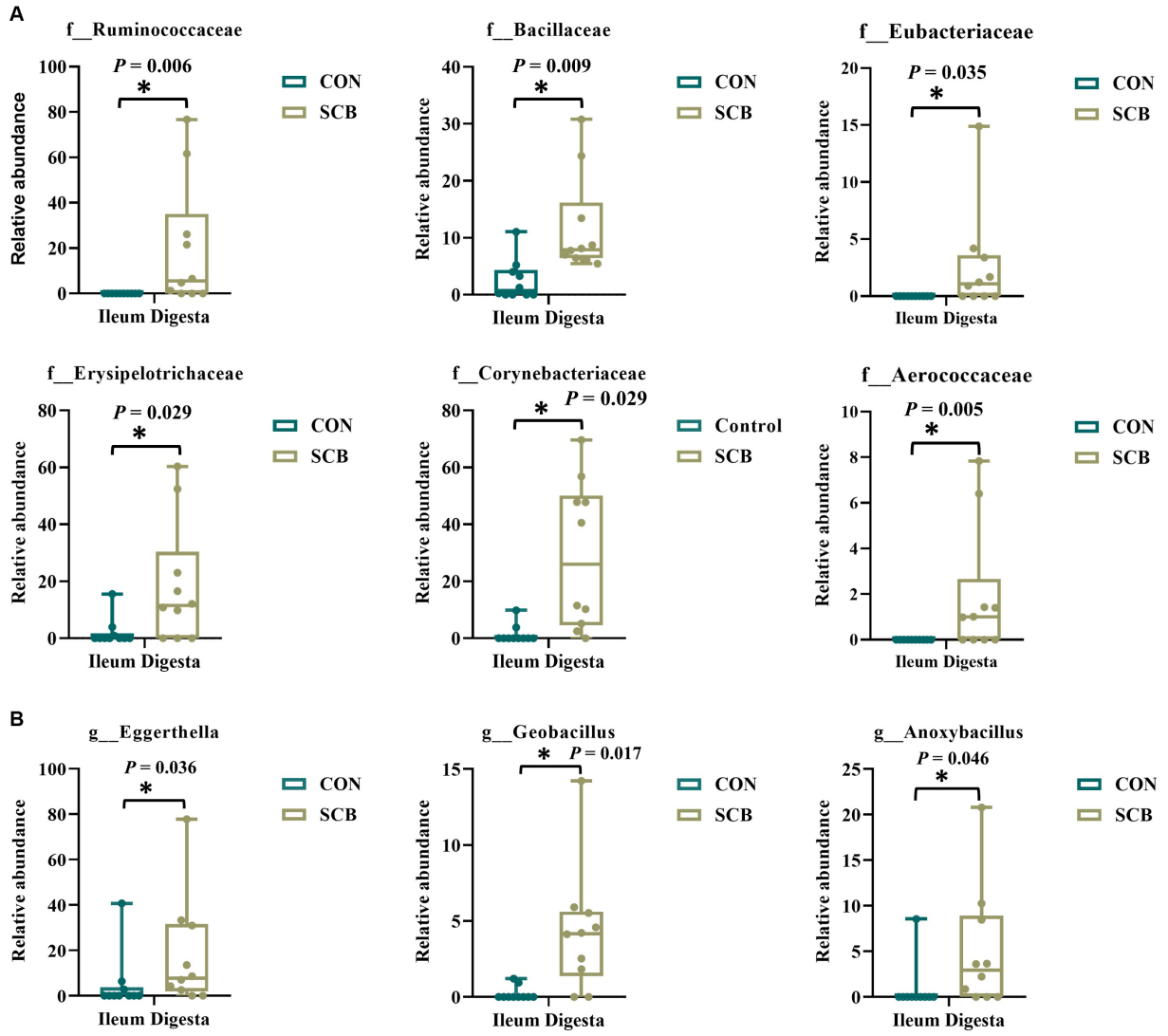


FIGURE 4
Relative abundance of discriminating taxa based on the LEfSe analysis at the family level **(A)**, and genus level **(B)** extracted from the ASV feature table compiled using the SILVA database from calves supplemented with *Saccharomyces cerevisiae boulardii* CNCM I-1079 at $10 \times 10^9$ cfu/d from birth to 1 week of age (SCB), and non-supplemented calves (CON). Relative abundance is presented as box and dot plots. The lines, boxes, and whiskers in the box plots represent the median, and 25th, and 75th percentiles, and the min-to-max distribution of replicate values, respectively. Data were analyzed using the Mann–Whitney *U* test. * Indicates a significant difference between CON and SCB groups ($p < 0.05$).

and metabolically active microbiota with the ability to ferment carbohydrates even before exposure to solid diet are present and are suggested to be vertically transferred by the dam (Malmuthuge et al., 2015). The small and large intestine of a newborn could also harbor functionally active SCFA producing bacteria even before exposure to solid diet.

We then identified keystone bacterial species that were central to the structure of the microbial community in the ileum digesta by using a co-occurrence network analysis, in addition to a multivariate associative test. In community ecology, keystone species are a group of species that play a crucial role in community organization, function, and diversity, and its loss results in the deterioration of the ecosystem function (Paine, 1966; Amit and Bashan, 2023). In our study, we observed that the keystone species were different between treatment groups and that SCB increased the number of different taxa that are central to the microbiota network. In addition, the level of interconnectivity within the network was higher in the SCB group compared to CON, suggesting a more stable microbial network, an important ecological feature that provides resistance to invasion by opportunistic pathogenic bacteria (Masi and Stewart, 2019; Samara et al., 2022). Furthermore, SCB keystone species were composed of members from the *Lactobacillus*, *Streptococcus*, *Lactococcus*, *Eubacterium*, *Lachnoclostridium*, *Actinomyces*, and *Pelomonas*, whereas non-supplemented calves' keystone species were only composed of members of the *Veillonella*, *Lactobacillus* and *Shigella* genus. Members of the *Shigella* genus contains pathogenic-facultative anaerobe bacteria that can promote intestinal tissue damage, and gastrointestinal disorders, and its relative abundance has been shown to be higher in diarrheic calves (Villot et al., 2019). Supplementation of SCB has been shown to reduce the pathogenicity of *Shigella*

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FIGURE 5
Co-occurrence network analysis using SPIEC-EASI used to infer the influence of *Saccharomyces cerevisiae boulardii* CNCM I-1079 supplementation on the microbial ecological networks in ileum digesta, at the phylum, and genus level. CON = non-supplemented calves at phylum level **(A)**, and at genus level **(C)**; SCB = calves supplemented with *Saccharomyces cerevisiae boulardii* CNCM I-1079 at $10 \times 10^9$ cfu/d from birth to 1 week of age at phylum level **(B)**, and at genus level **(D)**.

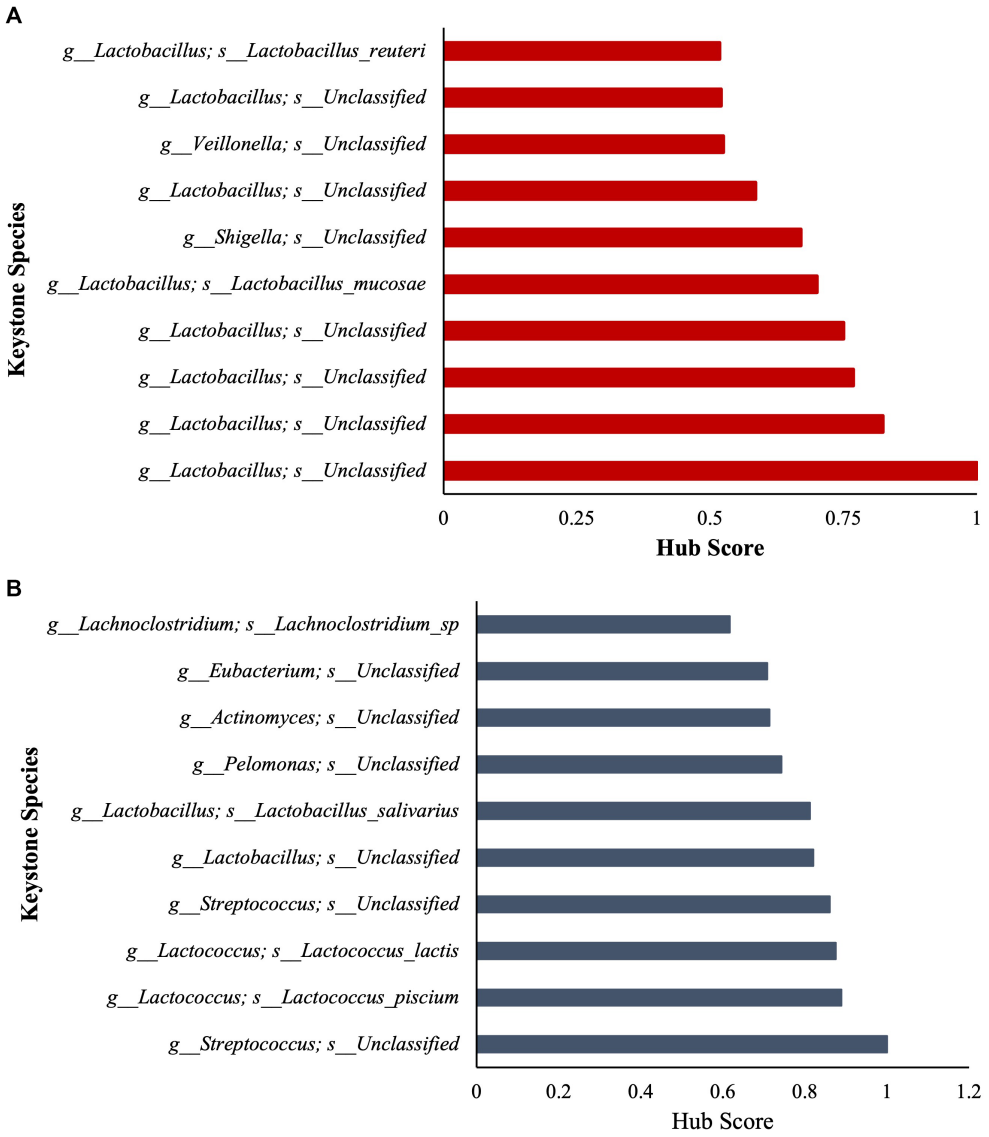


FIGURE 6
Stacked bar charts show the relative contribution of the 10 most important keystone species based on Hub score in ileum digesta within the microbiota of each treatment group (Higher hub score denotes an increased level of interconnectivity within the network). CON = non-supplemented calves **(A)**; SCB = calves supplemented with *Saccharomyces cerevisiae boulardii* CNCM I-1079 at $10 \times 10^9$ cfu/d **(B)** from birth to 1 week of age.

TABLE 2 Secretory IgA (sIgA) concentration in the different segments of the gut of 1-wk-old calves ($n = 20$) in response to supplementation of *Saccharomyces cerevisiae boulardii* CNCM I-1079.

| | Treatments[1] | | | *p*-value[2] | | |
|---|---|---|---|---|---|---|
| | CON | SCB | SEM | Location | TRT | TRT*Location |
| IgA, mg/g DM | | | | <0.001 | <0.001 | <0.001 |
| Distal-Jejunum | 0.2 | 0.22 | 0.12 | | | 0.938 |
| Ileum | 1.18 | 1.98 | 0.12 | | | <0.001 |
| Colon | 0.59 | 1.45 | 0.12 | | | <0.001 |
| Feces | 0.64 | 0.71 | 0.14 | | | 0.707 |

[1]CON = nonsupplemented calves; SCB = calves supplemented with *Saccharomyces cerevisiae boulardii* CNCM I-1079 at $10 \times 10^9$ cfu/d from birth until 1 week of age. Values are least square means.
[2]TRT = Treatment.

infection, but this protective effect seems to be mediated by a reduction in intestinal lesions and bacterial infiltration rather than by a direct reduction in this bacterial level in the intestinal population (Mumy et al., 2008). In this study, SCB appears to have modulated the establishment of keystone species in the neonatal calf's gut and increase the ecosystem resistance against *Shigella* infection. It has

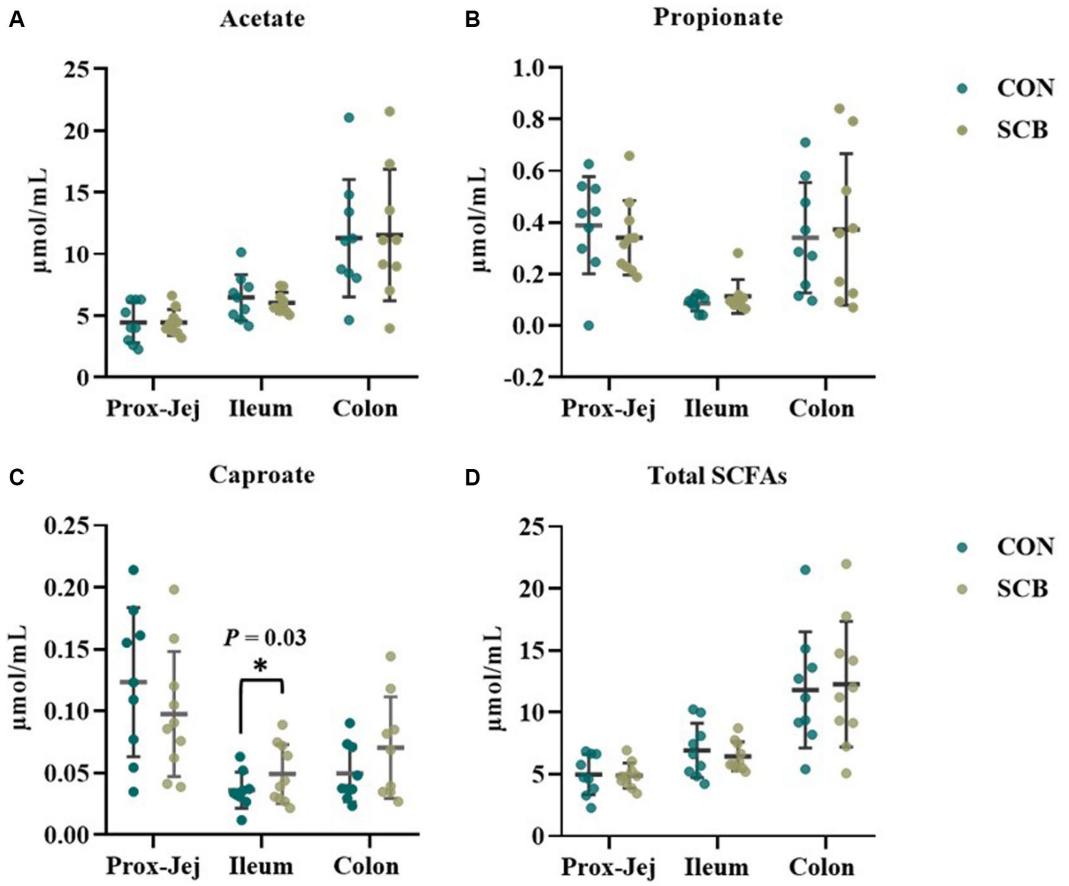


FIGURE 7
Short chain fatty acid (SCFA) profile in digesta from proximal jejunum (Prox-Jej), ileum, and colon in response to the supplementation of *Saccharomyces cerevisiae boulardii* CNCM I-1079 at $10 \times 10^9$ cfu/d (SCB) from birth until 1 week of age or not supplemented calves (CON). Values are means ± SD. * Indicates a significant difference between CON and SCB groups ($p < 0.05$).

been previously reported that *Saccharomyces cerevisiae* reduces oxygen levels and redox potential in the rumen of lambs (Chaucheyras-Durand and Fonty, 2002) and adult ruminants (Marden et al., 2008), thereby improving the conditions for strict anaerobes to thrive. In this study SCB could have reduced oxygen levels in the intestine and promote the establishment of strict anaerobic bacteria faster than in the non-supplemented group, preventing facultative anaerobic pathogens to grow. These results provide an explanation as to why SCB supplementation seems to function best during scenarios of high disease pressure (Villot et al., 2019) by creating a more stable bacterial community, therefore, preventing invasion with opportunistic pathogenic bacteria.

We observed that the probiotic-driven increased diversity of the intestinal bacterial microbiota was highly correlated with the enhanced secretory IgA capacity of the ileum. Furthermore, the relative abundance of the family *Corynebacteriaceae* and *Eubacteriaceae*, were among the discriminatory groups between treatments and an increase in relative abundance of these taxa in the SCB group was positively correlated with the concentration of sIgA in the ileum. This data suggests that the greater microbial diversity and the ecological attributes of a more diverse and interconnected microbiota promoted by SCB might have provided the necessary signals for stimulating the increased production of sIgA in the ileum. In ruminants, the ileum possesses continuous Peyer's patches that serve as an immune induction site for the intestinal mucosa (Snoeck et al., 2006; Fries et al., 2011; Villot et al., 2020). Activation and class switching of B cells occur in organized lymphoid structures, such as the Peyer patches, and its development and proper function depends on signals provided by the intestinal microbiota (Stavnezer et al., 2008; Vergani et al., 2022). Secretory IgA binds luminal bacteria and promotes its clearance from the gut, preventing direct contact of bacteria with the epithelial cell surface, to protect the gut mucosal surface from unnecessary immune activation and inflammation, in a process known as immune exclusion (Corthésy et al., 2006; Pabst et al., 2016). In addition, sIgA enhances transport of antigens and microorganisms from the lumen across the mucosal barrier through M cells into peyer's patches and mesenteric lymph nodes, which in turn, improves antigen presentation to T cells, thus promoting immune induction to specific non-invasive microorganisms (Mantis et al., 2002). Therefore, sIgA helps to modulate the composition of the intestinal microbiota, as sIgA deficient mice pups develop a different intestinal microbiota from that of normal non-deficient pups, and that difference persists into adulthood (Fransen et al., 2015).

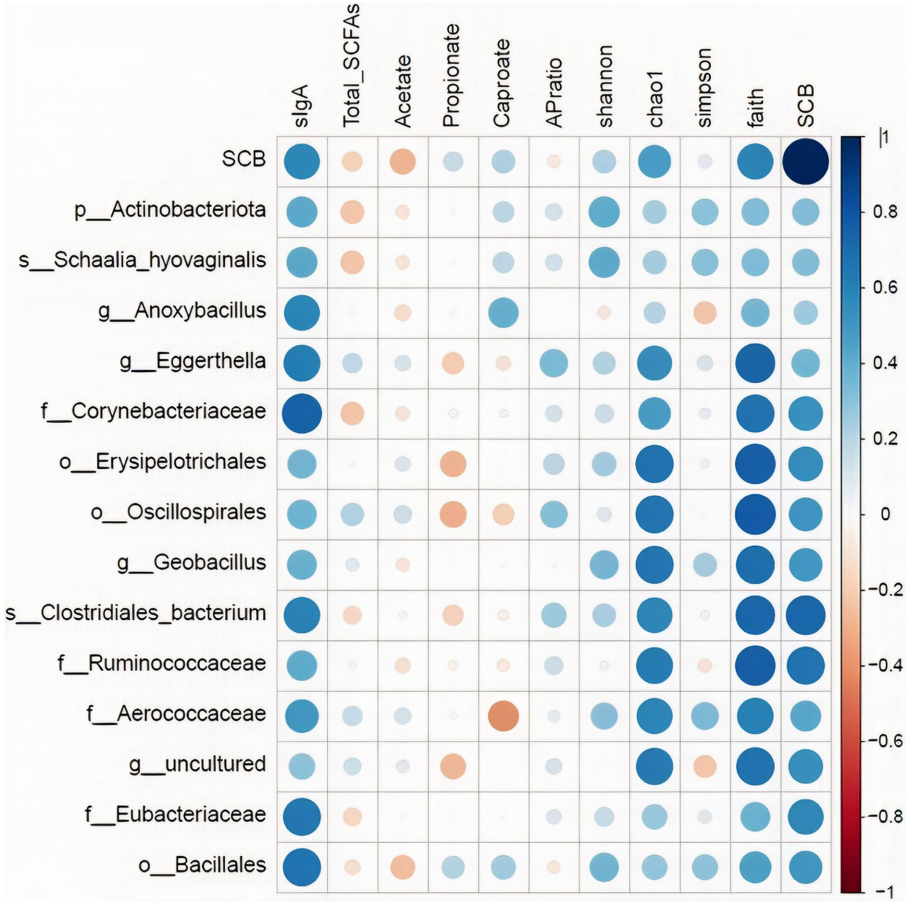


FIGURE 8
Spearman rank correlations of the relative abundance of the discriminatory taxa obtained in LEfSe in addition to the *Saccharomyces cerevisiae* gene copy numbers (SCB) in ileum digesta with: secretory IgA concentrations in ileum (sIgA-ileum mg/g DM); concentration of total short chain fatty acids (SCFAs), acetate (μmol/mL), propionate (μmol/mL), and caproate (μmol/mL) measured in ileum; and alpha diversity measures: Chao1, Shannon, Faith phylogenetic diversity (PD). Data is graphically presented in a dot plot heatmap. Strong correlations indicated by large circles and weaker correlations indicated by smaller circles. The scale colors denote whether the correlation is positive (closer to 1, blue circles) or negative (closer to –1, red circles).

Supplementation of SCB to neonates has been consistently shown to promote sIgA production in the intestine increasing the host's resistance to enteropathogenic bacterial infections (Rodrigues et al., 2000; Qamar et al., 2001), resulting in a reduction in the severity of diarrhea in calves (Villot et al., 2019). We speculate that the increase in microbial diversity stimulated by SCB supplementation in the ileum could have provided the necessary signals to stimulate sIgA production and secretion, and that the increase in sIgA could have partially mediated the changes in the composition of the ileal microbiota of the supplemented calves.

This study provides further evidence that supplementing newborn calves with SCB can promote the establishment of a healthy intestinal microbiota. Supplementation of SCB to newborn calves from birth until 1 week of life increased both the species richness and phylogenetic diversity of the infant bacterial microbiota in the ileum, resulting in a more mature and stable bacterial microbiota when compared to the non supplemented group. In addition, SCB modulated the establishment of keystone species in the neonatal calf's gut, possibly increasing resistance against opportunistic pathogenic bacteria, like *Escherichia Shigella*. The positive correlation between the increase in microbiota diversity and the increase in sIgA of the ileum mucosa provides further evidence that the calf's IgA secretory system relies on the development of a stable and diverse microbial community to provide the necessary signals to enhance IgA production and secretion. Future studies should focus on the long-term effects on the health and performance of these early life interventions.

## Data availability statement

The data presented in the study are deposited in the NCBI SRA repository, accession number PRJNA1013102.

## Ethics statement

The animal study was approved by Canadian Council of Animal Care. The study was conducted in accordance with the local legislation and institutional requirements.

## Author contributions

CV and MS conceived and designed the experiment. LR analyzed the data and wrote the manuscript. NM, LG, RG, and RA-H assisted with data analysis, data interpretation, and manuscript review. All authors contributed to the article and approved the submitted version.

## 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/fmicb.2023.1129250/full#supplementary-material

## References

Abe, F., Ishibashi, N., and Shimamura, S. (1995). Effect of administration of bifidobacteria and lactic acid bacteria to newborn calves and piglets. *J. Dairy Sci.* 78, 2838–2846. doi: 10.3168/jds. S0022-0302(95)76914-4

Aghakeshmiri, F., Azizzadeh, M., Farzaneh, N., and Gorjidooz, M. (2017). Effects of neonatal diarrhea and other conditions on subsequent productive and reproductive performance of heifer calves. *Vet. Res. Commun.* 41, 107–112. doi: 10.1007/s11259-017-9678-9

Amit, G., and Bashan, A. (2023). Top-down identification of keystone taxa in the microbiome. *Nat. Commun.* 14:3951. doi: 10.1038/s41467-023-39459-5

Bag, S., Ghosh, T. S., and Das, B. (2017). Complete Genome Sequence of Collinsella aerofaciens Isolated from the Gut of a Healthy Indian Subject. *Genome Announc*. doi: 10.1128/genomeA.01361-17

Barc, M. C., Charrin-Sarnel, C., Rochet, V., Bourlioux, F., Sandré, C., Boureau, H., et al. (2008). Molecular analysis of the digestive microbiota in a gnotobiotic mouse model during antibiotic treatment: influence of *saccharomyces boulardii*. *Anaerobe* 14, 229–233. doi: 10.1016/j.anaerobe.2008.04.003

Belkaid, Y., and Hand, T. W. (2014). Role of the microbiota in immunity and inflammation. *Cell* 157, 121–141. doi: 10.1016/j.cell.2014.03.011

Bolyen, E., Rideout, J. R., Dillon, M. R., Bokulich, N. A., Abnet, C. C., al-Ghalith, G. A., et al. (2019). Reproducible, interactive, scalable and extensible microbiome data science using QIIME 2. *Nat. Biotechnol.* 37, 852–857. doi: 10.1038/s41587-019-0209-9

Brousseau, J.-P., Talbot, G., Beaudoin, F., Lauzon, K., Roy, D., and Lessard, M. (2015). Effects of probiotics *Pediococcus acidilactici* strain MA18/5M and *Saccharomyces cerevisiae* ssp. *boulardii* strain SB- CNCM I-1079 on fecal and intestinal microbiota of nursing and weanling piglets. *J. Anim. Sci.* 93, 5313–5326. doi: 10.2527/jas.2015-9190

Buss, L. N., Yohe, T. T., Cangiano, L. R., Renaud, D. L., Keunen, A. J., Guan, L. L., et al. (2021). The effect of neomycin inclusion in milk replacer on the health, growth, and performance of male Holstein calves during preweaning. *J. Dairy Sci.* 104, 8188–8201. doi: 10.3168/jds.2020-19827

Cangiano, L. R., Yohe, T. T., Steele, M. A., and Renaud, D. L. (2020). Invited review: strategic use of microbial-based probiotics and prebiotics in dairy calf rearing. *Appl. Anim. Sci.* 36, 630–651. doi: 10.15232/aas.2020-02049

CCAC. (1993). *Guide to the Care and Use of Experimental Animals. Vol. 1*. Eds. E. D. Olfert, B. M. Cross and A. A. McWilliams (Canadian Council on Animal Care).

Chang, H.-W., Nam, Y.-D., Sung, Y., Kim, K.-H., Roh, S. W., Yoon, J.-H., et al. (2007). Quantitative real time PCR assays for the enumeration of *Saccharomyces cerevisiae* and the *Saccharomyces sensu stricto* complex in human feces. *J. Microbiol. Methods* 71, 191–201. doi: 10.1016/j.mimet.2007.08.013

Chaucheyras-Durand, F., and Fonty, G. (2002). Influence of a probiotic yeast (*Saccharomyces cerevisiae* CNCM I-1077) on microbial colonization and fermentations in the rumen of Newborn lambs. *Microb. Ecol. Health Dis.* 14, 30–36. doi: 10.1080/089106002760002739

Connell, J. H., and Slatyer, R. O. (1977). Mechanisms of succession in natural communities and their role in community stability and organization. *Am. Nat.* 111, 1119–1144. doi: 10.1086/283241

Corthésy, B., Benureau, Y., Perrier, C., Fourgeux, C., Parez, N., Greenberg, H., et al. (2006). Rotavirus anti-VP6 secretory immunoglobulin a contributes to protection via intracellular neutralization but not via immune exclusion. *J. Virol.* 80, 10692–10699. doi: 10.1128/JVI.00927-06

Czerucka, D., Piche, T., and Rampal, P. (2007). Review article: yeast as probiotics -- *saccharomyces boulardii*. *Aliment. Pharmacol. Ther.* 26, 767–778. doi: 10.1111/j.1365-2036.2007.03442.x.

Fransen, F., Zagato, E., Mazzini, E., Fosso, B., Manzari, C., El Aidy, S., et al. (2015). BALB/c and C57BL/6 mice differ in Polyreactive IgA abundance, which impacts the generation of antigen-specific IgA and microbiota diversity. *Immunity* 43, 527–540. doi: 10.1016/j.immuni.2015.08.011

Fries, P. N., Popowych, Y. I., Guan, L. L., and Griebel, P. J. (2011). Age-related changes in the distribution and frequency of myeloid and T cell populations in the small intestine of calves. *Cell. Immunol.* 271, 428–437. doi: 10.1016/j.cellimm.2011.08.012

Hooper, L. V., Littman, D. R., and Macpherson, A. J. (2012). Interactions between the microbiota and the immune system. *Science* 336, 1268–1273. doi: 10.1126/science.1223490

Kiros, T. G., Luise, D., Derakhshani, H., Petri, R., Trevisi, P., D'Inca, R., et al. (2019). Effect of live yeast *Saccharomyces cerevisiae* supplementation on the performance and cecum microbial profile of suckling piglets. *PLoS One* 14:e0219557. doi: 10.1371/journal.pone.0219557

Kroes, I., Lepp, P. W., and Relman, D. A. (1999). Bacterial diversity within the human subgingival crevice. *Proc. Natl. Acad. Sci. U. S. A.* 96, 14547–14552. doi: 10.1073/pnas.96.25.14547

Kurtz, Z. D., Müller, C. L., Miraldi, E. R., Littman, D. R., Blaser, M. J., and Bonneau, R. A. (2015). Sparse and compositionally robust inference of microbial ecological networks. *PLoS Comput. Biol.* 11:e1004226. doi: 10.1371/journal.pcbi.1004226

Laforest-Lapointe, I., and Arrieta, M. C. (2017). Patterns of early-life gut microbial colonization during human immune development: an ecological perspective. *Front. Immunol.* 8:788. doi: 10.3389/fimmu.2017.00788

Malmuthuge, N., Griebel, P. J., and Guan, L. L. (2015). The gut microbiome and its potential role in the development and function of the newborn calf gastrointestinal tract. *Front. Vet. Sci.* 2:36. doi: 10.3389/fvets.2015.00036

Malmuthuge, N., and Guan, L. L. (2017). Understanding host-microbial interactions in rumen: searching the best opportunity for microbiota manipulation. *J. Anim. Sci. Biotechnol.* 8, 8–7. doi: 10.1186/s40104-016-0135-3

Mantis, N. J., Cheung, M. C., Chintalacharuvu, K. R., Rey, J., Corthésy, B., and Neutra, M. R. (2002). Selective adherence of IgA to murine Peyer's patch M cells: evidence for a novel IgA receptor. *J. Immunol.* 169:1844. doi: 10.4049/jimmunol.169.4.1844

Mantis, N. J., Rol, N., and Corthesy, B. (2011). Secretory IgA's complex roles in immunity and mucosal homeostasis in the gut. *Mucosal Immunol.* 4, 603–611. doi: 10.1038/mi.2011.41

Marden, J. P., Julien, C., Monteils, V., Auclair, E., Moncoulon, R., and Bayourthe, C. (2008). How does live yeast differ from sodium bicarbonate to stabilize ruminal pH in high-yielding dairy cows? *Journal of Dairy Science* 91, 3528–3535. doi: 10.3168/jds.2007-0889

Marquez, C. J. (2014). *Calf intestinal health: assessment and dietary interventions for its improvement*. University of Illinois: Champaign, IL.

Martins, F. S., Silva, A. A., Vieira, A. T., Barbosa, F. H., Arantes, R. M., Teixeira, M. M., et al. (2009). Comparative study of *Bifidobacterium animalis*, *Escherichia coli*, *lactobacillus casei* and *saccharomyces boulardii* probiotic properties. *Arch. Microbiol.* 191, 623–630. doi: 10.1007/s00203-009-0491-x

Masi, A. C., and Stewart, C. J. (2019). The role of the preterm intestinal microbiome in sepsis and necrotising enterocolitis. *Early Hum. Dev.* 138:104854. doi: 10.1016/j.earlhumdev.2019.104854

Moré, M. I., and Swidsinski, A. (2015). *Saccharomyces boulardii* CNCM I-745 supports regeneration of the intestinal microbiota after diarrheic dysbiosis - a review. *Clin. Exp. Gastroenterol.* 8, 237–255. doi: 10.2147/CEG.S85574

Mumy, K. L., Chen, X., Kelly, C. P., and McCormick, B. A. (2008). *Saccharomyces boulardii* interferes with *Shigella* pathogenesis by post-invasion signaling events. *Am. J. Physiol. Gastrointest. Liver Physiol.* 294, G599–G609. doi: 10.1152/ajpgi.00391.2007

Pabst, O., Cerovic, V., and Hornef, M. (2016). Secretory IgA in the coordination of establishment and maintenance of the microbiota. *Trends Immunol.* 37, 287–296. doi: 10.1016/j.it.2016.03.002

Paine, R. T. (1966). Food web complexity and species diversity. *Am. Nat.* 100, 65–75. doi: 10.1086/282400

Pais, P., Almeida, V., Yılmaz, M., and Teixeira, M. C. (2020). *Saccharomyces boulardii*: what makes it tick as successful probiotic? *J. Fungi* 6:78. doi: 10.3390/jof6020078

Qamar, A., Aboudola, S., Warny, M., Michetti, P., Pothoulakis, C., LaMont, J. T., et al. (2001). 2001. *Saccharomyces boulardii* stimulates intestinal immunoglobulin a immune response to *Clostridium difficile* toxin a in mice. *Infect. Immun.* 69, 2762–2765. doi: 10.1128/IAI.69.4.2762-2765.2001

Rajput, I. R., Li, L. Y., Xin, X., Wu, B. B., Juan, Z. L., Cui, Z. W., et al. (2013). Effect of *saccharomyces boulardii* and B10 on intestinal ultrastructure modulation and mucosal immunity development mechanism in broiler chickens. *Poult. Sci.* 92, 956–965. doi: 10.3382/ps.2012-02845

Renaud, D. L., Kelton, D. F., Weese, J. S., Noble, C., and Duffield, T. F. (2019). Evaluation of a multispecies probiotic as a supportive treatment for diarrhea in dairy calves: a randomized clinical trial. *J. Dairy Sci.* 102, 4498–4505. doi: 10.3168/jds.2018-15793

Rodrigues, A. C. P., Cara, D., Fretez, S., Cunha, F., Vieira, E., Nicoli, J., et al. (2000). *Saccharomyces boulardii* stimulates sIgA production and the phagocytic system of gnotobiotic mice. *J. Appl. Microbiol.* 89, 404–414. doi: 10.1046/j.1365-2672.2000.01128. x

Samara, J., Moossavi, S., Alshaikh, B., Ortega, V. A., Pettersen, V. K., Ferdous, T., et al. (2022). Supplementation with a probiotic mixture accelerates gut microbiome maturation and reduces intestinal inflammation in extremely preterm infants. *Cell Host Microbe* 30, 696–711.e5. doi: 10.1016/j.chom.2022.04.005

Schlau, N., Guan, L. L., and Oba, M. (2012). The relationship between rumen acidosis resistance and expression of genes involved in regulation of intracellular pH and butyrate metabolism of ruminal epithelial cells in steers. *J. Dairy Sci.* 95, 5866–5875. doi: 10.3168/jds.2011-5167

Segata, N., Izard, J., Waldron, L., Gevers, D., Miropolsky, L., Garrett, W. S., et al (2011). Metagenomic biomarker discovery and explanation. *Genome Biol.* doi: 10.1186/gb-2011-12-6-r60

Snoeck, V., Peters, I. R., and Cox, E. (2006). The IgA system: a comparison of structure and function in different species. *Vet. Res.* 37, 455–467. doi: 10.1051/vetres:2006010

Stavnezer, J., Guikema, J. E. J., and Schrader, C. E. (2008). Mechanism and Regulation of Class Switch Recombination. *Annual Review of Immunology* 26, 261–292. doi: 10.1146/annurev.immunol.26.021607.090248

Thomas, S., Przesdzing, I., Metzke, D., Schmitz, J., Radbruch, A., and Baumgart, D. C. (2009). *Saccharomyces boulardii* inhibits lipopolysaccharide-induced activation of human dendritic cells and T cell proliferation. *Clin. Exp. Immunol.* 156, 78–87. doi: 10.1111/j.1365-2249.2009.03878. x

Urie, N. J., Lombard, J. E., Shivley, C. B., Kopral, C. A., Adams, A. E., Earleywine, T. J., et al. (2018). Preweaned heifer management on US dairy operations: part V. factors associated with morbidity and mortality in preweaned dairy heifer calves. *J. Dairy Sci.* 101, 9229–9244. doi: 10.3168/jds.2017-14019

Vergani, S., Muleta, K. G., da Silva, C., Doyle, A., Kristiansen, T. A., Sodini, S.et al. (2022). A self-sustaining layer of early-life-origin B cells drives steady-state IgA responses in the adult gut. *Immunity* 55, 1829–1842.e6. doi: 10.1016/j.immuni.2022.08.018

Villot, C., Chen, Y., Pedgerachny, K., Chaucheyras-Durand, F., Chevaux, E., Skidmore, A., et al. (2020). Early supplementation of *Saccharomyces cerevisiae boulardii* CNCM I-1079 in newborn dairy calves increases IgA production in the intestine at 1 week of age. *J. Dairy Sci.* 103, 8615–8628. doi: 10.3168/jds.2020-18274

Villot, C., Ma, T., Renaud, D. L., Ghaffari, M. H., Gibson, D. J., Skidmore, A., et al. (2019). *Saccharomyces cerevisiae boulardii* CNCM I-1079 affects health, growth, and fecal microbiota in milk-fed veal calves. *J. Dairy Sci.* 102, 7011–7025. doi: 10.3168/jds.2018-16149

Winder, C. B., Bauman, C. A., Duffield, T. F., Barkema, H. W., Keefe, G. P., Dubuc, J., et al. (2018). Canadian National Dairy Study: heifer calf management. *J. Dairy Sci.* 101, 10565–10579. doi: 10.3168/jds.2018-14680

Windeyer, M. C., Leslie, K. E., Godden, S. M., Hodgins, D. C., Lissemore, K. D., and LeBlanc, S. J. (2014). Factors associated with morbidity, mortality, and growth of dairy heifer calves up to 3 months of age. *Prev. Vet. Med.* 113, 231–240. doi: 10.1016/j.prevetmed.2013.10.019

Wopereis, H., Oozeer, R., Knipping, K., Belzer, C., and Knol, J. (2014). The first thousand days - intestinal microbiology of early life: establishing a symbiosis. *Pediatr. Allergy Immunol.* 25, 428–438. doi: 10.1111/pai.12232

Yang, L., Jin, R., Lu, D., and Ge, Q. (2020). T cell tolerance in early life. *Front. Immunol.* 11:576261. doi: 10.3389/fimmu.2020.576261

Yu, Z., and Morrison, M. (2004). Improved extraction of PCR-quality community DNA from digesta and fecal samples. *BioTechniques* 36, 808–812. doi: 10.2144/04365ST04

Zhang, W., Bao, C., Wang, J., Zang, J., and Cao, Y. (2020). Administration of *Saccharomyces boulardii* mafic-1701 improves feed conversion ratio, promotes antioxidant capacity, alleviates intestinal inflammation and modulates gut microbiota in weaned piglets. *J. Anim. Sci. Biotechnol.* 11:112. doi: 10.1186/s40104-020-00516-4

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# Combined network analysis and interpretable machine learning reveals the environmental adaptations of more than 10,000 ruminant microbial genomes

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**Background:** The ruminant gastrointestinal contains numerous microbiomes that serve a crucial role in sustaining the host's productivity and health. In recent times, numerous studies have revealed that variations in influencing factors, including the environment, diet, and host, contribute to the shaping of gastrointestinal microbial adaptation to specific states. Therefore, understanding how host and environmental factors affect gastrointestinal microbes will help to improve the sustainability of ruminant production systems.

**Results:** Based on a graphical analysis perspective, this study elucidates the microbial topology and robustness of the gastrointestinal of different ruminant species, showing that the microbial network is more resistant to random attacks. The risk of transmission of high-risk metagenome-assembled genome (MAG) was also demonstrated based on a large-scale survey of the distribution of antibiotic resistance genes (ARG) in the microbiota of most types of ecosystems. In addition, an interpretable machine learning framework was developed to study the complex, high-dimensional data of the gastrointestinal microbial genome. The evolution of gastrointestinal microbial adaptations to the environment in ruminants were analyzed and the adaptability changes of microorganisms to different altitudes were identified, including microbial transcriptional repair.

**Conclusion:** Our findings indicate that the environment has an impact on the functional features of microbiomes in ruminant. The findings provide a new insight for the future development of microbial resources for the sustainable development in agriculture.

KEYWORDS

**ruminants, metagenomics, machine learning, network, metagenome-assembled genome**

# 1. Introduction

Ruminants, as ancient animals, exhibit a wide range of morphological and ecological diversity (Mennecart et al., 2021). They have adapted to diverse habitats, from tropical jungles (Díaz-Céspedes et al., 2021) to the plateau (Guo et al., 2020); range in size from 2 kg (Pickford, 2001) to 1.5 tons (Sauer et al., 2016); show great variations in diet, feeding on objects ranging from moss (Ihl and Barboza, 2007) to ordinary standard feed (Li et al., 2019); and have adapted to almost all ecosystems on Earth. Ruminants are distinguished by their plant digestion patterns and have evolved the rumen. As one of the most vital organs, the rumen allows partial microbial digestion of feed before it enters the true stomach (van Lingen et al., 2017). The rumen is a crucial factor underlying the domestication of ruminants. The productivity of ruminant livestock depends on their gastrointestinal microbiota, which can transform plant material that humans cannot digest into easily accessible animal products (Shabat et al., 2016). The gastrointestinal microbiota of ruminants is characterized by its diversity and dynamic nature, making it prone to alterations due to changes in diet (Malmuthuge and Guan, 2016), environmental factors (Cholewinska et al., 2021), and the presence of enteric pathogens (Cortés et al., 2020). These perturbations play an integral role in host nutritional intake, behavior, metabolism, immunological function, and development. Natural selection has allowed hosts and symbiotic microbes to evolve as integrated systems.

In both the ecological and social realms, ruminants are immensely valuable. Due to rising consumer demand for animal products resulting from population growth (Seshadri et al., 2018), ruminants play an increasingly vital role in ensuring agricultural security. They generate a significant amount of the meat and milk that are the primary sources of protein in the human diet (Stewart et al., 2019). Nonetheless, sustainable manufacturing confronts significant obstacles due to the depletion of natural resources and the resulting rise in production costs.

The ability of ruminants to utilize microorganisms is one of their key traits. Microorganisms bring significant benefits to ruminant animals. However, due to the diverse functionalities and species diversity of microorganisms, they exhibit intricate physiological and biochemical characteristics, making their in-depth analysis quite challenging (Ban and Guan, 2021). We have uncovered inconsistencies in predicting microbial community structures, primarily stemming from a limited grasp of the mechanisms governing microbial community assembly. In order to mitigate this unpredictability, it is imperative to comprehensively understand the microbiome as a cohesive entity.

With the ongoing increase in the depth of metagenomic sequencing, the range of sequencing is progressively expanding (Vestergaard et al., 2017), while the cost of the technology is decreasing. Thus, large amounts of data can be generated for analysis. Consequently, substantial volumes of data can be generated for analysis. However, despite genomics being inherently data-driven, the resultant datasets are becoming both exceedingly large and complex, thereby giving rise to technological challenges. Recent publication of the most recent collection of gut microbial genomes includes a ruminant whole gastrointestinal tract microbial gene set and the reconstruction of over 10,000 nonredundant ruminant gastrointestinal microbial genomes (Xie et al., 2021). This represents a significant change in the ability to understand the ruminant microbiome. This study used public database collections to characterize the microbiomes and functional groups and applied a metagenomics approach to achieve the following objectives: (1) building microbial cooccurrence networks for exploring linkages in microbial communities, (2) the influence of the network's special microstructure on the survivability of gastrointestinal networks in ruminant was explored, (3) detecting antibiotic resistance in different ruminants on a large scale, and (4) exploring the adaptation of ruminant microbes to their environment.

# 2. Results

## 2.1. Microbiological characteristics of the gastrointestinal microbiota of ruminants

The microbes serve as an often overlooked yet independent source of data for understanding host evolution and ecological shifts. Based on Spearman's rank correlation coefficient matrix of the relative abundance of 488 collected microorganisms, a microbial network was constructed using seven microbiomes representing distinct ruminant gastrointestinal environments (Figure 1). To eliminate noise and false positive predictions, a conservative statistical cutoff was adopted to reject points with Spearman coefficients less than 0.85 and $p$-values less than 0.01 The number of modules in this global network was distributed with a long tail, with an average of 32 modules per network, while 80% of the vertices were concentrated in only 8 modules.

The organization of ruminant gastrointestinal microbial networks varies greatly, and there is minimal relationship between the various species (Figure 2A). Dairy cattle had the highest mean clustering coefficients, which indicated substantial clustering and indicated that the network's nodes tended to form relationships over shorter distances. Sheep, dairy cattle and water buffaloes all had more edges than the others. The quantity of edges varied notably among networks, with dairy cattle networks exhibiting three times the number of edges as compared to goat networks. Additionally, we found that all networks had a modular structure (modularity > 0.3) and dense connections between nodes within modules.

Figure 2B demonstrates that the random attack is less effective than the purposeful approach, showing that the microbial network is more resistant to random attacks. During simulations of purposeful attacks, the network stability rapidly degrades and shows a clear declining trend, illustrating the superiority of the DG (max degree graph node) and PG (max pagerank graph node) importance ranking-based techniques. Sheep had the most resilient microbial network regardless of the technique of hitting, while water deer and yak networks had the most susceptible microbial networks. The efficiency of network attacks using the same method varied in a multimethod robustness assessment of individual networks, with PG attacks performing better in elk and less successfully in deer.

## 2.2. The K-shell decomposition in gastrointestinal microbiota of ruminants

The K-shell technique is utilized to examine the network's hierarchical structure. The procedure is run repeatedly until the most concentrated core is discovered (Figure 3A). Nodes dispersed at the

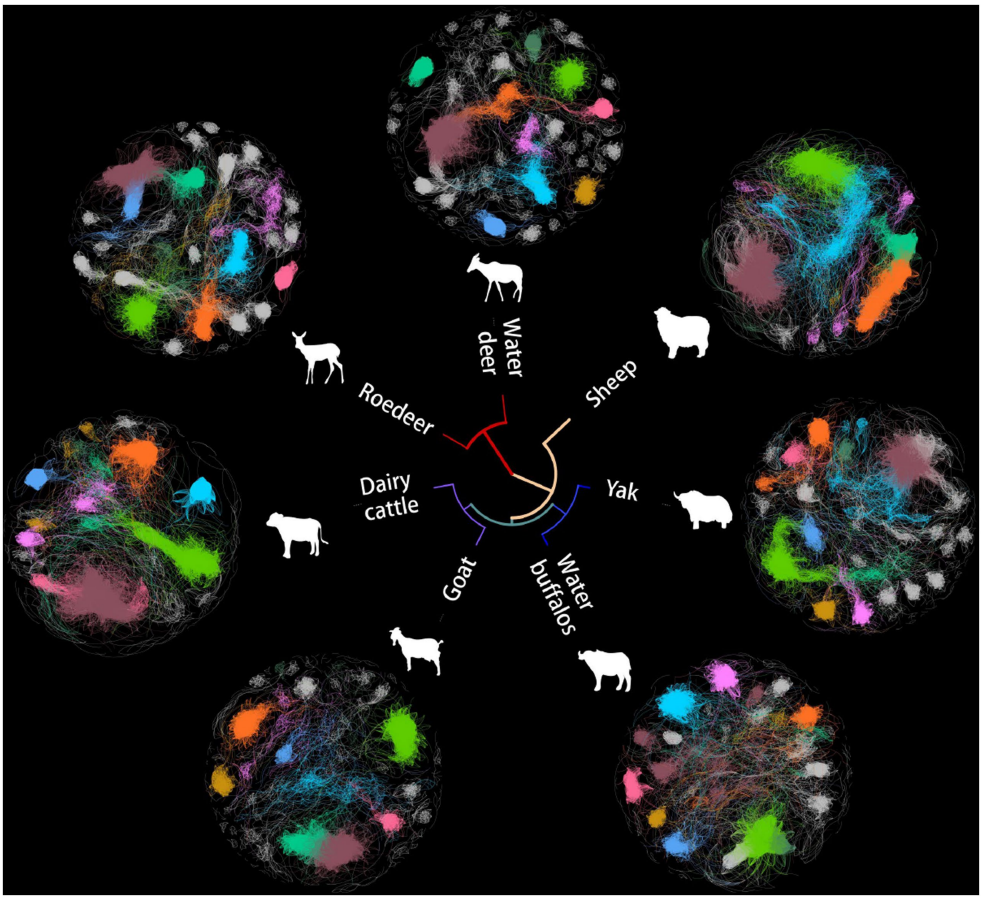


FIGURE 1
Networks of co-occurrence among seven ruminant microbes. Unrooted tree exhibiting a Bray-Curtis clustering tree of microbial genera. The first eight domain modules in the cooccurrence network are displayed in various colors in the outer circle, which displays various animal microbial cooccurrence networks.

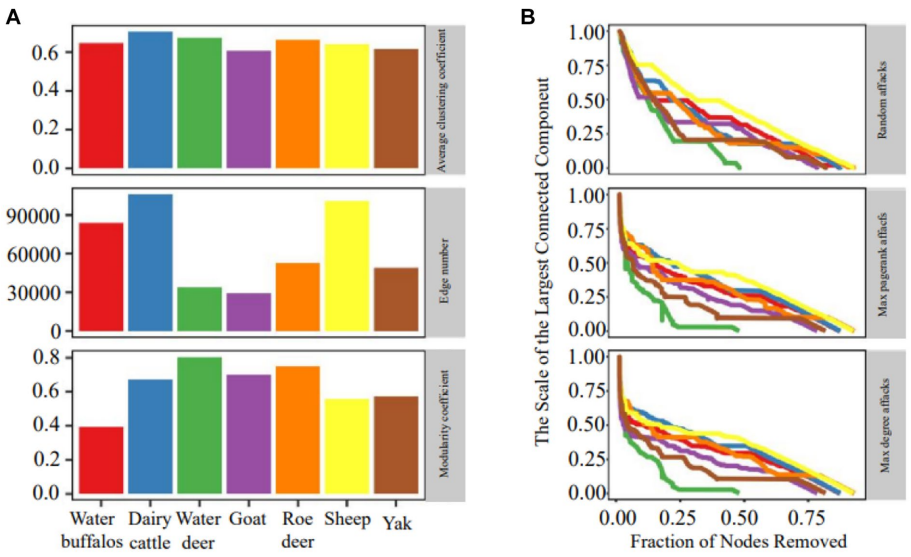


FIGURE 2
Network topology and robustness. **(A)** Microbial network topology inferred from a dataset of microbiome abundance of seven ruminant species. **(B)** The relationship between the proportion of removed nodes and the scale of the largest connected component.

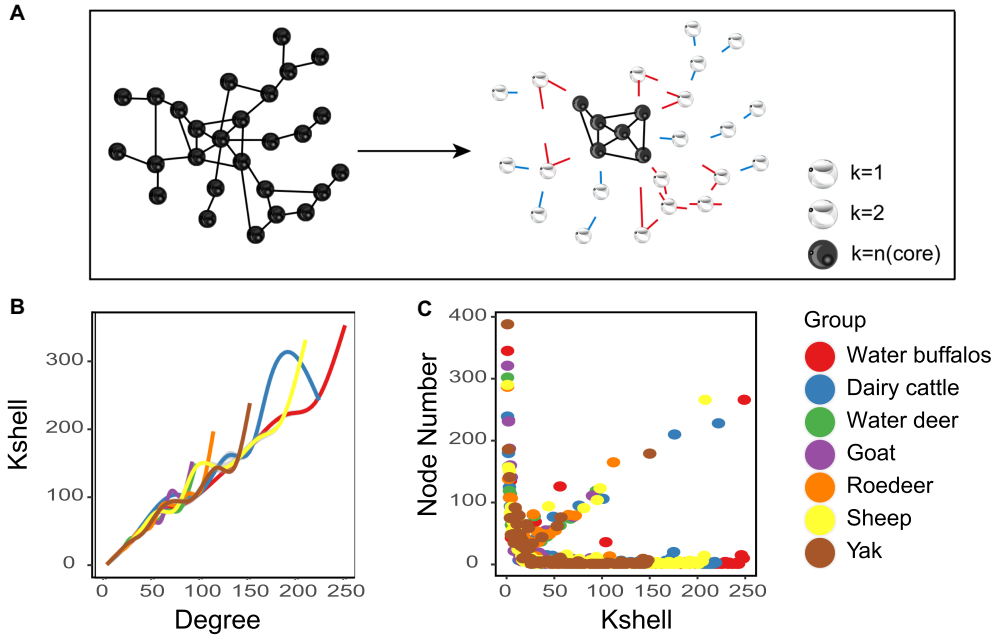


FIGURE 3
Network analyses of K-shell. **(A)** Schematic illustration of network layering using the k-shell decomposition method. **(B)** Distribution of the average degree of each shell. **(C)** each shell number.

network's periphery have a much lower average than nodes positioned close to its core (Figure 3B). The statistics suggest that inner layer nodes may be more efficient information emitters (Figure 3C). We also found that the overall structure of the microbial network topology is reminiscent of the internet network. While merely 0.5% of the nodes in the internet network constitute the nucleus, a mere 0.1% of the nodes comprise the core of the microbiological network.

## 3. Broad-spectrum profile of ARG abundance in ruminant gastrointestinal

Antibiotics stand as influential elements in microbial networks, exerting substantial impacts. Unveiling antibiotic resistance genes' presence holds the potential to influence the operational dynamics of microbial communities and the overall stability of ecosystems. We discovered a significant prevalence of antibiotic resistance genes (ARGs) in ruminant gastrointestinal microbes, with 6,268 (62%) of the 10,073 genomes tested in the microbial resistance gene analysis yielding positive results. Multiple ARGs demonstrated a high degree of variability within the ruminant resistance group (Figure 4). We also quantified the possible mechanisms of the identified ARGs. Multiple ARGs demonstrated a high degree of variability within the ruminant resistance group. We found that yaks had the fewest ARGs, with an average of 181 ARGs per sample. We hypothesize that this is because the Qinghai–Tibet Plateau is less contaminated as a result of human activities.

We further analyzed the detailed composition of ARGs in the gastrointestinal of seven ruminants. Among all the types of ARGs, uppP, tufab, tetW, tetT, tet37, rpsL, rpsJ, rpoC, rpoB, parE, nimJ, macB, and gyrAAPH (2″)-Ig, which had the highest prevalence, were found in the gastrointestinal tracts of all ruminants. We quantified the risk index of species containing risk genes using quantitative methods, and we characterized the risk level of MAG based on the risk score quartile. The risk level is divided down into 4 levels: 2611 (risk index 1), 1,491 (1 = risk index 10), 657 (10 = risk index 100), and 62 (risk index ≥100). The total number of level 1 microorganisms was 62, or 1.2%. We displayed the tick microbial symbiont risk indicator top 50 in. We discovered that among the top 10 MAGs of all hazards, there were members of Pseudomonas, Escherichia, and Acinetobacter.

Explanation of the machine learning-based analysis of the evolution of gastrointestinal microbial environmental suitability in ruminants.

Many creative strategies are supported by machine learning that can detect patterns and trends in huge data that cannot be identified using traditional analysis-based methods. To this end, we further investigated the differences between rumen microbes at different altitudes using the Kyoto Encyclopedia of Genes and Genomes (KEGG) database based on feature engineering. The 330 yak rumen microbial genomes were subjected to KEGG annotation with 1992 other ruminant rumen microbial genomes to generate a KEGG microbial function matrix (Figure 5) that indicated whether each genomic source was from a high altitude. Thus, the raw data were transformed into features that were more representative of potential problems with the prediction model. The ratio of the number of high-and low-elevation samples was approximately 1: 6. The significant imbalance between these two categories was evident. In order to balance the two sets of sample data, our study employed the synthetic minority oversampling technique (SMOTE) algorithm, which is a completely sampled synthetic data algorithm.

We developed four models of machine learning. The accuracy and applicability of the various techniques were assessed using

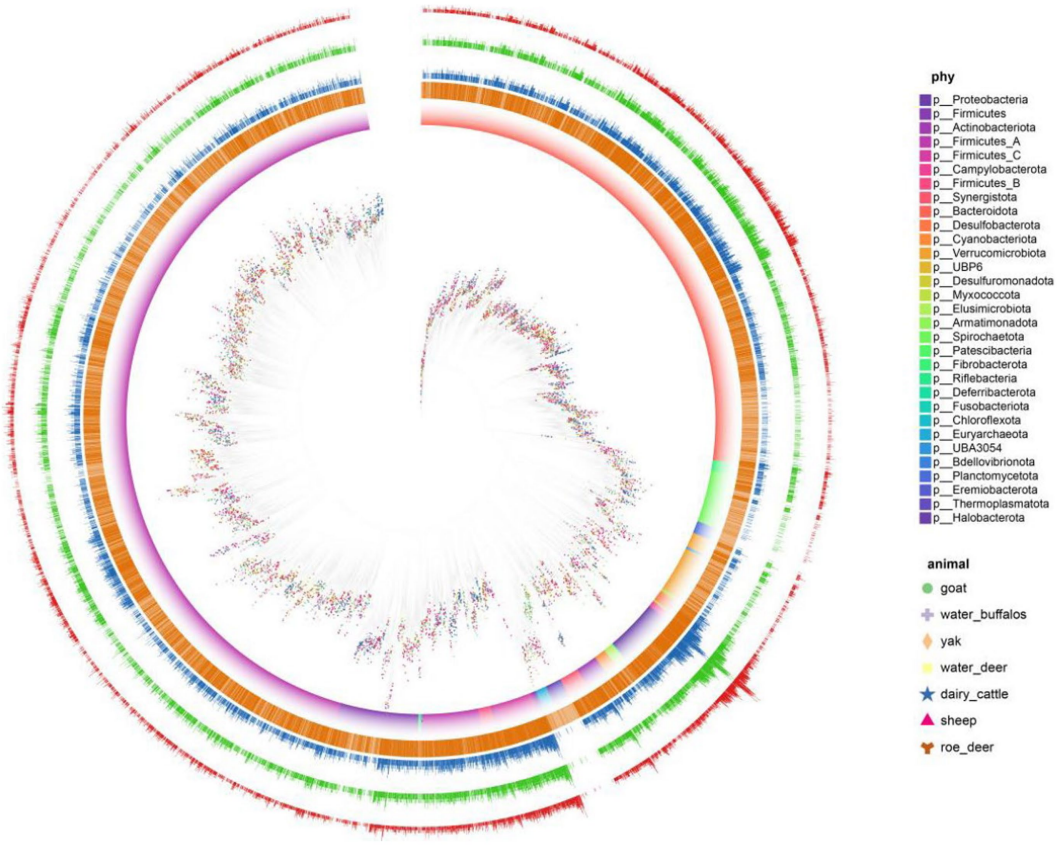


FIGURE 4
Antibiotic-resistance distribution in gastrointestinal microbes. Clades are 146 colored according to phyla and SHAP represents different hosts. The first layer uses 147 orange represents the genome of antibiotics. Second, three, and the four layers represent 148 the number of ARG, how many types and the ARG risk index.

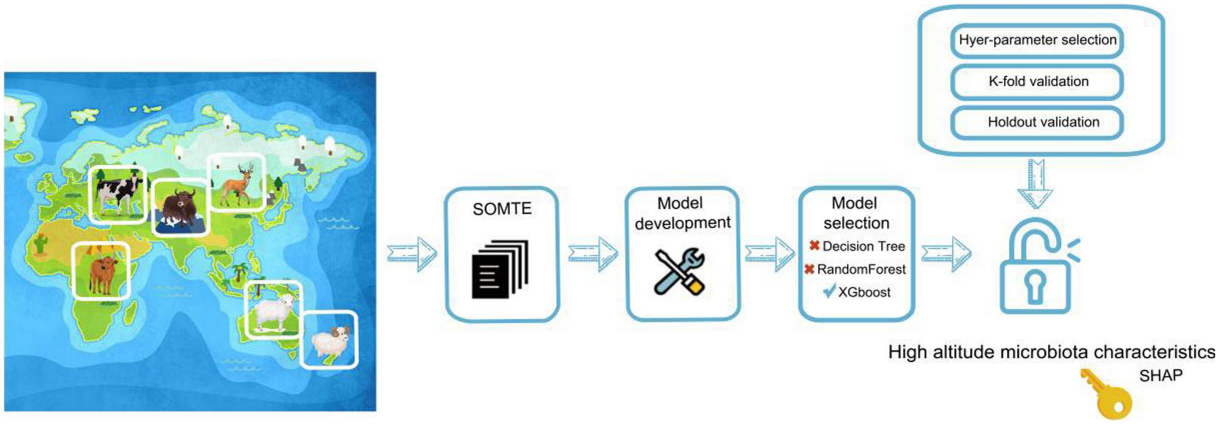


FIGURE 5
Flow chart of machine learning implementation for predicting the adaptive function of microorganisms.

cross-validation score, incorporating the evaluation of 10 distinct sets of cross-validation (Figure 6A). In the 10 cross-validations, the effect of the training and test sets became less effective as more tests were conducted, and the green line (lightGBM) outperformed all the other algorithms. Because of this, we ultimately chose lightGBM as the algorithm for the model. After choosing the LightGBM algorithm, hyperparameter training was performed. The input data were first divided into a test set and a training set, and then a search for hyperparameters was carried out. The appropriate parameters were selected using a plotted learning curve. By aggregating multiple

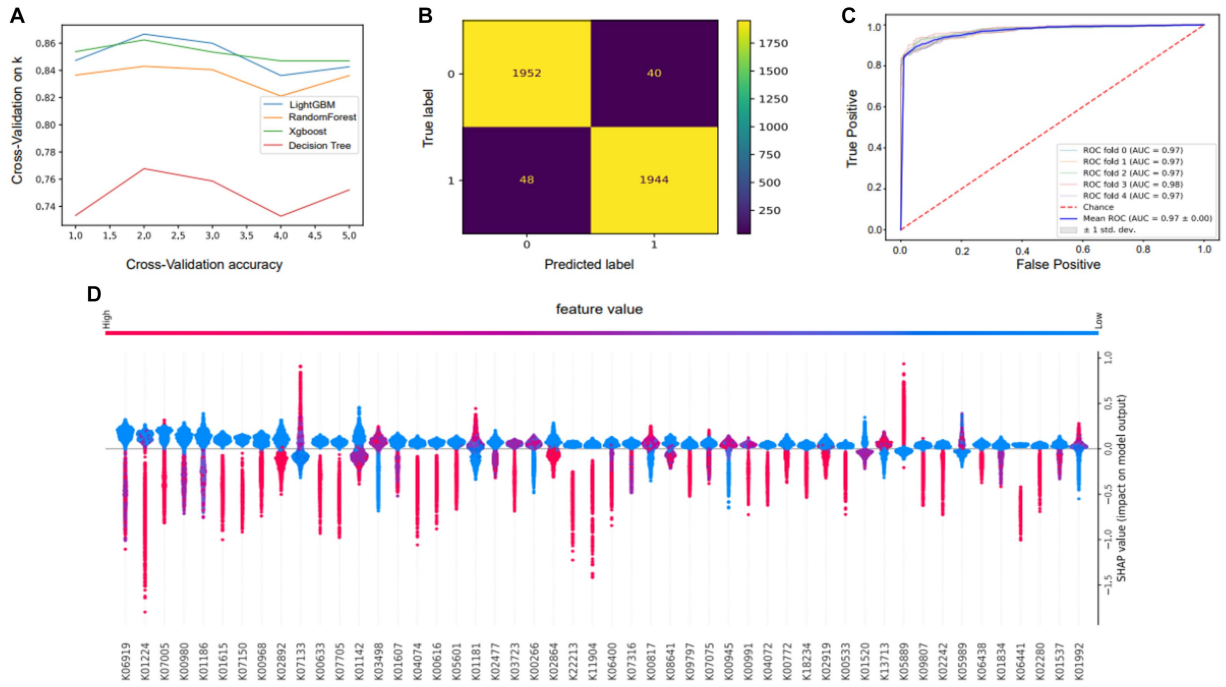


FIGURE 6
Performance and characteristics of the machine learning model. **(A)** Selection of model algorithms based on 10 cross-validation sets: lightGBM is the blue line, XGBoost is the green line, random forest is the yellow line and the decision tree is the red line. **(B)** Confusion matrix showing the performance of the LightGBM model. **(C)** Receiver operating characteristic (ROC) curve of the RF model. The horizontal coordinate of this curve is the false-positive rate (FPR), and the vertical coordinate is the true-positive rate (TPR). **(D)** Plots summarizing the SHAP values of all the samples were used for analysis to interpret key features.

hyperparameters, this model achieved an accuracy of 92.2%, representing an improvement of 13.68% over the lightGBM before tuning. To evaluate the efficacy of the model, a receiver operating characteristic (ROC) curve was constructed (Figure 6C). The closer the ROC curve is to the upper left corner, the better the performance of the classifier; the point on the ROC curve closest to the upper left corner is the best threshold for the lowest number of classification errors and the lowest number of false positives and false negatives. The ROC of the model is 0.97, which shows that the model has good prediction performance and is accurate and reliable and that there is no overfitting.

The mean absolute value of a feature's degree of influence on the target variable was utilized to determine its significance. Based on the Shapley additive explanation (SHAP) values (Figure 6D), the importance of features depicts the mean absolute SHAP values to illustrate the importance of global features. The abundance of genes in the KEGG orthology gene function category provides insight into the genetic underpinnings of adaptive phenotypic variation. The bee swarm plot is intended to show a summary of how the top characteristics of a data set influence the model's output. In each instance, the given explanation is represented by a single dot on each feature row. The x coordinate of the dot is determined by the SHAP value of that feature, and dots "pile up" along each feature row to represent density. Color is used to display the original value of a feature. Our investigation yielded insights into the genetic factors essential for the environmental adaptation of the yak gastrointestinal microbiota, highlighting the importance of the transcription-repair coupling factor (K03723), trk/ktr system potassium uptake protein (K03498), amino acid metabolism (K00817), and genes associated with polysaccharide and lipid metabolism (K05989, K01181). These identified genetic elements play pivotal roles in enabling the yak gut microbiota to effectively adapt to its surroundings. For example, the transcription-repair coupling factor likely contributes to genetic stability and maintenance in dynamic environments. Similarly, the trk/ktr system potassium uptake protein may support the maintenance of ionic balance critical for physiological functions. Amino acid metabolism, as well as polysaccharide and lipid metabolism genes, suggest the microbiota's ability to efficiently extract nutrients from its environment.

Enrichment analysis based on these gene revealed that the microbial community of yaks exhibits more robust specific pathways compared to other plains ruminants (Supplementary Figure S1). These pathways encompass carbohydrate metabolism, nucleotide metabolism, transport systems, and amino acid metabolism, potentially playing a pivotal role in their adaptation and survival within their specific environment. The heightened activity of carbohydrate metabolism suggests efficient energy extraction from their diet, supporting energy-intensive processes. Emphasis on nucleotide metabolism might indicate enhanced DNA and RNA synthesis, potentially aiding cellular growth and repair. The well-developed transport system could facilitate nutrient absorption and intercellular communication, thereby optimizing resource utilization. Overall, these findings shed light on the mechanisms by which the yak gut microbiota adapts to its habitat and offers valuable insights into the genetic underpinnings of its environmental resilience.

# 4. Discussion

Ruminants are among the most successful herbivorous mammals (Decker et al., 2009). In this study, we took full advantage of the largest and most comprehensive database of ruminant gastrointestinal microbes available. We employed a range of approaches, encompassing complex networks and interpretable machine learning, to characterize the state of environmental microbial populations.

In this study, we employ network science theory to examine the properties of gastrointestinal networks and evaluate the robustness of these networks by examining these properties in more detail. Diverse profiles of topological features in diverse environmental networks reveal the unique co-occurrence patterns of microorganisms in ruminant species. The prevalence of cross-feeding relationships in the network may be indicated by the high clustering coefficient, which suggests that these settings have abundant degradation pathways, niche filtering, or environmental unpredictability. Different models built to test the resilience of networks reveal that they are more resilient to random faults and more vulnerable to deliberate attacks. Understanding the resilience of networks and the various approaches to averting catastrophic failures of these networks is essential. Only a tiny number of significant species have been eliminated, which may have an effect on the general structure of the network of microbes in a healthy microbiome. This finding highlights the importance of using antibiotics sparingly once more.

By investigating antibiotic resistance genes, we gain insights into how microbial communities respond to antibiotics. This understanding is pivotal, as it unravels the intricate interplay among microorganisms and is fundamental to comprehending the intricate web of interactions that shape ecosystems (Sharland et al., 2015). The presence of antibiotic-resistant microbiomes (ARBs) and ARGs in supermarket meat and dairy products suggests that ARBs/ARGs from ruminants can penetrate the food system. It would be helpful to make an effort to compile a list of significant ARG-carrying species for monitoring and control based on the assessment of the total antibiotic resistance risk at the species level. It is alarming that the farming environment contains high-risk MAGs. Additionally, microbiomes communities frequently experience an increase or decrease in ARGs as a result of genetic changes or HGT. Human health would be seriously endangered by these high-risk MAGs.

The Qinghai–Tibet Plateau, sometimes known as the "Third Pole," is a huge, high-altitude region with a unique and fragile ecological environment (Yao et al., 2012). The region is characterized by a harsh climate of extreme cold, drought, high ultraviolet radiation and a lack of oxygen, making it a challenging living environment for humans and other mammals (Zhu et al., 2018; Pan et al., 2021; Shen et al., 2021). It is essential to determine precisely which genetic features give ruminants their exceptional digestive capacity and ability to live in harsh conditions (Zhu et al., 2020). To answer this question, we developed interpretable machine learning methods to deeply mine complex, high-dimensional metagenomic data. We found a significant increase in the transcription repair coupling factor (K03723) in the yak gastrointestinal microbiota. K03723 regulates transcriptional processes and recognizes DNA damage. In addition, such phenomena were also found in samples from plateau-based animals for *Rhodobacter* sp. (Pérez et al., 2018) and nitrogen-fixing microbiomes (Suyal et al., 2018). We hypothesize that this may be a common measure adopted by microorganisms facing extreme environments. In addition, we found that the K03498 trk/ktr system potassium uptake protein contributes significantly to plateau acclimatization. We speculate that the numerous high-salinity sites in the plateau region have resulted in a microbial response to salt stress (Li et al., 2021). Similar to previous studies (Guo et al., 2021),the results indicate a preference for an amino acid metabolism gene (K00817) and polysaccharide and lipid metabolism genes (K05989 and K01181) in the yak gastrointestinal microbiota. These pathways provide additional adaptive responses to the lack of energy intake in yaks. In conclusion, our study provides important insights into ruminant plateau adaptation and highlights the key role of the microbial genome as a "second genome" for adaptation, contributing to a more comprehensive understanding of mammals living in extreme environments.

# 5. Conclusion

In-depth exploration of ruminant gastrointestinal microbes is necessary to understand the function of the microbiome and its interactions with the host animal. This study enhances our comprehension of both the structure and function of the ruminant gastrointestinal microbiota, a critical aspect for investigating microbial-host symbiotic functional dynamics. Furthermore, it advances our understanding of the gastrointestinal microbiota adaptations necessary for herbivores. In addition, it informs strategies to decrease contamination and increase the robustness and efficiency of ruminants.

# 6. Methods

## 6.1. Data used in this study

The sequence files of 10,373 gastrointestinal microbial genomes of ruminants were downloaded in FASTA format from Figshare (DOI: 10.6084/m9.figshare.14176574). All the gene catalogs, annotation information, abundance profiles, assemblies, and predicted open reading frames (ORFs) from this study are available at https://microbiomejournal.biomedcentral.com/articles/10.1186/s40168-021-01078-x. The details of all samples used in this study are provided in Supplementary Table S1.

## 6.2. Network analysis

A Spearman correlation matrix was calculated based on the relative abundance of genera in each sample, and networks were graphed using Gephi (Bastian et al., 2009). Topological features were estimated with the igraph package (Csardi and Nepusz, 2006) (v1.4.1) in R 3.6.0.

The robustness of gastrointestinal networks can be regarded as the ability of the entire network to maintain the same performance when nodes in the network are affected by random factors and intentional damage. The survivability of networks can be measured by the change in network topological structure characteristics. We selected the relative size of the largest connected subgraph (RS) and relative connectivity efficiency (RE) after node failure as the measurement indexes of network survivability (Wei et al., 2021).

$$RS = \frac{N^t}{N}$$

where N is the number of nodes in the largest connected subgraph and N is the total number of nodes in the airline network. As the nodes in the network are attacked, the network splits into several subgraphs, and the relative size of the largest connected subgraph gradually decreases until the nodes are no longer connected with each other and finally become scattered nodes.

In this research, different attack strategies are developed to further understand the failure process of the network under different scenarios, specifically including random failures and targeted attacks (Wang et al., 2010). The strategies can be summarized as follows: First, there is the random attack strategy based on nodes. Second, the largest degree first attack strategy based on nodes. Third, the largest pagerank degree first attack strategy based on nodes.

# 7. Functional analysis of microbial genes

## 7.1. ARG annotation

The functional annotation of all identified proteins encoded by ARGs was based on sequence similarity searches carried out with DIAMOND BLASTP v2.0.9 (Buchfink et al., 2021) with the default settings against the HMD-ARG database (Li et al., 2021). ARGs were selected at a sequence similarity threshold of 75% and a score threshold of 60 (Yan et al., 2022). In our study, we utilized a set of equations to effectively quantify the antibiotic risk associated with the presence of ARGs within different Metagenome-MAGs in the context of the larger ecosystem. These equations, as described by equation (Zhang et al., 2022), were structured as follows:

$$Risk = \frac{num_ARG}{total_ARG} \times \frac{num_ARG_subtypes}{total_ARG_suntypes}$$

In this context, "num_ARG" stands for the count of ARGs detected within a specific MAG, while "total_ARG" refers to the overall count of ARGs encompassing the entire ecosystem under consideration. On the other hand, "num_ARG_subtypes" represents the tally of distinct ARG subtypes found within the same MAG, and "total_ARG_subtypes" denotes the comprehensive count of distinct ARG subtypes present within the entire ecosystem. By applying this approach, we aimed to evaluate and quantify the potential antibiotic risk posed by the presence of ARGs within each MAG, within the broader context of the ecological system. This method allowed us to gain insights into the degree of antibiotic resistance-related risk associated with specific MAGs and their ARG compositions, contributing to a more comprehensive understanding of the ecosystem's antibiotic resistance dynamics.

# 8. Machine learning model development

## 8.1. Data collection

In this experiment, 330 yak rumen microbial genomes were used as input data, along with 1992 other ruminant genomes. After comparing the protein sequences to the database using eggNOG, the proteins were grouped into different KOs (*KEGG Orthology*), with each cluster of KOs consisting of direct homologous sequences so that the function of the sequence could be inferred. The KO gene function matrix was built as an input file for machine learning.

## 8.2. Data preprocessing

Unbalanced data can pose challenges for machine learning models. Most machine learning models assume that the same number of samples is available for each class. Ignoring this problem can lead to errors in a few classes (and thus make the model sensitive to classification errors), causing ML models to ignore observations in a few classes. In the current work, the number of samples collected was uneven because the number of ruminant gastrointestinal samples varied from region to region. The synthetic minority oversampling technique (SMOTE) method was applied to overcome the adverse effect of learning data imbalance (Chawla et al., 2002).

## 8.3. Model development and tuning

Four machine learning models were developed to predict microbial plateau adaptive function in the Jupyter lab development environment (Perkel, 2018) using scikit-learn,[1] Numpy (v1.15.3), Pandas (v0.23.4), Matplotlib (v3.0.1), and Scipy (v1.1.0) for experiments. The machine learning algorithms used for classification in this work were random forest (Qi, 2012), decision tree (Somvanshi et al., 2016), light gradient boosting machine (lightGBM) (Ke et al., 2017), and XGBoost (Chen and Guestrin, 2016). These are all integrated tree-based learning methods and are rated by the machine learning community as the most popular nonlinear models today. LightGBM is a fast and efficient GBDT algorithm in the open-source promotion framework designed by Microsoft MSRA in 2016. The algorithm is used for many machine learning tasks, such as sorting, classification, and regression, and supports efficient parallel training.

## 8.4. Shapley additive explanation

SHAP quantifies the importance of variables by leveraging Shapley values, a concept originating from cooperative game theory introduced by Shapley in (Bouneder et al., 2020). SHAP's theoretical foundation is rooted in cooperative game theory, as highlighted by Lundberg and Lee in 2017 (Van den Broeck et al., 2022). The methodology explicates the model's predictions by embracing the idea of additive feature attribution. The fundamental principle of SHAP is to decompose the explanation of a prediction into contributions from each feature (Wang et al., 2022). It assigns each feature's contribution based on its Shapley value across different subsets of features, which is equivalent to a weighted average of feature contributions. Shapley values are a concept from cooperative game theory and denote the average contribution of a player across all possible coalition formations.

The SHAP value of feature i($\phi$i) can be computed using the following equation:

1 https://scikit-learn.org

$$\phi_i = \sum_{S \subseteq N \setminus \{i\}} \frac{|S|!(|M|-|S|-1)!}{|M|!} \left[ f_x\left(S \cup \{i\}\right) - f_x\left(S\right) \right]$$

Where: N represents the set of all features. *S is a subset of NN that does not include feature i.* $f\left(S\right)$ represents the model's prediction when considering only the feature set *S*. $f\left(S \cup \{i\}\right)$ is the model's prediction when feature ii is added to the features in subset S. The idea behind this formula is to consider all possible combinations of features, excluding feature ii, and calculate the difference in model predictions when feature ii is included in these combinations. The Shapley value concept assigns weights to these differences based on the number of ways a specific feature can contribute to different subsets of features. The summation calculates the weighted average of these differences, yielding the SHAP value for feature *i*.

In essence, the SHAP value quantifies the contribution of each feature to the model's prediction by considering how including or excluding that feature influences the model's output across various combinations of features.

## 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.

## Author contributions

YY conceived, designed the overall study, conceived, designed, and executed the bioinformatics analysis. XB and YG provided the genomic information. All authors contributed intellectually to the interpretation and presentation of the results in the manuscript, which was edited and approved by all authors.

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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/fmicb.2023.1147007/full#supplementary-material

## References

Ban, Y., and Guan, L. L. (2021). Implication and challenges of direct-fed microbial supplementation to improve ruminant production and health. *J. Anim. Sci. Biotechnol.* 12:109. doi: 10.1186/s40104-021-00630-x

Bastian, M., Heymann, S., and Jacomy, M. (2009). "Gephi: an open source software for exploring and manipulating networks" in *Proceedings of the International AAAI Conference on Web and Social Media*, 361–362.

Bouneder, L., Léo, Y., and Lachapelle, A. (2020). X-SHAP: towards multiplicative explainability of machine learning. *arXiv*. doi: 10.48550/arXiv.2006.04574

Buchfink, B., Reuter, K., and Drost, H.-G. J. (2021). Sensitive protein alignments at tree-of-life scale using DIAMOND. *Nat. Methods* 18, 366–368. doi: 10.1038/s41592-021-01101-x

Chawla, N. V., Bowyer, K. W., Hall, L. O., and Kegelmeyer, W. P. (2002). SMOTE: synthetic minority over-sampling technique. *J. Artif. Intell. Res.* 16, 321–357. doi: 10.1613/jair.953

Chen, T., and Guestrin, C. (2016) Xgboost: a scalable tree boosting system. *Proceedings of the 22nd ACM Sigkdd International Conference on Knowledge Discovery and Data Mining*, San Francisco, CA. (2016).

Cholewinska, P., Gorniak, W., and Wojnarowski, K. (2021). Impact of selected environmental factors on microbiome of the digestive tract of ruminants. *BMC Vet. Res.* 17:25. doi: 10.1186/s12917-021-02742-y

Cortés, A., Rooney, J., Bartley, D. J., Nisbet, A. J., and Cantacessi, C. (2020). Helminths, hosts, and their microbiota: new avenues for managing gastrointestinal helminthiases in ruminants. *Expert. Rev. Anti Infect. Ther.* 18, 977–985. doi: 10.1080/14787210.2020.1782188

Csardi, G., and Nepusz, T. J. I. (2006). The igraph software package for complex network research. *Inter J Compl Syst* 1695, 1–9.

Decker, J. E., Pires, J. C., Conant, G. C., McKay, S. D., Heaton, M. P., Chen, K., et al. (2009). Resolving the evolution of extant and extinct ruminants with high-throughput phylogenomics. *Proc. Natl. Acad. Sci. U. S. A.* 106, 18644–18649. doi: 10.1073/pnas.0904691106

Díaz-Céspedes, M., Hernández-Guevara, J. E., and Gómez, C. (2021). Enteric methane emissions by young Brahman bulls grazing tropical pastures at different rainfall seasons in the Peruvian jungle. *Trop. Anim. Health Prod.* 53:421. doi: 10.1007/s11250-021-02871-4

Guo, W., Wang, W., Bi, S., Long, R., Ullah, F., Shafiq, M., et al. (2020). Characterization of anaerobic rumen fungal community composition in yak, Tibetan sheep and small tail Han sheep grazing on the Qinghai-Tibetan plateau. *Animals* 10:144. doi: 10.3390/ani10010144

Guo, N., et al. (2021). Seasonal dynamics of diet–gut gastrointestinal microbiota interaction in adaptation of yaks to life at high altitude. *NPJ Biofilms Microb* 7:38. doi: 10.1038/s41522-021-00207-6

Sharland, M., Pulcini, C., Harbarth, S., Zeng, M., Gandra, S., Mathur, S., et al. (2015). Expert Committee on Selection and Use of Essential Medicines. Classifying antibiotics in the WHO Essential Medicines List for optimal use-be AWaRe. *Lancet. Infect. Dis.* 18, 18–20. doi: 10.1016/S1473-3099(17)30724-7

Ihl, C., and Barboza, P. S. (2007). Nutritional value of moss for arctic ruminants: a test with muskoxen. *J Wildl Manag* 71, 752–758. doi: 10.2193/2005-745

Ke, G., Meng, Q., Finley, T., Wang, T., Chen, W., Ma, W., et al. (2017). "LightGBM: a highly efficient gradient boosting decision tree" in *NIPS'17: Proceedings of the 31st International Conference on Neural Information Processing Systems*. Long Beach, CA, USA, 3149–3157.

Li, Z., Gao, Y., Wang, S., Lu, Y., Sun, K., Jia, J., et al. (2021). Phytoplankton community response to nutrients along lake salinity and altitude gradients on the Qinghai-Tibet plateau. *Ecol. Indic.* 128:107848. doi: 10.1016/j.ecolind.2021.107848

Li, F., Li, C., Chen, Y., Liu, J., Zhang, C., Irving, B., et al. (2019). Host genetics influence the rumen microbiota and heritable rumen microbial features associate with feed efficiency in cattle. *Microbiome* 7:92. doi: 10.1186/s40168-019-0699-1

Li, Y., Xu, Z., Han, W., Cao, H., Umarov, R., Yan, A., et al. (2021). HMD-ARG: hierarchical multi-task deep learning for annotating antibiotic resistance genes. *Microbiome* 9, 1–12. doi: 10.1186/s40168-021-01002-3

Malmuthuge, N., and Guan, L. L. (2016). Gastrointestinal microbiome and omics: a new definition to ruminant production and health. *Anim. Front.* 6, 8–12. doi: 10.2527/af.2016-0017

Mennecart, B., Aiglstorfer, M., Li, Y., Li, C., and Wang, S. (2021). Ruminants reveal Eocene Asiatic palaeobiogeographical provinces as the origin of diachronous mammalian Oligocene dispersals into Europe. *Sci. Rep.* 11:17710. doi: 10.1038/s41598-021-96221-x

Pan, X., Guo, X., Li, X., Niu, X., Yang, X., Feng, M., et al. (2021). National Tibetan Plateau Data Center: promoting earth system science on the third pole. *Am Meteorol Soc* 102, E2062–E2078. doi: 10.1175/BAMS-D-21-0004.1

Pérez, V., Dorador, C., Molina, V., Yáñez, C., Hengst, M. J. A. V., and SP, L. R. (2018). Rb3, an aerobic anoxygenic phototroph which thrives in the polyextreme ecosystem of the Salar de Huasco in The Chilean Altiplano. *Antonie Van Leeuwenhoek* 111, 1449–1465. doi: 10.1007/s10482-018-1067-z

Perkel, J. M. J. (2018). Why Jupyter is data scientists' computational notebook of choice. *Nature* 563, 145–147. doi: 10.1038/d41586-018-07196-1

Pickford, M. J. (2001). Africa's smallest ruminant: a new tragulid from the Miocene of Kenya and the biostratigraphy of east African Tragulidae. *Geobios* 34, 437–447. doi: 10.1016/S0016-6995(01)80007-3

Qi, Y. (2012). "Random forest for bioinformatics" in *Ensemble machine learning*. eds. C. Zhang and Y. Q. Ma (New York: Springer), 307–323.

Sauer, C., Bertelsen, M. F., Lund, P., Weisbjerg, M. R., and Clauss, M. (2016). Quantitative macroscopic anatomy of the giraffe (*Giraffa camelopardalis*) digestive tract. *Anat. Histol. Embryol.* 45, 338–349. doi: 10.1111/ahe.12201

Seshadri, R., Leahy, S. C., Attwood, G. T., Teh, K. H., Lambie, S. C., Cookson, A. L., et al. (2018). Cultivation and sequencing of rumen microbiome members from the Hungate1000 collection. *Nat. Biotechnol.* 36, 359–367. doi: 10.1038/nbt.4110

Shabat, S. K. B., Sasson, G., Doron-Faigenboim, A., Durman, T., Yaacoby, S., Miller, M. E. B., et al. (2016). Specific microbiome-dependent mechanisms underlie the energy harvest efficiency of ruminants. *ISME J.* 10, 2958–2972. doi: 10.1038/ismej.2016.62.

Shen, X., Huo, B., Li, Y., Song, C., Wu, T., and He, J. (2021). Response of the critically endangered Przewalski's gazelle (*Procapra przewalskii*) to selenium deprived environment. *J. Proteome* 241:104218. doi: 10.1016/j.jprot.2021.104218

Somvanshi, M., Chavan, P., Tambade, S., and Shinde, S. (2016). "A review of machine learning techniques using decision tree and support vector machine," in *2016 International Conference on Computing Communication Control and automation (ICCUBEA)*, Pune, India, pp. 1–7.

Stewart, R. D., Auffret, M. D., Warr, A., Walker, A. W., Roehe, R., and Watson, M. (2019). Compendium of 4,941 rumen metagenome-assembled genomes for rumen microbiome biology and enzyme discovery. *Nat. Biotechnol.* 37, 953–961. doi: 10.1038/s41587-019-0202-3

Suyal, D. C., Kumar, S., Joshi, D., Soni, R., and Goel, R. (2018). Quantitative proteomics of psychotrophic diazotroph in response to nitrogen deficiency and cold stress. *J. Proteome* 187, 235–242. doi: 10.1016/j.jprot.2018.08.005

Van den Broeck, G., Lykov, A., Schleich, M., and Suciu, D. J. J. (2022). On the tractability of SHAP explanations. *J. Artif. Intell. Res.* 74, 851–886. doi: 10.1613/jair.1.13283

van Lingen, H. J., Edwards, J. E., Vaidya, J. D., van Gastelen, S., Saccenti, E., van den Bogert, B., et al. (2017). Diurnal dynamics of gaseous and dissolved metabolites and microbiota composition in the bovine rumen. *Front. Microbiol.* 8:425. doi: 10.3389/fmicb.2017.00425

Vestergaard, G., Schulz, S., Schöler, A., Schloter, M. J. B., and Soils, F. O. (2017). Making big data smart—how to use metagenomics to understand soil quality. *Biol. Fertil. Soils* 53, 479–484. doi: 10.1007/s00374-017-1191-3

Wang, Z., Scaglione, A., and Thomas, R. J. (2010). Electrical centrality measures for electric power grid vulnerability analysis. Proceedings of the 49th IEEE Conference on Decision and Control, Atlanta: IEEE, 5792–5797.

Wang, D., Thunéll, S., Lindberg, U., Jiang, L., Trygg, J., and Tysklind, M. (2022). Towards better process management in wastewater treatment plants: process analytics based on SHAP values for tree-based machine learning methods. *J. Environ. Manag.* 301:113941. doi: 10.1016/j.jenvman.2021.113941

Wei, Y., Jin, Y., Ma, D., and Xiu, C. (2021). Impact of colored motif characteristics on the survivability of passenger airline networks in China. *Phys A* 566:125658. doi: 10.1016/j.physa.2020.125658

Xie, F., Jin, W., Si, H., Yuan, Y., Tao, Y., Liu, J., et al. (2021). An integrated gene catalog and over 10,000 metagenome-assembled genomes from the gastrointestinal microbiome of ruminants. *Microbiome* 9:137. doi: 10.1186/s40168-021-01078-x

Yan, Y., Li, H., Fayyaz, A., and Gai, Y. (2022). Metagenomic and network analysis revealed wide distribution of antibiotic resistance genes in monkey gut microbiota. *Microbiol. Res.* 254:126895. doi: 10.1016/j.micres.2021.126895

Yao, T., Thompson, L. G., Mosbrugger, V., Zhang, F., Ma, Y., Luo, T., et al. (2012). Third pole environment (TPE). *Environ Dev* 3, 52–64. doi: 10.1016/j.envdev.2012.04.002

Zhang, Z., Zhang, Q., Wang, T., Xu, N., Lu, T., Hong, W., et al. (2022). Assessment of global health risk of antibiotic resistance genes. *Nat. Commun.* 13:1553. doi: 10.1038/s41467-022-29283-8

Zhu, X., Guan, Y., Signore, A. V., Natarajan, C., DuBay, S. G., Cheng, Y., et al. (2018). Divergent and parallel routes of biochemical adaptation in high-altitude passerine birds from the Qinghai-Tibet plateau. *Proc. Natl. Acad. Sci. U. S. A.* 115, 1865–1870. doi: 10.1073/pnas.1720487115

Zhu, Z., Sun, Y., Zhu, F., Liu, Z., Pan, R., Teng, L., et al. (2020). Seasonal variation and sexual dimorphism of the microbiota in wild blue sheep (*Pseudois nayaur*). *Front. Microbiol.* 11:1260. doi: 10.3389/fmicb.2020.01260

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