frontiers | Research Topics

Crosslinking of feed nutrients, microbiome and production in ruminants

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Published in
Frontiers in Veterinary Science

ISSN 1664-8714
ISBN 978-2-8325-6578-0
DOI 10.3389/978-2-8325-6578-0

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Crosslinking of feed nutrients, microbiome and production in ruminants

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Citation

Xu, Q., Li, F., Zhao, S., Shen, J., Song, D., eds. (2025). *Crosslinking of feed nutrients, microbiome and production in ruminants*. Lausanne: Frontiers Media SA. doi: 10.3389/978-2-8325-6578-0

Table of contents

frontiers | Frontiers in Veterinary Science

TYPE Editorial
PUBLISHED 17 June 2025
DOI 10.3389/fvets.2025.1610490

OPEN ACCESS

EDITED AND REVIEWED BY
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RECEIVED 12 April 2025
ACCEPTED 02 June 2025
PUBLISHED 17 June 2025

CITATION
Du W, Xu W, Hu Y, Zhao S, Li F, Song D, Shen J and Xu Q (2025) Editorial: Crosslinking of feed nutrients, microbiome and production in ruminants. *Front. Vet. Sci.* 12:1610490. doi: 10.3389/fvets.2025.1610490

Editorial: Crosslinking of feed nutrients, microbiome and production in ruminants

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KEYWORDS

ruminants, feed nutrients, rumen microbiome, rumen fermentation, interactions

Editorial on the Research Topic
Crosslinking of feed nutrients, microbiome and production in ruminants

In ruminants, the rumen serves as the primary organ responsible for converting plant-based feed into nutrients and energy. Research indicates that microbial derivatives, dietary composition, and host metabolism collectively affect rumen metabolite concentrations and microbial community structure, shaping the mechanisms of host-microbiota interactions (1). Ruminants rely on symbiotic relationships with complex rumen microbial communities that specialize in degrading recalcitrant plant polymers such as cellulose and hemicellulose, converting them into digestible compounds. These microbes are critical for the productivity and health of ruminants because they directly contribute to volatile fatty acid (VFA) production and microbial protein biosynthesis, both of which are essential to milk efficiency (2). Additionally, bacterial communities influence growth performance as well as milk yield and composition in dairy cattle (3). Conversely, rumen microbiota structure is modulated by host species, dietary energy levels, and environmental factors (4). This Research Topic primarily explores nutritional interventions to regulate growth performance, rumen fermentation, and microbial composition in ruminants.

Diet and feed additives are potent modulators of rumen microbiota, serving as substrates for microbial metabolism and thereby altering rumen environments and species composition. Early dietary interventions may help establish rumen microbial communities, leading to long-term changes in community structure and function that ultimately affect host phenotypes (5). For instance, Liu J. et al. found that supplementing 0.3% moringa polysaccharides to the milk replacer of early-weaned goat kids increased their average daily gain (ADG), feed intake, serum immunoglobulins (IgA and IgM), rumen muscle thickness, rumen wall thickness, and rumen pH, while also enriching *Actinobacteria* and *Butyrivibrio* species in the rumen. Zhang S. et al. reported that adding 1,500 mg/kg of guanidinoacetic acid to the diet increased ruminal ammonia nitrogen concentration and total reducing sugar flow into the small intestine, thereby improving creatine levels, glucose utilization, and average daily gain (ADG) in lambs. Hou et al. demonstrated that supplementing 5% residual black wolfberry fruit enhanced

growth performance in Duolang sheep, optimized rumen fermentation parameters without negatively affecting microbial structure, and improved economic returns. Luo et al. found that nisin and monensin supplementation in fattening Hu sheep reduced ruminal acetate concentration and altered fermentation patterns, although it did not affect their growth performance or health.

Studies have suggested that fermented feed products can enhance antioxidant and immune capacity, improve rumen fermentation, and modulate microbial communities in ruminants (6). Cheng et al. reported that 15% fermented rice husk feed improved the growth performance, nutrient digestibility, and ruminal propionate, butyrate, and valerate concentrations of Hu sheep, while enriching fiber-degrading bacteria (e.g., *Ruminococcus*) and suppressing inefficient taxa (e.g., *Rikenellaceae RC9*). Thus, fermented rice husk represents a promising alternative to conventional roughage. Zhang J. et al. observed that fermented soybean meal did not affect milk yield in lactating cows but increased serum prolactin levels and altered rumen microbiota, potentially benefiting long-term health and productivity. Liu Y. et al. demonstrated that dietary fermented jujube powder (FJP) enhanced ADG and feed efficiency by promoting nutrient degradation and VFA production via microbial enzyme activity. Additionally, elevated serum total antioxidant capacity and reduced malondialdehyde levels indicated improved oxidative defense, underscoring FJP's potential as a functional feed additive.

Yeast culture (YC), a feed additive rich in yeast cell wall components (e.g., mannan oligosaccharides, and β-glucans) and fermentation metabolites (e.g., organic acids, B vitamins, and enzymes), stabilizes rumen pH and promotes fiber-degrading bacteria, enhancing feed efficiency (7). Li et al. found that 10 g/d YC supplementation in dairy goats increased milk yield and ruminal acetate, butyrate, and VFA concentrations while reducing NH3-N levels, suggesting improved microbial protein synthesis. Zhang L. et al. reported that *Saccharomyces cerevisiae*-fermented sorghum distillers' grains reduced weight loss in early-lactation goats and improved milk quality, likely via bile acid and caffeine metabolism pathways linked to energy and immune regulation.

Milk, a vital nutrient source for humans, contains lactose, triglycerides, proteins, minerals, and vitamins. β-casein, a major milk protein, exists in two primary genotypes (A1 and A2) that differ at position 67 (histidine in A1, proline in A2). A2 milk (from A2A2 genotype cows) is considered more digestible and health-promoting. Zhao et al. identified unique rumen microbial and metabolic profiles (e.g., arachidonic acid, adrenic acid, glycocholic acid, taurine, and *g_Acetobacter*) in A2A2 cows that correlated with higher milk fat content. Arachidonic acid, a key biomarker, may enhance milk fat synthesis by activating lipogenic genes.

Amino acids (AAs), the building blocks of proteins, are central to protein nutrition. Reducing dietary protein levels while supplementing limiting AAs (e.g., lysine and methionine) can meet ruminant requirements. Wang et al. showed that a 3: 1 ratio of rumen-protected Lys and Met in Holstein bulls improved nitrogen efficiency and stabilized rumen microbiota without compromising intake or digestibility, offering a strategy for stress-resistant feeding. L-carnosine, a dipeptide with antioxidant and anti-inflammatory properties, was shown by Meng et al. to enhance growth performance in fattening lambs by modulating gut microbiota and serum metabolites, thereby promoting protein synthesis and energy metabolism.

Alfalfa hay, a widely used forage in livestock production due to its high palatability, low fiber content, and high protein content (17%−22%), exhibits nutrient variability depending on cultivar, storage method, and harvest stage, which may influence dairy cow performance and rumen microbiota. La et al. observed that while alfalfa hay from different sources (Spanish SAH vs. American AAH) significantly altered the rumen microbial composition and function of dairy cows, these changes did not affect their production performance, nutrient digestibility, or blood biochemical parameters. This suggested SAH as a viable alternative to mitigate market supply fluctuations while maintaining productivity.

As the demand for high-quality dairy and beef rises, Holstein cattle—a globally dominant dairy breed—have been extensively studied, whereas indigenous breeds such as Chinese Sanhe cattle (which are dual-purpose for milk and meat production) remain under-researched. Liu Z. et al. compared rumen microbiota between multiparous Sanhe and Holstein cows, finding similar species compositions although there were variations in abundance by parity and breed. Rumen ecology was found to strongly correlate with metabolic patterns; however, breed remained the decisive factor for productivity. Crossbreeding, a strategy to enhance growth and feed efficiency, introduces superior traits into local breeds. Zhang R. et al. demonstrated that crossbreeding alters rumen microbiota and metabolites, significantly improving growth. ♂Poll Dorset × ♀Hu crosses were found to enhance fiber fermentation and energy supply, while ♂Southdown × ♀Hu crosses optimize amino acid metabolism for protein synthesis, providing insights for breeding and nutrition strategies.

In summary, ruminant digestion relies on an intricate host-microbe symbiosis, where feed nutrients shape microbial activity, influencing nutrient absorption and production outcomes. Future research should focus on precise microbiota modulation, functional feed development, and sustainable farming to enhance productivity, product quality, and environmental stewardship. These advances will revolutionize ruminant production systems.

Author contributions

WD: Writing – original draft, Writing – review & editing. WX: Writing – review & editing. YH: Writing – review & editing. SZ: Writing – review & editing. FL: Writing – review & editing. DS: Writing – review & editing. JS: Writing – review & editing. QX: Writing – review & editing.

Funding

The author(s) declare that financial support was received for the research and/or publication of this article. This work was funded by the grants from the National Key R&D Program of China (2022YFD1301004 and 2022YFD1300705), the Hubei Province Key R&D Program (2023DJC197), and the Fundamental Research Funds for the Central Universities (2662023DKPY002).

Acknowledgments

The editors thank the Frontiers admin team for their wonderful support with this Research Topic, along with the numerous peer reviewers involved.

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 author(s) declared that they were an editorial board member of Frontiers, at the time of submission. This had no impact on the peer review process and the final decision.

Publisher's note

References

1. Franzosa EA, Sirota-Madi A, Avila-Pacheco J, Fornelos N, Haiser HJ, Reinker S, et al. Gut microbiome structure and metabolic activity in inflammatory bowel disease. *Nat Microbiol.* (2019) 4:293–305. doi: 10.1038/s41564-018-0306-4

2. Krehbiel CR. Invited review: applied nutrition of ruminants: fermentation and digestive physiology1. *Prof Animal Sci.* (2014) 30:129–39. doi: 10.15232/S1080-7446(15)30100-5

3. Schären M, Frahm J, Kersten S, Meyer U, Hummel J, Breves G, et al. Interrelations between the rumen microbiota and production, behavioral, rumen fermentation, metabolic, and immunological attributes of dairy cows. *J Dairy Sci.* (2018) 101:4615–37. doi: 10.3168/jds.2017-13736

4. Weimer PJ, Cox MS, Vieira de Paula T, Lin M, Hall MB, Suen G. 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.* (2017) 100:7165–82. doi: 10.3168/jds.2017-12746

5. Fernando SC, Adams S, Lakamp A, Spangler ML. Stochastic and deterministic factors that shape the rumen microbiome. *J Dairy Sci.* (2025) S0022-0302(25)00121-3. doi: 10.3168/jds.2024-25797

6. Jiang X, Xu HJ, Ma GM, Sun YK, Li Y, Zhang YG. Digestibility, lactation performance, plasma metabolites, ruminal fermentation, and bacterial communities in Holstein cows fed a fermented corn gluten-wheat bran mixture as a substitute for soybean meal. *J Dairy Sci.* (2021) 104:2866–80. doi: 10.3168/jds.2020-19072

7. Carpinelli NA, Halfen J, Trevisi E, Chapman JD, Sharman ED, Anderson JL, et al. Effects of peripartal yeast culture supplementation on lactation performance, blood biomarkers, rumen fermentation, and rumen bacteria species in dairy cows. *J Dairy Sci.* (2021) 104:10727–43. doi: 10.3168/jds.2020-20002

frontiers | Frontiers in Veterinary Science

TYPE Original Research
PUBLISHED 31 July 2024
DOI 10.3389/fvets.2024.1442244

OPEN ACCESS

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RECEIVED 01 June 2024
ACCEPTED 15 July 2024
PUBLISHED 31 July 2024

CITATION
Liu Y, Wang G, Wang R, Zhang X, Feng C, He Y and Chu P (2024) Effects of fermented jujube powder on growth performance, rumen fermentation, and antioxidant properties of simmental bulls. *Front. Vet. Sci.* 11:1442244. doi: 10.3389/fvets.2024.1442244

Effects of fermented jujube powder on growth performance, rumen fermentation, and antioxidant properties of simmental bulls

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Introduction: Fermented jujube powder (FJP) promotes a balance between the intestinal microflora and immune factors in animals. In this study, we aimed to investigate the effects of FJP on the production performance, nutrient digestion, rumen fermentation, and antioxidant properties of bulls.

Methods: Forty Simmental bulls were randomly divided into four groups based on body weight and fed a basal diet with [5, 7.5, or 10% dry matter (DM)] or without FJP. The experimental period was 20 d for adaptation and 60 d for the feeding trial.

Results: Dietary FJP supplementation did not affect DM intake ($P > 0.05$) but increased the average daily gain quadratically ($P = 0.049$) and decreased the feed conversion ratio linearly ($P = 0.042$). FJP quadratically enhanced DM and crude protein digestibility ($P = 0.026$ and $P = 0.041$, respectively) and linearly enhanced acid detergent fiber digestibility ($P = 0.048$). It also increased the total volatile fatty acid concentration quadratically ($P = 0.037$), acetate molar percentage, and acetate-to-propionate ratio linearly ($P = 0.002$ and 0.001), and reduced the ammonia nitrogen concentration linearly ($P = 0.003$). Additionally, xylanase and protease activities and *Ruminococcus flavefaciens* abundance increased linearly ($P = 0.006$, 0.018, and 0.009, respectively), and total bacteria, *Ruminococcus albus,* and *Ruminobacter amylophilus* abundance increased quadratically ($P = 0.047$, 0.011, and 0.021, respectively). FJP linearly increased serum total protein concentration and antioxidant capacity ($P = 0.003$ and 0.018, respectively) and decreased malonaldehyde content ($P = 0.006$).

Discussion: FJP supplementation (7.5%) enhanced production performance, nutrient digestion, rumen fermentation, and serum antioxidant capacity in bulls. The improved nutrient digestion may be due to an increase in ruminal microorganisms and total volatile fatty acids from the FJP. High blood antioxidant levels indicate that FJP may preserve proteins, thereby boosting the production performance of bulls.

KEYWORDS

fermented jujube powder, production performance, rumen fermentation, antioxidant capacity, bulls

1 Introduction

Rising animal feed prices necessitate the inclusion of less expensive regionally produced ingredients as unconventional feedstocks (1–5). Jujube (*Ziziphus jujube*), also known as Chinese date, holds significant importance in nutritional, medicinal, industrial, and cultural domains (6). Jujube fruits are used in various food products, and industrial processing of jujube generates large amounts of by-products, which are rich sources of nutrients and exhibits potential health benefit (7). Therefore, consideration of jujube by-products as a feed source has gained increased interest. China is the leading producer of jujube fruit worldwide and produced 7.46 million tons in 2019; however, an estimated 1–1.5 million tons were not suitable for consumption (7). Therefore, using jujube as a feed source not only reduces waste but also alleviates feed resource shortages. Jujube is rich in sugars, vitamins, minerals, and amino acids. The key bioactive ingredients in jujube include polysaccharides, cyclic nucleotides, polyphenols, and flavonoids, which exhibit antioxidant, immunomodulatory, antitumor, and other physiological activities (8). Jujube powder improved average daily gain (ADG), nutrient digestibility, and muscle protein content in male goats (9). It also boosted total antioxidant levels in the milk and blood of dairy goats (10).

Fermentation of jujube powder eliminates anti-nutritional factors and enhances its overall benefits compared to those of powdered jujube when used as a feed supplement for livestock (11). For instance, supplementation with fermented jujube powder (FJP) promoted a balance between intestinal microflora and the immune system in animals (12). Moreover, dietary supplementation of FJP enhanced ADG, blood antioxidant levels, and immunological protein content in bulls (13). The bacterial protein in FJP contributes to nutrient utilization in animals by stimulating the secretion of various digestive enzymes (14). These studies suggest that FJP could improve the production performance of animals attributed to its nutrient profile and antioxidant properties.

The rumen microflora is crucial for optimal nutrient absorption and overall health in ruminants (15). A previous study has demonstrated that supplementation of *Bacillus licheniformis* in the feed of Chinese Holstein cows enhances the concentration of volatile fatty acids (VFAs) in ruminal fluid (16). However, the specific effects of FJP on rumen fermentation parameters in bulls remains unclear.

Building on these previous findings, in this study, we aimed to evaluate the effects of FJP supplementation on the production performance, rumen fermentation, and serum antioxidant activity of Simmental bulls. Additionally, we identified an optimal FJP replacement strategy for intensive cattle farming. Our findings highlight the value of including FJP in the diets of bulls to improve their production performance.

2 Materials and methods

2.1 FJP preparation

Dried dates were powdered and mixed with 50% bran, 16.7% soybean meal, 30% water, and 4% (g/ton) of bacterial solution (2×10^8 colony-forming units of *Candida utilis*, *Bacillus licheniformis*, and *Lactobacillus plantarum* at 1:1:2). Aerobic fermentation was conducted for 1 d, followed by sealed fermentation for 9 d at 37°C and drying at 65°C for 12 h. FJP comprised 89.91% dry matter (DM), 13.63% crude protein (CP), 3.38% ether extract (EE), 0.56% calcium, and 0.21% phosphorus. Additionally, the feed contained 45.82% neutral detergent fiber (NDF) and 35.99% acid detergent fiber (ADF).

TABLE 1 Ingredient and chemical composition of experimental diets (DM basis).

Items	Contents [g/kg]			
	Control	LFJP	MFJP	HFJP
Ingredients				
Corn silage	500	500	500	500
Corn grain, ground	275	242	225.5	209
Wheat bran	34	19.6	12.4	5.2
Soybean meal	120	118	117	116
Cottonseed meal	40	39.4	39.1	38.8
Fermented jujube powder	0	50	75	100
Calcium carbonate	5	5	5	5
Salt	5	5	5	5
Calcium biphosphate	15	15	15	15
Sodium bicarbonate	5	5	5	5
Mineral and vitamin premix[a]	1	1	1	1
Chemical composition				
NEm (MJ/kg)	7.53	7.53	7.53	7.53
NEg (MJ/kg)	5.21	5.21	5.21	5.21
Crude protein	159.1	159.1	159.1	159.1
Neutral detergent fiber	324.2	325.6	326.3	326.9
Acid detergent fiber	177.4	178.5	179.0	179.6
cAMP mg/kg	-	0.9	1.35	1.8
Polysaccharides g/kg	2.01	4.03	5.15	6.18
Polyphenols mg/kg	5.24	10.46	13.17	15.53
Flavonoids mg/kg	27.63	83.85	111.04	140.13
Calcium	6.8	6.9	7.0	7.1
Phosphorus	4.3	4.2	4.2	4.2

[a]Contained per kg premix: 100 mg Co, 8,500 mg Cu, 50,000 mg Fe, 30,000 mg Mn, 30,000 mg Zn, 300 mg I, 300 mg Se, 7,500,000 IU vitamin A, 1,200,000 IU vitamin D, and 40,000 IU vitamin E.

2.2 Animals and experimental design

The study protocol was approved by the Animal Care and Use Committee of Lyuliang University and was conducted in accordance with the Guide for the Care and Use of Agricultural Animals in Research and Teaching (https://www.asas.org/services/ag-guide). Forty Simmental bulls [mean age, 311 ± 15.6 d; mean body weight (BW), 402 ± 7.2 kg] were grouped according to BW

and randomly allocated to one of the four treatment groups. Bulls in the control group were fed a basal diet without FJP, whereas those in the low-, medium-, and high-dose FJP groups (LFJP, MFJP, and HFJP, respectively) were fed isoenergetic and isonitrogenous diets with 5, 7.5, and 10% FJP (DM basis), respectively. The composition and nutrient levels of the diets are shown in Table 1. The amount of FJP was determined based on a report by Zhang et al. (13). The diets were formulated based on "Nutrient Requirements of Beef Cattle: Eighth Revised Edition" (17). The experimental period was 80 d, consisting of adaptation for 20 d and a feeding trial for 60 d. The treatments were administered during the adaptation period. All bulls were housed individually, fed at 07:00 and 19:00 h daily, and had free access to feed and water.

2.3 Growth performance analysis

During the feeding trial, every bull was weighed for two consecutive days before the morning diet on days 1 and 60. For each bull, the daily DM intake (DMI) was calculated as the difference between the offered and remaining feed. The feed conversion rate (FCR) was calculated as DMI/ADG.

2.4 Feed and feces analysis

Feed and feces were sampled on days 51–57. Fecal samples (~200 g) were collected from each bull, as described by Liu et al. (18). Each sample was dried at 65°C for 48 h and filtered through a 1-mm screen for chemical analysis. Measurements of DM (method 934.01), ash (method 942.05), CP (method 976.05), and ADF (method 973.18) were performed according to the Association of Official Analytical Chemists' guidelines (19). The amount of NDF was measured as previously described by Van Soest et al. (20). The digestibility indicator (acid-insoluble ash) was calculated as previously described by Van Van Keulen and Young (21).

2.5 Ruminal fluid collection and analysis

On days 58–59, the ruminal fluid of each bull was sampled, as described by Liu et al. (18). The pH of the ruminal fluid samples was measured using a pH meter (Sartorius Lab Instruments GmbH & Co. KG, Göttingen, Germany). The ruminal fluid samples were filtered through four layers of cheesecloth and the filtrates were collected. The filtrate (5 mL) was premixed with meta-phosphoric acid (1 mL; 250 g/L) and analyzed using gas chromatography (Trace 1300 gas chromatograph; Thermo Fisher Scientific, Waltham, MA, USA) with 2-ethylbutyric acid as an internal standard to estimate total VFA content of the sample (18). The filtrate (5 mL) was mixed with H_2SO_4 (1 mL; 20 g/L) and the ammonia nitrogen (NH_3-N) content of the sample was estimated in accordance with the Association of Official Analytical Chemists guidelines (19). The enzyme activities of ruminal fluids were determined as described by Agarwal et al. (22).

Microbial DNA was isolated from 1 mL aliquots of blended ruminal filtrate samples as described in a previous study (23). The quality and quantity of the isolated DNA were determined using agarose gel electrophoresis and spectrophotometry (NanoDrop™ 2000 Spectrophotometer; Thermo Fisher Scientific). Microbial DNA was amplified using quantitative real-time polymerase chain reaction (RT-qPCR) carried out on a StepOne™ Real-Time PCR System (Thermo Fisher Scientific) with the primers listed in Table 2 (24). Each 20 μL RT-qPCR reaction volume included 10 μL of SYBR Green Premix Ex Taq™ II (2×; Takara Bio, Inc., Shiga, Japan), 2 μL of template DNA, 0.8 μL of forward and reverse primers (10 μM), 0.4 μL of ROX Reference Dye II (50×; Thermo Fisher Scientific), and 6 μL of ultrapure water. The PCR conditions included a denaturation step at 95°C for 2 min, followed by 45 cycles of annealing at 95°C for 15 s and elongation at 60°C for 1 min. RT-qPCR products were purified using the MiniBEST DNA Fragment Purification Kit Ver. 4.0 [Takara Biomedical Technology (Beijing) Co., Ltd., Beijing, China] and quantified using a spectrophotometer. For each standard derived from the sample, the concentration of copy numbers was determined based on the length and mass of the PCR products. Standard trajectories of specific microorganisms were charted using serial 10-fold dilutions (25). To assess amplification specificity, the dissociation patterns of the PCR end-products were determined by increasing the temperature from 60 to 95°C at 1°C/30 s.

2.6 Blood collection and analysis

On day 60, blood samples were collected from each bull as described by Liu et al. (18). Total protein concentration, total antioxidant capacity (T-AOC), superoxide dismutase (SOD) activity, glutathione peroxidase (GSH-Px) activity, and malondialdehyde (MDA) content were measured using appropriate enzyme-linked immunosorbent assay kits (Thermo Fisher Scientific).

2.7 Calculations and statistical analysis

Trial data were analyzed using SAS software (SAS Institute, Inc., Cary, NC, USA) with a one-way analysis of variance option of the generalized linear model, and both linear and quadratic impacts were examined using orthogonal polynomials (26). A randomized block experiment format was utilized, which incorporated multiple measurements, as described by the equation $Y_{ij} = \mu + F_i + \varepsilon_{ij}$, where Y_{ij} is the dependent variable, μ is the aggregate mean, F_i is the constant impact of FJP addition (i = 0, 5, 7.5, and 10%), and ε_{ij} is the remaining error. Each bull was treated as a single unit in the experiment. Duncan's method was used for multiple comparisons, and significant differences were identified at $P < 0.05$.

3 Results

3.1 Growth performance

As shown in Table 3, dietary FJP supplementation did not affect the DMI of the bulls ($P > 0.05$). The initial and final BWs of the bulls were similar among the treatment groups ($P > 0.05$). The

TABLE 2 PCR primers for amplification of microbial DNA.

Target species	Primer sequence (5′- 3′)	GenBank accession no.	Size (bp)
Total bacteria	F: CGGCAACGAGCGCAACCC	AY548787.1	147
	R: CCATTGTAGCACGTGTGTAGCC		
Total fungi	F: GAGGAAGTAAAAGTCGTAACAAGGTTTC	GQ355327.1	120
	R: CAAATTCACAAAGGGTAGGATGATT		
Total protozoa	F: GCTTTCGWTGGTAGTGTATT	HM212038.1	234
	R: CTTGCCCTCYAATCGTWCT		
Ruminococcus albus	F: CCCTAAAAGCAGTCTTAGTTCG	CP002403.1	176
	R: CCTCCTTGCGGTTAGAACA		
Ruminococcus flavefaciens	F: ATTGTCCCAGTTCAGATTGC	AB849343.1	173
	R: GGCGTCCTCATTGCTGTTAG		
Butyrivibrio fibrisolvens	F: ACCGCATAAGCGCACGGA	HQ404372.1	65
	R: CGGGTCCATCTTGTACCGATAAAT		
Fibrobacter succinogenes	F: GTTCGGAATTACTGGGCGTAAA	AB275512.1	121
	R: CGCCTGCCCCTGAACTATC		
Ruminobacter amylophilus	F: CTGGGGAGCTGCCTGAATG	MH708240.1	102
	R: GCATCTGAATGCGACTGGTTG		
Prevotella ruminicola	F: GAAAGTCGGATTAATGCTCTATGTTG	LT975683.1	74
	R: CATCCTATAGCGGTAAACCTTTGG		

TABLE 3 Effects of fermented jujube powder (FJP) on production performance of bulls ($n = 40$).

Item	Treatment[a]				SEM	*P*-values		
	Control	LFJP	MFJP	HFJP		Treatment	Linear	Quadratic
DMI (kg/d)	10.70	10.72	10.81	10.73	0.100	0.069	0.196	0.078
Body weight (kg)								
0 d	404.4	401.6	402.0	404.1	4.179	0.329	0.933	0.070
60 d	468.6	467.4	470.5	470.2	5.978	0.640	0.364	0.816
ADG (kg/d)	1.071^{b}	1.097^{ab}	1.143^{a}	1.101^{ab}	0.057	0.035	0.076	0.049
FCR (kg/kg)	10.02^{a}	9.80^{ab}	9.48^{b}	9.74^{ab}	0.429	0.033	0.042	0.060

[a]Control, LFJP, MFJP, and HFJP groups contained respectively 0, 5, 7.5, and 10 g FJP/100 g DM. Different superscript letters in the same row differed significantly.

ADG and FCR of the bulls showed a quadratic increase ($P = 0.049$) and linear reduction ($P = 0.042$) by dietary FJP supplementation, respectively, with higher ADG ($P = 0.035$) and lower FCR ($P = 0.033$) in the MFJP group than those in the control group.

3.2 Total-tract nutrient digestibility and ruminal fermentation

As shown in Table 4, DM and CP digestibility increased quadratically with FJP supplementation ($P = 0.026$ and 0.041, respectively) and was highest in the MFJP group ($P = 0.015$ and 0.026, respectively). The total-tract apparent digestibility of organic matter and NDF were similar among the treatment groups ($P > 0.05$). ADF digestibility showed a linear increase ($P = 0.048$) with FJP supplementation and was greater in the MFJP group than that in the control group ($P = 0.041$).

Ruminal pH was similar between the treatment groups ($P > 0.05$). The rumen total VFA concentration increased quadratically ($P = 0.037$) with dietary FJP supplementation and was the highest in the MFJP group ($P = 0.032$). The acetate molar proportion showed a linear increase ($P = 0.002$) with FJP supplementation and was higher in the LFJP, MFJP, and HFJP groups than that in the control group ($P = 0.004$). FJP supplementation did not affect the molar proportions of propionate, butyrate, valerate, isobutyrate, or isovalerate ($P > 0.05$). The rumen acetate-to-propionate ratio increased linearly ($P = 0.001$) with FJP supplementation and was greater in the MFJP and HFJP groups than that in the control group ($P = 0.001$). NH_3-N concentrations decreased linearly ($P = 0.003$) with FJP supplementation and were lower in the MFJP and HFJP groups than that in the control group ($P = 0.020$).

TABLE 4 Effects of fermented jujube powder (FJP) on ruminal fermentation and nutrient digestibility in bulls ($n = 40$).

Item	Treatment[a]				SEM	*P*-values		
	Control	LFJP	MFJP	HFJP		Treatment	Linear	Quadratic
Apparent digestibility								
Dry matter	67.11^{b}	67.42^{b}	67.98^{a}	67.45^{b}	0.640	0.015	0.059	0.026
Organic matter	74.36	74.92	75.12	74.83	0.801	0.182	0.152	0.093
Crude protein	72.15^{b}	72.39^{b}	72.95^{a}	72.43^{b}	0.621	0.026	0.097	0.041
Neutral detergent fiber	58.72	59.02	59.64	59.42	1.169	0.309	0.108	0.481
Acid detergent fiber	53.78^{b}	54.08^{ab}	54.51^{a}	54.16^{ab}	0.583	0.041	0.048	0.068
Ruminal fermentation								
pH	6.46	6.44	6.43	6.42	0.046	0.373	0.090	0.786
Total VFA (mM)	116.48^{b}	118.15^{b}	122.77^{a}	117.94^{b}	5.163	0.032	0.191	0.037
Mol/100 mol								
Acetate (A)	66.14^{b}	66.85^{a}	66.93^{a}	66.97^{a}	0.623	0.004	0.002	0.059
Propionate (P)	20.78	20.67	20.42	20.41	0.383	0.067	0.120	0.677
Butyrate	9.34	8.86	9.24	9.09	0.548	0.233	0.634	0.348
Valerate	1.72	1.70	1.67	1.64	0.168	0.761	0.291	0.942
Isobutyrate	0.85	0.84	0.80	0.85	0.088	0.620	0.760	0.411
Isovalerate	1.19	1.08	0.94	1.04	0.213	0.081	0.057	0.118
A:P	3.18^{b}	3.23^{ab}	3.28^{a}	3.28^{a}	0.067	0.001	0.001	0.184
Ammonia N (mg/100 mL)	10.03^{a}	9.85^{ab}	9.65^{b}	9.66^{b}	0.326	0.020	0.003	0.318

[a]Control, LFJP, MFJP, and HFJP groups contained respectively 0, 5, 7.5, and 10 g FJP/100 g DM. Different superscript letters in the same row differed significantly.

3.3 Ruminal enzyme activity and microflora

As shown in Table 5, the activities of carboxymethyl cellulase, cellobiase, pectinase, and α-amylase in the rumen were unaffected by FJP supplementation ($P > 0.05$). The activities of xylanase and protease increased linearly with dietary FJP supplementation ($P = 0.006$ and 0.018, respectively). Furthermore, xylanase activity was higher in the MFJP and HFJP groups than that in the control group ($P = 0.031$). Protease activity was also elevated in the MFJP group compared to that in the control group ($P = 0.044$). FJP supplementation quadratically increased the populations of total bacteria, *Ruminococcus albus*, and *Ruminobacter amylophilus* ($P = 0.047$, 0.011, and 0.021, respectively), and their counts were higher in the MFJP group than those in the control group ($P = 0.026$, 0.043, and 0.047, respectively). The abundance of *Ruminococcus flavefaciens* increased linearly ($P = 0.009$) and was higher in the LFJP, MFJP, and HFJP groups than that in the control group ($P = 0.027$). FJP supplementation did not affect the abundance of fungi, protozoa, *Fibrobacter succinogenes*, *Butyrivibrio fibrisolvens*, or *Prevotella ruminicola* ($P > 0.05$).

3.4 Blood antioxidant properties

As shown in Table 6, the total blood protein concentration and T-AOC increased linearly ($P = 0.003$ and 0.018, respectively) with FJP supplementation. The total protein concentrations were higher in the MFJP and HFJP groups than that in the control group ($P = 0.012$). Serum T-AOC was greater in the MFJP group than that in the control group ($P = 0.017$). The activities of SOD and GSH-Px were unaffected by FJP supplementation ($P > 0.05$). The blood MDA concentration decreased linearly ($P = 0.006$) with FJP supplementation and was lower in the LFJP, MFJP, and HFJP groups than that in the control group ($P = 0.012$).

4 Discussion

4.1 Growth performance

Dietary supplementation with FJP did not affect the DMI of bulls, which was consistent with the results of Zhang et al. (13). The observed increase in ADG could be due to improved nutrient digestibility and a higher total rumen VFA concentration. The decrease in FCR suggests that FJP supplementation enhanced the efficiency of nutrient utilization in the bulls. The positive effect of FJP on protein metabolism, indicated by an increase in total blood protein, likely contributed to the high ADG.

Cyclic adenosine monophosphate (cAMP) plays key roles in several biological processes. It alters energy partitioning in animals and energy availability is reduced by proteolysis, which subsequently increases protein deposition (27). Probiotic fermentation of jujube powder increases the concentration of cAMP in jujube (7), indicating the potency of FJP in improving the growth performance of bulls. Additionally, jujuboside, a

TABLE 5 Effects of fermented jujube powder (FJP) on rumen microbial enzyme activity and microflora in bulls ($n = 40$).

Item	Treatment[a]				SEM	*P*-values		
	Control	LFJP	MFJP	HFJP		Treatment	Linear	Quadratic
Microbial enzyme activity[b]								
Carboxymethyl-cellulase	0.254	0.262	0.265	0.264	0.016	0.459	0.167	0.424
Cellobiase	0.166	0.170	0.178	0.174	0.013	0.194	0.081	0.342
Xylanase	0.816^{b}	0.820^{ab}	0.828^{a}	0.828^{a}	0.012	0.031	0.006	0.487
Pectinase	0.458	0.470	0.472	0.470	0.021	0.433	0.226	0.278
α-amylase	0.464	0.472	0.474	0.470	0.022	0.782	0.559	0.401
Protease	0.346^{b}	0.352^{ab}	0.360^{a}	0.356^{ab}	0.011	0.044	0.018	0.192
Ruminal microflora (copies/mL)								
Total bacteria, $\times 10^{11}$	3.90^{b}	4.12^{ab}	4.50^{a}	4.18^{ab}	0.461	0.026	0.051	0.047
Total anaerobic fungi, $\times 10^{8}$	3.10	3.26	3.56	3.48	0.536	0.203	0.058	0.475
Total protozoa, $\times 10^{8}$	2.14	2.36	2.35	2.25	0.278	0.242	0.407	0.067
R. albus, $\times 10^{8}$	5.14^{b}	6.45^{ab}	7.07^{a}	5.78^{ab}	1.637	0.043	0.244	0.011
R. flavefaciens, $\times 10^{9}$	3.99^{b}	4.70^{a}	4.93^{a}	4.89^{a}	0.813	0.027	0.009	0.118
F. succinogenes, $\times 10^{10}$	2.05	2.18	2.87	2.30	0.764	0.073	0.160	0.134
B. fibrisolvens, $\times 10^{9}$	4.22	4.29	4.43	4.16	1.845	0.990	0.990	0.779
P. ruminicola, $\times 10^{10}$	1.34	1.52	1.72	1.52	0.602	0.589	0.408	0.330
Rb. amylophilus, $\times 10^{8}$	1.06^{b}	1.45^{ab}	1.68^{a}	1.34^{ab}	0.510	0.047	0.115	0.021

[a]Control, LFJP, MFJP, and HFJP groups contained respectively 0, 5, 7.5, and 10 g FJP/100 g DM. Different superscript letters in the same row differed significantly.
[b]Enzymatic activity units were as follows: carboxymethyl-cellulase (μmol glucose/min/mL), cellobiase (μmol glucose/min/mL), xylanase (μmol xylose/min/mL), pectinase (μmol D-galacturonic acid/min/mL), α-amylase (μmol glucose/min/mL), and protease (μg hydrolyzed protein/min/mL).

TABLE 6 Effects of fermented jujube powder (FJP) on blood antioxidant activity in bulls ($n = 40$).

Item	Treatment[a]				SEM	*P*-values		
	Control	LFJP	MFJP	HFJP		Treatment	Linear	Quadratic
Total protein (μg/mL)	725^{b}	764^{ab}	798^{a}	791^{a}	57.09	0.012	0.003	0.158
T-AOC (U/mL)	6.68^{b}	6.75^{b}	6.97^{a}	6.84^{ab}	0.218	0.017	0.018	0.123
SOD (U/mL)	58.86	60.49	63.20	61.22	3.603	0.209	0.087	0.250
GSH-Px (U/mL)	52.10	53.53	54.94	53.22	3.739	0.415	0.375	0.191
MDA (mmol/L)	2.36^{a}	2.02^{b}	1.88^{b}	1.95^{b}	0.366	0.012	0.006	0.055

[a]Control, LFJP, MFJP, and HFJP groups contained respectively 0, 5, 7.5, and 10 g FJP/100 g DM. Different superscript letters in the same row differed significantly.

bioactive compound found in jujube, promotes cell proliferation by regulating the cAMP-protein kinase A/cAMP-response element binding protein (PKA/CREB) signaling pathway and hormone secretion (6). Bioactive substances in jujube, including oligosaccharides and flavonoids, inhibit the proliferation of harmful bacteria, increase the abundance of probiotics in the intestine, inhibit the competition for nutrients between bacteria and the host, and improve the absorption and utilization of feed nutrients (9). A previous study on sows has demonstrated that FJP supplementation promotes protein utilization and ADG (11). However, in the present study, ADG did not increase further in the HFJP group, which is consistent with the observed nutrient digestibility and rumen VFA concentration. These findings suggest that the HFJP diet interferes with microbial growth in the rumen, resulting in decreased nutrient digestion in bulls. A previous study has shown that excessive FJP in diets result in high proliferation of *Lactobacillus* in the rumen, which could inhibit cell growth by disrupting the cell cycle (28). Moreover, Zhang et al. showed reduced ADG in Holstein dairy cattle fed 15% FJP diets compared with the control group (13). Together, these findings suggest that while FJP is beneficial in moderate amounts, excessive supplementation can be counterproductive. Therefore, careful consideration of the appropriate dosage is essential to maximize the benefits of FJP supplementation in animal diets.

4.2 Nutrient digestibility and ruminal fermentation

The increased digestibility of DM, CP, and ADF in the total tract was consistent with the increase in total VFA concentration and acetate molar proportion in the rumen, indicating that dietary

FJP supplementation enhanced rumen nutrient degradation. A previous study on ruminants has shown that FJP increases the effective degradability of CP, EE, and ADF in the rumen (14). Furthermore, FJP alleviates digestive tract injury and promotes nutrient digestion and absorption attributed to its polysaccharide, flavonoid, and cAMP contents (13, 29). Probiotics secrete various digestive enzymes that degrade macromolecules, and the resulting metabolites enhance gastrointestinal peristalsis (30). Together, these findings indicate that dietary FJP supplementation improves the apparent nutrient digestion in the total tract. Our findings also revealed that FJP maintained rumen pH in the range of 6.42–6.46, which is conducive to microbial growth (31). Furthermore, an increase in the acetate-to-propionate ratio indicates a shift in rumen fermentation toward more acetate production. High total VFA concentrations and acetate molar proportions in the rumen are associated with increased xylanase activity, as jujube is degraded to acetate by cellulolytic enzymes (15). Similarly, dietary probiotic complexes have been found to increase rumen VFA concentrations and acetate-to-propionate ratio in sheep (32). Rumen NH_3-N is mainly derived from feed CP degradation by rumen proteases (33). However, the decrease in rumen NH_3-N concentration by FJP supplementation was inconsistent with the observed increase in protease activity, which could be associated with increased microbial protein content, as cellulolytic bacteria utilize NH_3-N for protein synthesis (34). These findings collectively indicate that FJP supplementation positively influences rumen fermentation and nutrient digestibility, leading to better nutrient absorption and utilization in bulls.

4.3 Ruminal enzyme activity and microflora

The observed increase in xylanase activity coincided with an increase in the population of cellulolytic bacteria, indicating that FJP supplementation stimulated the secretion of enzymes by microbes. Cellulolytic bacteria produce enzymes that degrade fibers (15). The increase in protease activity is associated with an increase in the proportion of *Ruminobacter amylophilus*, which is responsible for protein degradation (35). These results suggest that dietary FJP supplementation promotes the growth of rumen microorganisms and enzyme secretion in bulls. Additionally, cAMP promotes cell proliferation via the PKA/CREB signaling pathway (6). FJP supplementation has been reported to promote the colonization of probiotics in the gastrointestinal environments and regulate the balance of microflora through biological oxygen capture and competitive inhibition (36). Probiotics can prevent the colonization of pathogens by niche pre-emption (37). Dietary supplementation with probiotic complexes has been found to increase the abundance of *Ruminococcus albus*, *F. succinogenes*, *Butyrivibrio fibrisolvens*, and *P. ruminicola* in the rumen of bulls (30). Consistently, a previous study has shown that probiotic supplementation increases the proportions of *Butyrivibrio fibrisolvens* and *Ruminococcus flavefaciens* in the rumen of sheep at a dietary concentrate-to-roughage ratio of 3:7 (38). Together, these findings suggest that FJP serves as a valuable dietary supplement to improve rumen microbes and rumen fermentation in livestock.

4.4 Blood antioxidant properties

Oxidative stress in farming bulls is a major concern because it negatively affects performance and meat quality and is associated with high morbidity. In this study, serum T-AOC significantly increased with FJP supplementation, consistent with the results of Zhang et al. (13). Additionally, Xu et al. (7) reported that the hydroxyl groups of polyphenols in jujube can chelate Cu^{2+} and Fe^{2+} to reduce oxidation rates, whereas the phenolic hydroxyl groups of flavonoids form stable semiquinone free radical structures, reducing free radical accumulation. The high polyphenol content in FJP is attributed to the release of bound phenols from the cell wall, which are degraded by cellulases secreted by *Bacillus* spp. and *Lactobacillus plantarum* (39). These findings suggest that an increase in T-AOC due to FJP supplementation may conserve the protein content and improve the production performance of bulls.

5 Conclusion

In conclusion, this study demonstrates that dietary supplementation with FJP has beneficial effects on multiple aspects of production performance, rumen fermentation, and antioxidant properties in Simmental bulls. Our findings indicate that FJP enhances ADG and improves feed conversion efficiency, suggesting improved nutrient utilization and growth. The observed changes in rumen fermentation, including increases in total VFA and acetate molar proportion, along with reduced NH3-N concentrations, highlight the role of FJP in optimizing digestive processes and nutrient metabolism. Furthermore, the significant improvements in blood T-AOC and reduction in MDA levels indicate enhanced antioxidant defenses, potentially reducing oxidative stress in supplemented bulls. These results underscore the potential of FJP as a valuable dietary supplement for enhancing growth performance in cattle farming practices. Future research should further explore optimal dosages and long-term effects of FJP supplementation across different cattle breeds and production environments to fully leverage its benefits in livestock management strategies.

Data availability statement

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

Ethics statement

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

Author contributions

YL: Conceptualization, Data curation, Formal analysis, Funding acquisition, Methodology, Software, Validation, Writing – original draft, Writing – review & editing. GW: Conceptualization, Supervision, Validation, Writing – review & editing. RW: Conceptualization, Data curation, Validation, Writing – original draft. XZ: Conceptualization, Validation, Writing – original draft. CF: Project administration, Resources, Writing – review & editing. YH: Investigation, Writing – original draft. PC: Formal analysis, Investigation, Supervision, Writing – review & editing.

Funding

The author(s) declare financial support was received for the research, authorship, and/or publication of this article. This work was supported by the Scientific and Technological Plan Programs of Lüliang City in Shanxi (2021NYGG-2–65).

Acknowledgments

The authors wish to thank the staff of the Hong Kang Mu Ye Farm (Fangshan, China) for assistance in feeding and care of the animals. We would like to thank Editage (www.editage.cn) for English language editing.

Conflict of interest

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

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fvets.2024.1442244/full#supplementary-material

References

1. Ay C, Ülger I, Kaliber M, Hizlisoy H, Ayasan T. Effects of Shiitake mushroom (*Lentinus edodes*) supplementation into quail diets on performance, blood serum parameters and intestine microbial populations. *J Hellenic Vet Med Soc.* (2023) 74:5705–12. doi: 10.12681/jhvms.30094

2. Ayasan T, Ulger I, Cil AN, Tufarelli V, Laudadio V, Palangi V. Estimation of chemical composition, *in vitro* gas production, metabolizable energy, net energy lactation values of different peanut varieties and line by Hohenheim *in vitro* gas production technique. *Semina: Ciências Agrárias.* (2021) 42:907–20. doi: 10.5433/1679-0359.2021v42n2p907

3. Ulger I, Kaliber M, Hizlisoy H, Ayasan T. Effects of Reishi mushroom (*Ganoderma lucidum*) powder inclusion into quail Diets on animal performance, carcass traits, intestinal microflora and serum parameters. *J Hellenic Vet Med Soc.* (2023) 74:5829–36. doi: 10.12681/jhvms.30440

4. Ülger I, Kaliber M, Beyzi SB, Konca Y. Possible ensiling of pumpkin (*Cucurbita pepo*) residues. *Turk J Vet Anim Sci.* (2020) 44:853–9. doi: 10.3906/vet-2002-81

5. Ekizoglu H, Ülger Y, Kaliber M, Ayaşan T. Effects of Spirulina (Algae) supplementation to Japanese Quail (Coturnix Japonica) diets on growth performance and carcass traits. *Indian J Anim Sci.* (2020) 90:923–7. doi: 10.56093/ijans.v90i6.105006

6. Chen JP, Liu XY, Li ZG, Qi AR, Yao P, Zhou ZY, et al. A review of dietary ziziphus jujuba fruit (jujube): developing health food supplements for brain protection. *Evid Based Complement Altern Med.* (2017) 2017:3019568. doi: 10.1155/2017/3019568

7. Xu T, Zhou XL, Degen A, Yin JL, Zhang SX, Chen N. The inclusion of jujube by-products in animal feed: a review. *Sustainability.* (2022) 14:7882. doi: 10.3390/su14137882

8. Gao QH, Wu CS, Wang M. The jujube (ziziphus jujuba mill) fruit: a review of current knowledge of fruit composition and health benefits. *J Agric Food Chem.* (2013) 61:3351–63. doi: 10.1021/jf4007032

9. Xie B, Zhang CX, Zhang JX. Growth performance, nutrient digestibility, carcass traits, body composition, and meat quality of goat fed Chinese jujube fruit as a replacement for maize in diet. *Anim Feed Sci Technol.* (2018) 246:127–36. doi: 10.1016/j.anifeedsci.2018.10.005

10. Sharifi M, Bashtani M, Naserian AA, Farhangfar H. The effect of feeding low quality date palm (*Phoenix dactylifera* L) on the performance, antioxidant status and ruminal fermentation of mid lactating saanen dairy goats. *Small Ruminant Res.* (2015) 130:95–100. doi: 10.1016/j.smallrumres.2015.07.031

11. Huang Y. *Effects of Jujube Powder and Fermented Jujube Powder on Production Performance, Blood Indicators and Fecal Microbes Flora in Sows* (Master's Thesis). Northwest A and F University, Xianyang, China (2018).

12. Zhang LH, Zhang M, Mujumdar AS. New technology to overcome defects in production of fermented plant products a review. *Trends Food Sci Technol.* (2021) 116:829–41. doi: 10.1016/j.tifs.2021.08.014

13. Zhang JP, Li RY, Li SJ, Li JG, Shen YZ, Li QF, et al. Effects of dietary fermented jujube powder on growth performance, immune performance and antioxidant performance of Holstein bulls during hot season. *Chin J Anim Nutr.* (2022) 34:1027–39. doi: 10.3969/j.issn.1006-267x.2022.02.034

14. Yan JL. *Effect of Fermented Jujube Powder on Growth Performance, Nutrient Apparent Digestibility and Slaughter Performance of Holstein Bulls in Fattening Period* (Master's Thesis). Agricultural University of Hebei, Baoding, China (2018).

15. Flint HJ. The rumen microbial ecosystem-some recent developments. *Trends Microbiol.* (1997) 5:483–8. doi: 10.1016/S0966-842X(97)01159-1

16. Qiao GH, Shan AS, Ma N, Ma QQ, Sun ZW. Effect of supplemental Bacillus cultures on rumen fermentation and milk yield in Chinese Holstein cows. *J Anim Physiol Anim Nutr.* (2010) 94:429–36. doi: 10.1111/j.1439-0396.2009.00926.x

17. National Academies Press. *Nutrient Requirements of Beef Cattle*. 8th ed. Washington, DC: National Academies Press (2016).

18. Liu YQ, Wang C, Liu C, Zhang J, Liu Q. Effects of coated folic acid and coated methionine on growth performance, nutrient digestibility and rumen fermentation in Simmental bulls. *Anim Feed Sci Technol.* (2023) 298:115596. doi: 10.1016/j.anifeedsci.2023.115596

19. Association of Official Analytical Chemists International. *Official Methods of Analysis*. 18th ed. Gaithersburg, MD: Association of Official Analytical Chemists International (2006).

20. Van Soest PJ, Robertson JB, Lewis BA. Methods for dietary fiber, neutral detergent fiber and non-starch polysaccharides in relation to animal nutrition. *J Dairy Sci.* (1991) 74:3583–97. doi: 10.3168/jds.S0022-0302(91)78551-2

21. Van Keulen J, Young BA. Evaluation of acid-insoluble ash as a natural marker in ruminant digestibility studies. *J Anim Sci.* (1977) 44:282–9. doi: 10.2527/jas1977.442282x

22. Agarwal N, Kamra DN, Chaudhary LC. Microbial status and rumen enzyme profile of crossbred calves fed on different microbial feed additives. *Lett Appl Microbiol.* (2002) 34:329–36. doi: 10.1046/j.1472-765X.2002.01092.x

23. Yu Z, Morrison M. Improved extraction of PCR-quality community DNA from digesta and fecal sample. *Bio Techniq.* (2004) 36:808–12. doi: 10.2144/04365ST04

24. Denman SE, McSweeney CS. Development of a real-time PCR assay for monitoring anaerobic fungal and cellulolytic bacterial populations within the rumen. *FEMS Microbiol Ecol.* (2006) 58:572–82. doi: 10.1111/j.1574-6941.2006.00190.x

25. Kongmun P, Wanapat M, Pakdee P, Navanukraw C. Effect of coconut oil and garlic powder on *in vitro* fermentation using gas production technique. *Livest Sci.* (2010) 127:38–44. doi: 10.1016/j.livsci.2009.08.008

26. SAS Institute Inc. *SAS/STAT® 9.0 User's Guide*. Cary, NC: SAS Institute Inc. (2002).

27. Aslam M, Ladilov Y. Emerging role of cAMP/AMPK signaling. *Cells.* (2022) 11:308. doi: 10.3390/cells11020308

28. Ahmed LA, Schirmer H, Berntsen GK. Self-reported diseases and the risk of non-vertebral fractures: the Tromsø study. *Osteoporosis Int.* (2006) 17:46–53. doi: 10.1007/s00198-005-1892-6

29. Mozsik G, Fiegler M, Lörincz P, Nagy L, Tornok F. The role of the ATP-adenylate cyclase-cAMP system and its pharmacological regulation in the development of gastric hypersecretion and ulceration. *Acta med Acad Sci Hung.* (1979) 36:427–48.

30. Liu YQ, Liu C, Feng ZW, Zhang K, Liu Q. Effects of complex probiotic preparation and inulin on growth performance, nutrient digestion, rumen fermentation and blood metabolism of finishing bulls. *Chin J Anim Nutr.* (2022) 34:2446–56. doi: 10.3969/j.issn.1006-267x.2022.04.039

31. Russell JB, Wilson DB. Why are ruminal cellulolytic bacteria unable to digest cellulose at low pH? *J Dairy Sci.* (1996) 79:1503–9. doi: 10.3168/jds.S0022-0302(96)76510-4

32. Zhang ZJ. *Effect of Dietary Compound Profosotks Oe Rumen Fermentation, Seriiib Biochemistry of Sheep and Productive Performance of Dairy Cow* (Master's Thesis). Xinjiang Agricultural University, Wulumuqi, China (2012).

33. Firkins JL, Yu Z, Morrison M. Ruminal nitrogen metabolism: perspectives for integration of microbiology and nutrition for dairy. *J Dairy Sci.* (2007) 90:E1–16. doi: 10.3168/jds.2006-518

34. Russell JB, O'Connor JD, Fox DG, Van Soest PJ, Sniffen CJ. A net carbohydrate and protein system for evaluating cattle diets: ruminal fermentation. *J Anim Sci.* (1992) 70:3551–61. doi: 10.2527/1992.70113551x

35. Schweikert M, Fujishima M, Görtz HD. Symbiotic associations between ciliates and prokaryotes. In: *The Prokaryotes.* Berlin; Heidelberg: Springer (2013). p. 427–63.

36. Vandana R, Yadav B, Lakhani G. Application of probiotic and prebiotic in animals production: a review. *Environ Ecol.* (2013) 31:873–6. Available online at: https://api.semanticscholar.org/CorpusID:85964792

37. Du YF, Gao Y, Hu MY, Hou JX, Yang LH, Wang XH, et al. Colonization and development of the gut microbiome in calves. *J Anim Sci Biotechnol.* (2023) 14:46. doi: 10.1186/s40104-023-00856-x

38. Xie MX, Li CR, Yang XD. Effects of compound probiotics on rumen microbial population in sheep fed different concentrate to roughage ratio diets. *Chin J Anim Nutr.* (2020) 32:4418–28. doi: 10.3969/j.issn.1006-267x.2020.09.053

39. De-Souza EL, De-Albuquerque TMR, Dos-Santos AS. Potential interactions among phenolic compounds and probiotics for mutual boosting of their health promoting properties and food functionalities, a review. *Crit Rev Food Sci Nutr.* (2019) 59:1645–59. doi: 10.1080/10408398.2018.1425285

frontiers | Frontiers in Veterinary Science

TYPE Original Research
PUBLISHED 01 August 2024
DOI 10.3389/fvets.2024.1441431

OPEN ACCESS

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RECEIVED 31 May 2024
ACCEPTED 22 July 2024
PUBLISHED 01 August 2024

CITATION
Luo J, Jiang J, Duan H, Zhang H, Sun M, Mao S and Shen J (2024) Comparative effects of nisin and monensin supplementation on growth performance, rumen fermentation, nutrient digestion, and plasma metabolites of fattening Hu sheep.
Front. Vet. Sci. 11:1441431.
doi: 10.3389/fvets.2024.1441431

Comparative effects of nisin and monensin supplementation on growth performance, rumen fermentation, nutrient digestion, and plasma metabolites of fattening Hu sheep

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Introduction: This study was conducted to compare the effects of nisin (NIS) and ionophore antibiotic monensin (MON) on the growth performance, rumen fermentation, nutrient digestion and plasma metabolites of fattening Hu sheep.

Methods: Thirty-six male Hu sheep (23.5 ± 1.0 kg) were divided into two blocks based on BW (low BW and high BW). Sheep within each block were then allotted to 9 pens respectively (two sheep/pen). Pens within each block were randomly assigned to one of three dietary treatments: (1) basal diet (CON); (2) basal diet + 40 mg/kg DM of MON; (3) basal diet + 274.5 mg/kg DM of NIS. The study lasted 9 weeks, with the initial 2 weeks for adaptation and the subsequent 7 weeks for treatment.

Results: The results showed that both NIS and MON addition had no impacts on average daily gain (ADG), dry matter intake (DMI), and feed conservation rate (G:F) of sheep ($p > 0.05$). The digestibility of ether extract (EE) was lower in the MON-fed and NIS-fed sheep ($p < 0.01$) than in the CON group, whereas crude protein (CP) digestibility was higher in the MON-fed sheep compared to those fed NIS ($p < 0.05$). Both NIS and MON supplementation decreased acetate levels and acetate/propionate ratio in the rumen of Hu sheep ($p < 0.05$). Sheep fed MON exhibited higher total cholesterol concentrations ($p < 0.05$) compared to the CON and NIS groups. However, there were no significant differences in other plasma metabolites, including blood urea nitrogen (BUN), total bile acid, triglyceride, total protein, albumin, globulin, glucose, etc., among the three groups ($p > 0.05$).

Discussion: In conclusion, dietary addition of NIS and MON altered the rumen fermentation mode by reducing acetate levels, with no discernible effects on the growth performance of the fattening Hu sheep.

KEYWORDS

nisin, fattening Hu sheep, growth performance, rumen fermentation, nutrient digestibility, monensin

1 Introduction

The increasing demand for livestock products such as milk, beef, and mutton has driven a shift toward large-scale and intensive livestock production, leading to higher livestock farming intensity and an elevated prevalence of diseases among livestock (1). Antibiotics are commonly utilized to enhance feed conservation rate and promote animal growth to meet societal demands for animal products and maximize economic benefits (2). In ruminant production, monensin (MON), the most widely employed antibiotic, is frequently used to modulate rumen fermentation, mitigate energy and nitrogen losses, and enhance feed conservation rate. Numerous studies have demonstrated that MON can optimize rumen fermentation patterns, suppress the production of methane, ammonia, and lactic acid in the rumen (3), and enhance the growth performance of ruminants (4). Nevertheless, the excessive use of antibiotics has led to the emergence of bacterial resistance and drug residue issues. In light of safety concerns, many countries have prohibited the use of antibiotics as dietary growth promoters for animals (5). Therefore, there is an urgent necessity to explore environmentally friendly, safe, effective, and residue-free alternatives to antibiotics to address this challenge.

Nisin (NIS), a 34-amino acid polypeptide antimicrobial substance, primarily inhibits gram-positive bacteria. It is the most extensively researched bacteriocin and has been approved by the FDA and the European Union as a food preservative (6, 7). Initially utilized in the food industry, NIS has demonstrated significant potential as an antibiotic alternative in recent years within the farming industry (8, 9). Prior studies have shown that NIS can enhance broiler growth performance through the modulation of gut microbiota composition, reduction of inflammatory responses, and intestinal apoptosis (7). Notably, Pogány Simonová et al. (8) observed a 9.4% increase in the average daily gain (ADG) of broiler rabbits fed NIS compared to a control group. Research in pigs has also indicated that NIS can influence the intestinal functional microbiota involved in acetate, butyrate, and propionate synthesis (10). Presently, most investigations on NIS in ruminants are conducted *in vitro* (11–13), and limited knowledge exists regarding its *in vivo* effects.

Previous *in vitro* studies have shown that both NIS and MON at appropriate concentrations can alter rumen fermentation patterns and increase propionate concentrations (11, 12). Unlike MON, NIS inhibited methane production without affecting dry matter disappearance rates (12). However, subsequent *in vivo* experiments, following the *in vitro* results, demonstrated that incorporating 30.5 mg/kg dry matter (DM) of NIS into the diet had no significant impact on the growth performance (14) and rumen microbiota of fattening Hu sheep (15). Notably, the *in vitro* artificial rumen lacks the complexity of the actual rumen environment in terms of substrates and microorganisms (16). It is hypothesized that the lack of the anticipated effects in previous animal experiments may be due to the insufficient dosage of NIS supplementation (14). Therefore, this study aimed to investigate the effects of a higher dose of NIS (274.5 mg/kg DM) on rumen fermentation and growth performance in fattening Hu sheep compared to MON.

2 Materials and methods

2.1 Animals, diets, and experimental design

The experimental procedures used in this study were approved by the Animal Care and Use Committee of Nanjing Agricultural University (protocol number: SYXK2017-0007).

Thirty-six healthy male Hu sheep (23.0 ± 1.0 kg) at the age of 3 months were divided into two blocks (18 sheep per block) based body weight (low BW: 22.3 ± 0.5 kg; high BW: 23.8 ± 0.6 kg). Within each block, the sheep were further divided into 9 pens (2 sheep/pen), and then randomly assigned to one of the three treatments: (1) basal diet (CON); (2) basal diet +40 mg/kg DM of MON; (3) basal diet +274.5 mg/kg DM of NIS. The NIS (Zhejiang New Yinxiang Bioengineering Co., Ltd.) used in this study contained 2.5% NIS with an activity of $\geq 1 \times 10^6$ IU/g. The MON premix (Shandong Shengli Bioengineering Co., Ltd.) added to the MON treatment consisted of monensin sodium at a concentration of 20 g per 100 g of premix.

The basal diet was designed in accordance with the Feeding Standard of Meat Sheep (2004) issued by the Ministry of Agriculture of the People's Republic of China (Table 1). The TMR was fed twice daily at 08:00 and 16:00 in all treatment groups, with a surplus of 5%–10% being ensured. All sheep were housed indoors pens (2 m × 4 m) with wooden slatted floors and had free access to drinking water. The study lasted for 9 weeks, incorporating a 2-week acclimatization phase followed by a 7-week experimental period.

2.2 Sampling and measurement

The diet provided and refusals were measured daily during weeks 2, 4, 5, and 7 to calculate dry matter intake (DMI). Prior to morning

TABLE 1 Composition and nutrient levels of basal diets (DM basis).

Item	Content
Ingredient (% of DM)	
Corn silage	23.00
Peanut vine	22.00
Corn grain	30.00
Soybean meal	7.00
DDGS	12.00
Wheat bran	2.00
Urea	0.75
NaCl	0.75
$NaHCO_3$	1.00
$CaCO_3$	1.00
$CaHPO_4$	0.37
Mineral Premix[1]	0.10
Vitamin Premix[2]	0.03
Nutrient composition, %	
DM	49.7
CP	15.71
NDF	30.38
ADF	17.92
EE	3.51
Ash	9.48
DE, MJ/kg[3]	13.25

[1]Trace mineral element content per kg of premix: Cu 18,000 mg, Fe 35,500 mg, Co 300 mg, I 1,650 mg, Se 340 mg, Zn 42,000 mg, Mn 36,000 mg. [2]Vitamin content per kg of premix: VA 20,040,000 IU, VD_3 6,600,000 IU, VE 200,000 IU. [3]DE was a calculated value, while the others were measured values.

feeding, the Hu sheep were weighed over 2 consecutive days in weeks 0, 4, and 7. These data were utilized to compute the average daily gain (ADG) of the sheep and the feed conservation rate (Gain:Feed, G:F), calculated as the ratio of ADG to DMI.

Ruminal fluid was obtained via an oral stomach tube around 3 h post morning feeding on the 5th day of weeks 4 and 7. The initial 50 mL of rumen fluid was discarded to minimize potential saliva contamination. Subsequently, the ruminal fluid was filtered through four layers of cheesecloth, and the pH of each sample was promptly measured using a portable pH-meter (Ecoscan pH-5, Eutech Instruments, Singapore). One milliliter of each ruminal fluid sample was preserved with the addition of 0.2 mL of 25% HPO_3 for VFA analysis using gas chromatography (GC-2014AFsc, Shimadzu, Japan) as described by Shen et al. (12). Another 1 mL of each ruminal fluid sample was stored at −20°C for subsequent analysis for ammonia nitrogen using a colorimetric method (17).

Diet and ort samples were collected continually for 2 days in weeks 2, 4, 5, 7. The feeds were mixed thoroughly using the quadrat method and stored in a sealed container at −20°C. Over the final 3 days of the study, feces samples were collected twice daily and stored in sealed containers at −20°C. The feed samples from each group and fecal samples from each sheep were combined for three consecutive days, subsequently dried in an oven for 48 h at 65°C. The dried samples were pulverized through a 1-mm screen utilizing a Cyclotec mill (Tecator 1093; Tecator AB, Höganäs, Sweden). Subsequently, all samples underwent analysis for dry matter (DM) (18), organic matter (OM) (18), and crude protein (CP) (18). The contents of ADF and NDF were determined according to Van Soest et al. (19). The acid-insoluble ash (AIA) in both the diet and fecal samples was measured according to the procedure outlined by Van Keulen and Young (20), which served as internal markers for estimating apparent nutrient digestibility.

On the 6th day of weeks 4 and 7 at approximately 3 h after morning feeding, blood samples were drawn from the jugular vein of each sheep into blood collection tube containing sodium heparin. The sample was then centrifuged at 3,000 × g/min for 10 min to separate plasma. Subsequently, all plasma samples were stored at −20°C for subsequent analysis of blood urea nitrogen (BUN), glucose, total protein (TP), albumin, creatinine (CRE), total cholesterol (TCHO), triglyceride (TG), total bile acid (TBA), alkaline phosphatase (ALP), aspartate aminotransferase (AST) and alanine aminotransferase (ALT) using commercial kits (Nanjing Jiancheng Technology Co., Ltd.). The Globulin levels were calculated by determining the difference between TP and albumin.

2.3 Statistical analyses

The statistical analyses were performed using the mixed model procedure of SAS version 9.4. The observational unit was defined as the pen when measurements were taken collectively for all aspects related to the pen, including DMI, ADG, feed conservation rate, nutrient intake, and apparent digestibility. Conversely, the observational unit was considered as the individual sheep when measurements were conducted on each lamb within the pen for parameters like BW, rumen fermentation characteristics, and plasma metabolites. Data for DMI, ADG, feed conservation rate, rumen fermentation characteristics, and plasma metabolites were analyzed with week as repeated measures. The statistical models incorporated fixed effects of treatment and week, together with their interaction, along with random effects of block, pen × block × treatment, and lambs within the pen × block × treatment. In contrast, data for BW, nutrients intake and apparent digestibility, the statistical models comprised fixed effects of treatment, along with random effects of block, pen × block × treatment, and lambs within the pen × block × treatment. When the pen served as the observational unit, the random effects in the aforementioned models were simplified to block and pen × block × treatment. Degrees of freedom were determined utilizing the Kenward-Roger option. Differences were considered to be statistically significant when the p-values were ≤ 0.05, and trends were declared at $0.05 < p \leq 0.10$. All reported values are least squares means unless otherwise stated.

3 Results

3.1 Growth performance

No difference ($p > 0.05$) in initial BW were observed across all treatments (Table 2). Dietary NIS and MON addition had no effect ($p > 0.05$) on the final BW, ADG, DMI and feed conservation rate of fattening Hu sheep. Nonetheless, the ADG of 267.8 g/d was the highest among fattening Hu sheep fed with MON compared to the other two groups.

3.2 Rumen fermentation characteristics

The inclusion of dietary NIS and MON resulted in a significant decrease ($p < 0.05$) in the concentration of acetate and the acetate/propionate ratio in the rumen of the fattening Hu sheep (Table 3), accompanied by a tendency toward decreased butyrate concentrations ($p = 0.07$). Notably, the NIS group exhibited significantly lower isobutyrate concentrations compared to the other treatment groups ($p < 0.05$). However, the dietary addition of NIS and MON had no impact on rumen pH, NH_3-N, TVFA, propionate, valerate, isovalerate and TBCFA concentrations in fattening Hu sheep ($p > 0.05$).

3.3 Nutrients intake and apparent digestibility

Dietary NIS and MON addition had no effect ($p > 0.05$) on the intake of DM, OM, CP, NDF, ADF and EE in fattening Hu sheep (Table 4). Both NIS and MON groups notably reduced the digestibility of EE ($p < 0.05$) in comparison to the CON group (Table 4). Furthermore, the MON group exhibited significantly higher digestibility of CP in contrast to the NIS group ($p < 0.05$). However, there were no differences in the digestibility of DM, OM, NDF and ADF among the treatments ($p > 0.05$).

3.4 Plasma metabolites

Compared with the CON and NIS groups, MON addition significantly elevated the blood levels of TCHO in Hu sheep ($p < 0.05$;

TABLE 2 Comparative effects of nisin and monensin supplementations on growth performance of fattening Hu sheep.

Item	CON	MON	NIS	SEM	*p*-value		
					Trt	Wk	Trt × Wk
BW, kg					-	-	-
Initial	22.92	23.01	23.17	0.792	0.54	-	-
Final	35.76	36.23	35.75	0.796	0.74	-	-
DMI, g/d	1194.9	1178.5	1180.8	46.88	0.95	<0.01	0.76
ADG, g/d	260.8	267.8	255.3	9.30	0.65	<0.01	0.79
G:F	0.22	0.23	0.22	0.006	0.37	<0.01	0.40

CON, control (no additives); MON, monensin, 40 mg/kg DM; NIS, nisin, 274.5 mg/kg DM; Trt, treatment; Wk, week; Trt × Wk, interaction of treatment and week; G:F, Gain:Feed.

TABLE 3 Comparative effects of nisin and monensin supplementations on ruminal fermentation of fattening Hu sheep.

Item	CON	MON	NIS	SEM	*p*-value		
					Trt	Wk	Trt × Wk
pH	6.37	6.46	6.52	0.079	0.20	0.49	0.89
NH_3-N, mg/dL	13.82	11.79	14.43	1.955	0.52	0.06	0.07
Total VFA, mM	97.9	90.8	88.6	3.27	0.16	0.83	0.86
Acetate, mM	62.0^{a}	55.4^{b}	54.8^{b}	2.03	0.05	0.92	0.88
Propionate, mM	20.9	22.2	20.6	0.94	0.47	0.95	0.64
A:P	2.99^{a}	2.52^{b}	2.70^{b}	0.082	<0.01	0.62	0.78
Butyrate, mM	11.91	10.09	10.27	0.532	0.07	0.14	0.35
Valerate, mM	1.08	0.95	0.94	0.059	0.21	0.97	0.89
Isobutyrate, mM	0.77^{a}	0.77^{a}	0.65^{b}	0.036	0.04	0.35	0.88
Isovalerate, mM	1.21	1.45	1.26	0.096	0.24	0.06	0.86
TBCVFA, mM	1.98	2.22	1.91	0.126	0.22	0.27	0.86

CON, control (no additives); MON, monensin, 40 mg/kg DM; NIS, nisin, 274.5 mg/kg DM; Trt, treatment; Wk, week; Trt × Wk, interaction of treatment and week; A:P, acetate:propionate; TBCVFA, total branched-chain VFA. ^{a,b}Means within a row with different superscripts differ ($p < 0.05$).

TABLE 4 Comparative effects of nisin and monensin supplementations on nutrient digestion of fattening Hu sheep.

Item	CON	MON	NIS	SEM	*p*-value
Intake, g/d					
DM	1302.5	1322.8	1289.9	90.5	0.92
OM	1179.3	1197.3	1167.8	81.91	0.92
CP	203.6	208.4	200.6	14.12	0.83
NDF	392.1	398.6	393.0	27.37	0.96
ADF	228.6	235.4	234.7	16.14	0.88
EE	45.6	46.2	45.1	3.16	0.93
Digestibility, %					
DM	72.7	73.0	72.5	0.80	0.93
OM	77.8	78.3	77.6	0.71	0.79
CP	73.9^{ab}	75.3^{a}	73.2^{b}	0.51	0.03
NDF	53.8	55.7	52.0	1.66	0.31
ADF	52.5	54.1	53.2	1.84	0.84
EE	84.9^{a}	80.0^{b}	78.0^{b}	1.25	<0.01

CON, control (no additives); MON, monensin, 40 mg/kg DM; NIS, nisin, 274.5 mg/kg DM. ^{a,b}Means within a row with different superscripts differ ($P < 0.05$).

Table 5). However, the addition of NIS and MON in the diet had no effect ($p > 0.05$) on the plasma levels of ALP, ALT, AST, BUN, CRE, glucose, TBA, TG, TP, albumin, and globulin in fattening Hu sheep (Table 5).

4 Discussion

4.1 Growth performance

MON is a commonly used antimicrobial growth promoter in livestock production aimed at enhancing feed conservation rate. The mechanism by which MON improves energy efficiency is primarily linked to increased ruminal propionate synthesis (21). Interestingly, in the current study, it was observed that dietary supplementation of MON had no impact on ADG and feed conservation rate in Hu sheep, which corresponded to the absence of changes in propionate concentration. The efficacy of MON on feed conservation rate is influenced by various factors, such as dosage, feeding strategies, and the dietary nutrient composition. Vyas et al. (22) reported that incorporating 33 mg/kg DM of MON into a high-forage (65%) diet led to enhanced feed conservation rate, whereas its addition to a

TABLE 5 Comparative effects of nisin and monensin supplementations on blood biochemical parameters of fattening Hu sheep.

Item	CON	MON	NIS	SEM	*p*-value		
					Trt	Wk	Trt × Wk
ALP, U/L	443.0	457.4	433.5	34.14	0.88	0.16	0.93
ALT, U/L	15.4	13.7	13.1	1.16	0.38	<0.01	0.56
AST, U/L	119.1	121.4	114.0	6.59	0.53	0.30	0.06
BUN, mg/dL	28.0	28.9	28.1	0.94	0.58	0.64	0.04
CRE, mg/dL	0.71	0.71	0.72	0.029	0.96	0.80	0.67
Glucose, mg/dL	64.5	66.0	65.7	1.35	0.50	<0.01	<0.01
TBA, μmol/L	10.61	10.32	12.70	1.279	0.37	0.31	0.86
TCHO, mg/dL	37.1^{b}	45.2^{a}	37.3^{b}	2.15	<0.01	<0.01	0.09
TG, mg/dL	33.5	34.5	36.2	0.95	0.14	0.28	0.46
TP, g/dL	4.88	4.81	4.83	0.104	0.83	0.97	0.06
Albumin, g/dL	1.92	2.00	1.96	0.024	0.10	<0.01	0.01
Globulin, g/dL	2.95	2.81	2.87	0.076	0.42	0.37	0.37
A:G	0.66	0.72	0.69	0.019	0.08	0.06	0.61

CON, control (no additives); MON, monensin, 40 mg/kg DM; NIS, nisin, 274.5 mg/kg DM; Trt, treatment; Wk, week; Trt × Wk, interaction of treatment and week; ALP, alkaline phosphatase; ALT, alanine aminotransferase; AST, aspartate aminotransferase; BUN, blood urea nitrogen; CRE, creatinine; TBA, total bile acid; TCHO, total cholesterol; TG, triglyceride; TP, total protein; A:G, Albumin:Globulin. ^{a,b}Means within a row with different superscripts differ ($P < 0.05$).

high-concentrate (92%) diet showed no effect on feed conservation rate. This discrepancy can be attributed to the higher energy content in high-grain diets, which stimulates a relatively higher production of ruminal propionate compared to high-forage diets (23). Consequently, the supplementation of MON in high-concentrate diets had no noticeable effect on feed conservation rate. In the current study, where the dietary composition was balanced (forage:concentrate = 45:55) to meet the energy requirements of Hu sheep, it is plausible that the feed conservation rate remained unaffected. Correspondingly, NIS exhibited a parallel effect to MON, consistent with the results of a previous study by Shen et al. (14), where supplementation with 30.5 mg/kg DM of NIS yielded similar results. The influence of NIS observed in this study may be influenced by the dietary forage-to-concentrate ratio similarly to MON, potentially limiting its ability to significantly enhance growth.

4.2 Rumen fermentation characteristics

In the present study, it was found that adding 274.5 mg/kg DM of NIS and 40 mg/kg DM of MON to the diet decreased acetate concentration and altered rumen fermentation pattern, which is consistent with the results of previous studies (12, 24). Acetate is primarily produced through the microbial fermentation of fibrous materials. It has been reported that both NIS and MON can inhibit acetate production by increasing the permeability of cell membranes to suppress gram-positive fiber-degrading bacteria in the rumen, such as Ruminococcus spp. and other major acetate-producing bacteria (12). The reduced acetate concentration in the present study indicated that the addition of NIS and MON may also inhibit the abundance of cellulolytic bacteria in Hu sheep. Further studies are warranted to assess the impact of NIS and MON on ruminal microorganisms.

Following the decrease in the acetate/propionate ratio, a shift in rumen fermentation mode is indicated. The VFA profile is closely related to the hydrogen metabolism pathway and methane production in the rumen. Acetate and butyrate formation releases hydrogen, aiding methane synthesis, whereas propionate formation competes for hydrogen utilization in the rumen. Although methane production was not quantified in the present study, an estimation based on the equation proposed by Moss et al. (25) indicated a reduction in methane production with NIS supplementation. Previous studies have shown that dietary NIS supplementation can inhibit rumen methane production (12, 13, 26). Therefore, direct measurement of methane production is advisable for validation. Furthermore, it is generally believed that inhibiting methane production can alter hydrogen utilization pathways, reduce energy loss and improve production performance (27). However, the present study did not find an improvement in ADG or feed conservation rate in fattening Hu sheep, aligning with the findings of a previous low-dose *in vivo* study (14). This suggests that the conserved energy is not digested, absorbed, and utilized effectively by the host.

Ruminal propionate is the principal substrate for hepatic gluconeogenesis, where glucose synthesis from propionate significantly contributes to the total energy production in ruminants, ranging from 24% to 61% (28). In the current study, the ruminal concentration of propionate in Hu sheep fed with NIS and MON did not exhibit significant variations. The results are in agreement with those of Santoso et al. (29) and Benchaar et al. (30), but inconsistent with previous reports that NIS and MON can enhance propionate production (12, 31). Several pathways, such as the succinate pathway, acrylate pathway, and tricarboxylic acid cycle, are involved in ruminal propionate synthesis (32). Shen et al. (12) reported that the addition of 1 μM and 5 μM nisin led to an increase in the relative abundance of genera with succinate as the metabolic end product, such as *Fibrobacter succinogenes* and Succinivibrio, and increased the concentration of propionate through the succinate pathway. It also reduces the relative abundance of streptococci involved in the

acrylate pathway, thereby weakening the production of propionate through the acrylate pathway. Therefore, the compensatory interactions between these metabolic pathways may explain the stable propionate levels observed in this study. Additionally, dietary composition, particularly the concentrate-to-forage ratio, plays a significant role in the effectiveness of additives. Ramanzin et al. (33) observed a more pronounced effect of MON on propionate concentration in diets with lower forage content (50%) compared to those with higher forage content (70%). Hence, the discrepancies in study results may be due to variations in dosage, dietary composition etc.

4.3 Nutrients intake and apparent digestibility

Shen et al. (14) previously reported that dietary supplementation with 30.5 mg/kg DM of NIS had no effect on the digestibility of DM, NDF, ADF, EE and CP in fattening sheep, which consistent with the results of the present study. This similarity in outcomes may be attributed to the maintenance or potential increase in the population of rumen protozoa and the relative abundance of major cellulolytic bacteria, as suggested by Shen et al. (12). However, Azzaz et al. (34) found that adding 500 U/kg NIS improved nutrient digestibility in ewes. Discrepancies in results observed across studies could be influenced by factors such as species differences, gender, and nutritional composition. Furthermore, in the current study, the inclusion of NIS significantly decreased the digestibility of EE. The rationale behind this finding remains unclear. Conducting further analysis on intestinal enzyme activity could potentially provide insights into the observed variations in EE digestibility.

This study found that dietary supplementation of MON had no effect on DM digestibility, which was consistent with previous study in cannulated wethers (35). In contrast, Soltan et al. (36) found that the addition of monensin led to reduced DM digestibility. The adverse effect of MON on feed digestion is primarily attributed to its inhibition of cellulolytic bacteria, such as *Fibrobacter succinogenes*. However, research has indicated that *Fibrobacter succinogenes* can develop resistance to MON over time. Thus potentially explaining the lack of effect on DM digestibility with MON supplementation (11, 37). Moreover, in the present study, the CP digestibility of the MON group exceeded that of the NIS group, consistent with the findings of Polizel et al. (35). MON may reduce MCP synthesis by decreasing the population of rumen bacteria, subsequently diminishing the proportion of RDP and elevating RUP levels (38). Studies have shown that RUP is more readily digested in the small intestine compared to microbial proteins (39), and the addition of MON can enhance amino acid absorption in the small intestine (40). Therefore, the increased CP digestibility in the MON group may be associated with heightened CP digestion and absorption rates in the small intestine.

4.4 Plasma metabolites

Blood metabolites are crucial indicators of the nutritional and health status of animals. In this study, the plasma metabolites of Hu sheep in each treatment were found to be within the normal range (41), suggesting that the dietary inclusion of NIS and MON did not have any adverse effects on the metabolism or health of the fattening Hu sheep. Shen et al. (14) reported that dietary supplementation of 30.5 mg/kg DM of NIS had no significant impact on the blood biochemical parameters of fattening sheep, consistent with our findings. Furthermore, previous studies have indicated that the levels of ALT, ALP, and AST in the blood are linked to inflammation, while the concentrations of globulin and albumin can serve as indicators of humoral immunity and protein synthesis in animals (42). These outcomes indirectly imply that the incorporation of MON and NIS into the diet does not affect the immune response of the Hu sheep.

The current study noted an elevation in the blood concentration of total cholesterol in Hu sheep supplemented with MON. Comparable findings have been documented by Duffield et al. (43) and O'Kelly and Spiers (44). It has been reported that MON can enhance energy metabolism by promoting fat export from the liver while decreasing the fat transported to the liver and reducing non-esterified fatty acids (NEFA), consequently mitigating liver fat accumulation and enhancing liver function (43). It is recognized that hepatic fat export is frequently released into the blood as lipoproteins, composed of triacylglycerol, cholesterol, and phospholipids synthesized in the liver with apolipoproteins. Hence, the rise in cholesterol levels triggered by MON supplementation may result from increased lipoprotein export from the liver. Further studies investigating the mechanisms of energy metabolism are needed to validate this hypothesis.

5 Conclusion

Both dietary supplements of NIS and MON reduced rumen acetate concentration and induced changes in the rumen fermentation pattern. However, neither of these additives impacted the growth performance or health status of the fattening Hu sheep. The regulatory mechanism of NIS and MON on rumen fermentation needs further investigation, especially through microbiome analysis. Moreover, the inclusion of MON in the diet led to elevated total cholesterol levels in the blood of Hu sheep in comparison to the other groups. The variations in cholesterol levels may be linked to energy metabolism, warranting further research on the specific metabolic pathways.

Data availability statement

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

Ethics statement

All animal protocols were approved by the Animal Care and Use Committee of Nanjing Agricultural University (protocol number: SYXK2017-0007). The studies were conducted in accordance with the local legislation and institutional requirements. Written informed consent was obtained from the owners for the participation of their animals in this study.

Author contributions

JL: Formal analysis, Writing – original draft. JJ: Writing – original draft, Formal analysis. HD: Writing – review & editing. HZ: Writing – review & editing. MS: Methodology, Writing – review & editing. SM: Resources, Writing – review & editing. JS: Conceptualization, Formal analysis, Funding acquisition, Supervision, Writing – review & editing.

Funding

The author(s) declare that financial support was received for the research, authorship, and/or publication of this article. This work was financially supported by and the National Key Research and Development Program of China (2023YFD1301801).

Conflict of interest

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

Publisher's note

References

1. Zamojska D, Nowak A, Nowak I, Macierzyńska-Piotrowska E. Probiotics and Postbiotics as substitutes of antibiotics in farm animals: a review. *Animals*. (2021) 11:3431. doi: 10.3390/ani11123431

2. Cheng G, Hao H, Xie S, Wang X, Dai M, Huang L, et al. Antibiotic alternatives: the substitution of antibiotics in animal husbandry? *Front Microbiol*. (2014) 5:217. doi: 10.3389/fmicb.2014.00217

3. Russell JB, Houlihan AJ. Ionophore resistance of ruminal bacteria and its potential impact on human health1. *FEMS Microbiol Rev*. (2003) 27:65–74. doi: 10.1016/s0168-6445(03)00019-6

4. Hales KE, Foote AP, Brown-Brandl TM, Freetly HC. The effects of feeding increasing concentrations of corn oil on energy metabolism and nutrient balance in finishing beef steers1. *J Anim Sci*. (2017) 95:939–48. doi: 10.2527/jas.2016.0902

5. Reddy S, Barathe P, Kaur K, Anand U, Shriram V, Kumar V. Antimicrobial resistance and medicinal plant products as potential alternatives to antibiotics in animal husbandry. *Antimicrob Resist*. (2022):357–84. doi: 10.1007/978-981-16-3120-7_13

6. Klaenhammer TR. Genetics of bacteriocins produced by lactic acid bacteria. *FEMS Microbiol Rev*. (1993) 12:39–85. doi: 10.1016/0168-6445(93)90057-g

7. Yuan H, Bai G, Lin Y, Yu X, Yang Q, Dou R, et al. Effects of dietary Nisin on growth performance, immune function and gut health of broilers challenged by *Clostridium perfringens*. *J Anim Sci*. (2024) 102:skae017. doi: 10.1093/jas/skae017

8. Pogány Simonová M, Chrastinová Ľ, Chrenková M, Formelová Z, Lauková A. Lantibiotic nisin applied in broiler rabbits and its effect on the growth performance and carcass quality. *Probiotics Antimicrob Proteins*. (2019) 11:1414–7. doi: 10.1007/s12602-019-9525-0

9. Kierończyk B, Rawski M, Mikołajczak Z, Świątkiewicz S, Józefiak D. Nisin as a novel feed additive: the effects on gut microbial modulation and activity, histological parameters, and growth performance of broiler chickens. *Animals*. (2020) 10:101. doi: 10.3390/ani10010101

10. O'Reilly C, Grimaud GM, Coakley M, O'Connor PM, Mathur H, Peterson VL, et al. Modulation of the gut microbiome with nisin. *Sci Rep*. (2023) 13:7899. doi: 10.1038/s41598-023-34586-x

11. Callaway TR, Carneiro De Melo AM, Russell JB. The effect of nisin and monensin on ruminal fermentations in vitro. *Curr Microbiol*. (1997) 35:90–6. doi: 10.1007/s002849900218

12. Shen J, Liu Z, Yu Z, Zhu W. Monensin and nisin affect rumen fermentation and microbiota differently in vitro. *Front Microbiol*. (2017) 8:1111. doi: 10.3389/fmicb.2017.01111

13. Li Z, Wang M, Usman S, Khan A, Zhang Y, Li F, et al. Effects of nisin on bacterial community and fermentation profiles, in vitro rumen fermentation, microbiota, and methane emission of alfalfa silage. *J Sci Food Agric*. (2023) 103:6706–18. doi: 10.1002/jsfa.12765

14. Shen J, Chen Y, Moraes LE, Yu Z, Zhu W. Effects of dietary protein sources and nisin on rumen fermentation, nutrient digestion, plasma metabolites, nitrogen utilization, and growth performance in growing lambs. *J Anim Sci*. (2018) 96:1929–38. doi: 10.1093/jas/sky086

15. Jiang J, Sun M, Shen J, Diao Q, Zhu W. Effects of different dietary protein sources supplemented with Nisin on rumen fermentation and rumen microbiota of fattening Hu sheep. *Act Vet Zootechn Sin*. (2021) 52:2534–44. doi: 10.11843/j.issn.0366-6964.2021.09.016

16. Patra AK, Yu Z. Essential oils affect populations of some rumen bacteria in vitro as revealed by microarray (RumenBactArray) analysis. *Front Microbiol*. (2015) 6:297. doi: 10.3389/fmicb.2015.00297

17. Chaney AL, Marbach EP. Modification reagents for determination of urea and ammonia. *Clin Chem*. (1962) 8:130–2. doi: 10.1093/clinchem/8.2.130

18. Hasan MT. Official methods of analysis. *15th* ed. Virginia, USA: Association of Official Analytical Chemists, Artington (1990). 2015 p.

19. Pv VS, Robertson JB, Lewis BA. Methods for dietary fiber, neutral detergent fiber, and nonstarch polysaccharides in relation to animal nutrition. *J Dairy Sci*. (1991) 74:3583–97. doi: 10.3168/jds.s0022-0302(91)78551-2

20. Van Keulen J, Young BA. Evaluation of acid-insoluble ash as a natural marker in ruminant digestibility studies. *J Anim Sci*. (1977) 44:282–7. doi: 10.2527/jas1977.442282x

21. Ellis JL, Dijkstra J, Bannink A, Kebreab E, Hook SE, Archibeque S, et al. Quantifying the effect of monensin dose on the rumen volatile fatty acid profile in high-grain-fed beef cattle. *J Anim Sci*. (2012) 90:2717–26. doi: 10.2527/jas.2011-3966

22. Vyas D, Alemu AW, McGinn SM, Duval SM, Kindermann M, Beauchemin KA. The combined effects of supplementing monensin and 3-nitrooxypropanol on methane emissions, growth rate, and feed conversion efficiency in beef cattle fed high-forage and high-grain diets. *J Anim Sci*. (2018) 96:2923–38. doi: 10.1093/jas/sky174

23. Duffield T, Merrill J, Bagg R. Meta-analysis of the effects of monensin in beef cattle on feed efficiency, body weight gain, and dry matter intake. *J Anim Sci*. (2012) 90:4583–92. doi: 10.2527/jas.2011-5018

24. Wu J, Guo J, Liu T, Chen H, Bai Y, Casper DP. Feeding a calf starter containing monensin alone or in combination with an oregano, and cobalt blend to Holstein calves. *J Anim Sci*. (2020) 98:skaa214. doi: 10.1093/jas/skaa214

25. Moss AR, Jouany JP, Newbold J. Methane production by ruminants: its contribution to global warming. *Ann Zootech*. (2000) 49:231–53. doi: 10.1051/animres:2000119

26. Santoso B, Mwenya B, Sar C, Gamo Y, Kobayashi T, Morikawa R, et al. Effects of supplementing galacto-oligosaccharides, *Yucca schidigera* or nisin on rumen methanogenesis, nitrogen and energy metabolism in sheep. *Livest Prod Sci*. (2004) 91:209–17. doi: 10.1016/j.livprodsci.2004.08.004

27. Lan W, Yang C. Ruminal methane production: associated microorganisms and the potential of applying hydrogen-utilizing bacteria for mitigation. *Sci Total Environ*. (2019) 654:1270–83. doi: 10.1016/j.scitotenv.2018.11.180

28. Bergman E. Energy contributions of volatile fatty acids from the gastrointestinal tract in various species. *Physiol Rev*. (1990) 70:567–90. doi: 10.1152/physrev.1990.70.2.567

29. Santoso B, Mwenya B, Sar C, Takahashi J. Ruminal fermentation and nitrogen metabolism in sheep fed a silage-based diet supplemented with *Yucca schidigera* or Y. Schidigera and nisin. *Anim Feed Sci Technol*. (2006) 129:187–95. doi: 10.1016/j.anifeedsci.2006.01.001

30. Benchaar C, Petit H, Berthiaume R, Whyte T, Chouinard P. Effects of addition of essential oils and monensin premix on digestion, ruminal fermentation, milk production, and milk composition in dairy cows. *J Dairy Sci*. (2006) 89:4352–64. doi: 10.3168/jds.s0022-0302(06)72482-1

31. Jalc D, Lauková A. Effect of nisin and monensin on rumen fermentation in the artificial rumen. *Berl Munch Tierarztl*. (2002) 115:6–10.

32. Jeyanathan J, Martin C, Morgavi D. The use of direct-fed microbials for mitigation of ruminant methane emissions: a review. *Animal*. (2014) 8:250–61. doi: 10.1017/s1751731113002085

33. Ramanzin M, Bailoni L, Schiavon S, Bittante G. Effect of monensin on milk production and efficiency of dairy cows fed two diets differing in forage to concentrate ratios. *J Dairy Sci*. (1997) 80:1136–42. doi: 10.3168/jds.s0022-0302(97)76040-5

34. Azzaz HH, Kholif AE, Abd El Tawab AM, El-Sherbiny M, Murad HA, Hassaan NA, et al. Lactation performance and feed utilization of Rahmani ewes fed with either a newly produced bacteriocin-like substance or a commercial bacteriocin. *Transl. Anim Sci*. (2023) 7:txad010. doi: 10.1093/tas/txad010

35. Polizel DM, Martins AS, Miszura AA, Ferraz Júnior MVC, Bertoloni AV, Oliveira GB, et al. Low doses of monensin for lambs fed diets containing high level of ground flint corn. *Sci Agric*. (2020) 78:78. doi: 10.1590/1678-992X-2019-0263

36. Soltan YA, Hashem NM, Morsy AS, El-Azrak KM, El-Din AN, Sallam SM. Comparative effects of *Moringa oleifera* root bark and monensin supplementations on ruminal fermentation, nutrient digestibility and growth performance of growing lambs. *Anim Feed Sci Technol*. (2018) 235:189–201. doi: 10.1016/j.anifeedsci.2017.11.021

37. Guo X, Liu Y, Jiang Y, Yao J, Li Z. Ruminal bacterial community successions in response to Monensin supplementation in goats. *Animals*. (2022) 12:2291. doi: 10.3390/ani12172291

38. Bergen WG, Bates DB. Ionophores: their effect on production efficiency and mode of action. *J Anim Sci*. (1984) 58:1465–83. doi: 10.2527/jas1984.5861465x

39. Salles MSV, Zanetti MA, Titto EAL, Conti RMC. Effect of monensin on performance in growing ruminants reared under different environmental temperatures. *Anim Feed Sci Technol*. (2008) 147:279–91. doi: 10.1016/j.anifeedsci.2008.01.008

40. Santos MCB, Araújo APC, Venturelli BC, Freitas JE Jr, Barletta RV, Gandra JR, et al. Effects of increasing monensin doses on performance of mid-lactating Holstein cows. *J Appl Anim Res*. (2019) 47:297–302. doi: 10.1080/09712119.2019.1629298

41. Peng K, Shirley DC, Xu Z, Huang Q, McAllister TA, Chaves AV, et al. Effect of purple prairie clover (*Dalea purpurea* vent.) hay and its condensed tannins on growth performance, wool growth, nutrient digestibility, blood metabolites and ruminal fermentation in lambs fed total mixed rations. *Anim Feed Sci Technol*. (2016) 222:100–10. doi: 10.1016/j.anifeedsci.2016.10.012

42. Chen G, Song S, Wang B, Zhang Z, Peng Z, Guo C, et al. Effects of forage: concentrate ratio on growth performance, ruminal fermentation and blood metabolites in housing-feeding yaks. *Asian Australas J Anim Sci*. (2015) 28:1736–41. doi: 10.5713/ajas.15.0419

43. Duffield T, LeBlanc S, Bagg R, Leslie K, Ten Hag J, Dick P. Effect of a monensin controlled release capsule on metabolic parameters in transition dairy cows. *J Dairy Sci*. (2003) 86:1171–6. doi: 10.3168/jds.s0022-0302(03)73700-x

44. O'Kelly J, Spiers W. Monensin induced metabolic changes in cattle fed a restricted intake of lucerne hay. *J Agric Sci*. (1988) 111:403–6. doi: 10.1017/s0021859600083374

frontiers | Frontiers in Veterinary Science

TYPE Original Research
PUBLISHED 07 August 2024
DOI 10.3389/fvets.2024.1447238

OPEN ACCESS

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RECEIVED 11 June 2024
ACCEPTED 31 July 2024
PUBLISHED 07 August 2024

CITATION
Li Z, Hu Y, Li H, Lin Y, Cheng M, Zhu F and Guo Y (2024) Effects of yeast culture supplementation on milk yield, rumen fermentation, metabolism, and bacterial composition in dairy goats.
Front. Vet. Sci. 11:1447238.
doi: 10.3389/fvets.2024.1447238

Effects of yeast culture supplementation on milk yield, rumen fermentation, metabolism, and bacterial composition in dairy goats

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The effects of yeast culture (YC) on dairy goat milk yield and potential effects of rumen microbial population changes on rumen fermentation are poorly understood. This study aimed to evaluate the effects of YC on milk yield and rumen fermentation in dairy goats and explore the potential microbial mechanisms. Forty Laoshan dairy goats with a weight of 51.23 ± 2.23 kg and daily milk yield of 1.41 ± 0.26 kg were randomly divided into 4 groups: control (no YC), YC1 (10 g/day per goat), YC2 (25 g/day per goat), and YC3 (40 g/day per goat). The pre-feeding period was 15 days, and the official period was 60 days. Laoshan dairy goats were milked twice daily, and the individual milk yield was recorded. On the last day of the official period, rumen fluid was collected to measure rumen fermentation, perform quantitative polymerase chain reaction (PCR), and detect metabolites. Compared to the control group, the YC group had greater milk yield; higher acetic acid, butyric acid, and total volatile fatty acid contents; and lower ammonia-N (NH_3-N) content in the rumen ($p < 0.05$). YC increased the abundance of *Clostridia_UCG-014* and *Paraprevotella* ($p < 0.05$). Differential metabolites L-leucine and aspartic acid were screened. This study revealed the microbial mechanisms linking the relative abundance of *Paraprevotella* and *Clostridia_UCG-014* to L-leucine and aspartic acid utilization. These results describe the potential benefits of supplementing 10 g/day per goat YC in the diets of Laoshan dairy goats for improving the rumen environment and milk yield.

KEYWORDS

dairy goats, production performance, rumen fermentation parameters, rumen microflora, rumen metabolism

1 Introduction

Increasing dairy production efficiency is a key goal in dairy goat nutrition, and dietary interventions play a crucial role. Yeast culture (YC) has been reported to improve milk yield (1, 2), stabilize rumen pH, and promote consistent environments for rumen fermentation (3–5).

Commercial YC and yeast-containing feed ingredients vary in many characteristics, including the yeast strain, viability, culture and associated media, and post-fermentation processing [e.g., fractionated yeast (6)]. YC is unique among yeast products because it contains yeast biomass and

fermentation metabolites (6). The composition and characteristics of the fermentation metabolites are highly dependent on the medium used to grow the yeast. The freeze-drying method of yeast culture can also retain a small amount of active yeast and the fermentation activity of yeast (6, 7). The benefits of YC have been attributed to the presence of functional metabolites (e.g., organic acids, B vitamins, and enzymes) that may influence ruminal fermentation by supplying key nutrients that are otherwise scarce in the ruminal environment (8, 9).

The abundance of various rumen bacterial taxa is correlated with production performance and rumen fermentation parameters, indicating that bacterial communities play important roles in regulating host physiological parameters (10, 11). According to Chaucheyras-Durand et al. (12), the main effects of YC are related to rumen fermentation and benefit key microbial populations and their metabolism, increase fiber degradation, and stabilize rumen pH. *In vivo* and *in vitro* experiments have shown that YC can stimulate the growth of rumen cellulolytic bacteria (13), promote fiber degrading bacteria establishment in the digestive tract of lambs, and accelerate microbial activity in the rumen (14). Rumen cellulolytic bacteria can decompose dietary macromolecular carbohydrates into glucose, which is then fermented to produce volatile fatty acids (VFAs), such as acetic acid, propionic acid, and butyric acid. These VFAs contain large amounts of energy and are the main energy sources for dairy cows (15, 16). In addition, studies have reported that the ratios of these VFAs are affected by changes in microbial metabolism and species (17). However, the effects of YC on the production performance and rumen fermentation parameters of dairy goats and the exact microbial mechanisms underlying these effects are unclear. Therefore, understanding the microbial mechanisms underlying the effects of YC on the rumen is important for optimizing the utilization of YC for ruminant nutrition.

Therefore, the current study evaluated the effects of YC on the production performance, rumen fermentation characteristics, rumen microorganisms, and metabolites of dairy goats. Moreover, the main metabolites and metabolic pathways affecting rumen fermentation parameters were explored through the weighted gene correlation network analysis (WGCNA) method to further understand the microbial mechanisms underlying the effect of YC on rumen fermentation parameters in dairy goats.

2 Materials and methods

2.1 Ethical considerations and location

This study was conducted at the Aote Breeder Goat Co., Ltd., Qingdao City, Shandong Province (120°36′E, 36°58′N), and the experiments were performed in strict accordance with the guidance of the National Council for the Control of Animal Experimentation under an experimental protocol approved by the Animal Science and Technology College of Qingdao Agricultural University, Qingdao, Shandong (protocol code DKY20200701, dated July 1, 2020).

2.2 Diet and livestock management

The studied YC, which was provided by Danongwei Technology (Shenzhen) Co., Ltd., is a concentrated and dried product of *Saccharomyces cerevisiae* after fermentation. It is mainly composed of yeast cell lysate, yeast cell wall (e.g., mannan-oligosaccharide, β-glucan, and chitin), post-fermentation denaturation medium, and extracellular metabolites (e.g., nucleosides, organic acids, proteins, peptides, plant Zi alcohol, natural antioxidants, and digestive enzymes). The crude protein (CP) content was 16.2%, crude fat (EE) content was 1.53%, ash content was 7.71%, and moisture content was 9.87%.

A completely randomized experimental design was adopted. Forty Laoshan adult dairy goats (born in late April 2020) that had previously given birth twice and exhibited good body condition, (51.23 ± 2.23 kg body weight; 40 ± 4 days in milk; 1.41 ± 0.26 kg/d milk yield) were selected and randomly divided into four groups. Each treatment consisted of 10 Laoshan dairy goats.

The diets were formulated based on the nutrient requirements of lactating goats reported by Cannas et al. (18, 19). The goats were fed a basal diet *ad libitum* and allowed approximately 5% orts twice a day at 08:00 and 14:30. The daily feeding quantity was 3.5% of the body weight. The dietary composition and nutritional levels are shown in Table 1.

The control group (CON) was fed a basic diet, while the YC1, YC2, and YC3 groups were fed the basic diet and 10, 25, and 40 g YC/day per goat, respectively. To ensure that the YC groups ingested sufficient YC per day per goat, YC was mixed with a small amount of basic diet and fed to each goat separately every morning. After each goat had finished all of the basic diet with YC, the remaining feed would be given to them. The YC dose was based on the recommendations of the supplier, which suggested a range of 10–40 g/day per goat. The adaptation period was 15 days, and the experimental period was 60 days. During the test period, all Laoshan dairy goats were allocated to individual pens with natural light and cool environmental temperatures and provided *ad libitum* access to drinking water.

TABLE 1 Ingredients and chemical composition of diets (DM basis).

Item	g/100 g
Ingredients	
Corn silage	16.46
Peanut vine	20.77
Pomace	6.21
Garlic stalks	3.53
Hay	2.16
NaCl	0.29
Mineral-vitamin-protein mix[1]	50.58
Total	100
Nutrient content[2], dry matter basis	
DE, MJ/kg	13.85
CP, %	13.95
NDF, %	47.59
ADF, %	22.19

[1]Mineral-vitamin-protein mix was purchased from Oulifeide Feed Science and Technology Co, Ltd. (Shandong, China), and it contained corn, corn dry alcohol grains, spray corn husk, corn germ meal, soybean meal, bran, cotton meal, calcium bicarbonate, stone powder, sodium chloride, sugarcane molasses, palm fat powder, sodium bicarbonate, minerals, and vitamin ingredients. The feed had the following contents, crude protein (CP), 20.1%; Ash, 9.7% and NaCl, 1%.

[2]For the nutrient levels, the digestible energy (DE) was based on the calculated value. CP, neutral detergent fiber (NDF), and acid detergent fiber (ADF) were the measured value.

2.3 Data and sample collection

Laoshan dairy goats were milked twice daily at approximately 06:00 and 18:00, and individual milk yield was recorded using an Afikim milking system (AfiFlo milk meters, S.A.E. Afikim, Israel). The milk samples (morning and afternoon) of each animal were mixed to form a composite sample, which was placed in plastic containers with Brono-pol® preservative and stored in a freezer at −20°C until the chemical composition (50 mL sample) was analyzed. Milk fat concentrations were analyzed once weekly using the Gerber method (20). The 4% fat corrected milk (FCM) yield was calculated as follows: [(0.4 × milk yield) + (15 × milk fat percentage × milk yield)] (21).

The rumen fluid was collected from 40 Laoshan dairy goats on the final day of the study period. Laoshan dairy goats were sampled in the afternoon via an esophageal tube at 14:00. The esophageal tubing apparatus was assembled by coupling the esophageal tube to a metal strainer (22) at one end and the opposite handle side of a manual vacuum pump (Med-Eze stomach pump, MAI Animal Health, China) at the other end. The rumen fluid samples were collected by passing the fluid through the hollow shaft of the pump into a plastic beaker. After discarding the first 200 mL of fluid to minimize salivary contamination, approximately 50 mL of rumen fluid was collected. After collection, the pH was immediately measured using a pH meter (Waterproof pH Testr 30, Oakton Instruments, United States), and two aliquots (10 mL) were acidified with either 200 μL of 50% sulfuric acid or 2 mL of 25% meta-phosphoric acid and stored at −20°C until analysis of ammonia-N (NH_3-N) and VFAs, respectively. In addition, 2 mL of rumen fluid samples were collected and immediately frozen in liquid nitrogen and stored at −80°C until DNA isolation and subsequent relative abundance analysis of bacteria species, which was performed via the quantitative polymerase chain reaction (qPCR) method. Detection of metabolites was performed via non-targeted metabolomics analysis on Ultra-high-performance Liquid Chromatography–Tandem Mass Spectrometry (UHPLC–MS/MS) system (ExionLC AD, SCIEX, United States; QTRAP®, SCIEX, United States).

Rumen fluid samples preserved in 50% sulfuric acid and 25% meta-phosphoric acid were thawed and transferred into 2 mL microcentrifuge tubes. Then, the samples were centrifuged at 30,000 × *g* for 20 min at 4°C (model 5403, Eppendorf, Germany), and the supernatant from samples in sulfuric acid was used to analyze NH_3-N using the colorimetric assay described by Chaney and Marbach (23). The supernatant of rumen fluid containing 25% meta-phosphoric acid was analyzed for the acetic acid, propionic acid, butyric acid, and total VFA (TVFA) concentrations using an automated gas chromatograph (model 689, Hewlett-Packard, Juyi Hui supply chain Co., Ltd., China) equipped with a 0.25 mm i.d × 15-m column (Nukol 24106-U, Supelco, Inc., United States), and the internal standard was 2-ethylbutyrate.

2.4 Ruminal bacteria DNA isolation and qPCR amplification of 16S rDNA genes

Microbiome DNA was extracted from each sample (250 μL of rumen fluid was used) using a PowerSoil DNA Isolation Kit (MoBio Laboratories, Inc., Canada), following the manufacturer's instructions. The extracted DNA was detected using a NanoDrop 2000 (ThermoFisher Scientific, Inc., United States) to determine the DNA quality and concentration. The samples qualified for quality inspection were stored at −20°C for use in follow-up experiments.

The V3-V4 region of the 16Sr RNA gene of bacteria was amplified by primers 338F (5′-ACTCCTACGGGAGGCAGCAG-3′) and 806R (5′-GGACTACHVGGGTWTCTAAT-3′). Eight base-pair barcode sequences were added to the 5′ ends of upstream and down-stream primers to distinguish different samples. The PCR reaction system contained the following (25 μL total volume): 12.5 μL 2× Taq Plus Master Mix II (Vazyme Biotech Co., Ltd., China), 3 μL BSA (2 ng/μL), 1 μL forward primer (5 μM), 1 μL reverse [rimer (5 μM), 2 μL DNA (total amount of added DNA was 30 ng)], and 5.5 μL dd H_2O to a volume of 25 μL. The reaction parameters were pre-denatured at 95°C for 45 min, denatured at 95°C for 45 s, annealed at 55°C for 50 s, annealed at 55°C for 45 s, extended at 72°C for 45 s for 28 cycles, and extended at 72°C for 10 min. The PCR products were amplified using an ABI 9700 PCR instrument (Thermo Fisher Scientific, Inc., United States). The size of the amplified bands was detected by 1% agarose gel electrophoresis, and the bands were purified using an Agencourt AMPure XP nucleic acid purification kit (Beckman Coulter, Inc., United States).

The library was constructed using the NEB Next Ultra II DNA Library Prep Kit (New England Biolabs, Inc., United States), which is a library building kit, and paired-end sequencing was performed using the Illumina MiSeq PE300 (Illumina, Inc., United States) high-throughput sequencing platform. Trimmomatic software was used to control the quality of Fastq data, and it used the sliding window strategy, a window size of 50 mbp, average quality value of 20, and minimum reserved sequence length of 120. Using Pear (v0.9.6), the minimum overlap was set to 10 bp, and the mismatch rate was 0.1. After splicing, Vsearch (v2.7.1) software was used to remove sequences whose length was less than 230 bp, and the UCHIME method was used to remove chimera sequences according to the Gold Database. Using the Vsearch (v2.7.1) software uparse algorithm for operational taxonomic unit (OTU) clustering of high-quality sequences. Valid tags with sequence similarity thresholds ≥97% were assigned to the same taxon (OTU), and the tag sequence with the highest abundance was selected as the representative sequence in each OTU cluster. The BLAST algorithm with Silva138 was used to annotate the species classification. The α diversity index results were analyzed by QIIME (v2.0.0) software, and the α diversity among groups was compared by Wilcoxon rank test using the R package ggpubr (0.4.0). Based on the species annotation and relative abundance results, R (v3.6.0) software was used to analyze the histogram of species composition. QIIME (v2.0.0) was used to calculate the beta diversity distance matrix. NMDS analysis and mapping were performed using the RPG plot2 (3.3.2) and vegan software packages. The Sankey diagram of the species community composition was visualized using the R-package ggplot2. Short time-series expression miner (STEM) (24). The abundance distributions of all OTUs were analyzed. Co-occurrence network analysis was performed using the R-package Psych and visualized using the Cytoscape software (version 3.7.1).

2.5 Metabolite extraction and UHPLC–MS/MS analysis

Rumen fluid samples were collected in 1.5 mL Eppendorf miniature centrifuge tubes, and 1 mL 70% methanol internal standard extract was added. The samples were oscillated for 5 min, maintained on ice for 15 min, centrifuged at 12000 × *g* for 10 min at 4°C. The

supernatant was then extracted, and 400 μL was placed in a corresponding EP tube after centrifugation. The supernatant was then placed in a −20°C refrigerator overnight. Then, it was centrifuged at 12000 × *g* for 3 min at 4°C. Subsequently, 200 μL of supernatant was placed into the inner liner of the corresponding injection bottle for UHPLC–MS/MS analysis, which combined ultra-performance liquid chromatography (UPLC) (ExionLC AD, SCIEX, United States) with MS/MS (QTRAP®, SCIEX, United States). Samples collected from the supernatant mixture were used as quality control samples. During the instrument analysis, one quality control sample was injected into every six test samples to monitor the repeatability and stability of the instrument.

The original data obtained from the UHPLC–MS/MS platform were converted into the TXT format using MSconverter software. Based on the self-built target database MWDB (software database), the software Analyst1.6.3 was used to qualitatively analyze the information and secondary spectrum data according to the retention time (RT), parent ion pair, and secondary spectrum data. Heat maps of different metabolites were drawn using the R-package pheatmap. Metabolites were enriched using MetaboAnalyst4.0.[1]

The WGCNA method is a correlation-based method that describes and visualizes networks of data points, regardless of whether they are estimates of gene expression, metabolite concentration, or other phenotypic data (25, 26). WGCNA can identify a module by building a metabolite correlation network and deriving the characteristic metabolite score (related to the first principal component) (27) from the identified module. To explore the mechanism of rumen acid-related metabolism, WGCNA was used to identify highly related metabolite modules based on annotated metabolites. These modules were associated with the rumen acid indexes determined in this study. The parameters used were a soft power of five and a minimum module size of 10 metabolites. The modules with $p < 0.05$ and $R > 0.5$ were considered to be related to the rumen acid index. The molecule pathway database MetaboAnalyst4.0[2] was used to analyze the pathway enrichment of metabolites in the rumen acid-related modules. This pathway was defined as significantly enriched at $p < 0.05$. Coefficients of the Spearman correlation of acetic acid, propionic acid, and butyric acid with key metabolites were analyzed using the R-package Psych (2.0.7) and visualized using the R-package pheatmap (1.0.12). This correlation was statistically significant ($p < 0.05$).

2.6 Statistical analysis

Data on the production performance and rumen fermentation parameters were analyzed using one-way ANOVA and Tukey's honest significant difference test using SPSS statistical software (version 20.0; SPSS Inc., Chicago, IL, USA). Statistical significance was set at $p < 0.05$. The results are expressed as the mean ± standard deviation. The experimental units were replicates, and the statistical model used was as follows:

$$Y_{ij} = \mu + A_i + e_{ij}$$

where Y_{ij} represents an observation, μ is the overall mean, A_i represents the effect of YC, and e_{ij} represents random error.

3 Results

3.1 Milk yield

The effects of YC on the average daily 4% FCM yield of Laoshan dairy goats are presented in Table 2. At weeks 4, 5, and 10, the average daily 4% FCM yield of YC1 was significantly greater than that of CON, YC2, and YC3 ($p < 0.05$). At week 4, the average daily 4% FCM yield of YC1 surpassed that of CON, YC2, and YC3 by 12.94, 14.29, and 9.09%, respectively; at week 5, it reached 16.77, 17.47, and 10.80%, respectively; and at week 10, it further rose to 24.55, 26.06, and 18.18%, respectively. At the 9th week, the average daily 4% FCM yield of YC1 was significantly greater than that of CON and YC2 ($p < 0.05$), with increases of 23.84 and 18.99%, respectively. However, no significant differences were observed between the YC1 and YC3 groups ($p > 0.05$).

3.2 Rumen fermentation

The effects of YC on ruminal fermentation parameters in Laoshan dairy goats are shown in Table 3. Significant differences in ruminal pH were not observed between the CON, YC1, YC2, and YC3 groups ($p > 0.05$). Compared with the CON group, a significant decrease in ruminal NH_3-N content was observed in the YC2 group ($p < 0.05$). However, significant differences were not observed in ruminal NH_3-N content between the YC1, YC2, and YC3 groups ($p > 0.05$). Compared with the CON group, significant increases in ruminal acetic acid, butyric acid, and TVFA contents were observed among the YC1, YC2, and YC3 groups ($p < 0.05$), whereas no significant differences in ruminal acetic acid, butyric acid, and TVFA contents were observed between the YC1, YC2, and YC3 groups ($p > 0.05$).

TABLE 2 Effects of yeast culture on the milk yield of Laoshan dairy goats, kg/d.

Items[1]	CON	YC1	YC2	YC3	*p*-value
1st week	1.75 ± 0.17	1.78 ± 0.06	1.76 ± 0.03	1.83 ± 0.09	0.76
2nd week	1.76 ± 0.17	1.73 ± 0.12	1.71 ± 0.14	1.82 ± 0.17	0.81
3rd week	1.87 ± 0.31	1.78 ± 0.09	1.69 ± 0.13	1.88 ± 0.12	0.56
4th week	1.70 ± 0.04^{b}	1.92 ± 0.10^{a}	1.68 ± 0.59^{b}	1.76 ± 0.12^{b}	<0.05
5th week	1.67 ± 0.15^{b}	1.95 ± 0.03^{a}	1.66 ± 0.07^{b}	1.76 ± 0.04^{b}	<0.05
6th week	1.77 ± 0.90	1.95 ± 0.11	1.76 ± 0.11	1.83 ± 0.20	0.21
7th week	1.74 ± 0.07	2.07 ± 0.27	1.74 ± 0.11	1.85 ± 0.20	0.12
8th week	1.74 ± 0.08	2.18 ± 0.30	1.84 ± 0.11	1.90 ± 0.86	0.07
9th week	1.72 ± 0.13^{b}	2.13 ± 0.23^{a}	1.79 ± 0.08^{b}	1.89 ± 0.20^{ab}	<0.05
10th week	1.67 ± 0.16^{b}	2.08 ± 0.17^{a}	1.65 ± 0.11^{b}	1.76 ± 0.69^{b}	<0.05

^{a,b}Values within the same row with different superscripts are significantly different ($p < 0.05$).
[1]CON, control diet; YC1, CON + 10 g YC/d/goat; YC2, CON + 25 g YC/d/goat; YC3, CON + 40 g YC/d/goat; YC; yeast culture.

1 www.metaboanalyst.ca/

2 https://smpdb.ca/

TABLE 3 Effects of yeast culture on the rumen fermentation parameters of Laoshan dairy goats.

Items[1]	CON	YC1	YC2	YC3	*p*-value
pH	5.90 ± 0.18	5.89 ± 0.22	6.03 ± 0.16	6.22 ± 0.35	0.22
NH_3-N, mg/dL	13.72 ± 2.42[a]	11.01 ± 1.97[ab]	9.28 ± 1.24[b]	11.04 ± 0.44[ab]	<0.05
Acetic, mmol/L	36.83 ± 6.09[b]	51.94 ± 6.82[a]	50.39 ± 6.57[a]	52.67 ± 11.33[a]	<0.05
Propionate, mmol/L	16.21 ± 2.11	22.05 ± 3.67	21.21 ± 2.90	21.33 ± 3.51	0.07
Butyrate, mmol/L	11.33 ± 1.21[b]	16.85 ± 3.48[a]	15.11 ± 1.94[a]	16.48 ± 2.14[a]	<0.05
TVFA, mmol/L	64.39 ± 9.35[b]	90.84 ± 13.92[a]	86.72 ± 11.25[a]	90.48 ± 16.96[a]	<0.05
Acetic/Propionate	2.26 ± 0.13	2.37 ± 0.08	2.38 ± 0.13	2.45 ± 0.13	0.21

[a,b]Values within the same row with different superscripts are significantly different ($p < 0.05$).
[1]CON, control diet; YC1, CON + 10 g YC/d/goat; YC2, CON + 25 g YC/d/goat; YC3, CON + 40 g YC/d/goat; YC, yeast culture; NH_3-N, ammonia-N; TVFA, total volatile fatty acids.

3.3 Characteristic analysis of OTUs

A total of 1,734,238 tags were generated from 16 samples (with an average of 106,831 tags). These samples were obtained by randomly selecting four samples from each treatment group. After performing quality control, denoising, splicing, and de-chimerism, and eliminating singleton OTUs, 16 samples (average 96,824) generated 1,564,718 high-quality data points (valid tags). At a 97% similarity level, a total of 33,640 OTUs were described. The abundance of 33,640 OTUs was analyzed using STEM software. Six significantly enriched modules were identified (Figure 1A). Upset diagrams (Figure 1B) depict the number of OTUs that are unique to each group or shared among multiple groups. The CON, YC1, YC2, and YC3 groups had 17,820, 18,883, 19,329, and 17,254 unique OTUs, respectively, with 7,961 OTUs in total.

3.4 Abundance of ruminal bacteria

The α diversity of microbiota in the rumen fluid of dairy goats was analyzed by calculating the Chao1, Simpson, Shannon, Observed_species, PD_whole_tree, and Goods_coverage indexes. The results showed that the six α diversity indexes did not significantly differ among the different groups ($p > 0.05$) (Figure 2A). β diversity analyses were then performed, and the results showed that there were significant differences in phylogenetic distance among the four groups. Principal component analysis (PCA) showed that CON, YC1, YC2, and YC3 could not be distinguished, indicating that different treatments did not change the microbial diversity of the rumen fluid of dairy goats as a whole (Figure 2B). However, the partial least squares discriminant analysis (PLS-DA) model showed significant differences among CON, YC1, YC2, and YC3 (Figure 2C).

Changes in the composition of different microorganisms were analyzed at the phylum and genus levels. The results showed that Bacteroidota and Firmicutes were the two main phyla observed, and in CON, YC1, YC2, and YC3. Compared to that in CON, YC1 and YC2 showed relative increases in the abundance of Bacteroidota but decreases in the abundance of Firmicutes (Figure 2D). At the genus level, compared with the CON group, the relative abundance of *Prevotella* increased in the YC1 and YC2 groups but decreased in YC3, whereas the relative abundance of *Rikenellaceae_RC9_gut_group* increased in YC3. The relative abundances of *NK4A214_group* and *F082* were stable (Figure 2E). In summary, YC supplementation increased the abundance of the phylum Bacteroidota and genera *Prevotella* and *Rikenellaceae_RC9_gut_group* in the rumen of Laoshan dairy goats. Microbial co-occurrence networks have been widely used to explore the relationships in microbial communities. The Circos species relationship diagram at the phylum level of microbial community composition (Figure 1C) revealed that the CON, YC1, YC2, and YC3 groups mainly contained Bacteroidota and Firmicutes. An analysis of changes in relative abundance revealed that the genera *Prevotella*, *Rikenellaceae_RC9_gut_group*, *Bacteroi-dales_RF16_group*, *F082*, *p_251_o5*, *Muribaculaceae*, and *Prevotellaceae_UCG_001* of the phylum Bacteroidota and genera *NK4A214_group*, *Christensenellaceae_R_7_group*, and *Ruminococcus* of the phylum Firmicutes were highly abundant (Figure 1D). As shown in Figure 1D, the red lines between phyla represent positive correlations. Notably, the network demonstrated a multitude of positive correlations, as indicated by the dense web of red edges, indicating the existence of synergistic interactions among the phyla Firmicutes, Spirochaetota, Bacteroidota, Patescibacteria, and Synergistota.

3.5 Rumen fluid metabolic spectrum

In this study, a UPLC-MS/MS non-targeted metabolomics analysis method was used to study the rumen fluid samples from dairy goats subjected to different treatments. Based on the results of secondary quality determination, 366 metabolites were annotated, of which 224 were annotated based on the HMDB database,[3] and 203 were annotated based on the Kyoto Encyclopedia of Genes and Genomes (KEGG) database. A heat map of the expression profiles of the 366 metabolites was generated (Figure 3A). The PCA and PLS-DA maps were consistent with the results for the microorganisms (Figures 3B,C). The KEGG enrichment analysis of 336 metabolites showed that the metabolites were mainly concentrated in the primary metabolic pathways. The pathways with significant enrichment were purine metabolism; valine, leucine, and isoleucine bio-synthesis; phenylalanine metabolism; pyrimidine metabolism; aminoacyl-tRNA biosynthesis; alanine, aspartate, and glutamate metabolism; arginine biosynthesis; histidine metabolism; vitamin B6 metabolism; glycine, serine, and threonine

3 https://hmdb.ca/metabolites

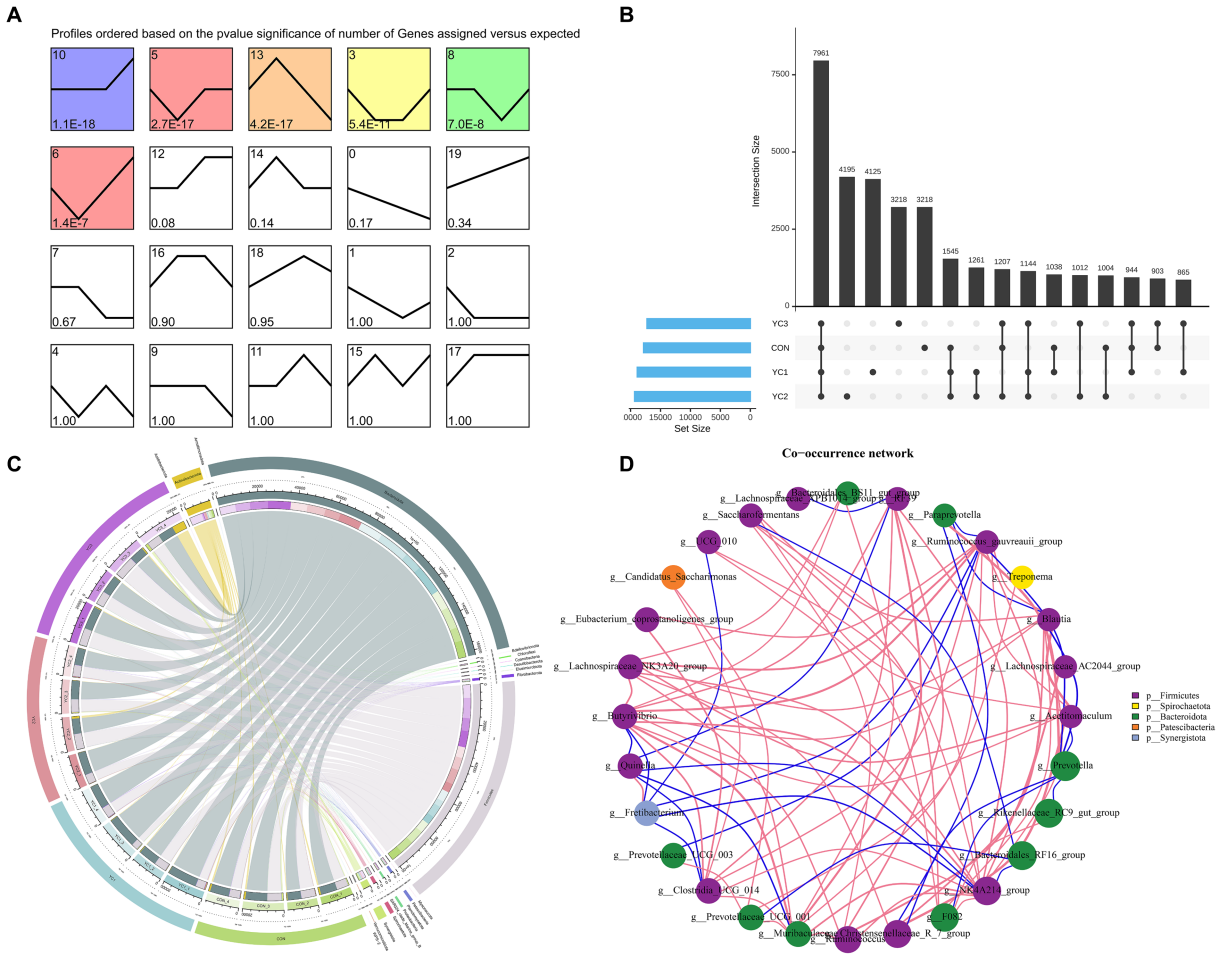

FIGURE 1
Characteristic analysis of operational taxonomic units (OTUs). **(A)** Temporal expression cluster analysis of OTUs. The color module showed a significant enrichment trend ($p < 0.05$). **(B)** Upset map of rumen fluid OTUs. The left bar chart shows the total elements in each original data set; vertical lines connect points to show intersections between data sets, and values represent common OTUs. **(C)** Circos map of OTUs in rumen fluid samples. The outermost circle on the left is sample grouping, on the right is phylum species, and the innermost part is the relative abundance percentage circle. The lines indicate the species and relative abundance information in the samples. **(D)** Change in the relative abundance of the genus. Using the Spearman test method, the top 20 genera in samples were selected for correlation analysis, and corresponding phyla were used as the legend. Results with *p* values greater than 0.05 were filtered out. The size of the point represents abundance, the thickness of the line represents correlation, and the color of the point represents the phylum. Red lines indicate positive correlations and blue lines indicate negative correlations.

metabolism; phenyl-alanine, tyrosine, and tryptophan biosynthesis; and pantothenate and CoA biosynthesis (Figure 3D).

3.6 Weighted co-expression network analysis of metabolomics and rumen fermentation parameters

To explore the relationship between metabolites and rumen acid indexes, 366 metabolites were analyzed using WGCNA, and highly related metabolite modules were identified and correlated with the acetic acid, propionic acid, and butyric acid contents. WGC-NA used a soft threshold (power) of 5 to cluster 366 metabolites into 14 modules (Figures 4A,B). Among the 14 modules, 21 metabolites in the red module were significantly correlated with acetic, propionic, and butyric acids ($p < 0.05$, $|r| > 0.5$) (Figure 4C). The red module was negatively correlated with acetic, propionic, and butyric acids, indicating that the metabolites in this module may be the substrates used in the synthesis of acetic, propionic, and butyric acids. The abundances of 21 metabolites in the red module that were significantly related to rumen fluid were plotted. The results showed that the aspartic acid and L-leucine levels in the YC1 group were significantly lower than those in the CON group (Figure 4D).

3.7 Pathway enrichment analysis of important modules

Twenty metabolic pathways were identified, among which five were significant ($p < 0.05$) (Figure 5A): aminoacyl-tRNA biosynthesis; valine, leucine, and isoleucine biosynthesis; valine, leucine, and isoleucine degradation; arginine biosynthesis; and histidine metabolism. To further explore the potential correlation between these metabolites and related bacteria, R-package Psych software was

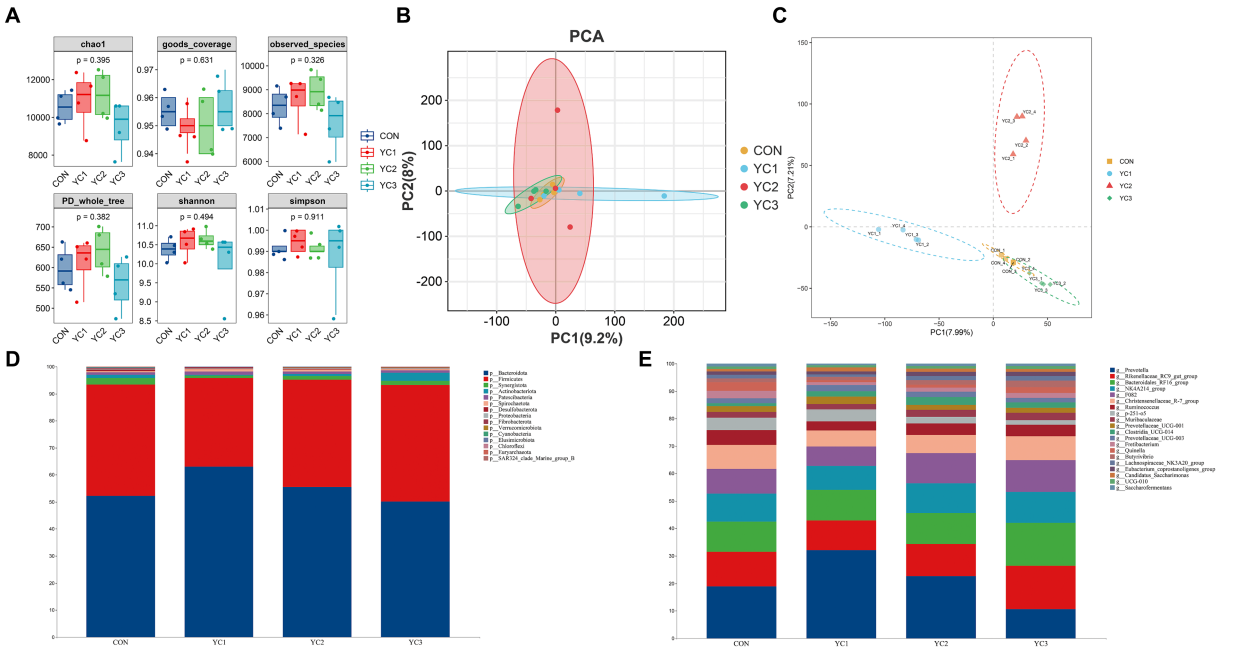

FIGURE 2
Abundance of ruminal bacteria. **(A)** Alpha diversity of rumen fluid microbiota of dairy goats with different treatments. The horizontal bar in the box represents the average. The top and bottom of the box represent the upper and lower quartiles, respectively. Single asterisk (*) means $p < 0.05$, and no asterisk means $p > 0.05$. **(B)** Principal component analysis (PCA) and **(C)** Partial least squares discriminant analysis (PLS-DA) models. Different colors represent samples from different groups of rumen liquid from dairy goats. The distance between the points on the map represents the similarity of all samples in terms of microflora composition and abundance. **(D)** Sankey diagram of species composition at the phylum level. Different colors represent different phyla. **(E)** Sankey diagrams of species composition at the genus level. Different colors represent different genera.

used to calculate the Spearman correlation coefficients. Figure 5B shows the relationship between the key metabolites and bacteria. The results revealed a significant positive correlation between *Acetitomaculum* and aspartic acid ($p < 0.05$), a significant negative correlation between *Clostridia_UCG-014* and aspartic acid ($p < 0.05$), a significant positive correlation between *Acetitomaculum*, *Blautia*, *Lachno-spiraceae_NK3A20_group*, and *Butyrivibrio* and L-leucine ($p < 0.05$); and a significant negative correlation between *Paraprevotella* and L-leucine ($p < 0.05$). As shown in Table 4, the abundance of *Acetitomaculum* in the YC1, YC2, and YC3 groups was significantly greater than that in the CON group ($p < 0.05$); the abundance of *Clostridia_UCG-014* in the YC3 group was significantly greater than that in the CON group ($p < 0.05$); and the abundance of *Blautia* and *Paraprevotella* in the YC1 and YC2 groups was significantly greater than that in the CON group ($p < 0.05$).

4 Discussion

The findings showed that dietary supplementation with YC has positive and significant effects on the milk yield of Laoshan dairy goats, which is consistent with the results of Khan et al. (28), who studied the effects of yeast supplementation on Beetal goats during early lactation and concluded that dietary yeast supplementation had beneficial effects on the milk yield. Moreover, the finding is consistent with that of Zaworski et al. (29), Dias et al. (4, 5), Nocek et al. (30), Halfen et al. (31), and Shi et al. (32) for cows and Baiomy (33) and Zicarelli et al. (34) for goats. In this study, the increased milk yield of dairy goats owing to YC supplementation was attributed to elevated levels of acetate, propionate, butyrate, and TVFAs in the rumen, which enhanced the fermentation activity of cellulolytic bacteria (35). In contrast, our findings are inconsistent with the results of Hadjipanayiotou et al. (36), who evaluated the effect of YC on milk yield in Damascus goats and reported that the milk yield did not differ between animals. The variance in results might be attributed to differences in the yeast strains and animal species.

Dietary carbohydrates are fermented by ruminal bacteria, fungi, and protozoa into end products, including VFA (e.g., acetate, propionate, and butyrate), which constitute nearly 50% of the energy requirements for ruminants (37). In this study, dietary YC supplementation increased the ruminal acetic, butyrate, and TVFA contents. These findings are consistent with those of Carpinelli et al. (38), Zhu et al. (14), Sun et al. (39) for cows, and Xue et al. (40), Özsoy et al. (41), and Ogbuewu et al. (42) for goats. In our study, we observed that YC supplementation increased the abundance of fiber degrading bacteria (Bacteroidetes and *Prevotella*) in the goat rumen, thereby promoting cellulose metabolism, which ultimately led to an increase in the ruminal acetate, butyrate, and TVFA contents (43). Studies have shown that YC supplementation reduces NH_3-N levels in ruminants. For instance, a study on dairy cows observed a tendency for lower rumen NH_3-N concentrations with YC compared to the control (44). In this study, among the 21 metabolites that presented significant correlations with rumen fermentation parameters, aspartic acid and L-leucine contents in the rumen of the YC group were significantly reduced. Furthermore, KEGG analysis of these

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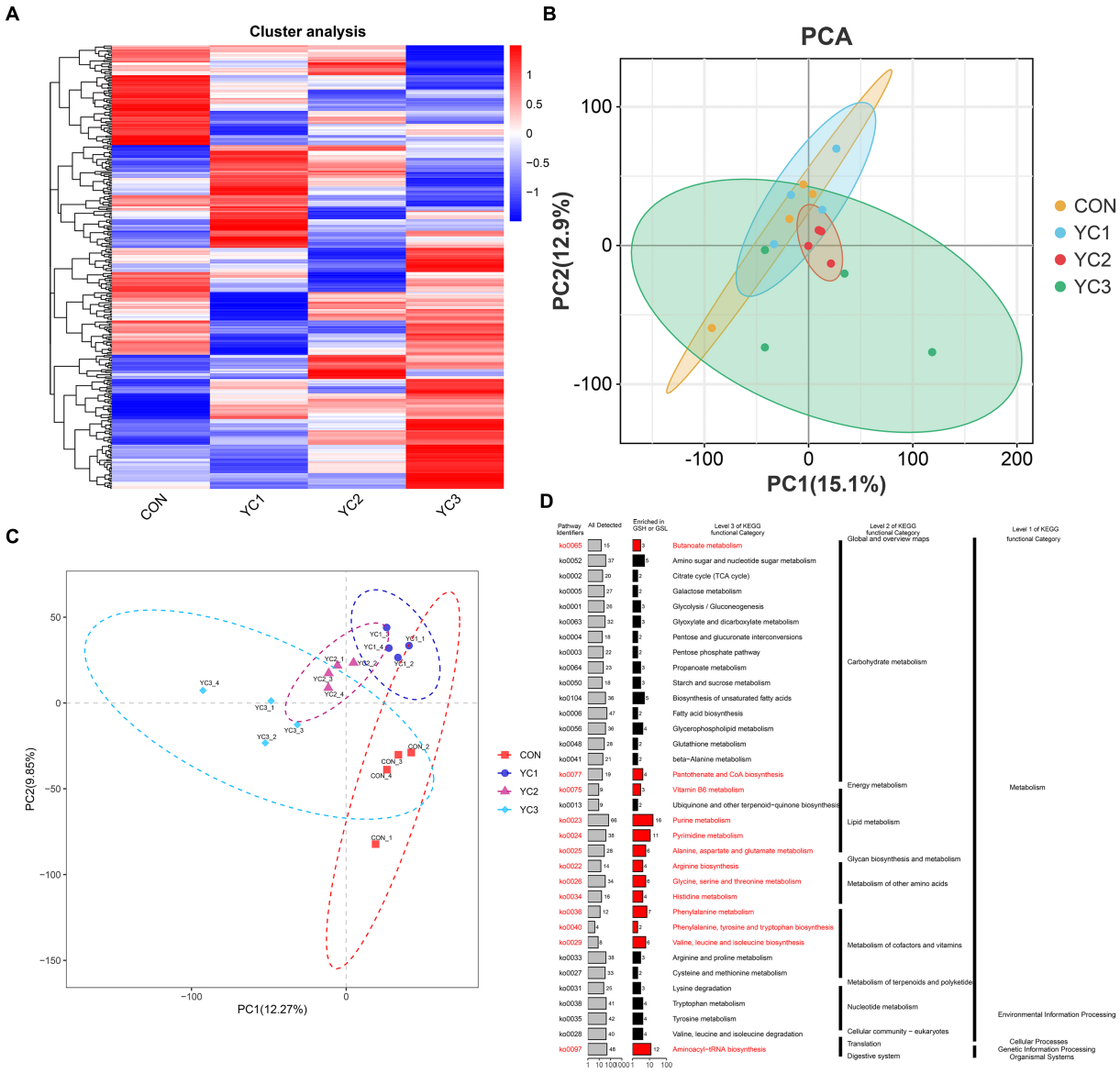

FIGURE 3
Rumen fluid metabolic spectrum. **(A)** Cluster analysis. Metabolite changes from green to red are shown for different samples. Darker red indicates greater abundance, darker green indicates lower abundance. **(B)** PCA and **(C)** PLS-DA diagrams. Different colors represent different groups of rumen fluid samples from dairy goats. The distance between points represents the similarity in microflora composition and abundance. **(D)** Kyoto encyclopedia of genes and genomes (KEGG) enrichment analysis, showing primary and tertiary pathways and KOID for each metabolic pathway. "All detected" indicates the number of metabolites annotated by KEGG data; "enrichment" indicates the number of differential metabolites enriched in the rumen acid pathway.

21 metabolites highlighted significant amino acid synthesis pathways, including aminoacyl-tRNA biosynthesis, valine, leucine, arginine biosynthesis, and histidine metabolism. These results suggest that YC supplementation enhances the efficiency of microbial protein synthesis in the rumen, thereby enabling more effective utilization of NH_3-N (45).

Higher microbial diversity in the mammalian gastrointestinal system is often associated with a stronger metabolic capacity (46). In this study, β-diversity analyses showed that microbial diversity was increased in the YC groups. Furthermore, studies have shown that feed efficiency in dairy cows was significantly associated with lower microbial diversity in the rumen (47). However, YC supplementation increased the abundance of the phylum Bacteroidota and genera *Prevotella* and *Rikenellaceae_RC9_gut_group* in the rumen of goats, mitigating the negative impacts on milk production and feed efficiency. The rumen is a complex microbial anaerobic fermentation chamber that harbors one of the most diverse intestinal microbial communities in the animal kingdom (48). Firmicutes and Bacteroidota are the dominant species in the goat rumen (49). The positive correlations observed among the phyla Firmicutes, Spirochaetota, Bacteroidota, Patescibacteria, and Synergistetes in this study are consistent with the synergistic interactions among related taxa identified in previous studies (50). These relationships may arise from shared metabolic pathways or

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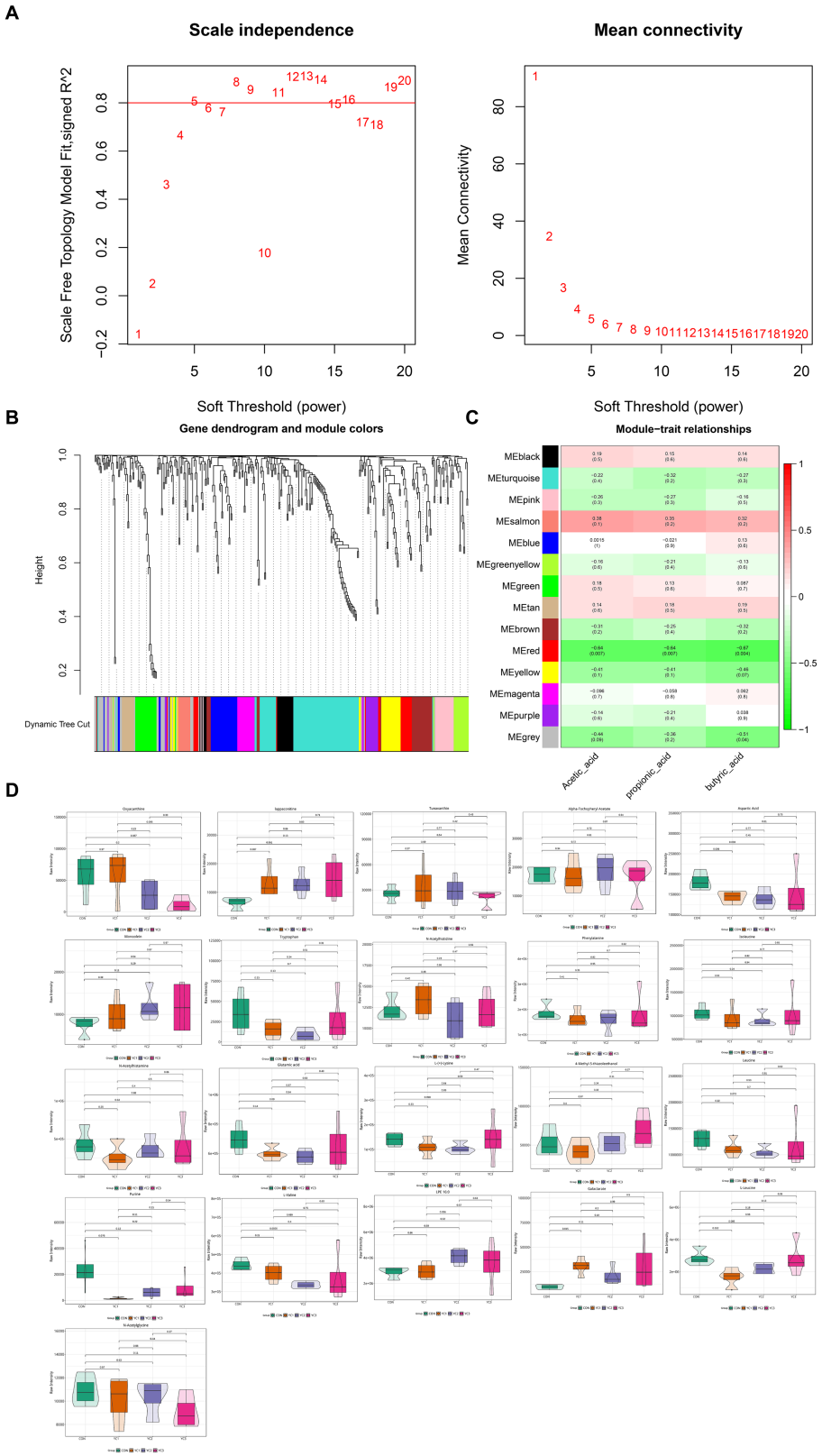

FIGURE 4
Weighted co-expression network analysis (WGCNA) of metabonomic and rumen fermentation parameters. **(A)** Determination of the soft threshold for WGCNA analysis. The scale-free fitting index and average connectivity show that a soft threshold greater than 5 satisfies a scale-free topology greater than 0.8. **(B)** Clustering tree map of different metabolites based on topological overlap. The dynamic cutting method identifies modules, shown in different colors below the tree view. **(C)** Heatmap diagram visualization module-feature association. Each row is a module characteristic, and each column is a feature, with correlation and *p* value in each unit. Red and green represent positive and negative correlations, respectively, with darker colors indicating stronger correlations. **(D)** Violin diagram of the abundance of 21 metabolites. $p < 0.05$ indicates a significant difference.

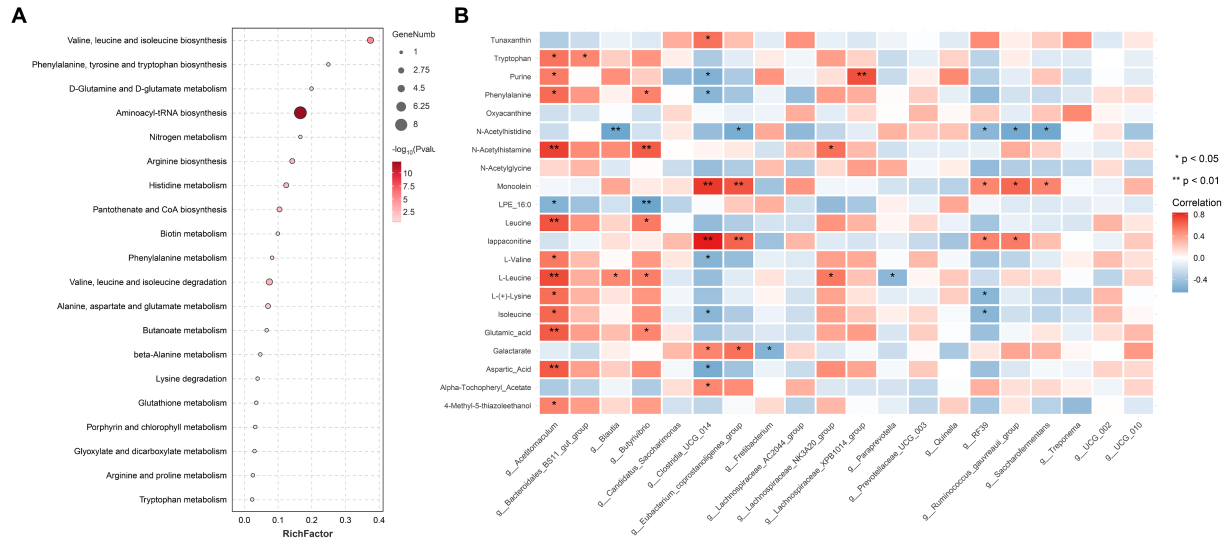

FIGURE 5
Pathway enrichment analysis of important modules. **(A)** KEGG enrichment analysis of differential metabolites significantly related to rumen acid. The *x*-axis shows the rich factor of each pathway, the *y*-axis shows the pathway names, and the color of the dot is the *p* value. A redder color indicates more significant enrichment. The size of the point represents the number of differential metabolites enriched. **(B)** Correlation analysis between rumen fluid-related metabolites and top 20 most abundant bacteria. Blue represents a negative correlation, red represents a positive correlation, and a darker color indicates a more significant correlation. An asterisk (*) indicates significance at $p < 0.05$, and no asterisk indicates $p > 0.05$.

TABLE 4 Differences in abundance of bacteria related to aspartic acid and L-leucine.

Items[1]	CON	YC1	YC2	YC3	*p*-value
Acetitomaculum	1.32 ± 0.03^{c}	1.58 ± 0.07^{a}	1.48 ± 0.04^{b}	1.43 ± 0.05^{b}	<0.01
Clostridia_UCG-014	2.22 ± 0.16^{b}	2.42 ± 0.03^{b}	2.33 ± 0.05^{b}	3.01 ± 0.14^{a}	<0.01
Blautia	1.32 ± 0.09^{b}	1.63 ± 0.11^{a}	1.57 ± 0.06^{a}	1.36 ± 0.11^{b}	0.01
Lachnospiraceae_NK3A20_group	2.65 ± 0.55	2.06 ± 0.91	3.06 ± 0.97	3.02 ± 0.85	0.47
Butyrivibrio	2.77 ± 0.70	2.76 ± 0.83	3.50 ± 0.99	4.83 ± 0.83	0.06
Paraprevotella	1.42 ± 0.01^{b}	1.86 ± 0.09^{a}	1.84 ± 0.01^{a}	1.07 ± 0.04^{c}	<0.01

[a,b]Values within the same row with different superscripts are significantly different ($p < 0.05$).
[1]CON, control diet; YC1, CON + 10 g YC/d/goat; YC2, CON + 25 g YC/d/goat; YC3, CON + 25 g YC/d/goat; YC; yeast culture.

mutualistic interactions, such as cross-feeding, in which one genus produces a metabolite utilized by another (51). Moreover, the relative abundance of Bacteroidetes increased in the rumen of the dairy goats treated with YC in this study, which is similar to the findings of Li et al. (52) regarding cows. The positive effects of YC on *Prevotella* growth have also been previously documented (53) and supported by the results of the current study. Such effects are related to YC growth factors (i.e., organic acids, B vitamins, and AA) that stimulate fiber-digesting bacteria, such as Bacteroidetes and *Prevotella* (54, 55).

The metabolites in the rumen mainly include nutrients that can be used by the host and rumen microorganisms, and differences in the levels of ruminal metabolites are associated with changes in the microbiota (56). We identified 13 tertiary metabolic pathways, which primarily included secondary metabolic pathways of lipid metabolism, glycan biosynthesis and metabolism, amino acid metabolism, and cofactor and vitamin metabolism. These findings are consistent with the results of Li et al. (57). As the main pathways of microbial VFA production, lipid metabolism, glycan biosynthesis and metabolism, and carbohydrate metabolism play important roles in the rumen (3, 58, 59). In addition, microorganisms satisfy the host's nutritional needs by performing amino acid metabolism and cofactor and vitamin metabolism to produce amino acids, vitamins, and cofactors (60). This study observed the consistency between the differences in metabolite types between the YC and CON groups and the differences in microbial populations, indicating that the diversity of microbial populations also affects the diversity of rumen metabolite types. Similarly, Xue et al. (61) also reached similar conclusions in a study on the impact of different feed types on the rumen microbiome and serum metabolome in lambs.

Goat rumen metabolites were significantly negatively correlated with acetate, butyrate, and propionate, which is consistent with a previous study in which negative correlations were observed between specific metabolites and rumen fermentation parameters (62). This study found that among the 21

metabolites negatively correlated with ruminal acetate, propionate, and butyrate, YC supplementation significantly reduced the ruminal aspartic acid and L-leucine. This observation is in line with a previous study in which YC significantly influence the metabolite profiles in the rumen of dairy cows, thereby affecting the fermentation processes (63). Studies have also shown that L-leucine could be converted into branched chain VFAs, such as acetic acid and butyric acid, during oxidative deamination (64). In addition, Jalc and Ceresnáková (65) investigated the effect of aspartic acid on rumen fermentation and found that aspartate influenced propionate production during rumen fermentation. To explore the microbes that caused the differences in ruminal aspartic acid and L-leucine levels, this study conducted a correlation analysis between aspartic acid and L-leucine and the top 20 most abundant genera in the rumen, and then it performed an analysis of variance among the groups for the correlated microbial populations. The negative correlation between aspartic acid and L-leucine and *Clostrid-ia_UCG-014* and *Paraprevotella* and the greater abundance of *Clostridia_UCG-014* and *Paraprevotella* in the YC groups revealed that differences in these genera in the rumen lead to the differences in ruminal aspartic acid and L-leucine levels.

Further investigation into these specific metabolites could reveal more about their roles and how they influence the rumen's acidic environment. Among the five significant pathways identified in this study, aspartic acid directly participates in the amino-acyl-tRNA biosynthesis and arginine biosynthesis pathways and L-leucine directly participates in the aminoacyl-tRNA biosynthesis, valine, leucine, and isoleucine biosynthesis, and valine, leucine, and isoleucine degradation pathways. Moreover, research has shown that aminoacyl-tRNA biosynthesis is the foundation of microbial protein synthesis in the rumen, supporting their growth and metabolism, which in turn influences the fermentation process in the rumen (66). In addition, microorganisms can use L-leucine to produce propionyl-CoA and acetyl-CoA through valine, leucine, and isoleucine degradation metabolic pathways, and then propionyl-CoA and acetyl-CoA can be further metabolized into VFAs, such as propionic acid and acetate, thus affecting rumen fermentation parameters (67). Integrating the findings of this study with previous research reveals that the impact of YC on ruminal fermentation parameters in goats was attributed to its promotional effect on *Clostridia_UCG-014* and *Paraprevotella*, which then utilize more aspartic acid and L-leucine through pathways such as aminoacyl-tRNA biosynthesis and valine, leucine, and isoleucine degradation. These changes ultimately lead to alterations in ruminal fer-mentation parameters.

5 Conclusion

Yeast culture dietary supplementation at 10 g/day per goat improved the milk yield and ruminal fermentation parameters in Laoshan dairy goats. Moreover, YC increased the ruminal *Clostridia_UCG-014* and *Paraprevotella* abundance, which facilitated aspartic acid and L-leucine utilization by these genera, thereby enhancing the ruminal acetic, butyrate, and TVFA contents and reducing the ruminal NH_3-N content.

Data availability statement

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

Ethics statement

The animal study was approved by the Animal Administration and Ethics Committee of Qingdao Agricultural University, Animal Science and Technology College. The study was conducted in accordance with the local legislation and institutional requirements.

Author contributions

ZL: Writing – original draft, Writing – review & editing. YH: Data curation, Formal analysis, Writing – review & editing. HL: Investigation, Writing – review & editing. YL: Funding acquisition, Project administration, Resources, Writing – review & editing. MC: Supervision, Writing – review & editing. FZ: Methodology, Writing – review & editing. YG: Methodology, Project administration, Writing – review & editing.

Funding

The author(s) declare that financial support was received for the research, authorship, and/or publication of this article. This study was financially supported by the Technological System of Modern Agricultural Industry in Shandong Province Goat Industry Innovation team (SDAIT-10), the Qingdao Agricultural University Doctoral Start Up Fund (663/1120009), and the Major Scientific and Technological Innovation Project of Shandong Province (2019JZZY020609-02).

Conflict of interest

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

Publisher's note

References

1. Wohlt JE, Finkelstein AD, Chung CH. Yeast culture to improve intake, nutrient digestibility, and performance by dairy cattle during early lactation. *J Dairy Sci*. (1991) 74:1395–400. doi: 10.3168/jds.S0022-0302(91)78294-5

2. Acharya S, Pretz JP, Yoon I, Scott MF, Casper DP. Effects of *Saccharomyces cerevisiae* fermentation products on the lactational performance of mid-lactation dairy cows. *Transl Anim Sci*. (2017) 1:221–8. doi: 10.2527/tas2017.0028

3. Bach KKE, Lærke HN, Hedemann MS, Nielsen TS, Ingerslev AK, Gundelund NDS, et al. Impact of diet-modulated butyrate production on intestinal barrier function and inflammation. *Nutrients*. (2018) 15:1499–517. doi: 10.3390/nu10101499

4. Dias ALG, Freitas JA, Micai B, Azevedo RA, Greco LF, Santos JEP. Effect of supplemental yeast culture and dietary starch content on rumen fermentation and digestion in dairy cows. *J Dairy Sci*. (2018) 101:201–21. doi: 10.3168/jds.2017-13241

5. Dias JDL, Silva RB, Fernandes T, Barbosa EF, Graças LEC, Araujo RC, et al. Yeast culture increased plasma niacin concentration, evaporative heat loss, and feed efficiency of dairy cows in a hot environment. *J Dairy Sci*. (2018) 101:5924–36. doi: 10.3168/jds.2017-14315

6. Shurson GC. Yeast and yeast derivatives in feed additives and ingredients: sources, characteristics, animal responses, quantification methods. *Anim Feed Sci Technol*. (2018) 235:60–76. doi: 10.1016/j.anifeedsci.2017.11.010

7. AAFCO. Official publication. Atlanta, GA: Association of American Feed Control Officials (2020).

8. Oeztuerk H. Effects of live and autoclaved yeast cultures on ruminal fermentation in vitro. *J Anim Feed Sci*. (2009) 18:142–50. doi: 10.22358/jafs/66378/2009

9. Opsi F, Fortina R, Tassone S, Bodas R, López S. Effects of inactivated and live cells of *Saccharomyces cerevisiae* on in vitro ruminal fermentation of diets with different forage: concentrate ratio. *J Agricul Sci*. (2012) 150:271–83. doi: 10.1017/S0021859611000578

10. Min BR, Gurung N, Shange R, Solaiman S. 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 pyrosequencing1. *J Anim Sci*. (2019) 97:3523–34. doi: 10.1093/jas/skz193

11. Wang D, Tang G, Wang Y, Yu J, Chen L, Chen J, et al. Rumen bacterial cluster identification and its influence on rumen metabolites and growth performance of young goats. *Anim Nutr*. (2023) 15:34–44. doi: 10.1016/j.aninu.2023.05.013

12. Chaucheyras DF, Walker ND, Bach A. Effects of active dry yeasts on the rumen microbial ecosystem: past, pre-sent and future. *Anim Feed Sci Technol*. (2008) 145:5–26. doi: 10.1016/j.anifeedsci.2007.04.019

13. Harrison GA, Hemken RW, Dawson KA, Harmon RJ, Barker BK. Influence of addition of yeast culture supplement to diets of lactating cows on ruminal fermentation and microbial population. *J Dairy Sci*. (1988) 71:2967–75. doi: 10.3168/jds.S0022-0302(88)79894-X

14. Zhu W, Wei Z, Xu N, Yang F, Yoon I, Chung Y, et al. Effects of *Saccharomyces cerevisiae* fermentation products on performance and rumen fermentation and microbiota in dairy cows fed a diet containing low quality forage. *J Anim Sci Biotechnol*. (2017) 8:36. doi: 10.1186/s40104-017-0167-3

15. Kholif AE, Hamdon HA, Kassab AY, Farahat ESA, Azzaz HH, Matloup OH, et al. *Chlorella vulgaris* microalgae and/or copper supplementation enhanced feed intake, nutrient digestibility, ruminal fermentation, blood metabolites and lactational performance of Boer goat. *J Anim Physiol Anim Nutr*. (2020) 104:1595–605. doi: 10.1111/jpn.13378

16. Sofyan A, Mitsumori M, Ohmori H, Uyeno Y, Hasunuma T, Akiyama K, et al. Differences in rumen fermentation characteristics between low-yield and high-yield dairy cows in early lactation. *Anim Sci J*. (2017) 88:974–82. doi: 10.1111/asj.12745

17. Russell JB, Hespell RB. Microbial rumen fermentation. *J Dairy Sci*. (1981) 64:1153–69. doi: 10.3168/jds.S0022-0302(81)82694-X

18. Cannas A, Nudda A, Pulina G. Nutritional strategies to improve lactation persistency in dairy ewes. Dairy Sheep Symposium Proceedings. (2002).

19. Cannas A, Tedeschi LO, Atzori AS, Lunesu MF. How can nutrition models increase the production efficiency of sheep and goat operations? *Anim Front*. (2019) 9:33–44. doi: 10.1093/af/vfz005

20. Instituto AL. Métodos físico-químicos para análise de alimentos. São Paulo, Brazil: Instituto Adolfo Lutz (2005).

21. NRC ed. Nutrient requirements of dairy cattle. *7th rev.* ed. Washington, DC: NRC (2001).

22. Cunha LL, Monteiro HF, Figueiredo CC, Canisso IF, Bicalho RC, Cardoso FC, et al. Characterization of rumen microbiome and metabolome from oro-esophageal tubing and rumen cannula in Holstein dairy cows. *Sci Rep*. (2023) 13:58–4. doi: 10.1038/s41598-023-33067-5

23. Chaney AL, Marbach EP. Modified reagents for determination of urea and ammonia. *Clin Chem*. (1962) 8:130–2. doi: 10.1093/clinchem/8.2.130

24. Kuczynski J, Stombaugh J, Walters WA, González A, Caporaso JG, Knight R. Using QIIME to analyze 16S rRNA gene sequences from microbial communities. *Curr Protoc Bioinformatics*. (2011) 10:10.7.1–10.7.20. doi: 10.1002/0471250953.bi1007s36

25. Dileo MV, Strahan GD, Bakker M, Hoekenga OA. Weighted correlation network analysis (WGCNA) applied to the tomato fruit metabolome. *PLoS One*. (2011) 6:e26683. doi: 10.1371/journal.pone.0026683

26. Chen PY, Cripps AW, West NP, Cox AJ, Zhang P. A correlation-based network for biomarker discovery in obesity with metabolic syndrome. *BMC Bioinformatics*. (2019) 20:477. doi: 10.1186/s12859-019-3064-2

27. Langfelder P, Horvath S. WGCNA: an R-package for weighted correlation network analysis. *BMC Bioinformatics*. (2008) 9:559. doi: 10.1186/1471-2105-9-559

28. Khan MJ, Ahmad N, Asif AR, Saleem AH. Effect of yeast supplementation on growth and production performance in beetal goats. *Pak J Agr Sci*. (2020) 57:251–4.

29. Zaworski EM, Shriver-Munsch CM, Fadden NA, Sanchez WK, Yoon I, Bobe G. Effects of feeding various dosages of *Saccharomyces cerevisiae* fermentation product in transition dairy cows. *J Dairy Sci*. (2014) 97:3081–98. doi: 10.3168/jds.2013-7692

30. Nocek JE, Holt MG, Oppy J. Effects of supplementation with yeast culture and enzymatically hydrolyzed yeast on performance of early lactation dairy cattle. *J Dairy Sci*. (2011) 94:4046–56. doi: 10.3168/jds.2011-4277

31. Halfen J, Carpinelli N, Del Pino FAB, Chapman JD, Sharman ED, Anderson JL, et al. Effects of yeast culture supplementation on lactation performance and rumen fermentation profile and microbial abundance in mid-lactation Holstein dairy cows. *J Dairy Sci*. (2021) 104:11580–92. doi: 10.3168/jds.2020-19996

32. Shi W, Knoblock CE, Murphy KV, Bruinje TC, Yoon I, Ambrose DJ, et al. Effects of supplementing a *Saccharomyces cerevisiae* fermentation product during the periparturient period on performance of dairy cows fed fresh diets differing in starch content. *J Dairy Sci*. (2019) 102:3082–96. doi: 10.3168/jds.2018-15307

33. Baiomy AA. Influence of live yeast culture on milk production, composition and some blood metabolites of Ossimi ewes during the milking period. *J Anim Poult Prod*. (2010) 1:469–80. doi: 10.21608/jappmu.2010.86260

34. Zicarelli F, Addi L, Tudisco R, Calabrò S, Lombardi P, Cutrignelli MI, et al. The influence of diet supple-mentation with *Saccharomyces cerevisiae* or *Saccharomyces cerevisiae* plus aspergillus oryzae on milk yield of Cilentana grazing dairy goats. *Small Rumin Res*. (2016) 135:90–4. doi: 10.1016/j.smallrumres.2015.12.018

35. Xu Q, Li Y, Du W, Zheng N, Wang J, Zhao S. Effect of dietary biochanin a on lactation performance, antioxidant capacity, rumen fermentation and rumen microbiome of dairy goat. *Front Microbiol*. (2023) 14:1101849. doi: 10.3389/fmicb.2023.1101849

36. Hadjipanayiotou M, Antoniou I, Photiou A. Effects of the inclusion of yeast culture on the performance of dairy ewes and goats and the degradation of feedstuffs. *Livest Prod Sci*. (1997) 48:129–34.

37. Bergman EN. Energy contributions of volatile fatty acids from the gastrointestinal tract in various species. *Physiol Rev*. (1990) 70:567–90. doi: 10.1152/physrev.1990.70.2.567

38. Carpinelli NA, Halfen J, Trevisi E, Chapman JD, Sharman ED, Anderson JL, et al. Effects of peripartal yeast culture supplementation on lactation performance, blood biomarkers, rumen fermentation, and rumen bacteria species in dairy cows. *J Dairy Sci*. (2021) 104:10727–43. doi: 10.3168/jds.2020-20002

39. Sun X, Wang Y, Wang E, Zhang S, Wang Q, Zhang Y, et al. Effects of *Saccharomyces cerevisiae* culture on ruminal fermentation, blood metabolism, and performance of high-yield dairy cows. *Animals*. (2021) 11:2401. doi: 10.3390/ani11082401

40. Xue L, Zhou S, Wang D, Zhang F, Li J, Cai L. The low dose of *Saccharomyces cerevisiae* is beneficial for rumen fermen-tation (both in vivo and in vitro) and the growth performance of heat-stressed goats. *Microorganisms*. (2022) 10:1877. doi: 10.3390/microorganisms10101877

41. Özsoy B, Yalçın S, Erdoğan Z, Cantekin Z, Aksu T. Effects of dietary live yeast culture on fattening performance on some blood and rumen fluid parameters in goats. *Rev Med Vet*. (2013) 164:263–71.

42. Ogbuewu IP, Mbajiorgu CA. Meta-analysis of *Saccharomyces cerevisiae* on enhancement of growth performance, rumen fermentation and haemato-biochemical characteristics of growing goats. *Heliyon*. (2023) 9:e14178. doi: 10.1016/j.heliyon.2023.e14178

43. Tun HM, Li S, Yoon I, Meale SJ, Azevedo PA, Khafipour E, et al. *Saccharomyces cerevisiae* fermentation products (SCFP) stabilize the ruminal microbiota of lactating dairy cows during periods of a depressed rumen pH. *BMC Vet Res*. (2020) 16:237. doi: 10.1186/s12917-020-02437-w

44. Hristov AN, Varga G, Cassidy T, Long M, Heyler K, Karnati SK, et al. Effect of *Saccharomyces cerevisiae* fermentation product on ruminal fermentation and nutrient utilization in dairy cows. *J Dairy Sci*. (2010) 93:682–92. doi: 10.3168/jds.2009-2379

45. Erasmus LJ, Botha PM, Kistner A. Effect of yeast culture supplement on production, rumen fermentation, duodenal nitrogen flow in dairy cows. *J Dairy Sci*. (1992) 75:3056–65. doi: 10.3168/jds.S0022-0302(92)78069-2

46. Li H, Zhou R, Zhu J, Huang X, Qu J. Environmental filtering increases with elevation for the assembly of gut micro-biota in wild pikas. *Microb Biotechnol*. (2019) 12:976–92. doi: 10.1111/1751-7915.13450

47. Monteiro HF, Zhou Z, Gomes MS, Peixoto PMG, Bonsaglia ECR, Canisso IF, et al. Rumen and lower gut microbiomes relationship with feed efficiency and production

traits throughout the lactation of Holstein dairy cows. *Sci Rep*. (2022) 12:4904. doi: 10.1038/s41598-022-08761-5

48. Li C, Li Y, Li X, Ma X, Ru S, Qiu T. Veterinary antibiotics and estrogen hormones in manures from concentrated animal feedlots and their potential ecological risks. *Environ Res*. (2021) 198:110463. doi: 10.1016/j.envres.2020.110463

49. Klassen L, Reintjes G, Tingley JP, Jones DR, Hehemann J, Smith AD. Quantifying fluorescent glycan uptake to elucidate strain-level variability in foraging behaviors of rumen bacteria. *Microbiome*. (2021) 9:23. doi: 10.1186/s40168-020-00975-x

50. Schink B. Synergistic interactions in the microbial world. *Antonie Van Leeuwenhoek*. (2002) 81:257–61. doi: 10.1023/a:1020579004534

51. Culp EJ, Goodman AL. Cross-feeding in the gut microbiome: ecology and mechanisms. *Cell Host Microbe*. (2023) 31:485–99. doi: 10.1016/j.chom.2023.03.016

52. Li Z, Fan Y, Bai H, Zhang J, Mao S, Jin W. Live yeast supplementation altered the bacterial community's composition and function in rumen and hindgut and alleviated the detrimental effects of heat stress on dairy cows. *J Anim Sci*. (2023) 101:skac410. doi: 10.1093/jas/skac410

53. Mao HL, Mao HL, Wang JK, Liu JX, Yoon I. Effects of *Saccharomyces cerevisiae* fermentation product on in vitro fer-mentation and microbial communities of low-quality forages and mixed diets. *J Anim Sci*. (2013) 91:3291–8. doi: 10.2527/jas.2012-5851

54. Aschalew ND, Zhang L, Wang Z, Xia Y, Yin G, Dong J, et al. Effects of yeast culture and oxalic acid supplementation on in vitro nutrient disappearance, rumen fermentation, and bacterial community composition. *Front Vet Sci*. (2024) 10:1330841. doi: 10.3389/fvets.2023.1330841

55. Rabee AE, Younan BR, Kewan KZ, Sabra EA, Lamara M. Modulation of rumen bacterial community and feed utilization in camel and sheep using combined supplementation of live yeast and microalgae. *Sci Rep*. (2022) 12:12990. doi: 10.1038/s41598-022-16988-5

56. Zhu W, Liu T, Deng J, Wei CC, Zhang ZJ, Wang DM, et al. Microbiome-metabolomics analysis of the effects of decreasing dietary crude protein content on goat rumen mictobiota and metabolites. *Anim Biosci*. (2022) 35:1535–44. doi: 10.5713/ab.21.0411

57. Li YQ, Xi YM, Wang ZD, Zeng HF, Han ZY. Combined signature of rumen microbiome and metabolome in dairy cows with different feed intake levels. *J Anim Sci*. (2020) 98:skaa070. doi: 10.1093/jas/skaa070

58. Flint HJ, Bayer EA, Rincon MT, Lamed R, White BA. Polysaccharide utilization by gut bacteria: potential for new insights from genomic analysis. *Nat Rev Microbiol*. (2008) 6:121–31. doi: 10.1038/nrmicro1817

59. Andersen TO, Altshuler I, Vera-Ponce de León A, Walter JM, McGovern E, Keogh K, et al. Metabolic influence of core ciliates within the rumen microbiome. *ISME J*. (2023) 17:1128–40. doi: 10.1038/s41396-023-01407-y

60. Newbold CJ, Ramos-Morales E. Review: ruminal microbiome and microbial metabolome: effects of diet and ruminant host. *Animal*. (2020) 14:s78–86. doi: 10.1017/S1751731119003252

61. Xue MY, Sun HZ, Wu XH, Liu JX, Guan LL. Multi omics reveals that the rumen microbiome and its metabolome together with the host metabolome contribute to individualized dairy cow performance. *Microbiome*. (2020) 8:64. doi: 10.1186/s40168-020-00819-8

62. Wang Y, Nan X, Zhao Y, Jiang L, Wang M, Wang H, et al. Rumen microbiome structure and metabolites activity in dairy cows with clinical and subclinical mastitis. *J Anim Sci Biotechnol*. (2021) 12:36. doi: 10.1186/s40104-020-00543-1

63. Du D, Jiang W, Feng L, Zhang Y, Chen P, Wang C, et al. Effect of *Saccharomyces cerevisiae* culture mitigates heat stress-related dame in dairy cows by multi-omics. *Front Microbiol*. (2022) 13:935004. doi: 10.3389/fmicb.2022.935004

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

65. Jalc D, Ceresnáková Z. Effect of plant oils and aspartate on rumen fermentation in vitro. *J Anim Physiol Anim Nutr*. (2001) 85:378–84. doi: 10.1046/j.1439-0396.2001.00349.x

66. Lu Z, Xu Z, Shen Z, Tian Y, Shen H. Dietary energy level promotes rumen microbial protein synthesis by improving the energy productivity of the ruminal microbiome. *Front Microbiol*. (2019) 10:847. doi: 10.3389/fmicb.2019.00847

67. National Center for Biotechnology Information. PubChem pathway summary for pathway SMP0063689, valine, leucine, and isoleucine degradation. Source: PathBank. (2023). Available at: https://pubchem.ncbi.nlm.nih.gov/pathway/PathBank:SMP0063689

frontiers | Frontiers in Veterinary Science

TYPE Original Research
PUBLISHED 25 September 2024
DOI 10.3389/fvets.2024.1455029

OPEN ACCESS

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RECEIVED 26 June 2024
ACCEPTED 26 August 2024
PUBLISHED 25 September 2024

CITATION
Zhang R, Zhang L, An X, Li J, Niu C, Zhang J, Geng Z, Xu T, Yang B, Xu Z and Yue Y (2024) Hybridization promotes growth performance by altering rumen microbiota and metabolites in sheep.
Front. Vet. Sci. 11:1455029.
doi: 10.3389/fvets.2024.1455029

Hybridization promotes growth performance by altering rumen microbiota and metabolites in sheep

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Hybridization can substantially improve growth performance. This study used metagenomics and metabolome sequencing to examine whether the rumen microbiota and its metabolites contributed to this phenomenon. We selected 48 approximately 3 month-old male ♂Hu × ♀Hu (HH, $n = 16$), ♂Poll Dorset × ♀Hu (DH, $n = 16$), and ♂Southdown × ♀Hu (SH, $n = 16$) lambs having similar body weight. The sheep were fed individually under the same nutritional and management conditions for 95 days. After completion of the trial, seven sheep close to the average weight per group were slaughtered to collect rumen tissue and content samples to measure rumen epithelial parameters, fermentation patterns, microbiota, and metabolite profiles. The final body weight (FBW), average daily gain (ADG), and dry matter intake (DMI) values in the DH and SH groups were significantly higher and the feed-to-gain ratio (F/G) significantly lower than the value in the HH group; additionally, the papilla height in the DH group was higher than that in the HH group. Acetate, propionate, and total volatile fatty acid (VFA) concentrations in the DH group were higher than those in the HH group, whereas NH_3-N concentration decreased in the DH and SH groups. Metagenomic analysis revealed that several *Prevotella* and *Fibrobacter* species were significantly more abundant in the DH group, contributing to an increased ability to degrade dietary cellulose and enrich their functions in enzymes involved in carbohydrate breakdown. *Bacteroidaceae bacterium* was higher in the SH group, indicating a greater ability to digest dietary fiber. Metabolomic analysis revealed that the concentrations of rumen metabolites (mainly lysophosphatidylethanolamines [LPEs]) were higher in the DH group, and microbiome-related metabolite analysis indicated that *Treponema bryantii* and *Fibrobacter succinogenes* were positively correlated with the LPEs. Moreover, we found methionine sulfoxide and N-methyl-4-aminobutyric acid were characteristic metabolites in the DH and SH groups, respectively, and are related to oxidative stress, indicating that the environmental adaptability of crossbred sheep needs to be further improved. These findings substantially deepen the general understanding of how hybridization promotes growth performance from the perspective of rumen microbiota, this is vital for the cultivation of new species and the formulation of precision nutrition strategies for sheep.

KEYWORDS

hybridization, rumen, metagenome, growth performance, sheep

1 Introduction

Hybridization is an important method of breeding sheep that can produce heterosis, resulting in a new breed that is stronger or performs better than its parents (1). Binary hybridization in the cultivation of new varieties of mutton sheep has been frequently reported in China (2). Even though three-way crossbreeding is commonly used in pig production and for developing new breeds (3, 4), relevant research is still in the early stages of selecting suitable species on mutton sheep industry (5). Compared with the former, the latter could effectively utilize the heterosis of three hybrid individuals, producing a significantly stronger effect. Hu is a native sheep breed with perennial estrus, high fecundity, high lactation, good parenting traits, and strong adaptability; therefore, they are often used as crossbreeding ewes for mutton sheep, however, their meat production rate and dressing percentage are unsatisfactory (6, 7). Poll Dorset is native to Australia and New Zealand, has the characteristics of fast growth, early maturity, and perennial estrus, and is considered the main parent for hybridization in the Chinese sheep breeding industry (8). Southdown is considered the best breed for meat quality in the United Kingdom due to its ideal meat structure, early maturity, easy breeding, good carcass quality, and dressing percentage > 55% (9, 10). Southdown and Poll Dorset are small sheep species that grow rapidly in the early stages and are suitable for producing high-grade fat lambs, which aligns with the current market demand for mutton in China. Therefore, we attempted to cultivate a new breed of sheep by using Poll Dorset and Southdown as sires, crossing them with Hu sheep, and monitoring the production traits of the F_1 generation.

Growth performance is the main selection trait for breeding new sheep varieties. The rumen microbiota is associated with animal growth and health (11). The rumen is colonized by bacteria, protozoa, archaea, fungi, and viruses, which degrade complex plant fibers and polysaccharides to produce volatile fatty acids (VFAs), microbial proteins, and vitamins, in turn providing nutrients to meet host requirements for maintenance and growth (12, 13). Although diet plays a major role in shaping the gastrointestinal microbiota, recent evidence has revealed host genetics to be an important factor in determining the gut microbiota composition in cattle (13–15). However, research on the effects of host genetics on the rumen microbiota composition of hybridized sheep is limited.

Therefore, in this study, we hypothesized that rumen microbiota composition and function in sheep are affected by host genetics, affecting growth performance in turn. We assessed the rumen microbiota and metabolite profiles of different hybridized sheep raised in the same farm environment, aiming to elucidate the underlying molecular mechanisms from the perspective of rumen microbiota and thus provide a theoretical and practical basis for improving the growth performance of hybridized sheep.

2 Materials and methods

2.1 Animals, experimental design, and sample collection

This study was conducted at the Qinghuan Mutton Sheep Breeding Company in Huanxian County, Gansu, China from October 2022 to January 2023. The experimental design, procedures, and methods were approved by the Animal Administration and Ethics Committee of the Lanzhou Institute of Husbandry and Pharmaceutical Science of the Chinese Academy of Agricultural Science under permission number 2022–018 and followed the Chinese standards for the use and care of animals. Forty-eight approximately 3 month-old male ♂Hu × ♀Hu (HH, $n = 16$), ♂Poll Dorset × ♀Hu (DH, $n = 16$), and ♂Southdown × ♀Hu (SH, $n = 16$) lambs were selected and raised in a single pen under the same nutritional and management conditions for 95 days (including a 15 day pre-test period). Hu sheep were chosen from 216 approximately 3 month-old male lambs with an average body weight of 22.98 ± 6.37 kg, and F_1 generation sheep of similar body weight were selected. Poll Dorset and Southdown sires (aged 31 months) generated from embryo transplantation and Hu sires (aged 24 months) were crossed with Hu ewes (parity one or two) by estrus synchronization and artificial insemination. The offspring (one, in case of twins) were confirmed to stem from different fathers. All sheep were fed the same total mixed ratio composed of oat hay (6.08% DM), *Leymus chinensis* (13.04% DM), corn silage (13.53% DM), corn (32.78% DM), megalac (6.08% DM) and grain mixtures (28.49% DM) twice daily at 08:00 and 15:00 (Supplementary Table S1). During the experimental period, all animals had access to feed and water *ad libitum*. Feed intake was recorded daily based on the feed offered and refusals to calculate the average dry matter intake (DMI). Body weight was recorded every 20 d using a digital livestock scale (TCS Electronic Platform Scale, Rongcheng, China) to calculate the average daily gain (ADG) and feed-to-gain ratio (F/G).

At the end of the experiment, seven sheep close to the average weight per group were selected and fasted for 12 h before harvesting. Harvesting was based on standard commercial procedures, individually restrained, exsanguinated, skinned, and gutted. Rumen tissue samples of approximately 2 cm × 2 cm were carefully separated from the left dorsal sac to fixed in 4% paraformaldehyde solution for hematoxylin–eosin (H&E) staining. Then, two portions of 5 mL rumen content were collected from each animal, transferred into a sterile tube, immediately frozen using liquid nitrogen, and stored at −80°C for DNA and metabolite extraction. Another 15 mL rumen content was sampled and stored in a sterilized container at −20°C for evaluation of fermentation parameters. The rumen contents of all animals were collected from the left dorsal sac as a mixture of liquid and solid components. All samples were collected within 30 min of slaughter.

2.2 Rumen epithelial parameters

Rumen epithelial parameters, including papilla height, papilla width, and muscle thickness, were obtained using the H&E staining technique (16). The fixed rumen tissues were dehydrated in alcohol, cleared in xylene, and embedded in paraffin. Then the cooling concretionary samples were sectioned at 5 μm thickness and mounted on glass slides. Paraffin-embedded sections were dewaxed with xylene, passed through a graded ethanol series to remove the xylene, rinsed with distilled water, and stained with H&E. Subsequently, the paraffin was sealed with neutral gum after dehydration and immediately examined. Finally, two digital slides were acquired from each animal using a Slide Viewer device (3D HISTECH Ltd.) at two-fold magnification; then, five papillae (excluding damaged ones) were chosen randomly from each digital slide to measure papilla height using ImageJ software (National Institutes of Health, Bethesda, MD, United States). Within those papillae measured for height, three different regions (apical, middle, and basal) were identified to measure papilla width. Each digital slide was divided into five equal parts to measure muscle thickness.

2.3 Rumen fermentation parameters

The pH of the rumen was measured immediately after the sheep were slaughtered using an Ark Technology PHS-10 portable acidity meter (Chengdu, China). The NH_3-N concentration was determined by colorimetry. Briefly, 2 g of rumen content was accurately weighed and mixed with deionized water at a 1:5 ratio. The mixed solution was shaken for 1 h at 105 r/min and centrifuged at 12,000 rpm at 4°C for 20 min. The supernatant was then passed through a 0.45 μm polymer fiber filter membrane and stored for further analysis. Subsequently, the NH_3-N concentration was measured using a kit (Biosino Biotechnology Co. Ltd., Beijing, China) according to the manufacturer's instructions and a microplate reader (DR-200BS; Hiwell-Diatek, Wuxi, China). VFA concentrations were measured as previously described (16). Briefly, rumen contents were centrifuged at 5,400 rpm for 10 min. We subsequently uniformly mixed 1 mL of the resulting supernatant and a 0.2 mL 25% metaphosphate solution containing 2-ethylbutyric acid as an internal standard in a new centrifuge tube. This reaction tube was then immersed in an ice bath (30 min) and centrifuged at 10,000 rpm for another 10 min. The supernatant was passed through a 0.22 μm organic phase filter membrane and stored in 2 mL bottles for subsequent analysis. A gas chromatograph (GC 7890A; Agilent Technologies, Santa Clara, CA, United States) fitted with an AT-FFAP capillary column (50 m × 0.32 mm × 0.25 μm; Agilent Technologies) was used to determine VFA concentrations. The column temperature was maintained at 60°C for 1 min, raised by 5°C/min to 115°C, and increased by 15°C/min to 180°C. Notably, the detector and injector temperatures were 260°C and 250°C, respectively.

2.4 Metagenome sequencing and bioinformatics analysis

Total genomic DNA was extracted from rumen content sample using the E.Z.N.A. Soil DNA Kit (Omega Biotek, Norcross, GA, United States). The DNA concentration and purity were determined using TBS-380 mini-fluorometer (Promega, Madison, WI, United States) and NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, United States), respectively. The DNA was fragmented to approximately 400 bp for paired-end library construction using a Covaris M220 (Gene Company Limited, Hong Kong, China). Metagenome libraries sequencing was performed using an Illumina NovaSeq 6,000 platform (Illumina Inc., San Diego, CA, United States).

Quality control of the metagenomic sequence reads was performed using the Fastp software (version 0.20.0) to trim the 3′-end and 5′-end of reads, cut low-quality bases, and remove short reads and "N" records (17). The quality-filtered reads were then aligned to the *Ovis aries* reference genome using BWA v 0.7.1 to filter out the host DNA (18). The remaining reads were assembled using Multiple Megahit (version 1.1.2) (19), and contigs with lengths ≥300 bp were selected as the final assembling result for the prediction of open reading frames (ORF) using MetaGene v 0.3.38 (20). Non-redundant contigs were identified using CD-HIT with 95% sequence identity and 90% coverage (21). Original sequencing reads were mapped to predicted genes to estimate their abundance using SOAPAligner v. 2.21 (22). Subsequently, the non-redundant gene catalog was aligned to the NCBI non-redundant protein sequence database using BLASTP (version 2.2.28+) to obtain taxonomic annotations and species abundances (23). Taxonomic profiles were conducted at domain, phylum, genus and species levels, with relative abundances calculated. The PCoA based on Bray–Curtis dissimilarity matrices at domain level was performed. Bacteria with a relative abundance >0.01% and archaea with a relative abundance >0.001% were analyzed. Finally, a BLAST search (version 2.2.28+) with an optimization criterion cutoff of $1e^{-5}$ was annotated against the Kyoto Encyclopedia of Genes and Genomes (KEGG) database (24). Carbohydrate-active enzyme (CAZyme) annotation was conducted using hmmscan[1] against CAZy database version 5.0[2] with an e-value cutoff of $1e^{-5}$.

2.5 Metabolome sequencing and bioinformatics analysis

Rumen sample metabolite extraction was based on previously published procedures (16). The metabolome of the rumen content was analyzed by ultra-performance liquid chromatography (UPLC; Shim-pack UFLC Shimadzu CBM30A; Shimadzu, Kyoto, Japan) and tandem mass spectrometry (MS/MS, QTRAP® 6,500+, SCIEX, Framingham, MA, United States) (25). After obtaining the liquid chromatography–mass spectrometry data of the samples, the extracted ion chromatographic peaks of all metabolites were integrated using MultiQuant software (Applied Biosystems, Foster City, CA, United States). The chromatographic peaks of the metabolites in different samples were corrected using integration (26). The relative concentrations of rumen metabolites were screened by fold change (FC) (FC ≥ 2 or FC ≤ 0.5) and variable importance in projection (VIP) (VIP ≥ 1) to identify the different metabolites. Identified metabolites were annotated using the KEGG compound database (27).

2.6 Statistical analysis

All data were checked for normality and outliers using SPSS version 26.0 (IBM Corp., Armonk, NY, United States) before any statistical analyses were conducted. One-way ANOVA and LSD *post-hoc* test was used to analyze the data of growth performance as well as rumen epithelial and fermentation parameters. Spearman's correlation test was used for correlation analyses. Statistical significance was set to $p < 0.05$. GraphPad Prism version 8.5 (GraphPad Software, La Jolla, CA, United States) drew the statistical maps.

3 Results

3.1 Hybridization promoted growth performance in Hu sheep

A summary of the growth performance data is presented in Table 1. The initial body weight (IBM) did not differ significantly among the three groups ($p = 0.156$). The DH and SH groups exhibited greater ADG than did the HH group ($p = 0.008$) but did not differ from each other. The final body weight (FBW) was greater in the DH and SH groups than in the HH

1 http://hmmer.janelia.org/search/hmmscan

2 http://www.cazy.org/

TABLE 1 Hybridization promoted growth performance in Hu sheep.

Measurements	HH	DH	SH	SEM	*p* value
Initial body weight (IBM, kg)	23.36	23.64	24.21	0.43	0.156
Final body weight (FBW, kg)	44.33^{b}	49.31^{a}	49.16^{a}	0.81	<0.001
Average daily gain (ADG, g/d)	223.57^{b}	259.29^{a}	258.66^{a}	11.36	0.008
Dry matter intake (DMI, kg/d)	0.86^{b}	0.88^{a}	0.89^{a}	0.01	0.001
Feed/gain (F/G)	7.84^{a}	6.86^{b}	6.90^{b}	0.33	0.013

Different lower-case letters indicate significant differences between groups. HH = ♂Hu × ♀Hu; DH = ♂Poll Dorset × ♀Hu; SH = ♂Southdown × ♀Hu; SEM = standard error of the mean.

group ($p < 0.001$). DMI was also greater in the DH and SH groups, resulting in a lower F/G value than that in the HH group ($p = 0.013$).

3.2 Hybridization increased rumen epithelial development in Hu sheep

Three rumen epithelial development indices, including papilla height, papilla width, and muscle thickness, were measured using H&E staining (Figures 1A–C). Hybridization had no effect on rumen papilla width or muscle thickness ($p > 0.128$; Figures 1E,F). The largest difference was observed in papilla height, the DH group exhibited a greater height than the HH group ($p = 0.046$; Figure 1D).

3.3 Hybridization altered rumen fermentation parameters in Hu sheep

The NH_3-N concentration was significantly lower in the SH and DH groups than in the HH group ($p = 0.023$; Figure 2). Acetate and propionate concentrations in the DH group were significantly higher than those in the HH group ($p < 0.041$), resulting in higher total VFAs ($p = 0.048$). The pH value as well as butyrate, valerate, isobutyrate, isovalerate, and acetate/propionate concentrations remained unchanged.

3.4 Profiling of the rumen metagenome

Metagenome sequencing generated a total of 1,443,800,424 raw reads, with an average of 68,752,401 ± 5,953,935 reads (mean ± SD) per sample. After quality control and removing host genes, a total of 1,284,320,034 reads were retained, or 61,158,097 ± 5,264,516 per sample. After assembly, a total of 19,962,258 contigs were generated (with an average N50 length of 825 ± 60), with 950,584 ± 91,196 per sample (Supplementary Table S2). The results indicated that the sequencing data were credible and could be used for subsequent bioinformatics investigations. The α-diversity analysis using Kruskal–Wallis Test revealed that the Shannon index was significantly increased in the DH group compared to that in the SH group ($p = 0.016$; Figure 3A), while the Ace and Simpson indices did not differ among the three groups (Supplementary Table S3). The rumen metagenome comprised 97.18% bacteria (591,631,456 sequences), 1.60% archaea (9,758,146 sequences), 0.43% eukaryotes (2,601,458 sequences), and 0.78% viruses (4,731,066 sequences). Principal coordinate analysis (PCoA) at the domain level revealed a separation between the HH and DH groups and HH and SH groups based on bacteria (Figure 3B), as well as between the SH and HH groups and SH and DH groups based on archaea (Figure 3C), no separation was observed based on eukaryotes or viruses (Supplementary Figure S1). Therefore, the comparative analysis of rumen microbial taxa among the three groups focused only on bacteria and archaea.

3.5 Compositional profiles of the rumen microbiome and taxonomic differences

The dominant bacterial phyla in the rumen were Bacteroidetes (HH: 44.39%; DH: 44.87%; SH: 45.53%) and Firmicutes (HH: 42.72%; DH: 40.33%; SH: 43.24%), followed by Spirochaetes (HH: 2.81%; DH: 4.64%; SH: 2.55%); however, no significant differences were found among the three groups ($p > 0.05$; Supplementary Figure S2A). A total of 5,413 genera were identified. Among them, *unclassified o Bacteroidales* (HH: 19.53%; DH: 14.50%; SH: 20.21%), *Prevotella* (HH: 14.31%; DH: 19.93%; SH: 14.87%), and *unclassified c Clostridia* (HH: 8.33%; DH: 7.03%; SH: 8.83%) were the predominant bacteria in the rumen samples (Supplementary Figure S2B). A total of 20,943 species were found, among which the dominant bacteria were the *Bacteroidales bacterium* (HH: 19.35%; DH: 14.42%; SH: 20.00%), *Prevotella* sp. (HH: 12.37%; DH: 16.29%; SH: 12.71%) and *Clostridia bacterium* (HH: 8.33%; DH: 7.03%; SH: 8.83%) (Supplementary Figure S2C).

Differential analysis revealed a total of 28 significantly different bacterial species between the DH and HH groups. Among them, 18 exhibited significantly higher abundances in the DH group. The relative abundances of *Treponema bryantii*, *Fibrobacter* sp., *Prevotella* sp. *tc2-28*, *Spirochaetia bacterium*, and *Alloprevotella* sp. were the top five most significantly increased. Additionally, the relative abundances of *Prevotella copri*, *Prevotella* sp. *BP1-148*, *Prevotella* sp. *E13-3*, *Fibrobacter succinogenes*, *Fibrobacter* sp. *UWB4*, *Fibrobacter* sp. *UWB2*, *Fibrobacter* sp. *UWR2*, *Fibrobacter* sp. *UWOV1*, and *Fibrobacter* sp. *UWB1* was also significantly increased in the DH group. Ten bacteria were significantly enriched in the HH group, mainly *Bacteroidales bacterium, Anaerolineaceae bacterium, Sarcina* sp., *Sarcina* sp. *DSM 11001*, and *Flexilinea* sp. (Figure 4A). Compared to levels in the HH group, only one bacterium (*Bacteroidaceae bacterium*) was significantly enriched in the SH group, whereas three (*Succiniclasticum ruminis*, *Sarcina* sp. *DSM 11001*, and *Ruminococcus* sp. M6(2020)) were significantly more abundant in the HH group (Figure 4B). When compared to levels in the DH group, 19 bacterial abundances were significantly different, including six (*Bacteroidales bacterium*, *Pyramidobacter* sp., *Clostridiaceae bacterium*, *Bacteroidales bacterium WCE2004*, *Bacteroidetes bacterium*, and *unclassified p Firmicutes*) with higher abundances in the SH group and 13 (*Treponema* sp., *Acidaminococcaceae bacterium*, *Fibrobacter* sp., *Treponema bryantii*, *Schwartzia* sp., *Prevotella* sp.*tc2-28*, *Spirochaetia bacterium*, *Schwartzia succinivorans*,

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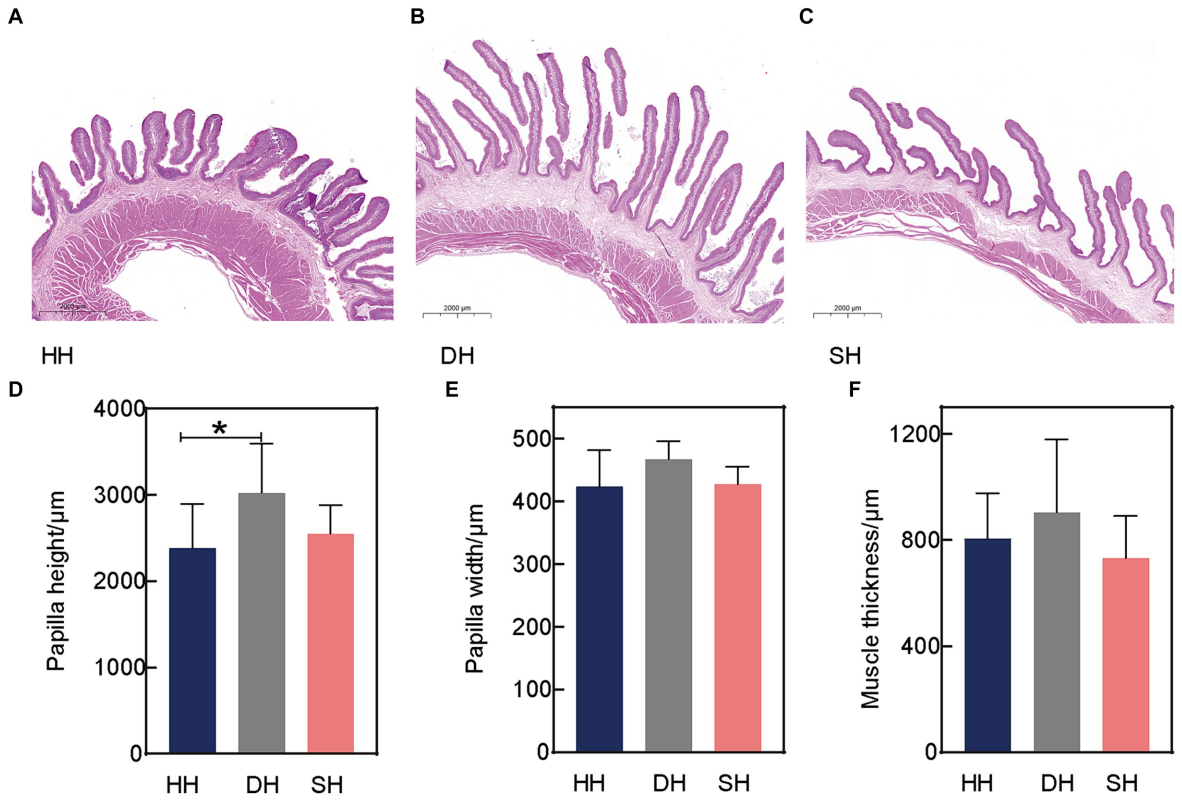

FIGURE 1
Hybridization promoted rumen epithelial development in Hu sheep. **(A)** Rumen epithelium hematoxylin and eosin (H&E) staining in the HH group. **(B)** Rumen epithelium H&E staining in the DH group. **(C)** Rumen epithelium H&E staining in the SH group. **(D)** Rumen papilla height. **(E)** Rumen papilla width. **(F)** Muscle thickness. $n = 7$ individuals/group; * indicates $p < 0.05$.

Fibrobacter succinogenes, *Fibrobacter* sp. *UWB2*, *Fibrobacter* sp. *UWB3*, *Eubacterium ruminantium*, and *Fibrobacter* sp. *UWB12*) with higher abundances in the DH group (Supplementary Figure S3A).

At the species level, the abundance of two archaeal species (*Nitrosopumilales archaeon* and *Candidatus Methanomethylophilus alvus*) was significantly enriched and one (*Methanophagales archaeon*) significantly decreased in the DH group compared to levels in the HH group (Figure 4C). The abundance of three archaea (*Methanomicrobium* sp., *Methanolobus bombayensis*, and *Methanococcus voltae*) was significantly enriched and three (*Candidatus Helarchaeota archaeon*, *Thermoplasmata archaeon M8B2D*, and *Theionarchaea archaeon*) decreased in the SH group compared to levels in the HH group (Figure 4D). When compared with levels in the DH group, the abundances of *Methanocorpusculum* sp. and *Candidatus Altiarchaeales archaeon WOR SM1* 86-2 were significantly higher, whereas the abundances of *Candidatus Methanomethylophilaceae archaeon*, *Candidatus Methanomethylophilus* sp., *Methanomicrobium* sp., *Methanobacterium* sp. *ERen5*, *unclassified g Methanobacterium*, and *Methanolobus bombayensis* were significantly lower than those in the SH group (Supplementary Figure S3B).

3.6 Functional profiles and differences of the rumen microbiome

The functions of the rumen microbiome were determined using KEGG profiles and genes encoding CAZymes. For KEGG profiles, a total of six pathways were annotated at the first level among the three groups, including "metabolism" (HH: 49.78%; DH: 50.16%; SH: 50.85%), "genetic information processing" (HH: 16.78%; DH: 16.71%; SH: 17.05%), "environmental information processing" (HH: 12.92%; DH: 12.98%; SH: 12.77%), "cellular processes" (HH: 9.71%; DH: 9.68%; SH: 9.42%), "human diseases" (HH: 6.67%; DH: 6.47%; SH: 6.22%) and "organismal systems" (HH: 4.15%; DH: 4.00%; SH: 3.70%). At the second level, we obtained only one profile, "metabolism of cofactors and vitamins," that was significantly enriched in the DH group compared to level in the HH groups (Supplementary Figure S4A). Nine KEGG profiles were significantly enriched in the SH group compared to levels in the HH group, among them, "replication and repair," "nucleotide metabolism," and "biosynthesis of other secondary metabolites" were the most dominant (Supplementary Figure S4B). Seven KEGG profiles were significantly different between the DH and SH groups. "glycan biosynthesis and metabolism" and "nucleotide metabolism" were the most enriched in the SH group, while "signal transduction" was enriched in the DH group (Supplementary Figure S4C). When comparing the identified KEGG pathways at the third level, "biofilm formation—pseudomonas aeruginosa," "bacterial chemotaxis," and "riboflavin metabolism" were significantly enriched in the DH group, while the "lysosome" and "biosynthesis of various plant secondary metabolites" pathways were significantly decreased compared to levels in the HH group (Figure 5A). A total of 21 differential pathways were identified in the HH and SH groups. Among them, 13 were significantly enriched in the SH group, mainly including "biosynthesis of amino acids,"

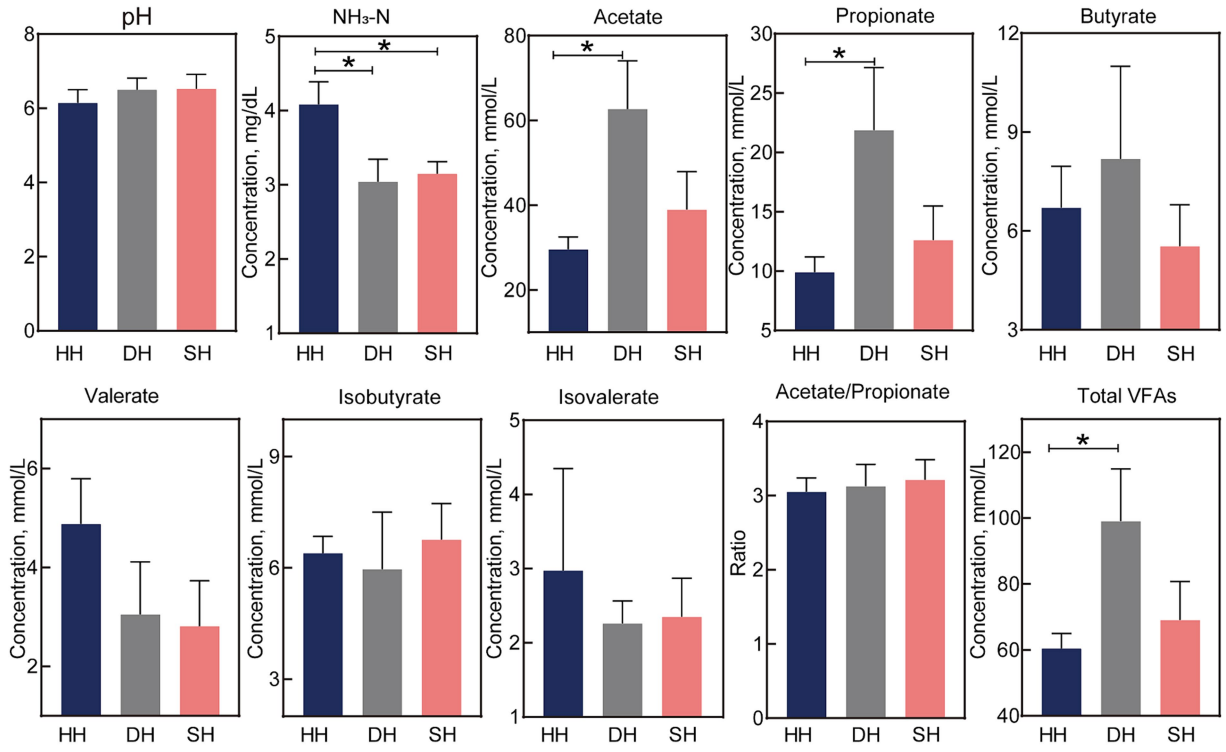

FIGURE 2
Hybridization altered rumen fermentation parameters in Hu sheep. $n = 7$ individuals/group; * indicates $p < 0.05$.

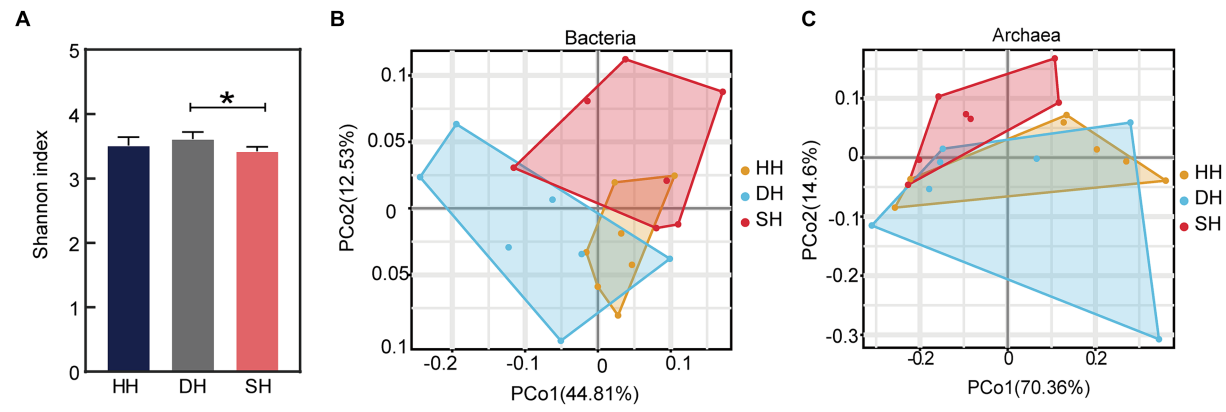

FIGURE 3
Alpha diversity index and microbial compositional profiles of rumen sample principal coordinate analysis (PCoA). **(A)** Alpha diversity as presented by the Shannon index. **(B)** PCoA based on bacterial domain. **(C)** PCoA based on archaeal domain. $n = 7$ individuals/group; * indicates $p < 0.05$.

"homologous recombination," "peptidoglycan biosynthesis," "nucleotide metabolism," "pyrimidine metabolism," "mismatch repair," "DNA replication," "alanine, aspartate and glutamate metabolism," "carbon fixation pathways in prokaryotes," "beta-lactam resistance," "lysine biosynthesis," "glycine, serine and threonine metabolism," and "one carbon pool by folate"; whereas eight were significantly enriched in the HH group (Figure 5B). Twenty differential pathways were identified in the DH and SH groups. Among them, five were significantly enriched in the DH group and 15 in the SH group. "Carbon metabolism," "aminoacyl-tRNA biosynthesis," "nucleotide metabolism," "pyrimidine metabolism," and "other glycan degradation" were the top five pathways the most significantly enriched in the SH group (Supplementary Figure S5A).

For the CAZyme profiles, 55 differentially expressed genes encoding CAZymes were identified between the HH and DH groups at the family level (Figure 5C). Among them, 23 involved in the breakdown of carbohydrates (including cellulose, hemicellulose, starch, protein, and lignin) were enriched in the DH group (2 carbohydrate esterases [CEs], 18 glycoside hydrolases [GHs], and 3 polysaccharide lyases [PLs]), and 18 in the HH group (2 CEs, 15 GHs,

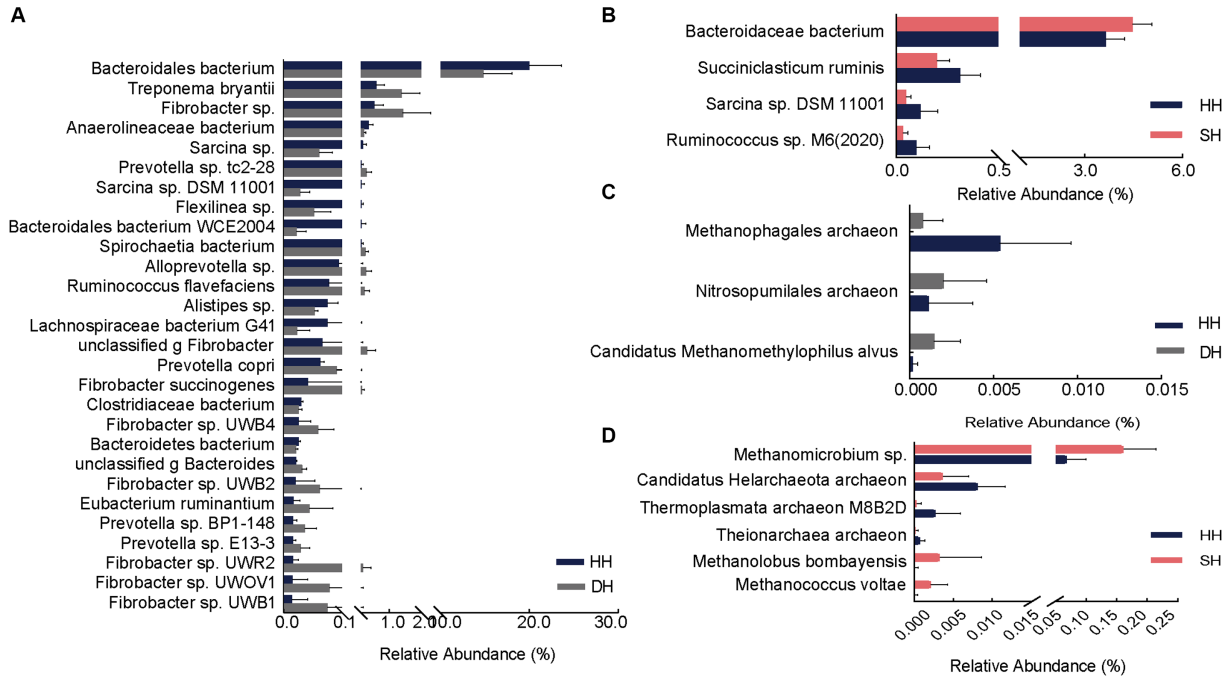

FIGURE 4
Differential rumen bacterial and archaeal species. **(A)** Significantly different bacterial species between the HH and DH groups. **(B)** Significantly different bacterial species between the HH and SH groups. **(C)** Significantly different archaeal species between the HH and DH groups. **(D)** Significantly different archaeal species between the HH and SH groups. *n* = 7 individuals/group.

and 1 PL). Five glycosyltransferases (GTs) involved in carbohydrate synthesis were enriched in the DH group, while three were enriched in the HH group. Several non-catalytic CAZymes were involved in the degradation of complex carbohydrates as parts of carbohydrate-binding modules (CBMs), including two enriched in the DH group and four in the HH group. A total of six differentially expressed genes encoding CAZymes were observed between the HH and SH groups (Figure 5D). Among these, 2 GHs and 1 GT were enriched in the SH group, whereas 1 GH and 2 CBMs were enriched in the HH group. We obtained 41 differential genes encoding CAZymes between the DH and SH groups (Supplementary Figure S5B). Among those involved in carbohydrate breakdown, 21 were enriched in the SH group (2 CEs, 15 GHs, 2 GLs, and 2 PLs), whereas 12 were enriched in the DH group (2 CEs, 8 GHs, and 2 PLs). Additionally, 3 GTs were enriched in the DH group.

3.7 Correlation analysis between phenotype and microbiome

Spearman's correlation coefficient analysis identified correlations between growth performance, rumen epithelial parameters, fermentation parameters, and significantly different microbiomes ($|r| > 0.5$; $p < 0.05$). When we compared the HH and DH groups, the relative abundance of *Alloprevotella* sp. was positively correlated with FBW, as well as the relative abundance of *Fibrobacter* sp. *UWR2* and *Fibrobacter* sp. were positively correlated with DMI, and the relative abundance of *Alistipes* sp. was negatively correlated with FBW, ADG, and DMI (Figure 6A). When we compared the HH and SH groups, the relative abundance of *Bacteroidaceae bacterium* was positively correlated with FBW and DMI; however, the relative abundances of *Ruminococcus* sp. M6(2020) and *Theionarchaea archaeon* were negatively correlated with NH_3-N concentration (Figure 6B). When we compared the DH and SH groups, we found that *Pyramidobacter* sp. was negatively correlated to acetate, propionate, and butyrate concentrations, the relative abundances of *Candidatus Methanomethylophilus* sp. and *Candidatus Methanomethylophilaceae archaeon* were negatively related to acetate, propionate, and total VFA concentrations (Supplementary Figure S6).

3.8 Rumen metabolome analysis

In total, 947 metabolites were identified in the 21 rumen samples. The orthogonal projections to latent structures-discriminant analysis (OPLS-DA) score plots showed good separation of metabolites between the HH and DH ($R^2X = 0.419$, $R^2Y = 0.994$, $Q^2 = 0.345$), HH and SH ($R^2X = 0.495$, $R^2Y = 0.992$, $Q^2 = 0.580$), and DH and SH ($R^2X = 0.451$, $R^2Y = 0.991$, $Q^2 = 0.315$) groups (Figure 7A). After screening the relative concentrations of rumen metabolites by FC ($FC \geq 2$ and $FC \leq 0.5$) and VIP ($VIP \geq 1$), we obtained 14 metabolites significantly differently enriched in the HH and DH groups, including nine upregulated and five downregulated metabolites (Figure 7B). Levels of lysophosphatidylethanolamine (LPE) (P-18:1), (P-17:0), (O-17:1), (P-17:1), and methionine sulfoxide were significantly increased in the DH group, while thr-tyr, cyclo (his-pro) and heparin was significantly decreased. Interestingly, methionine sulfoxide was only present in the DH group. A total of 35 metabolites were significantly differently enriched between the HH and SH groups, with19 upregulated and 16 downregulated (Figure 7C). Levels of 6

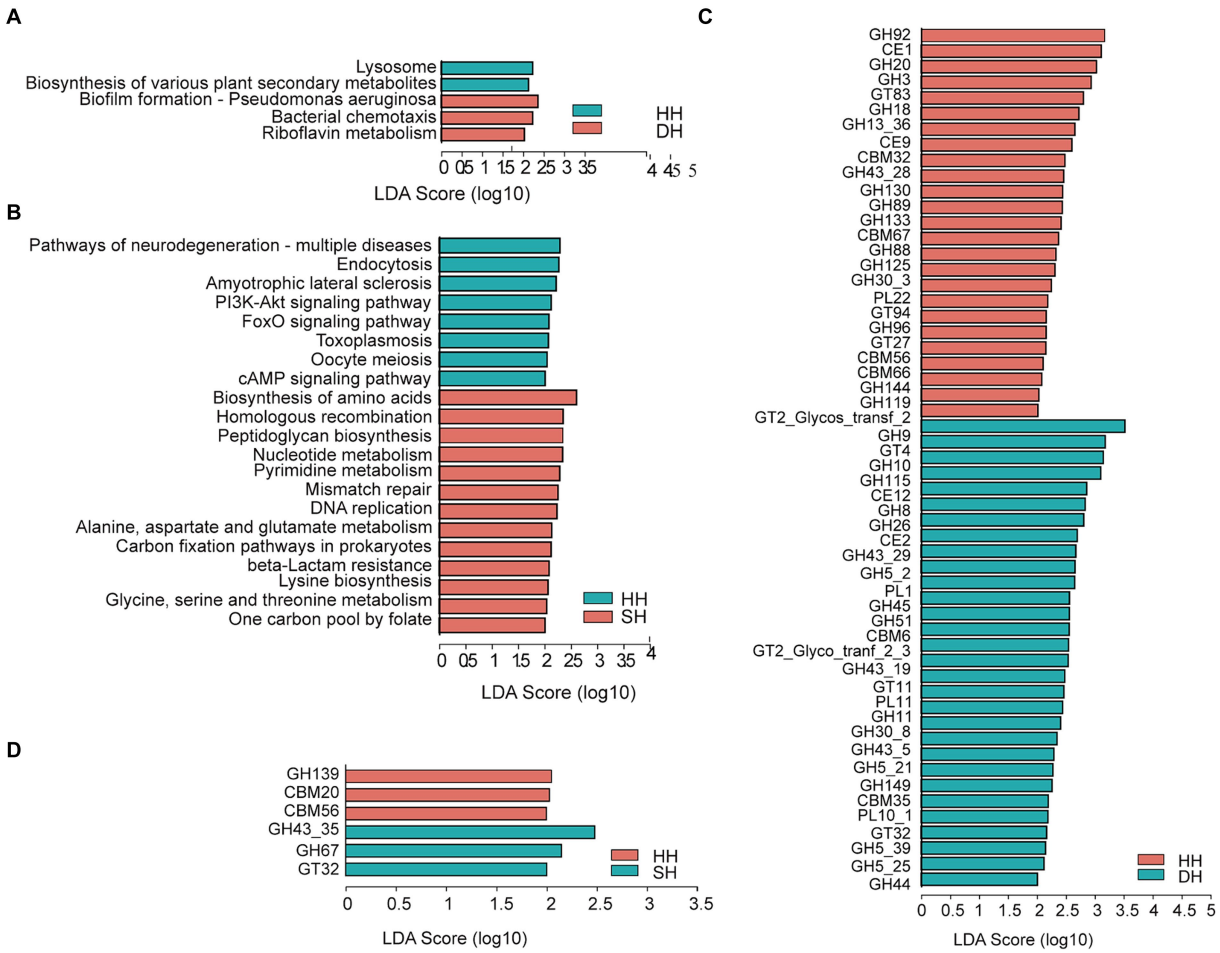

FIGURE 5
Differential rumen microbiome functions. **(A)** Significantly differential KEGG functions at the third level between the HH and DH groups. **(B)** Significantly differential KEGG functions at the third level between the HH and SH groups. **(C)** Significantly differential CAZy functions at the family level between the HH and DH groups. **(D)** Significantly differential CAZy functions at the family level between the HH and SH groups. Significant differences were tested by linear discriminant analysis effect size (LEfSe) analysis, with linear discriminant analysis (LDA) score > 2 and $p < 0.05$. $n = 7$ individuals/group.

from organic acid and its derivatives, 3 from fatty acyls (FA), and 2 from amino acid and its derivatives were significantly increased in the SH group, while 6 from nucleotides and their metabolites, 2 from heterocyclic compounds, 3 from amino acids and its derivatives were significantly decreased. N-methyl-4-aminobutyric acid was identified only in the SH group. Between the DH and SH groups, 21 metabolites with significant differences were observed, including 3 upregulated and 18 downregulated. Levels of 3-hydroxy-3-methyl butyric acid, 7-ketocholesterol, and lysophosphatidylcholine (LPC) (14:0/0:0) were significantly increased, while 4 from nucleotides and their metabolites, 10 from glycerophospholipids (GP), 2 from alcohol and amines metabolites were significantly decreased (Supplementary Figure S7).

KEGG pathway analysis revealed that of these differential metabolites, six were annotated to 16 pathways between the HH and DH groups (Figure 8A). "Cysteine and methionine metabolism," "phenylalanine metabolism," "glycine, serine, and threonine metabolism," "biosynthesis of amino acids," "glycolysis/gluconeogenesis," and "pentose phosphate pathway" were significantly enriched in the DH group, however, "Fc epsilon RI signaling pathway" was enriched in the HH group. Twelve metabolites were annotated in 48 pathways between the HH and SH groups (Figure 8B). Among them, relating to amino acid metabolism, bile acid metabolism and tricarboxylic acid cycle pathways were mainly enriched in the SH group, for example, "phenylalanine metabolism," "histidine metabolism," "glycine, serine and threonine metabolism," "taurine and hypo taurine metabolism," "bile secretion," and "citrate cycle (TCA cycle)." The HH group enriched nucleotide metabolism and ABC transporters related to purine metabolism. Seven metabolites were annotated in 12 pathways between the DH and SH groups (Supplementary Figure S8). "Fat digestion and absorption," "glycerolipid metabolism," and "vitamin digestion and absorption" were mainly enriched in the DH group, while "valine, leucine and isoleucine degradation" and "choline metabolism in cancer" were mainly enriched in the SH group.

3.9 Microbiome-related metabolite analysis

Spearman's correlation coefficient analysis identified correlations between differential microbiomes and metabolites ($|r| > 0.8$; $p < 0.05$).

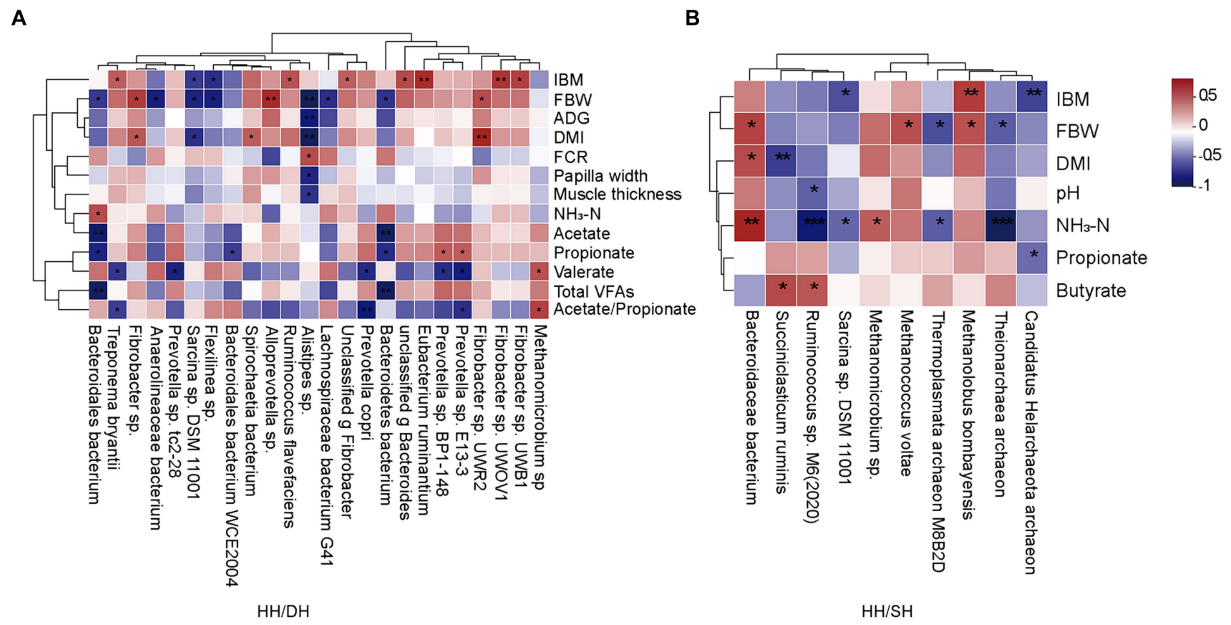

FIGURE 6
Correlation analysis between growth performance, rumen epithelial parameters, fermentation parameters, and significantly different microbiomes. ($|r| > 0.5$). **(A)** Between the HH and DH groups. **(B)** Between the HH and SH groups. $n = 7$ individuals/group. *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$.

Treponema bryantii, *Fibrobacter succinogenes*, *Fibrobacter* sp. *UWB4* and *Fibrobacter* sp. levels were positively correlated with LPE (P-17:0); *Alloprevotella* sp.was positively correlated with 4-hydroxyquinoline; and *Alistipes* sp. was negatively correlated with hydroxypiperazic acid when we compared the HH and DH groups (Figure 9A). *Bacteroidales bacterium* and *Ruminococcus* sp. M6 (2020) were positively correlated with 3-hydroxy-3-methyl butyric acid and dopaquinone, respectively; whereas *Theionarchaea archaeon* and *Thermoplasmata archaeon M8B2D* were negatively correlated with tetradecanedioic acid in the HH and SH groups (Figure 9B). *Candidatus Altiarchaeales archaeon WOR SM1* 86-2 was positively correlated with LPE (16:0/0:0), LPE (0:0/16:0), LPE (O-18:2), phosphatidylcholine (PC) (O-1:0/O-16:0), lysophosphatidylserine (LPS) (20:0), lysophosphatidic acid (LPA) (22:6); 7-ketocholesterol was positively correlated with *Candidatus Methanomethylophilaceae archaeon*, *Candidatus Methanomethylophilus* sp., and *Bacteroidales bacterium* between the DH and SH groups (Supplementary Figure S8).

4 Discussion

From the perspective of genetics and breeding, introducing a favorable exogenous gene may be a better choice for improving the low to moderate heritability of traits (28). In this study, we screened Poll Dorset and Southdown sheep as sires to improve the growth performance of Hu sheep, focusing on how hybridization affects growth performance and investigating the regulation mechanism of the rumen microbiome and its metabolites.

Hybridization results in heterosis and improves animal growth. As expected, our data showed that hybridization increased DMI and ADG, resulting in higher FBW and lower F/G. Our previous study indicated that F_1 hybrid offspring grew faster and became larger than did Hu sheep, particularly during the later periods (29). Another study on a three-way cross system between Angus, Qaidam, and yak indicated that the F_2 offsprings were all significantly better than those of the yak and 1/2 yak (28). These results suggest the importance of hybridization for improving growth performance of animals.

Rumen microecology is closely associated with animal growth. In this study, hybridization significantly changed the rumen fermentation pattern in sheep, especially the NH_3-N concentration. NH_3-N is mainly derived from microbe-mediated amino acid deamination and non-protein nitrogen hydrolysis in the rumen (30). The rumen microbiome utilizes NH_3-N to provide primary protein synthesis precursors for the host. NH_3-N also has a feedback effect on rumen microbiota structure and epithelial function, affecting rumen fermentation and host health (31). The NH_3-N concentration was significantly lower in the SH and DH groups than in the HH group, indicating that the hybrid offspring had a higher utilization efficiency of nitrogen sources to synthesize bacterial proteins. VFAs are the main energy source for ruminants, which ferment plant materials into VFAs that regulate a variety of physiological functions in the rumen (32). Acetate, propionate, and butyrate are the three major VFAs and provide approximately 70% of energy requirements. Acetate concentration is mainly affected by the microbial degradation of fibrous substances. Reports have shown that rumen acetate concentration increases when ruminants are fed a high-fiber diet (33) or when the rumen microbiota is dominated by *Prevotella* (34). Of the acetate in the rumen, 67% is used for oxidative energy, while the rest is used for body fat synthesis. Propionate contributes to energy supplementation via the gluconeogenic pathway to promote glucose synthesis (35). In this study, acetate and propionate levels in the DH group significantly increased, resulting in higher total VFAs, indicating a higher energy supply to the host and improved growth performance.

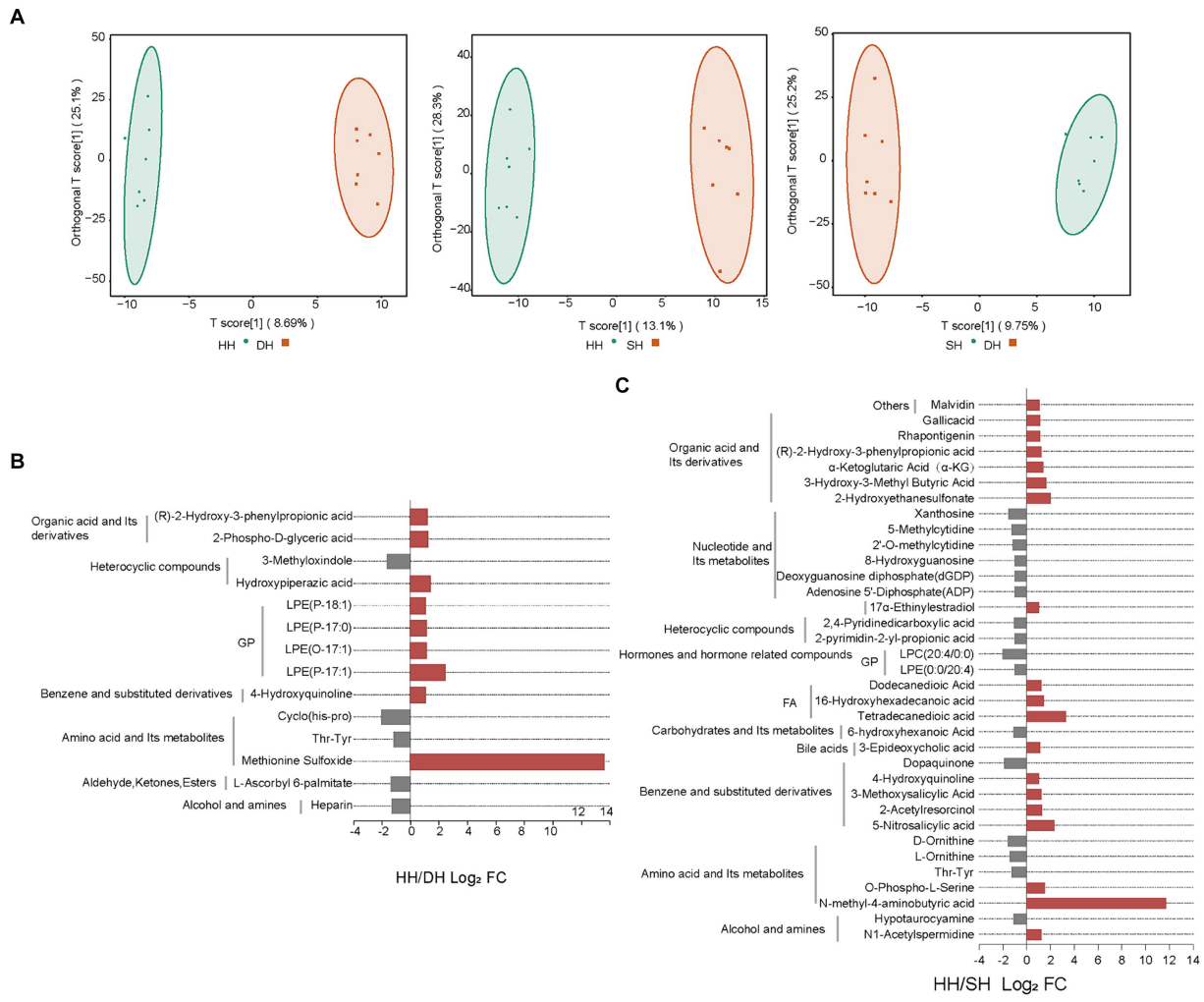

FIGURE 7
Change in rumen content metabolite levels. **(A)** OPLS-DA analysis. **(B)** Significantly differential metabolites between the HH and DH groups. **(C)** Significantly differential metabolites between the HH and SH groups. *n* = 7 individuals/group.

The rumen epithelium is an important indicator of rumen development and is responsible for the absorption and metabolism of nutrients and microbial byproducts. Evidence has shown that VFAs are absorbed through the rumen epithelium and that their absorption capacity is mainly related to the surface area and expression of VFA transporter vectors in the epithelium (36). The rumen epithelium is abundant in mitochondria, powering epithelial metabolism; a thicker epithelium indicates more efficient feed utilization in cattle (37). Additionally, butyrate stimulates epithelial cellular proliferation (38). Our results indicate that hybridization increased papilla height, while no change was observed in papilla width or muscle thickness, which suggests an increased nutrient contact surface area to completely crush the feed to promote digestion and feed efficiency (39). Moreover, these variations in rumen epithelial physical structures are considered to potentially influence the rumen microbiota.

Using PCoA, the present study found significant differences in the rumen microbiome between the F_1 hybrid offspring and Hu sheep. Hybridization can produce new gene flow and different gene expression patterns in offspring. Sika and elk deer have different rumen microbiota than do their hybrid offspring; pure and hybrid mice have different microbial clustering characteristics suggesting a significant effect of host genetics on the rumen microbiome that may result from vertical transmission (40–42). Moreover, we found that Bacteroidetes and Firmicutes were the dominant bacteria at the phylum level, however, hybridization had no considerable effect on their relative abundances. Evidence has shown that Bacteroidetes have low heritability estimates, while Firmicutes have moderate heritability estimates, suggesting that they are largely affected by environmental factors, such as diet (15, 43). Concurrently, the different heritability estimates indicate that host effects are not equal for different rumen microbial phylotypes. At the species level, we observed that bacteria related to fiber degradation were more abundant in the DH group, *Treponema bryantii*, *Prevotella copri*, and *Fibrobacter succinogenes* were particularly abundant. *Treponema bryantii*, when present with cellulolytic species such as *Bacteroides succinogenes*, can promote the digestion of cellulosic materials to increase feed efficiency (44). A higher abundance of *Treponema bryantii* and *Bacteroides* sp. were found in the cecum of pigs with high feed efficiency (45). *Prevotella* are considered the most important bacteria for polysaccharide degradation and fermentation based on their genomic glycoside hydrolase profiles,

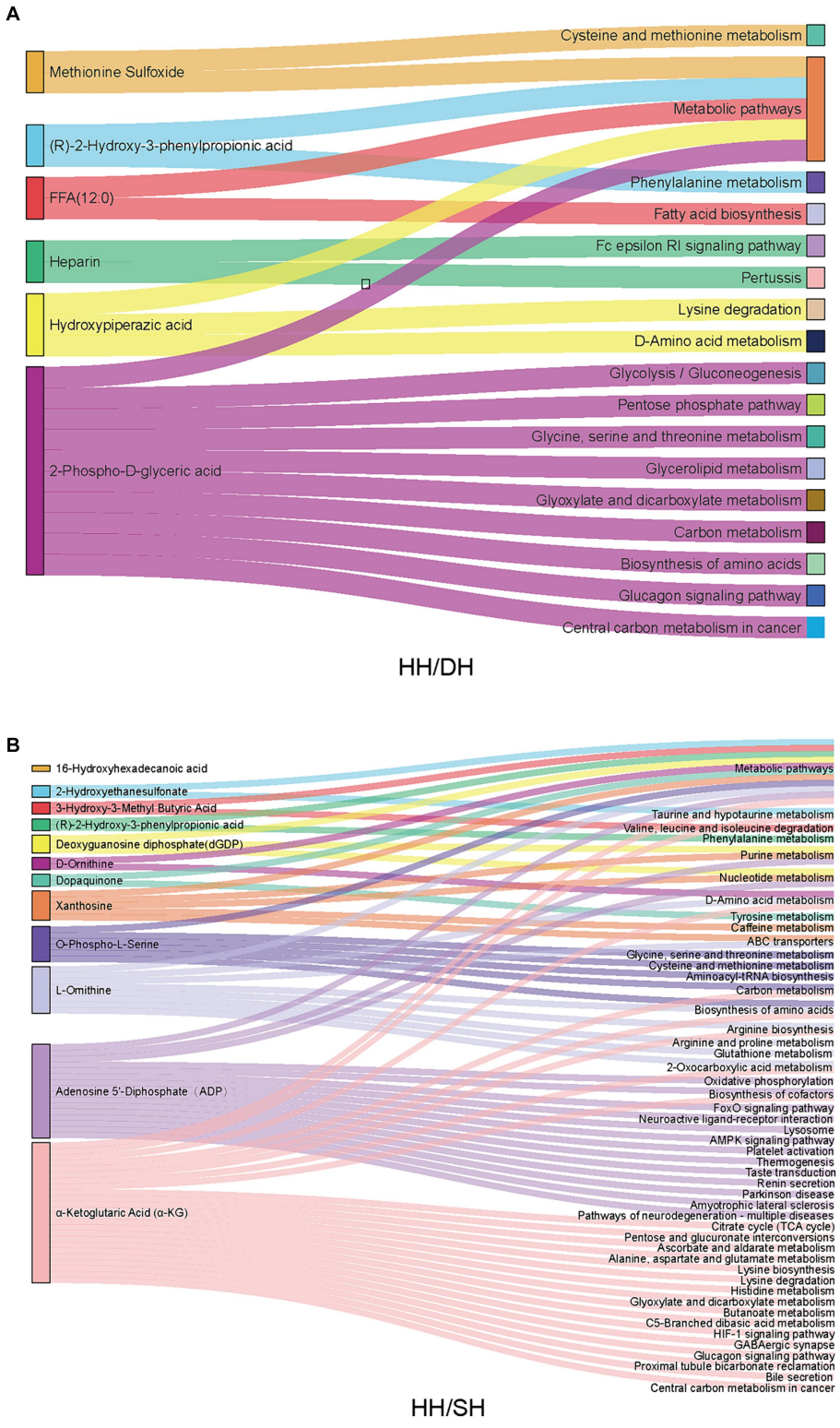

FIGURE 8
Metabolic pathway enrichment analysis. **(A)** Between the HH and DH groups. **(B)** Between the HH and SH groups. $n = 7$ individuals/group.

gene expression, and abundance in the rumen (46). *Prevotella copri* might be the keystone bacterial species associated with host feed intake and fat metabolism, and evidence shows that its abundance increases in pigs with high average daily feed intake (47) and promotes fat accumulation in pigs fed formula diets (48). Studies in humans indicate that *Prevotella copri* largely increases the microbial potential for branched-chain amino acid biosynthesis (49), which is beneficial for milk protein yield in dairy cattle and animal growth (50, 51).

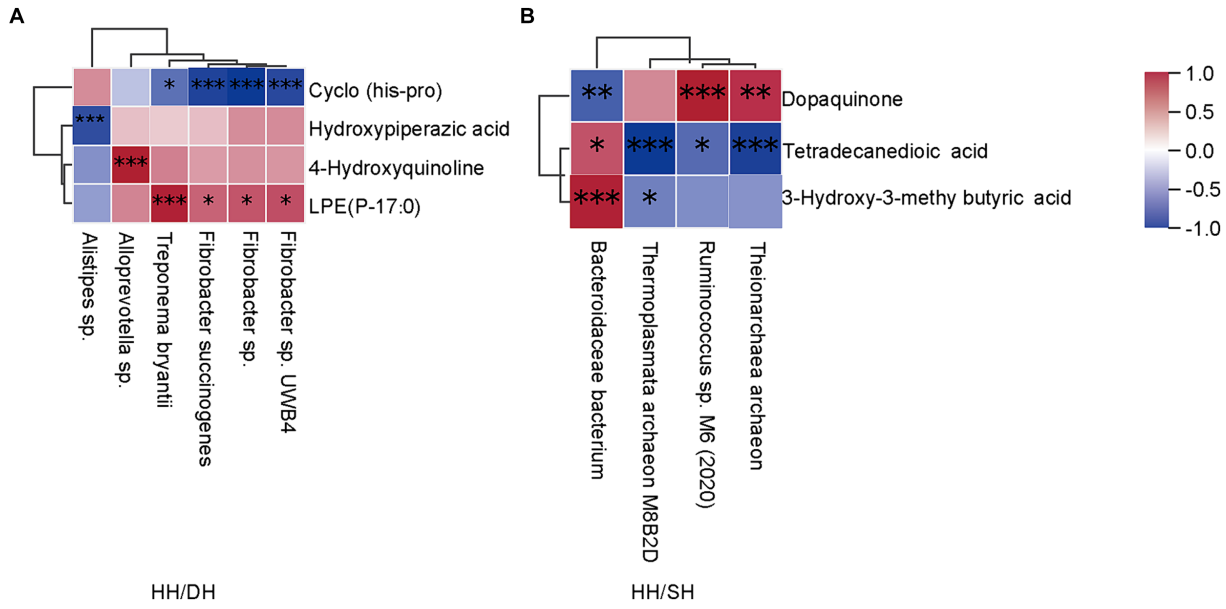

FIGURE 9
Correlation analysis between microbiome and metabolite profiles in rumen samples. ($|r| > 0.8$). **(A)** Between the HH and DH groups. **(B)** Between the HH and SH groups. $n = 7$ individuals/group. *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$.

Moreover, *Prevotella copri* is heritable ($h^2 > 0.15$) in the cecum and feces of pigs (52). *Fibrobacter succinogenes* is regarded as the most highly cellulolytic bacterium in ruminal microbiomes and specializes in the production of succinate, acetate, and formate (53). An increase in the *Fibrobacter succinogenes* population in the rumen is attributed to an increase in dry matter degradability (54). In the present study, the relative abundances of *Fibrobacter* sp. *UWR2* and *Fibrobacter* sp. were positively linked to DMI, suggesting their essential roles in DMI and dry matter degradability. In the SH group, the abundance of the *Bacteroidaceae bacterium* was higher than that of the HH group. *Bacteroidaceae bacterium* is also a rumen cellulolytic bacterium that can convert succinate to propionate as the sole energy source (55). Therefore, the higher abundance of polysaccharide-degrading bacteria in the rumen of hybrid sheep enhances the degradation of dietary carbohydrates, thus promoting digestive ability and growth performance. This may be attributed to host genetics because we excluded the effects of environmental factors. However, this speculation needs to be verified by monitoring the rumen microbiota of Poll Dorset and Southdown sheep. The DH group lacked archaea related to nutrient digestion and absorption, however, the abundance of methanogenic bacteria (*Methanomicrobium* sp., *Methanolobus bombayensis*, and *Methanococcus voltae*) increased in the SH group compared to levels in the HH group, and methane production is an energy-intensive process, resulting in low production performance. However, in this study, the abundance of these methanogenic bacteria was quite low. We also found that DMI and feed protein utilization efficiency in the SH group were higher than those in the HH group, which suggests that the contributions of DMI and feed protein utilization efficiency surpassed the methane production rate, which may be the reason for the FBW gain in the SH group. Moreover, Xue et al. found that some methanogenic bacteria with low abundance were enriched in the rumens of dairy cows with high milk protein yields (50); however, further studies are needed to validate our hypotheses.

KEGG functional taxonomic analysis revealed that amino acid biosynthesis and metabolism were enriched in the SH group. Metabolic products such as lysine, aspartate, glycine, alanine, glutamate, serine, and threonine are important contributors to rumen microbial protein synthesis, constituting 90% of the microbial proteins that arrive in the small intestine and provide a protein source for the host (56). Our previous study identified that the muscle protein content was higher in the SH group than in the HH group (29), which is in line with the results of the rumen microbiota. Genes encoding CAZymes involved in deconstructing carbohydrates (GHs, CEs, PLs, and CBMs) were enriched in the DH group, indicating that these sheep were more capable of degrading complex substrates to promote microbial fermentation, which is consistent with our VFA results.

In addition to affecting the composition and function of the rumen microbiome, hybridization alters rumen metabolites and metabolic pathways. First, LPEs are cell membrane components and are the second most abundant *in vivo* after LPCs (57, 58). LPEs reportedly induce the activation of mitogen-activated protein kinase signaling and exhibit anti-apoptotic effects in pheochromocytoma cells (59). LPE (P-18:1) has oleic acid as its acyl chain, stimulating neurite outgrowth and neuroprotective effects against glutamate-induced excitotoxicity (60). Additionally, LPEs are involved in lipid droplet formation by suppressing lipolysis and fatty acid biosynthesis, thereby promoting the absorption of lipid substances (61). This evidence indicates that LPEs enriched in the DH group benefited from fat deposition, resulting in FBW gain. Interestingly, methionine sulfoxide was only present in the DH group. The exposure of proteins to reactive oxygen species and hydrogen peroxide may lead to the oxidation of free methionine and methionine residues, forming methionine sulfoxide (62). Methionine sulfoxide levels are elevated during oxidative stress, aging, and inflammation. The results of this study imply that the sheep in the

DH group had poor environmental adaptability. However, methionine sulfoxide can be diastereoselectively repaired by methionine sulfoxide reductase to produce methionine (63). Methionine augments the endogenous antioxidant capacity of rice proteins by stimulating methionine sulfoxide reductase expression and enhancing glutathione synthesis via the Nrf2-ARE pathway (64). The beneficial effects of methionine supplementation on the antioxidant activity of heat-stressed birds have been previously demonstrated (65). The present study shows that although sheep in the DH group were in a state of oxidative stress, this phenomenon can be improved by regulating the methionine sulfoxide reductase system, which will be our next research direction. N-methyl-4-aminobutyric acid was only present in the SH group. The metabolic product aminobutyric acid acts as a key inhibitory neurotransmitter of the central nervous system and functions via sedation, relieving fever and decreasing heat production (66). Aminobutyric acid reduces stress caused by various environmental conditions and improves productivity (67). For example, aminobutyric acid improves growth performance, meat quality, and feed intake by promoting the activation of characteristic enzymes and improving the absorption and immune function of the intestinal mucosa under heat-stress conditions (68, 69). The characteristic metabolites in both the DH and SH groups were related to oxidative stress, indicating that the environmental adaptability of the F_1 generation sheep needs improvement, which we expect to occur in the F_2 generation. α-Ketoglutaric acid is an intermediate metabolite of the tricarboxylic acid cycle and is generated from glucose and glutamine, providing substrates for the synthesis of carbohydrates, amino acids, fats, and other biomolecules (70). In this study, α-ketoglutarate was significantly increased in the SH group compared to levels in the HH group, further enhancing amino acid metabolism ability. Our results were consistent with a previous report by Kong (71), who confirmed the tricarboxylic acid cycle pathways were enhanced in the muscle of Southdown × Hu F_1 generation sheep.

Four metabolites and six microbiomes showed regulatory relationships in growth performance between the DH and HH groups. *Alloprevotella* sp. can ferment carbohydrates to produce acetate and succinate as end-metabolites and is positively correlated with 4-hydroxyquinoline, which has antibacterial and anti-inflammatory effects that maintain rumen health (72). *Treponema bryantii*, *Fibrobacter succinogenes*, *Fibrobacter* sp., and *Fibrobacter* sp.*UWB4* are associated with fiber degradation and production of propionate, which are positively correlated with LPE (P-17:0). Propionate can promote glucose synthesis via the gluconeogenic pathway in the rumen, and glucose is a precursor for the form of glycerol and fatty acids, which further help format LPEs. LPEs originate in part from the lipids in the diet, but also from gut microbial synthesis. Phospholipid levels in the host cells of germ-free mice differ from those in the host cells of conventional mice, suggesting a relationship between gut microbes and glycerophospholipids (73). The relative abundance of *Faecalibacterium* and *Prevotella* is related to circulating lipids, particularly LEPs and phosphatidylglycerol (74); however, we found in the present study that *Fibrobacter* was also related to the levels of LPE (P-17:0). *Bacteroidales bacterium* was positively correlated with 3-hydroxy-3-methyl butyric acid between the SH and HH groups. 3-Hydroxy-3-methyl butyric acid is a leucine metabolite that stimulates the growth hormone/insulin-like growth factor-1 axis (75). These results indicate that the rumen microbiota of sheep in the DH group were mainly involved in body fat deposition by increasing DMI and dry matter degradability, whereas that in the SH group mainly participated in amino acid metabolism to provide energy. However, the causal relationship between the microbiota and metabolites requires further research. In addition, the rumen microbiomes and metabolites data of Poll Dorset and Southdown sheep should be considered and further elucidated in future studies.

5 Conclusion

The results of this study support the feasibility of using crossbreeding programs to promote growth and increase feed efficiency by altering the rumen microbiota and metabolites in sheep. In the DH group, *Treponema bryantii*, *Prevotella copri*, and *Fibrobacter succinogenes* levels increased, and their functions were mainly enriched at the CAZyme level, contributing to higher acetate, propionate, total VFA, and LPE levels to further promote growth. *Bacteroidaceae bacterium* was enriched in the SH group and is involved in amino acid metabolism, fulfilling the demand for ruminal microbial proteins that are utilized by hosts to promote growth. Additionally, methionine sulfoxide and N-methyl-4-aminobutyric acid were characteristic metabolites in the DH and SH groups, respectively, indicating that the F_1 generation crossbred sheep had poor environmental adaptability. Taken together, these findings provide new ideas and nutritional strategies for the cultivation of new sheep breeds.

Data availability statement

The data presented in the study are deposited in the SRA repository, accession number PRJNA1111660.

Ethics statement

The animal study was approved by Animal Administration and Ethics Committee of the Lanzhou Institute of Husbandry and Pharmaceutical Science of the Chinese Academy of Agricultural Science. The study was conducted in accordance with the local legislation and institutional requirements.

Author contributions

RZ: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Visualization, Writing – original draft, Writing – review & editing. LZ: Data curation, Investigation, Methodology, Writing – original draft. XA: Data curation, Investigation, Methodology, Writing – review & editing. JL: Data curation, Methodology, Resources, Writing – review & editing. CN: Investigation, Validation, Visualization, Writing – review & editing. JZ: Data curation, Methodology, Visualization, Writing – review & editing. ZG: Data curation, Methodology, Visualization, Writing – review & editing. TX: Data curation, Investigation, Writing – review & editing. BY: Conceptualization, Formal analysis, Writing – review & editing. ZX: Conceptualization, Investigation, Project administration, Supervision, Writing – original draft, Writing – review

& editing. YY: Conceptualization, Funding acquisition, Investigation, Project administration, Supervision, Writing – original draft, Writing – review & editing.

Funding

The author(s) declare that financial support was received for the research, authorship, and/or publication of this article. This study was funded by the Major Scientific Research Task of the Science and Technology Innovation Project of the Chinese Academy of Agricultural Sciences (CAAS-ZDRW202106), the Central Public interest Scientific Institution Basal Research Fund (1610322023014, 1610322024020), and the Science and Technology Program of Gansu Province (22ZD6NA037, 21ZD11NM001).

Acknowledgments

We are grateful to the Qinghuan Mutton Sheep Breeding Company for their work including the care and feeding of animals.

Conflict of interest

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

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Supplementary material

The Supplementary material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fvets.2024.1455029/full#supplementary-material

References

1. Bunning H, Wall E, Chagunda M, Banos G, Simm G. Heterosis in cattle crossbreeding schemes in tropical regions: meta-analysis of effects of breed combination, trait type, and climate on level of heterosis. *J Anim Sci*. (2019) 97:29–34. doi: 10.1093/jas/sky406

2. Liu A, Chen X, Liu M, Zhang L, Ma X, Tian S. Differential expression and functional analysis of CircRNA in the ovaries of low and high fecundity Hanper sheep. *Animals (Basel)*. (2021) 11:71863. doi: 10.3390/ani11071863

3. Yang Y, Shen L, Gao H, Ran J, Li X, Jiang H, et al. Comparison of cecal microbiota composition in hybrid pigs from two separate three-way crosses. *Anim Biosci*. (2021) 34:1202–9. doi: 10.5713/ab.20.0681

4. Christensen OF, Legarra A, Lund MS, Su G. Genetic evaluation for three-way crossbreeding. *Genet Sel Evol*. (2015) 47:98. doi: 10.1186/s12711-015-0177-6

5. Quan K, Li J, Han H, Wei H, Zhao J, Si HA, et al. Review of Huang-Huai sheep, a new multiparous mutton sheep breed first identified in China. *Trop Anim Health Prod*. (2020) 53:35. doi: 10.1007/s11250-020-02453-w

6. Lu Z, Yue Y, Shi H, Zhang J, Liu T, Liu J, et al. Effects of sheep sires on muscle fiber characteristics, fatty acid composition and volatile flavor compounds in F_1 crossbred lambs. *Food Secur*. (2022) 11:4076. doi: 10.3390/foods11244076

7. Zhao L, Zhang D, Li X, Zhang Y, Zhao Y, Xu D, et al. Comparative proteomics reveals genetic mechanisms of body weight in Hu sheep and Dorper sheep. *J Proteome*. (2022) 267:104699. doi: 10.1016/j.jprot.2022.104699

8. Quan F, Zhang Z, An Z, Hua S, Zhao X, Zhang Y. Multiple factors affecting superovulation in poll Dorset in China. *Reprod Domest Anim*. (2011) 46:39–44. doi: 10.1111/j.1439-0531.2009.01551.x

9. Hall SJG. Conserving and developing minority British breeds of sheep: the example of the Southdown. *J Agric Sci*. (1989) 112:39–45. doi: 10.1017/S0021859600084082

10. Dai R, Zhou H, Fang Q, Zhou P, Yang Y, et al. Variation in ovine DGAT1 and its association with carcass muscle traits in Southdown sheep. *Genes (Basel)*. (2022) 13:13. doi: 10.3390/genes13091670

11. Cheng J, Zhang X, Xu D, Zhang D, Zhang Y, Song Q, et al. Relationship between rumen microbial differences and traits among Hu sheep, Tan sheep, and Dorper sheep. *J Anim Sci*. (2022) 100:100. doi: 10.1093/jas/skac261

12. Firkins JL, Yu Z. Ruminant nutrition symposium: how to use data on the rumen microbiome to improve our understanding of ruminant nutrition. *J Anim Sci*. (2015) 93:1450–70. doi: 10.2527/jas.2014-8754

13. Li F, Hitch T, Chen Y, Creevey CJ, Guan LL. Comparative metagenomic and metatranscriptomic analyses reveal the breed effect on the rumen microbiome and its associations with feed efficiency in beef cattle. *Microbiome*. (2019) 7:6. doi: 10.1186/s40168-019-0618-5

14. Cersosimo LM, Bainbridge ML, Wright AD, Kraft J. Breed and lactation stage alter the rumen protozoal fatty acid profiles and community structures in Primiparous dairy cattle. *J Agric Food Chem*. (2016) 64:2021–9. doi: 10.1021/acs.jafc.5b05310

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

16. Zhang R, Wu J, Lei Y, Bai Y, Jia L, Li Z, et al. Oregano essential oils promote rumen digestive ability by modulating epithelial development and microbiota composition in beef cattle. *Front Nutr*. (2021) 8:722557. doi: 10.3389/fnut.2021.722557

17. Chen S, Zhou Y, Chen Y, Gu J. Fastp: an ultra-fast all-in-one FASTQ preprocessor. *Bioinformatics*. (2018) 34:i884–90. doi: 10.1093/bioinformatics/bty560

18. Li H, Durbin R. Fast and accurate short read alignment with burrows-wheeler transform. *Bioinformatics*. (2009) 25:1754–60. doi: 10.1093/bioinformatics/btp324

19. Li D, Liu CM, Luo R, Sadakane K, Lam TW. Megahit: an ultra-fast single-node solution for large and complex metagenomics assembly via succinct de Bruijn graph. *Bioinformatics*. (2015) 31:1674–6. doi: 10.1093/bioinformatics/btv033

20. Noguchi H, Park J, Takagi T. MetaGene: prokaryotic gene finding from environmental genome shotgun sequences. *Nucleic Acids Res*. (2006) 34:5623–30. doi: 10.1093/nar/gkl723

21. Fu L, Niu B, Zhu Z, Wu S, Li W. CD-HIT: accelerated for clustering the next-generation sequencing data. *Bioinformatics*. (2012) 28:3150–2. doi: 10.1093/bioinformatics/bts565

22. Li R, Li Y, Kristiansen K, Wang J. SOAP: short oligonucleotide alignment program. *Bioinformatics*. (2008) 24:713–4. doi: 10.1093/bioinformatics/btn025

23. Altschul SF, Madden TL, Schaffer AA, Zhang J, Zhang Z, Miller W, et al. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Res*. (1997) 25:3389–402. doi: 10.1093/nar/25.17.3389

24. Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ. Basic local alignment search tool. *J Mol Biol*. (1990) 215:403–10. doi: 10.1016/S0022-2836(05)80360-2

25. Sun HZ, Wang DM, Wang B, Wang JK, Liu HY, Guan LL, et al. Metabolomics of four biofluids from dairy cows: potential biomarkers for milk production and quality. *J Proteome Res*. (2015) 14:1287–98. doi: 10.1021/pr501305g

26. De Almeida R, Do PR, Porto C, Dos SG, Huws SA, Pilau EJ. Exploring the rumen fluid metabolome using liquid chromatography-high-resolution mass spectrometry and molecular networking. *Sci Rep*. (2018) 8:17971. doi: 10.1038/s41598-018-36196-4

27. Kanehisa M, Goto S. KEGG: Kyoto encyclopedia of genes and genomes. *Nucleic Acids Res*. (2000) 28:27–30. doi: 10.1093/nar/28.1.27

28. Cao XK, Cheng J, Huang YZ, Wang XG, Ma YL, Peng SJ, et al. Growth performance and meat quality evaluations in three-way cross cattle developed for the Tibetan plateau and their molecular understanding by integrative omics analysis. *J Agric Food Chem*. (2019) 67:541–50. doi: 10.1021/acs.jafc.8b05477

29. Zhang R, An X, Li J, Lu Z, Niu C, Xu Z, et al. Comparative analysis of growth performance, meat productivity, and meat quality in Hu sheep and its hybrids. *Acta Pratacul Sin*. (2024) 33:186–97. doi: 10.11686/cyxb2023157

30. Schwab CG, Broderick GA. A 100-year review: protein and amino acid nutrition in dairy cows. *J Dairy Sci*. (2017) 100:10094–112. doi: 10.3168/jds.2017-13320

31. Shen J, Zheng W, Xu Y, Yu Z. The inhibition of high ammonia to in vitro rumen fermentation is pH dependent. *Front Vet Sci*. (2023) 10:163021. doi: 10.3389/fvets.2023.1163021

32. Shen H, Xu Z, Shen Z, Lu Z. The regulation of ruminal short-chain fatty acids on the functions of rumen barriers. *Front Physiol*. (2019) 10:1305. doi: 10.3389/fphys.2019.01305

33. Li QS, Wang R, Ma ZY, Zhang XM, Jiao JZ, Zhang ZG, et al. Dietary selection of metabolically distinct microorganisms drives hydrogen metabolism in ruminants. *ISME J*. (2022) 16:2535–46. doi: 10.1038/s41396-022-01294-9

34. Wang D, Tang G, Wang Y, Yu J, Chen L, Chen J, et al. Rumen bacterial cluster identification and its influence on rumen metabolites and growth performance of young goats. *Anim Nutr*. (2023) 15:34–44. doi: 10.1016/j.aninu.2023.05.013

35. De Vadder F, Kovatcheva-Datchary P, Zitoun C, Duchampt A, Bäckhed F, Mithieux G. Microbiota-produced succinate improves glucose homeostasis via intestinal gluconeogenesis. *Cell Metab*. (2016) 24:151–7. doi: 10.1016/j.cmet.2016.06.013

36. Chai J, Lv X, Diao Q, Usdrowski H, Zhuang Y, Huang W, et al. Solid diet manipulates rumen epithelial microbiota and its interactions with host transcriptomic in young ruminants. *Environ Microbiol*. (2021) 23:6557–68. doi: 10.1111/1462-2920.15757

37. Lam S, Munro JC, Zhou M, Guan LL, Schenkel FS, Steele MA, et al. Associations of rumen parameters with feed efficiency and sampling routine in beef cattle. *Animal*. (2018) 12:1442–50. doi: 10.1017/S1751731117002750

38. Zhang K, Xu Y, Yang Y, Guo M, Zhang T, Zong B, et al. Gut microbiota-derived metabolites contribute negatively to hindgut barrier function development at the early weaning goat model. *Anim Nutr*. (2022) 10:111–23. doi: 10.1016/j.aninu.2022.04.004

39. Liu H, Hu J, Mahfuz S, Piao X. Effects of hydrolysable tannins as zinc oxide substitutes on antioxidant status, immune function, intestinal morphology, and digestive enzyme activities in weaned piglets. *Animals*. (2020) 10:757. doi: 10.3390/ani10050757

40. Li Z, Wright ADG, Si H, Wang X, Qian W, Zhang Z, et al. Changes in the rumen microbiome and metabolites reveal the effect of host genetics on hybrid crosses. *Environ Microbiol Rep*. (2016) 8:1016–23. doi: 10.1111/1758-2229.12482

41. Kreisinger J, Ížková D, Vohánka J, Piálek J. Gastrointestinal microbiota of wild and inbred individuals of two house mouse subspecies assessed using high-throughput parallel pyrosequencing. *Mol Ecol*. (2014) 23:5048. doi: 10.1111/mec.12909

42. Wang J, Kalyan S, Steck N, Turner LM, Harr B, Künzel S, et al. Analysis of intestinal microbiota in hybrid house mice reveals evolutionary divergence in a vertebrate hologenome. *Nat Commun*. (2015) 6:6440. doi: 10.1038/ncomms7440

43. Goodrich JK, Davenport ER, Beaumont M, Jackson MA, Knight R, Ober C, et al. Genetic determinants of the gut microbiome in UK twins. *Cell Host Microbe*. (2016) 19:731–43. doi: 10.1016/j.chom.2016.04.017

44. Kudo H, Cheng KJ, Costerton JW. Interactions between Treponema bryantii and cellulolytic bacteria in the in vitro degradation of straw cellulose. *Can J Microbiol*. (1987) 33:244–8. doi: 10.1139/m87-041

45. Quan J, Wu Z, Ye Y, Peng L, Wu J, Ruan D, et al. Metagenomic characterization of intestinal regions in pigs with contrasting feed efficiency. *Front Microbiol*. (2020) 11:32. doi: 10.3389/fmicb.2020.00032

46. Shinkai T, Ikeyama N, Kumagai M, Ohmori H, Sakamoto M, Ohkuma M, et al. Prevotella lacticifex sp. Nov., isolated from the rumen of cows. *Int J Syst Evol Microbiol*. (2022) 72:5278. doi: 10.1099/ijsem.0.005278

47. Yang H, Yang M, Fang S, Huang X, He M, Ke S, et al. Evaluating the profound effect of gut microbiome on host appetite in pigs. *BMC Microbiol*. (2018) 18:215. doi: 10.1186/s12866-018-1364-8

48. Chen C, Fang S, Wei H, He M, Fu H, Xiong X, et al. Prevotella copri increases fat accumulation in pigs fed with formula diets. *Microbiome*. (2021) 9:175–5. doi: 10.1186/s40168-021-01110-0

49. Pedersen HK, Gudmundsdottir V, Nielsen HB, Hyotylainen T, Nielsen T, Jensen BAH, et al. Human gut microbes impact host serum metabolome and insulin sensitivity. *Nature (London)*. (2016) 535:376–81. doi: 10.1038/nature18646

50. Xue MY, Sun HZ, Wu XH, Liu JX, Guan LL. Multi-omics reveals that the rumen microbiome and its metabolome together with the host metabolome contribute to individualized dairy cow performance. *Microbiome*. (2020) 8:64. doi: 10.1186/s40168-020-00819-8

51. Pezeshki A. The role of gut microbiota on regulation of growth and metabolism by branched-chain amino acids. *J Anim Sci*. (2024) 102:199. doi: 10.1093/jas/skae102.222

52. Chen C, Huang X, Fang S, Yang H, He M, Zhao Y, et al. Contribution of host genetics to the variation of microbial composition of cecum lumen and feces in pigs. *Front Microbiol*. (2018) 9:2626. doi: 10.3389/fmicb.2018.02626

53. Suen G, Weimer PJ, Stevenson DM, Aylward FO, Boyum J, Deneke J, et al. The complete genome sequence of Fibrobacter succinogenes S85 reveals a cellulolytic and metabolic specialist. *PLoS One*. (2011) 6:e18814. doi: 10.1371/journal.pone.0018814

54. Astuti WD, Ridwan R, Fidriyanto R, Rohmatussolihat R, Sari NF, Sarwono KA, et al. Changes in rumen fermentation and bacterial profiles after administering Lactiplantibacillus plantarum as a probiotic. *Vet World*. (2022) 15:1969–74. doi: 10.14202/vetworld.2022.1969-1974

55. Van Gylswyk NO. Succiniclasticum ruminis gen. Nov., Sp. Nov., a ruminal bacterium converting succinate to propionate as the sole energy-yielding mechanism. *Int J Syst Bacteriol*. (1995) 45:297–300. doi: 10.1099/00207713-45-2-297

56. Li Z, Shi J, Lei Y, Wu J, Zhang R, Zhang X, et al. Castration alters the cecal microbiota and inhibits growth in Holstein cattle. *J Anim Sci*. (2022) 100:100. doi: 10.1093/jas/skac367

57. Koistinen KM, Suoniemi M, Simolin H, Ekroos K. Quantitative lysophospholipidomics in human plasma and skin by LC–MS/MS. *Anal Bioanal Chem*. (2015) 407:5091–9. doi: 10.1007/s00216-014-8453-9

58. Ukanović N, Obradović S, Zdravković M, Urašević S, Stojković M, Tosti T, et al. Lipids and antiplatelet therapy: important considerations and future perspectives. *Int J Mol Sci*. (2021) 22:3180. doi: 10.3390/ijms22063180

59. Inoue N, Sakurai T, Yamamoto Y, Chiba H, Hui SP. Profiling of lysophosphatidylethanolamine molecular species in human serum and in silico prediction of the binding site on albumin. *Biofactors*. (2022) 48:1076–88. doi: 10.1002/biof.1868

60. Hisano K, Yoshida H, Kawase S, Mimura T, Haniu H, Tsukahara T, et al. Abundant oleoyl-lysophosphatidylethanolamine in brain stimulates neurite outgrowth and protects against glutamate toxicity in cultured cortical neurons. *J Biochem*. (2021) 170:327–36. doi: 10.1093/jb/mvab046

61. Yamamoto Y, Sakurai T, Chen Z, Inoue N, Chiba H, Hui S. Lysophosphatidylethanolamine affects lipid accumulation and metabolism in a human liver-derived cell line. *Nutrients*. (2022) 14:579. doi: 10.3390/nu14030579

62. Moskovitz J, Smith A. Methionine sulfoxide and the methionine sulfoxide reductase system as modulators of signal transduction pathways: a review. *Amino Acids*. (2021) 53:1011–20. doi: 10.1007/s00726-021-03020-9

63. Sharov VS, Schoneich C. Diastereoselective protein methionine oxidation by reactive oxygen species and diastereoselective repair by methionine sulfoxide reductase. *Free Radic Biol Med*. (2000) 29:986–94. doi: 10.1016/s0891-5849(00)00400-7

64. Li H, Cai L, Liang M, Wang Z, Zhang Y, Wu Q, et al. Methionine augments endogenous antioxidant capacity of rice protein through stimulating MSR antioxidant system and activating Nrf2-are pathway in growing and adult rats. *Eur Food Res Technol*. (2020) 246:1051–63. doi: 10.1007/s00217-020-03464-5

65. De Freitas DA, de Souza KA, Alcalde CR, Gasparino E, Feihrmann AC. Supplementation with free methionine or methionine dipeptide improves meat quality in broilers exposed to heat stress. *J Food Sci Technol*. (2021) 58:205–15. doi: 10.1007/s13197-020-04530-2

66. Hu H, Bai X, Shah AA, Wen AY, Hua JL, Che CY, et al. Dietary supplementation with glutamine and γ-aminobutyric acid improves growth performance and serum parameters in 22- to 35-day-old broilers exposed to hot environment. *J Anim Physiol Anim Nutr*. (2016) 100:361–70. doi: 10.1111/jpn.12346

67. Park K, Oh M, Joo Y, Han J. Effects of gamma aminobutyric acid on performance, blood cell of broiler subjected to multi-stress environments. *Anim Biosci*. (2023) 36:248–55. doi: 10.5713/ab.22.0031

68. Dai SF, Gao F, Zhang WH, Song SX, Xu XL, Zhou GH. Effects of dietary glutamine and gamma-aminobutyric acid on performance, carcass characteristics and serum parameters in broilers under circular heat stress. *Anim Feed Sci Technol*. (2011) 168:51–60. doi: 10.1016/j.anifeedsci.2011.03.005

69. Chen Z, Xie J, Wang B, Tang J. Effect of gamma-aminobutyric acid on digestive enzymes, absorption function, and immune function of intestinal mucosa in heat-stressed chicken. *Poult Sci*. (2014) 93:2490–500. doi: 10.3382/ps.2013-03398

70. Inigo M, Deja SA, Burgess SC. Ins and outs of the TCA cycle: the central role of anaplerosis. *Annu Rev Nutr*. (2021) 41:19–47. doi: 10.1146/annurev-nutr-120420-025558

71. Kong L, Yue Y, Li J, Yang B, Chen B, Liu J, et al. Transcriptomics and metabolomics reveal improved performance of Hu sheep on hybridization with Southdown sheep. *Food Res Int*. (2023) 173:113240. doi: 10.1016/j.foodres.2023.113240

72. Oien NL, Brideau RJ, Hopkins TA, Wieber JL, Knechtel ML, Shelly JA, et al. Broad-spectrum antiherpes activities of 4-Hydroxyquinoline carboxamides, a novel class of herpesvirus polymerase inhibitors. *Antimicrob Agents Chemother*. (2002) 46:724–30. doi: 10.1128/AAC.46.3.724-730.2002

73. Schoeler M, Caesar R. Dietary lipids, gut microbiota and lipid metabolism. *Rev Endocr Metab Dis*. (2019) 20:461–72. doi: 10.1007/s11154-019-09512-0

74. Brown EM, Clardy J, Xavier RJ. Gut microbiome lipid metabolism and its impact on host physiology. *Cell Host Microbe*. (2023) 31:173–86. doi: 10.1016/j.chom.2023.01.009

75. Kaczka P, Michalczyk MM, Jastrząb R, Gawelczyk M, Kubicka K. Mechanism of action and the effect of beta-hydroxy-beta-methyl butyrate (HMB) supplementation on different types of physical performance-a systematic review. *J Hum Kinet*. (2019) 68:211–22. doi: 10.2478/hukin-2019-0070

frontiers | Frontiers in Veterinary Science

TYPE Original Research
PUBLISHED 01 October 2024
DOI 10.3389/fvets.2024.1438717

OPEN ACCESS

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RECEIVED 26 May 2024
ACCEPTED 03 September 2024
PUBLISHED 01 October 2024

CITATION
Zhao J, Wang C, Hu J, Ma R, Yu B, Zhao W, Wang H, Gu Y and Zhang J (2024) Integrated metagenomics and metabolomics analyses revealed biomarkers in β-casein A2A2-type cows.
Front. Vet. Sci. 11:1438717.
doi: 10.3389/fvets.2024.1438717

Integrated metagenomics and metabolomics analyses revealed biomarkers in β-casein A2A2-type cows

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In Holstein cows, β-casein, one of the most critical proteins in milk, exists in two main genotypes, A1 and A2. Herein, 45 Holstein cows [categorized into three groups based on β-casein A1A1, A1A2, and A2A2 genotypes ($N = 15$)] with the same feeding management and litter size were enrolled to explore differences in rumen microflora and metabolites across various β-casein genotypes. Rumen fluids were collected for metagenomics and metabolomics analyses. Metabolomics and weighted gene co-expression network analysis (WGCNA) revealed that arachidonic acid (AA), adrenic acid (AdA), glycocholic acid (GCA), and taurocholic acid (TCA) were significantly and positively correlated with milk fat % in dairy cows ($p < 0.05$). Furthermore, macro-genomics and Spearman's correlation analysis revealed significant positive correlations ($p < 0.05$) between the characteristic flora (*g_Acetobacter*, *g_Pseudoxanthomonas*, *g_Streptococcus*, and *g_Pediococcus*) and the five characteristic metabolites in the rumen of A2A2 dairy cows. Moreover, functional enrichment analysis revealed more genes enriched to the TRP channel's inflammatory mediator-regulated pathway and the mTOR signaling pathway in A2A2 genotyped cows. Additionally, the regulatory effects of AA on bovine mammary epithelial cells (BMECs) were examined using CCK-8, EdU, and qRT-PCR assays, revealing that AA promoted triglyceride (TG) synthesis and upregulated the milk fat marker genes including *SREBF1*, *ACSS2*, *AGPAT6*, and *FASN*. Overall, we identified characteristic microorganisms and metabolites in A2A2 Holstein cows and established that AA could be a biomarker for higher milk fat %.

KEYWORDS

Holstein dairy cows, beta-casein, A2A2, milk fat percentage, metabolomics, metagenomics

Background

Milk, one of the body's primary sources of nutrients, is rich in lactose, triglycerides (TGs), proteins, minerals, and vitamins (1). Milk proteins are classified based on their solubility into casein (~80%), whey proteins (~14%), and fat globule membrane proteins (~6%), with casein-soluble proteins being the most abundant and further classified into four categories: α1-casein, α2-casein, β-casein, and κ-casein (2). The structure of

β-casein depends on the dairy cow's breed and genotype, with A1 and A2 as the two main isoforms (3). The disparity between the two isoforms stems from a mutation at position 67, which induces a transformation of the amino acid from histidine (in A1) to proline (in A2), and this is attributed to genetic predetermination (4, 5). Cows with the β-casein A2A2 genotype produce the popular "A2 milk" (3). Additionally, A2A2 genotyped cows have a higher milk fat % than their A1A1 and A1A2 counterparts (6). Furthermore, during digestion and metabolism, A1 β-casein produces β-casein-7, which has been linked with Gastrointestinal (GI) issues and lactose intolerance disorders in humans (7, 8). It is also noteworthy that A1 β-casein possesses pro-inflammatory properties that can synergize, negatively affecting GI, endocrine, neurological, and cardiovascular systems. On the other hand, A2 milk (9, 10), which is free of A1 β-casein, has beneficial effects on human health and is easier to digest in lactose-intolerant individuals (11), making it a feasible alternative solution for individuals with pertinent GI disorders (12).

In ruminants, the rumen is the primary organ responsible for converting plant feeds into nutrients and energy (13, 14). According to research, microorganism derivatives, diet composition, and host metabolism influence rumen metabolite concentrations and colony structure, with all three factors collectively shaping the mechanisms underlying microbiota-host interactions (15). Rumen microbes have been established to be crucially involved in ruminant productivity and health (16). For instance, cows with mastitis exhibited significant alterations in inflammation-associated microbial communities and metabolite abundance in their rumen (17). Additionally, Zhang et al. (18) employed rumen fluid metabolomics to identify potential milk production biomarkers in high- and low-yielding cows. Biohydrogenation-linked rumen microbial populations were also associated with individual milk fat % in dairy cows (19). Although scholars both at home and abroad have extensively assessed A2 β-casein genotypes in cows, they mostly used milk or genetic tests (3, 20). Furthermore, to the best of our knowledge, no studies have characterized biomarkers and their roles in the rumen of A2-type β-casein dairy cows. Consequently, we explored the rumen microbiomes and metabolomes of different genotyped cows and examined the roles of characteristic metabolites and microorganisms in A2A2 genotyped cows using an integrated approach involving weighted gene co-expression network analysis (WGCNA) and Spearman correlation analysis.

Herein, 45 Holstein cows of three different β-casein genotypes [A1A1, A1A2, and A2A2 ($N = 15$)] from the Ningxia Nongken Helanshan dairy farm were included. Their rumen fluids were analyzed using metagenomics and metabolomics techniques. Metabolite clustering analysis and association analysis of characteristic metabolites with their characteristic microorganisms were performed using the WGCNA-Spearman integrated approach to further elucidate the contribution of metabolites to milk fat synthesis. Additionally, the regulatory role of AA on BMECs was explored using CCK-8, EdU, and qRT-PCR assays. Our analysis of the differences between rumen flora composition and metabolic pathways in cows of different β-casein genotypes could provide an essential reference for subsequent studies on the molecular genetic mechanisms of the characteristics of Holstein cows with the A2 pure genotype.

Materials and methods

Animals and experimental design

Holstein cow rumen fluids were collected from the Ningxia Nongken Helanshan dairy farm. The experimental cows were fed the same balanced total mixed ration (TMR) diet (Supplementary Table S1). Notably, the cows were previously typed using the competitive allele-specific PCR (KASP), and three genotypes were obtained: A1A1, A1A2, and A2A2 (21). For each genotype, 15 Holstein cows were selected in good condition and in their first lactation, in which the milk fat and protein content were similar across the three groups (Supplementary Table S2).

Sample collection

Two hours after the morning feeding, the rumen contents were collected using a rumen fluid collection tube. Specifically, after inserting the rumen fluid collection tube, rumen vesicle contents were aspirated and collected under negative pressure. To avoid contamination with saliva, the first 150 mL of the collected rumen contents were discarded. Subsequently, 100 mL rumen content was collected and filtered using four sterile gauze layers, portioned, quickly frozen in liquid nitrogen, and stored in a −80°C refrigerator, awaiting further use. The Institutional Animal Care Committee of Ningxia University approved our experimental protocol (Approval Number: NXU-2024-065).

Microbiota analysis

First, total DNA was extracted from the rumen fluid and purified using a DNA extraction kit (TruSeq Nano DNA LT Sample Preparation Kit, Illumina, United States), following the manufacturer's instructions. Subsequently, DNA concentration and quality were assessed using 1.0% agarose gel electrophoresis and a NanoDrop spectrophotometer. Following that, purified and tested DNA samples underwent fragmentation and end repair using the Covaris S220 before attaching the Y-junctions to the sample ends. We then performed PCR amplification to recover the target fragments and create a library. Subsequently, the libraries were sequenced on the Illumina HiSeq 2000 platform at the Shanghai Ouyi Biomedical Technology Co., Ltd. Following that, the genes were filtered and quality-controlled using Trimmomatic (v0.36) and Bowtie2 (v2.2.9) before splicing the sequences using MEGAHIT (v1.1.2) software. The spliced contigs' open reading frames (ORFs) were predicted using Prodigal (v2.6.3) software. Finally, clustering was performed using CDHIT (v4). After predicting the ORF of the spliced contig, we constructed the non-redundant gene set of the predicted genes using CDHIT (v4.5.7) software.

The obtained set of non-redundant genes was compared to the GeneBank non-redundant (NR) database of nucleic acid sequences[1] using DIAMOND (v0.9.7) software. The sequences with an *e*-value

1 https://www.ncbi.nlm.nih.gov/guide/taxonomy/

$\leq 1 \times 10^{-5}$ were considered meaningful for obtaining species annotation information. Differences in α-diversity indices, including Shannon, Simpson, and ACE, were examined to detect the median, dispersion, maximum, minimum, and outliers of species diversity, yielding insights into rumen microbial diversity. The rumen fluid characteristic microorganisms of dairy cows across the three genotypes were screened using the Linear discriminant analysis Effect Size (LEfSe) approach based on the LDA >2 and $p < 0.05$ criteria. Finally, the predicted genes were integrated with the Kyoto Encyclopedia of Genes and Genomes (KEGG) database[2] to obtain the gene function annotation information.

Untargeted metabolomics

First, the stored sample was thawed slowly on ice before obtaining 1 mL from the SPE solid-phase column and precisely adding 3 mL methanol. After blow drying, the sample was further dried through nitrogen blowing using a nitrogen blowing instrument before adding 300 μL methanol-water mixture (V:V = 4:1, containing L-2-chlorophenylalanine, 4 μg/mL) to redissolve it. Subsequently, the sample was vortexed for 1 min, sonicated for 10 min in an ice-water bath, and incubated at −40°C for 30 min. The sample was then centrifuged at 12,000 rpm for 10 min at 4°C before aspirating 150 μL of the supernatant using a syringe, which was filtered through a 0.22 μm organic-phase pinhole membrane, transferred to a liquid chromatography-mass spectrometry (LC-MS) injection vial, and stored at −80°C, awaiting LC-MS analysis.

Metabolite detection was performed using a liquid-mass spectrometry platform comprising an ACQUITY UPLC I-Class plus ultrahigh-performance liquid chromatography-tandem system and a QE plus high-resolution mass spectrometer. The LC-MS instrument was equipped with a preset ACQUITY UPLC HSS T3 chromatography column (100 mm × 2.1 mm, 1.8 μm), operated at a flow rate of 0.35 mL/min and a temperature of 45°C. Mobile phase A consisted of water and 0.1% formic acid, whereas mobile phase B comprised 100% acetonitrile. Supplementary Table S3 shows the elution process of the mobile phases. Each sample (2 μL) was injected into an autosampler set at 4°C. The spray voltages for the positive and negative modes were set at 3.8 kV and 3.0 kV, respectively. Other parameters were the same for both the positive and negative modes (capillary temperature = 320°C; aux gas heater temperature = 350°C). The raw peaks were extracted, analyzed, and quantified using the LECO-Fiehn Rtx5 database, and normalization analyses were performed (22). To obtain precise qualitative and relative quantitative results, the peaks were compared to those in various databases such as mzCloud,[3] mzVault, and MassList. Statistical analyses were performed using R v3.43, Python v276, and Cent vOS66 software.

MetaX, a metabolomics data processing software, was used to perform principal component analysis (PCA) and partial least squares discriminant analysis (PLS-DA). Statistical significance (*p*-value) was evaluated using one-way analysis of variance, and marker metabolite screening was aided by the variable importance for the projection (VIP) of the (O)PLS-DA model variables. Metabolites with VIP >1, $p < 0.05$, FC ≥2, or FC ≤0.5 were considered differential expressed. Annotation and metabolite pathway analysis was performed using metabolites obtained from the KEGG (see text footnote 2), HMDB,[4] and LIPID MAPS[5] databases.

Metabolite co-expression module construction

To obtain precise qualitative and relative quantitative results, the peaks were compared to those in databases such as mzCloud (see text footnote 3), mzVault, and MassList. Statistical analyses were performed using R v3.43, Python v276, and CentOS66 software (23). For network construction, we used the soft threshold power (β) based on an *R*-value of 0.96. The smallest module comprised 35 genes (minimum module size = 35), and the merged module had a height of 0.25. Correlations between modules and cow milk fat % were determined to identify modules that affect milk fat, and metabolomics was used to enrich for metabolites within those modules.

Statistical analysis

Univariate ANOVA (*t*-test) was used to assess statistical significance (*p*-value), with $p < 0.05$ and $p < 0.01$ indicating significant and highly significant differences, respectively. GraphPad Prism 8 was used to plot histograms. Dominant rumen flora ($R > 0.6$, $p < 0.05$) were correlated with milk fat-related metabolites using Spearman correlation analysis, and all significant correlation networks were visualized using Cytoscape (3.8.2). Receiver operating characteristic (ROC) curves were plotted, and the corresponding area under the curve (AUC) values were computed using the ROCR software package (24).

Cell culture

Mammary epithelial cell lines from dairy cows were cultured and frozen in the preliminary phase of this experiment. Specifically, BMECs were grown in a DMEM/F12 growth medium supplemented with 10% fetal bovine serum (FBS) (Cell Max, Beijing, China) in a 5% CO_2 and 37°C incubator. Passaging and culture treatments were performed at ~70–80% cell density.

AA master mix configuration

To prepare AA mother liquor at a 10 mM concentration, 10 mg AA dry powder (Sigma, America) was first weighed and then transferred into a 5 mL centrifuge tube before adding 3.28 mL anhydrous ethanol to dissolve it at room temperature (RT). After thorough mixing, the solution was filtered to remove bacteria,

2 http://www.genome.jp/kegg/pathway.html
3 https://www.mzcloud.org/
4 https://hmdb.ca/metabolites
5 http://www.lipidmaps.org/

dispensed into 200 μL centrifuge tubes, and stored at −20°C in a refrigerator in the dark for spare use. The experimental group received AA at final concentrations of 1, 5, and 10 μM, whereas the control group (NC) received anhydrous ethanol.

CCK-8 cell viability and cell proliferation EdU assays

First, cells were inoculated into 96-well plates, and the optimal concentrations from the AA treatment and experimental groups were selected for the EdU assay. Following the instructions in the EdU assay kit (Beyotime, Shanghai, China), the 2xEdU working solution was prepared in equal volumes and added into petri dishes after seeding cells in optimal growth conditions into 6-well plates. The cells were then observed under an inverted fluorescence microscope DMi8 (Leica, Germany) and counted using ImageJ software.

TG content determination

The treated cells were tested for TG content using the cell-specific high-fat sample TG enzymatic assay kit (E1025, Prilosec, Beijing, China). Based on the reagent instructions, the lysed supernatant was added to the prepared working solution, and the reaction was conducted at 37°C for 15 min. Each tube's optical density (OD) value was detected at 550 nm, and the TG content was adjusted based on protein concentration per mg.

RT-qPCR-related gene expression detection

Total RNA was extracted using TRizol reagent (Invitrogen, Thermo Fisher, United States) and then reverse-transcribed into complementary DNA (cDNA) using Prime Script RT Reagent Kit (Takara, Dalian, China). Following the manufacturer's instructions, SYBR Premix Ex Taq™ II (Takara, Dalian, China) was used to extract RNA from the cells for RT-qPCR on the Bio-Rad CFX96 Touch™ Real-Time PCR Detection System (Bio-Rad, Hercules, CA, United States). The primers used were designed using the Primer Premier 5.0 system (Supplementary Table S4). The $2^{-\Delta\Delta Ct}$ technique was used to analyze the relative mRNA expression in different treatment groups. Gene expression was normalized to *GAPDH*, and all results were subjected to ANOVA using SAS software (version 9.2, SAS Institute, Cary, NC). Three replicates were set up for each gene, and results or differences with $p < 0.05$ and $p < 0.01$ were considered significant and highly significant, respectively.

Results

Structural analysis of microbial communities

Herein, rumen fluid samples from 45 Holstein cows were subjected to macro genome sequencing. According to the results, the samples' clean reads and contig N50 statistics were distributed in the 10.01–16.63 G and 299–475 bp ranges, respectively, and the number of ORFs in the constructed gene catalog (non-redundant genes) after de-redundancy was 15,361,808 (Supplementary Table S5). Furthermore, sample size significance was determined using core-pan gene dilution curve analysis. According to the results, the number of subjects selected for the study ($n = 45$) was sufficient (Supplementary Figure S1A). A comparison of specific genes across the three groups (A1A1, A1A2, and A2A2) revealed that their proportions were 2.23, 3.29, and 2.58%, respectively (Supplementary Figure S1B). Additionally, we determined the rumen flora α diversity indices based on species abundance (Supplementary Table S6). According to the results, the three groups showed no significant differences in the three α diversity indices (Shannon, Simpson, and ACE) ($p > 0.05$). Conversely, PCA revealed microbial β diversity differences in the rumen fluid of the dairy cows with different genotypes (Supplementary Figure S1C). Furthermore, Analysis of Similarities (ANOSIM) revealed that the differences among the three groups were significantly greater than those within each group, indicating meaningful subgroup distinctions ($R = 0.078$, $p < 0.05$; Supplementary Figure S1D). The top 15 most abundant phyla and genera among the 45 cows were plotted using a species relative abundance bar chart (Figures 1A,B). The dominant phylum- and genus-level microorganisms were *p_Bacteroidetes* and *p_Firmicutes* and *g_Prevotella* and *g_Clostridium*, respectively. The potential biomarkers in the rumen of the three different dairy cow genotypes were further examined through LEfSe analysis. According to the results, 55 characterized microorganisms were enriched in A2A2 cows (Supplementary Table S7), of which the key biomarker genera were *g_Stenotrophomonas*, *g_Fusobacterium*, *g_Mannheimia*, *g_Acetobacter*, *g_Xanthomonas*, *g_Pichia*, *g_Pseudoxanthomonas*, *g_Pediococcus*, *g_Gluconobacter*, *g_Komagataeibacter*, *g_Glomus*, *g_Luteimonas*, *g_Pasteurella*, and *g_Streptococcus* (Figures 1C,D).

Analysis of marker bacteria in the rumen of dairy cows across the three different genotypes

Differential KEGG functional enrichment through STAMP analysis revealed that arrhythmogenic right ventricular cardiomyopathy (ARVC) and jak-STAT signaling pathway, among others, were the functions enriched in A1A1 and A1A2 genotyped cows. On the other hand, TRP channels' inflammatory mediator regulation and the mTOR signaling pathway, among others, were the functions enriched in A2A2 genotyped cows (Figure 2).

Analysis of metabolomics results

Differentially expressed metabolites (DEMs) in the rumens of Holstein cows across the three different genotypes were detected using UPLC-MS metabolomics technology. A total ion chromatogram (TIC) overlap plot was obtained via superimposition of the mass spectra of the QC samples from the positive and negative ion detection modes on the TIC data (Supplementary Figures S2A,B), revealing that the peaks' response

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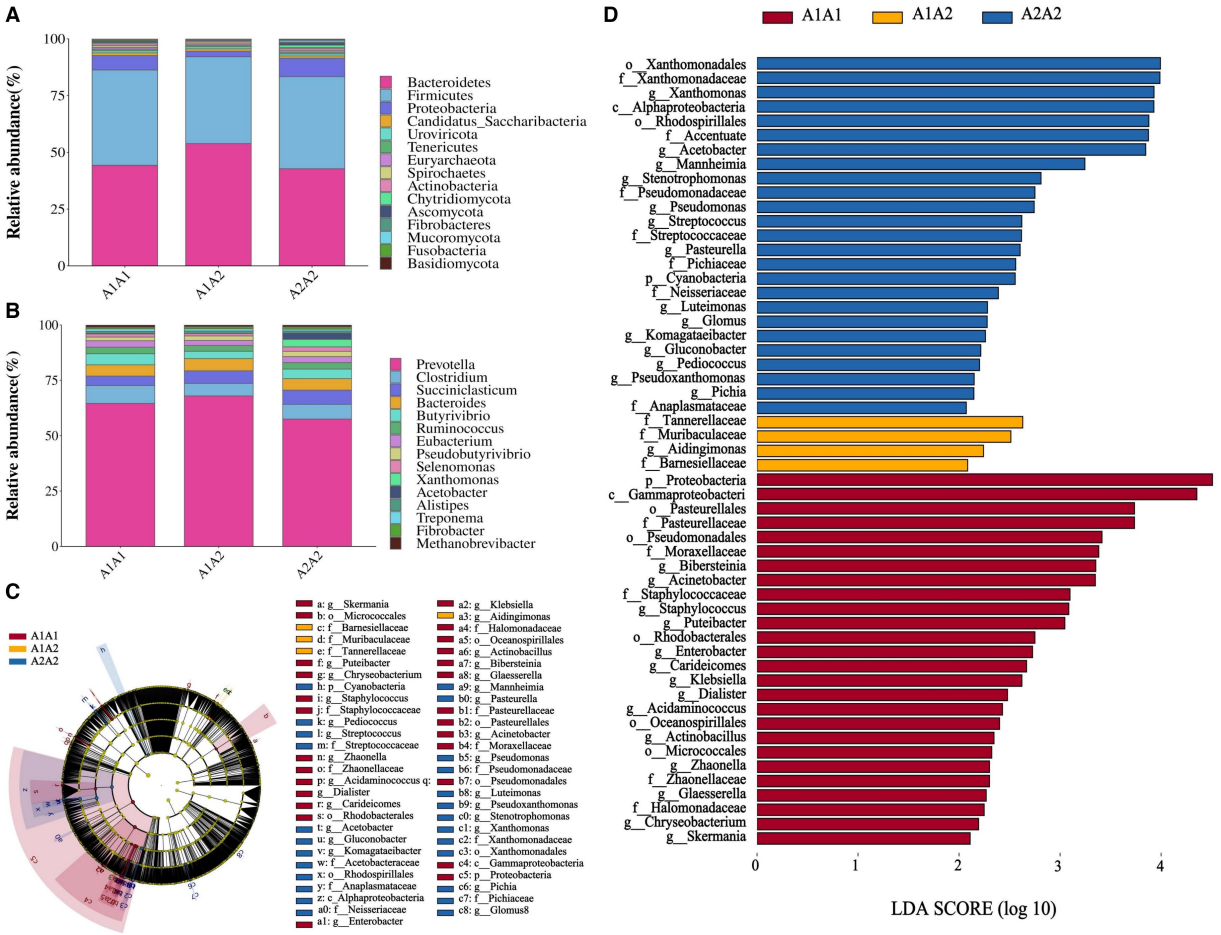

FIGURE 1
Structural analysis of rumen microbial communities. **(A)** The relative abundance of the 15 most abundant bacteria at the phylum level. **(B)** The relative abundance of the 15 most abundant bacteria at the genus level. **(C)** Branch diagram of LEfSe analysis of the three groups. **(D)** Histogram showing the distribution of LDA values among the three groups; higher LDA scores indicate greater importance of the bacteria.

intensities and retention times overlapped and had stable baseline values. These findings confirmed the reliability of the experimental data. According to the PCA and PLS-DA results, metabolites in the three dairy cow genotypes showed inter- and intra-group disparities (Supplementary Figures S2C,D). Furthermore, the OPLS-DA score plots of metabolites showed significant differences across the rumen metabolite groups of the three dairy cow genotypes, and the permutation test revealed that all OPLS-DA models were reliable and did not overfit (Supplementary Figures S3A–F). Venn diagrams of DEMs and differential metabolic pathways among the three groups showed that the two comparison groups overlapped significantly (Supplementary Figures S2E,F). Statistical analysis of mass spectrometry-identified metabolites revealed 691 DEMs in the rumen fluid of the A1A1 and A1A2 dairy cow groups (Figure 3A and Supplementary Table S8) and 283 DEMs in the rumen fluid of the A1A1 and A2A2 dairy cow groups (Figure 3B and Supplementary Table S9). Furthermore, the analysis of the rumen fluid of the A1A2 and A2A2 dairy cow groups revealed 1,025 DEMs (Figure 3C and Supplementary Table S10). The top 50 DEMs were then analyzed for their respective enrichments (Supplementary Figures S4A–C). The substances enriched in the A2A2 group were arachidonic acid (AA), adrenic acid (AdA), taurocholic acid (TCA), and glycocholic acid (GCA). To better understand differential metabolite enrichment across the three groups, the top 20 metabolic pathways of differential metabolites were identified (Figures 3D–F), including those related to cholesterol metabolism, vascular smooth muscle contraction, ovarian steroidogenesis, primary bile acid biosynthesis, the GnRH signaling pathway, AA metabolism, Fc gamma R-mediated phagocytosis, and necroptosis.

WGCNA

We constructed co-expression networks between the identified metabolites and milk fat % using WGCNA to better understand the relationship between metabolites and milk fat % in Holstein cows. Ten co-expression modules were identified after merging modules with similar characteristics (Figure 4A). Furthermore, the results showed that MEturquoise correlated positively with milk fat content (Figure 4B) and 1,308 metabolites from this module were selected for further analysis.

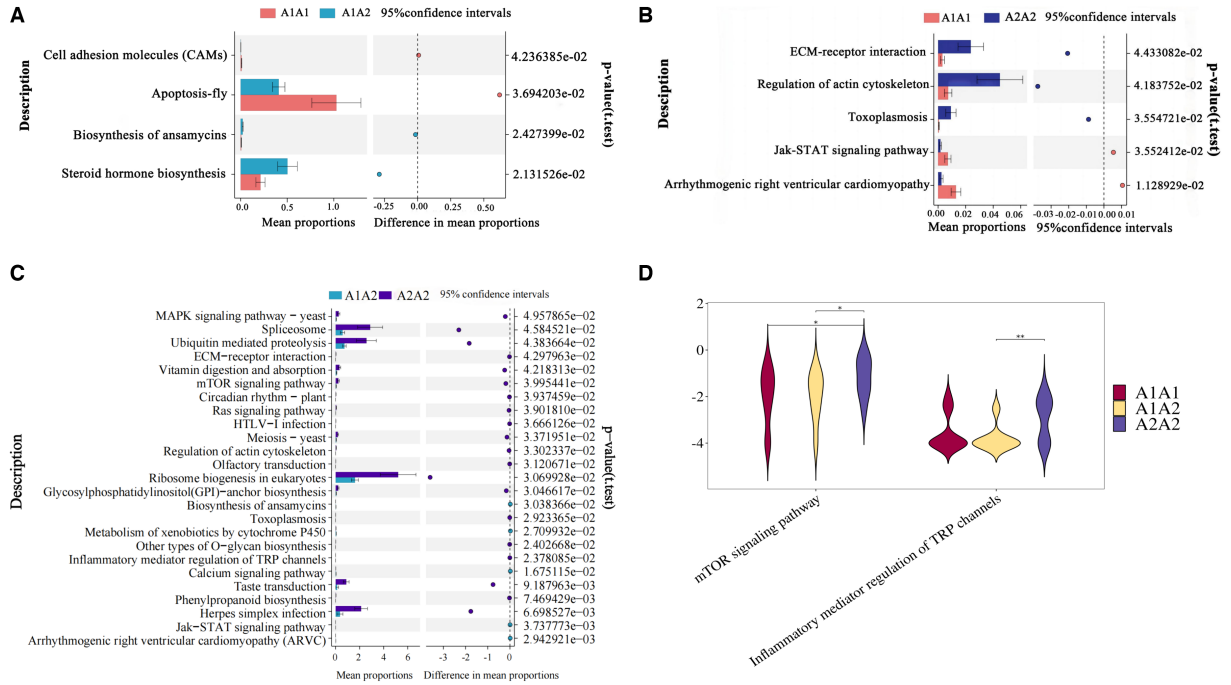

FIGURE 2
Functional analysis of rumen microorganisms in dairy cows. **(A–C)** The proportion of abundance of different KEGG functional entries between groups at the functional level, with the proportion of differences within the 95% confidence interval shown in the middle, and the rightmost value is the *p*-value, with $p < 0.05$ indicating a significant difference. **(D)** Violin plot of the key KEGG functional entries in cows of the A2A2 genotype.

Metabolic pathway analysis

MEturquoise metabolites were significantly positively correlated with milk fat % ($cor = 0.6$, $p = 7.7 \times 10^{-48}$) (Supplementary Figure S5A). Notably, enrichment analysis of the 1,308 MEturquoise metabolites yielded 35 DEMs (VIP >1.00, $p < 0.05$), including AA, GCA, and TCA (Supplementary Figure S5C). All 35 DEMs, as well as those between the three groups, were subjected to metabolic pathway enrichment (661 metabolites, VIP >1, $p < 0.05$) (Figures 5A,B). According to the results, pathways such as cholesterol metabolism, vascular smooth muscle contraction, ovarian steroidogenesis, ferroptosis, primary bile acid biosynthesis, the GnRH signaling pathway, AA metabolism, Fc gamma R-mediated phagocytosis, and necroptosis were co-enriched (Supplementary Figure S5B). Furthermore, the metabolites included in the co-enrichment pathway were AA, AdA, GCA, TCA, and 8,9-Epoxyeicosatrienoic Acid (8,9-EET) (Supplementary Table S11). Specifically, AA, AdA, GCA, and TCA were significantly enriched in A2A2 cows, whereas 8,9-EET was significantly enriched in both A2A2 and A1A1 cows ($p < 0.05$) (Figures 5D–I).

Correlation analysis of key metabolites in rumen milk fat with characteristic flora in A2A2 genotyped dairy cows

Herein, we employed Spearman's correlation analysis to examine the relationship between characteristic genera (LDA >2; $p < 0.05$) and key differential rumen metabolites of milk fat in the rumen fluid of A2A2 dairy cows in order to characterize the relationship between the rumen flora and key metabolites of milk fat (Figure 6A). According to the results, some of the genera and metabolites correlated significantly and strongly ($|R| > 0.6$, $p < 0.01$) (Figure 6B). Among them, *g_Acetobacter*, *g_Pseudoxanthomonas*, *g_Streptococcus*, *g_Pediococcus*, *g_Mannheimia*, *g_Stenotrophomonas*, *g_Komagataeibacter*, *g_Gluconobacter*, and *g_Luteimonas* correlated significantly positively with both primary bile acid biosynthesis (GCA and TCA) and AA metabolism (AA and 8,9-EET). It has been established that AA regulates milk lipid synthesis and secretion (25). In this regard, it is noteworthy that biomarker prediction using ROC curves to identify significantly enriched characteristic metabolites and flora in A2A2 cows revealed that the model had a good prognostic effect, with AUC values for AA of 0.713 in groups A1A1 vs. A1A2 and 0.846 in groups A1A2 vs. A1A2 (Supplementary Figure S6). We will focus more on AA in the subsequent sections.

Effects of the candidate marker metabolite AA on BMEC proliferation

After culturing BMECs *in vitro* for 12 and 24 h, different AA concentrations were added to the culture medium, and their effects on BMEC viability were evaluated using the CCK-8 assay. According to the results, 5 μM AA was the optimal concentration, with the cells reaching the highest viability after 24 h (Figures 7A,B). Furthermore, the EdU results showed that the 5 μM AA-treated

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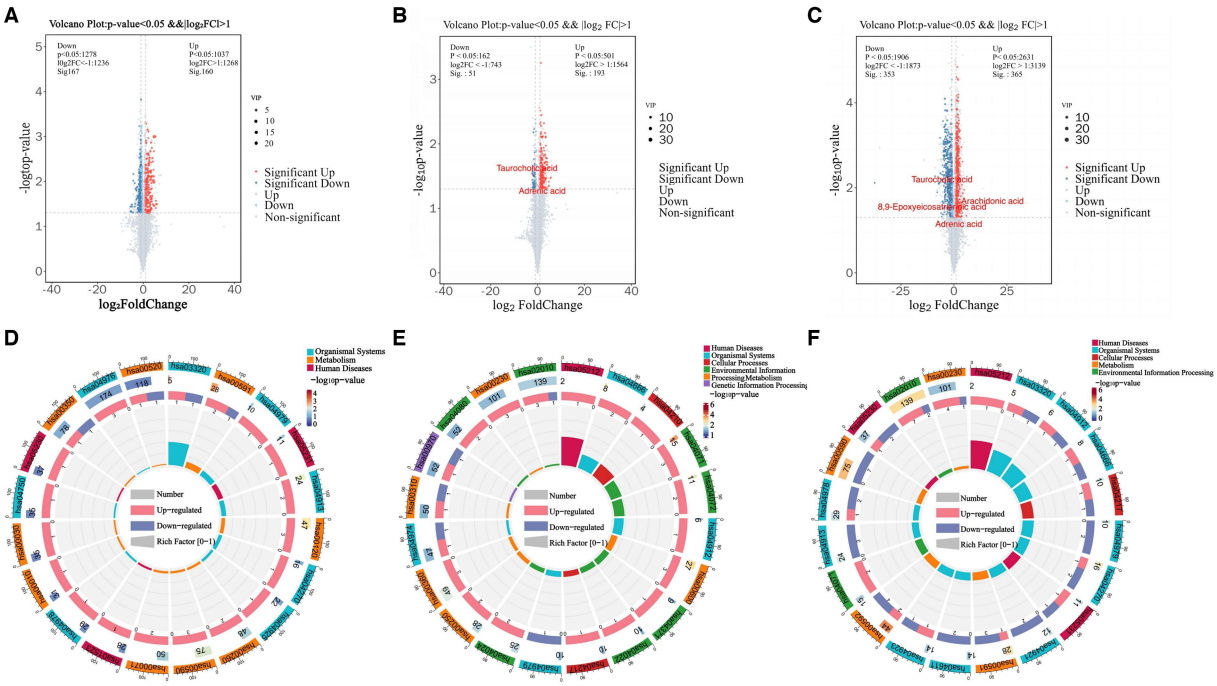

FIGURE 3
Rumen metabolome analysis. **(A)** Volcano plots of differential metabolites between A1A1 and A1A2 groups, **(B)** A1A1 and A2A2, **(C)** A1A2 and A2A2. **(D)** The circle plots of KEGG enrichment analysis between groups A1A1 and A1A2, **(E)** A1A1 and A2A2, **(F)** A1A2 and A2A2. There are 4 circles from the outside to the inside: the first circle: the classification of enrichment, the outside of the circle is the scale of the number of metabolites, different colors represent different classifications; the second circle: the number of the classification in the background metabolism and the *p*-value. The more metabolites the longer the bar is, the smaller the value the redder the color is, and the bigger the value the bluer the color is; the third circle: the bar of the proportion of the metabolism in the up and down-regulated metabolism, light red represents the proportion of the metabolism in the up-regulated metabolism, light blue represents the proportion of metabolism in the down-regulated metabolism; the specific values are shown below; and the specific values are presented below. Proportions, specific values are shown below; fourth circle: RichFactor values for each category, each cell of the background auxiliary line represents 0.2.

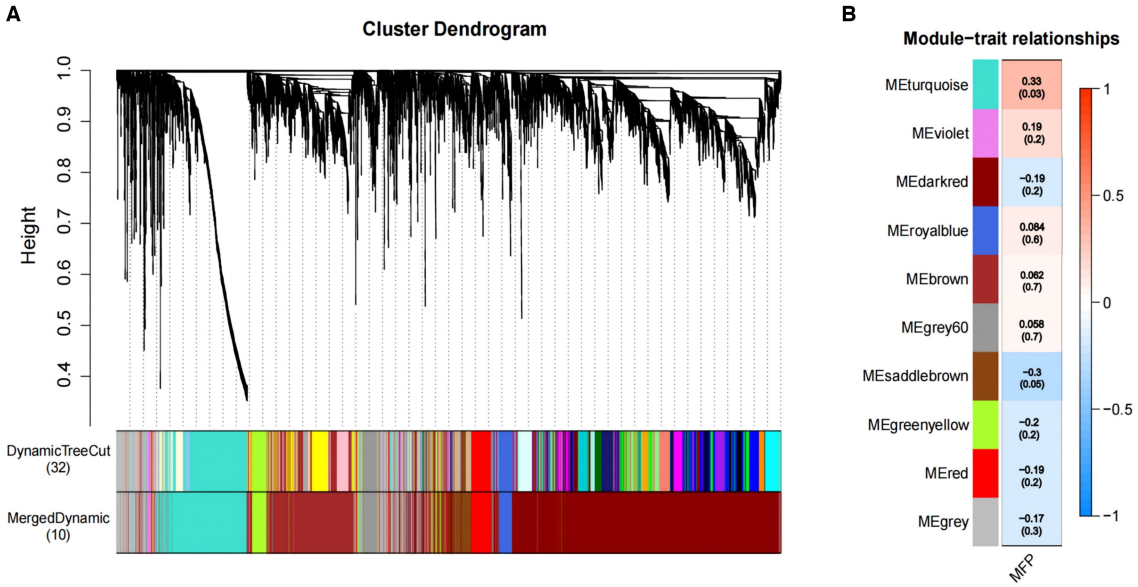

FIGURE 4
Association of metabolites with MFP based on WGCNA. **(A)** The clustering dendrogram of the average network adjacency for the identification of metabolite co-expression modules. **(B)** Heatmap of the correlation of module trait genes with MFP. Each row corresponds to a trait module, and the each column represents a trait. The plot is color-coded by correlation according to the color legend, and each module contains the corresponding correlation and *p*-value. MFP, milk fat percentage.

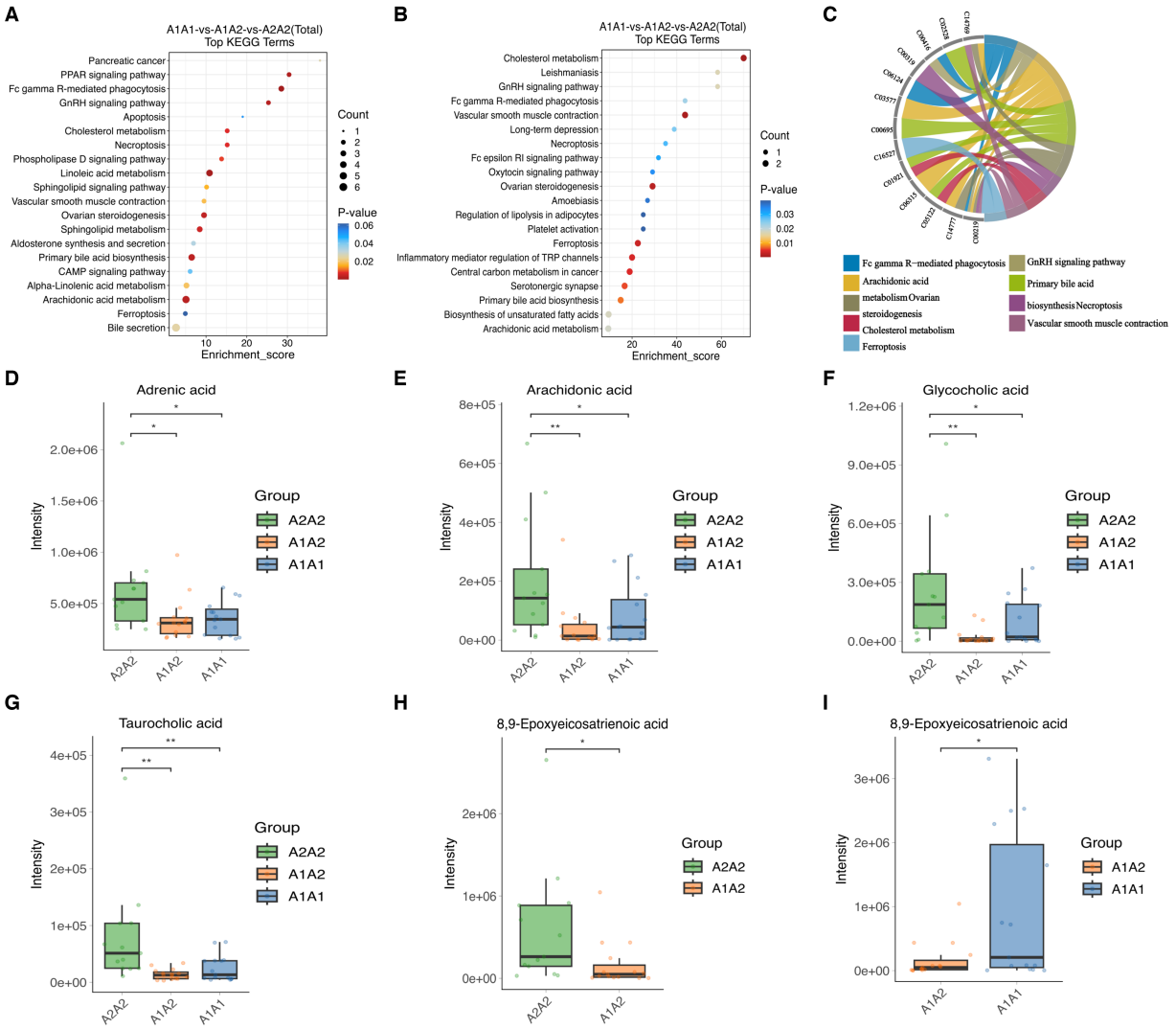

FIGURE 5
Differential metabolites and differential metabolic pathway analysis. **(A)** Top 20 differential metabolic pathways enriched in differential metabolites among the three groups (MEturquoise). **(B)** Top 20 differential metabolic pathways enriched in differential metabolites among the three groups; color gradient and circle size indicate the significance of pathways sorted by *p*-value (red: higher *p*-value, blue: lower *p*-value) and pathway impact scores (the larger the circle, the higher the impact score), respectively. **(C)** Nine co-enrichment pathways corresponding to metabolite chordograms. **(D–I)** Levels of adrenic acid, AA, glycocholic acid, 8,9-EET, and taurocholic acid in the rumen of dairy cows of three genotypes.

group exhibited enhanced BMEC proliferation after 24 h of incubation (Figures 7C,D).

Effects of the candidate marker metabolite AA on milk fat synthesis

We treated BMECs with 5 μM AA for 24 h to further determine whether AA influences the cells' milk lipid synthesis. We then assessed TG concentration and expression levels of milk lipid marker genes. According to the results, the 5 μM concentration AA-treated group exhibited a significantly elevated TG concentration ($p < 0.01$) (Figure 8A), as well as the upregulation of lactolipid marker genes, including SREBF1, *ACSS2*, *AGPAT6*, and *FASN* (Figures 8B–E).

Discussion

A triad of flora, metabolites, and organismal immunity has been established to regulate the internal rumen environment, significantly impacting dairy cow health and performance (15). In the rumen, flora digest polysaccharides from feeds into Short-Chain Fatty Acids (SCFA) such as acetate, butyrate, and propionate, contributing up to 70% of animals' total energy intake (14). Metabolites are important markers of biochemical reactions in the rumen microecosystem and are sensitive to changes in rumen microbiology (26). According to research, β-casein is essential for individual cow health and lactation performance (27, 28), a phenomenon consistent with our findings, which demonstrated that A2A2 genotyped cows exhibited a higher milk fat % than the other two genotyped groups. Furthermore, A1β-casein and

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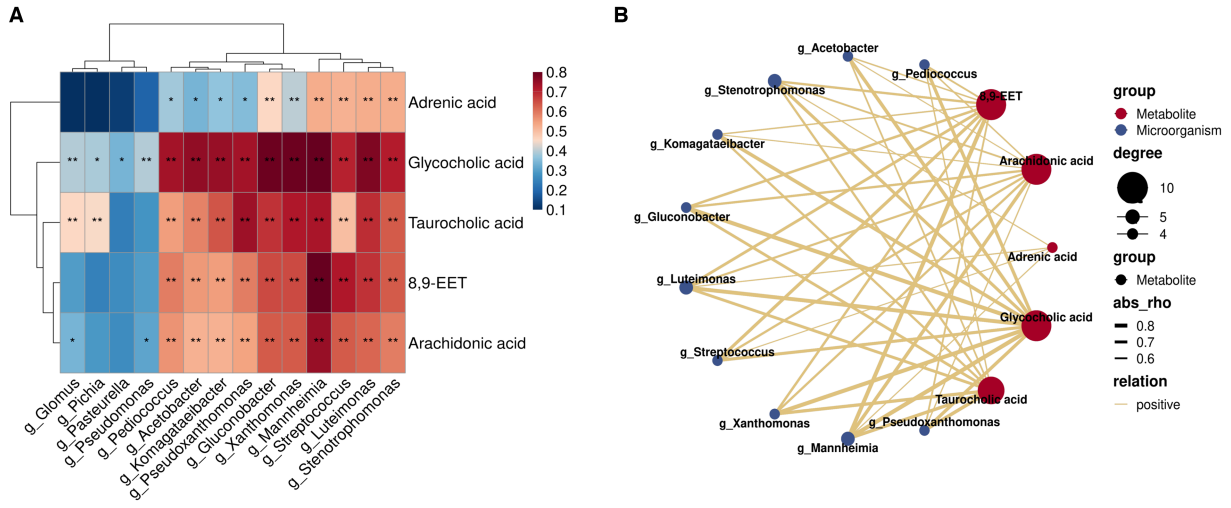

FIGURE 6
Correlation analysis of key flora and metabolites for differences among groups. **(A)** Heatmap of the Spearman correlation coefficient matrix between key rumen metabolites for milk fat synthesis and key microorganisms of group A2A2; ** represents a significant $p < 0.01$, * represents a significant $p < 0.05$. **(B)** A correlation network map; the colors of the lines represent positive and negative values of the correlation coefficients between the two (blue for negative correlation, red for positive correlation, only correlation with $|R| > 0.6$ and $p < 0.01$), and the thickness of the lines is directly proportional to the absolute value of the correlation coefficients; degree: centrality, the number of other nodes to which each node is connected, gradient according to the size of the centrality value.

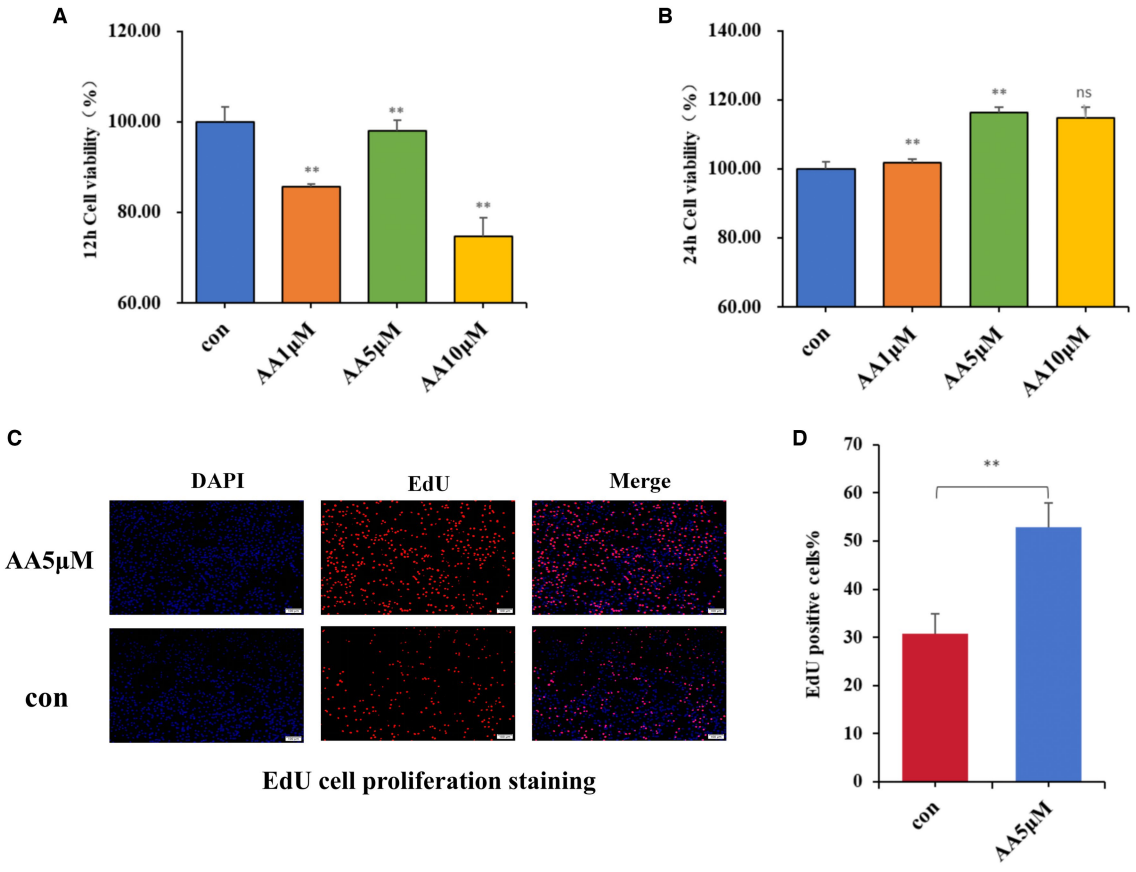

FIGURE 7
The effects of AA on cell proliferation. **(A,B)** Effects of different concentrations of AA on the viability of BMECs at 12 h and 24 h. **(C)** EdU cell proliferation staining images. **(D)** EdU proliferating cell counting analysis.

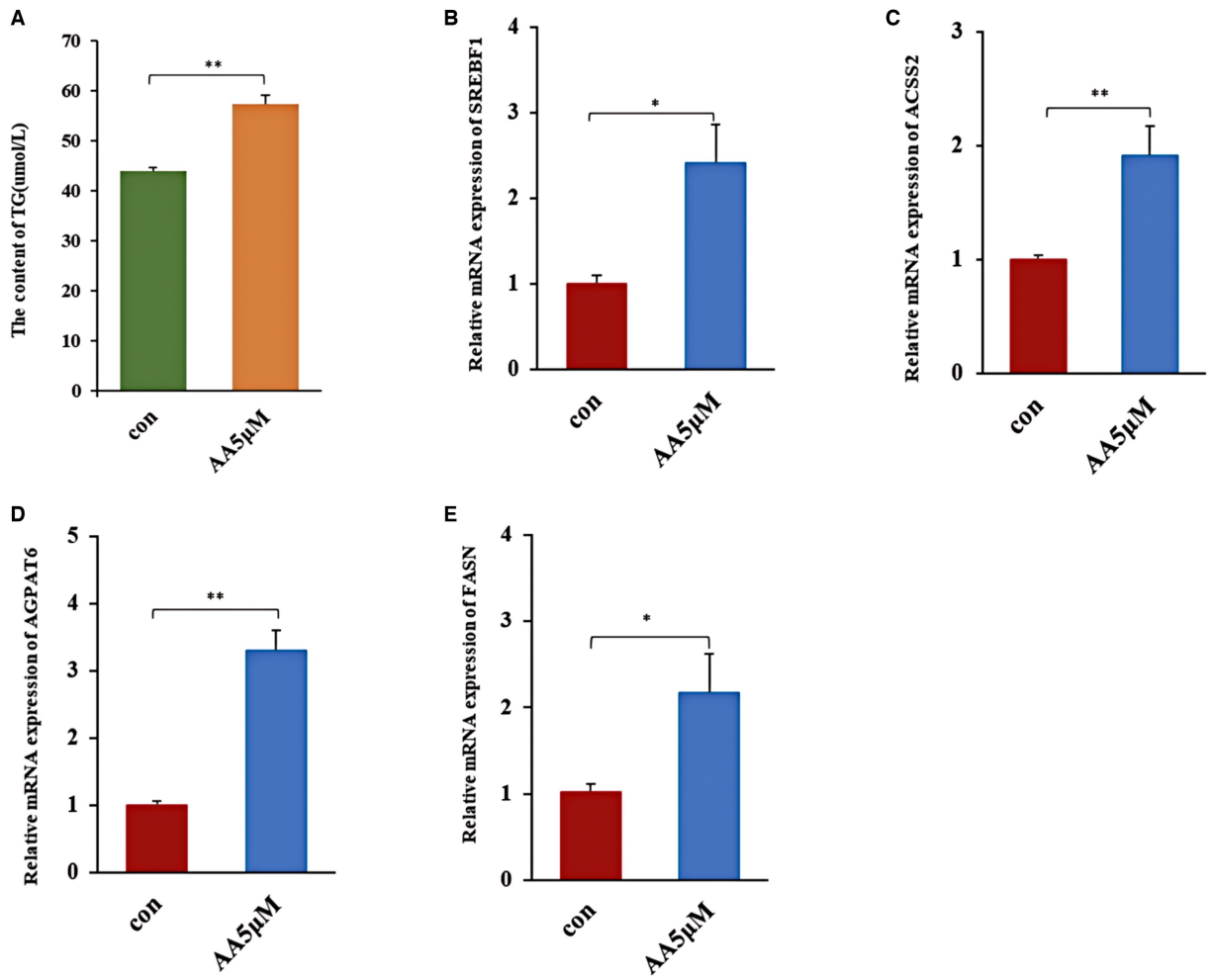

FIGURE 8
The effect of AA on milk fat synthesis. **(A)** TG content; **(B–E)** relative expression of milk lipid synthesis marker genes *SREBF1*, *ACSS2*, *AGPAT6* and *FASN*. ** In the figure represents a significance $p < 0.01$, and * in the figure represents a significance $p < 0.05$.

A2β-casein can impact colony fermentation, and the rumen flora structure could affect colony metabolites and milk fat synthesis processes (29, 30). Nonetheless, whether rumen flora and metabolites differ in β-casein A1A1, A1A2, and A2A2 genotyped cows remained unclear. Consequently, we screened the characteristic metabolites related to milk fat synthesis in A2A2 genotyped cows using an integrated metabolomics and WGCNA approach. We then examined the rumen characteristic flora and functions in cows with different β-casein genotypes using metagenomics technology. Finally, Spearman correlation analysis was employed to determine the relationships between characteristic flora and metabolites of A2A2 genotyped cows.

Our findings revealed that AA, AdA, GCA, and TCA were significantly upregulated in A2A2 cows ($p < 0.05$). According to research, AdA, as a substance downstream of AA, has anti-inflammatory effects (31, 32). On the other hand, TCA and GCA are mainly enriched in the primary bile acid synthesis and cholesterol metabolism pathways, which are critically involved in lipid homeostasis and inflammation regulation (33, 34). Furthermore, TCA can inhibit the production of inflammatory mediators such as nitric oxide (NO), prostaglandin E2 (PGE2), and histamine, exerting anti-inflammatory effects (35). Additionally, TCA can regulate *ACACA*, *FASN*, *AACS*, and *LPL* expression, potentially promoting adipogenesis. On the other hand, GCA has been established to lower the serum levels of NO and Leukotriene B4 (LTB4), as well as PGE2 levels in inflammatory tissues, exerting an anti-inflammatory effect (36). Notably, AA is enriched in the AA metabolic pathway (part of the lipid metabolic pathway) and is closely related to lipid synthesis (37). According to research, AA can be converted to PGE2 and LTB4 via the cyclooxygenase pathway, inhibiting inflammatory cell migration and activation, thus exerting anti-inflammatory effects (38, 39). Furthermore, as an ω-6 Polyunsaturated Fatty Acid (PUFA), AA regulates milk lipid synthesis and secretion via *PPARγ* activity modulation (25). Research has also shown that AA can act via a specific G Protein-Coupled Receptor (*GPR120*) (40), and *GPR120* activators can promote milk fat synthesis through *SREBP1* and *FASN* upregulation (41). Herein, the AA-treated group exhibited *SREBF1* and *FASN* upregulation. Similarly, *AGPAT6* and *ACSS2*, key genes involved in fatty acid and TG synthesis regulation (42–44), were upregulated in the AA-treated group, resulting in enhanced TG synthesis. Based on these findings, we deduced that AA, a characteristic metabolite in the rumen of A2A2 genotyped cows, promotes milk fat synthesis. We further hypothesized that the higher milk fat % and

anti-inflammatory effect of "A2 milk" on the GI tract of A2A2 genotyped cows may be related to AA, AdA, GCA, and TCA enrichment.

Rumen microbiota composition has been established to significantly impact milk production and composition in dairy cows (29). Herein, consistent with previous research (45, 46), the dominant phylum- and genus-level microorganisms in the flora content were *p_Bacteroidetes* and *g_Prevotella*, respectively. At the genus level, several significantly differentially expressed species were identified in the rumen of A2A2 cows, primarily belonging to genera *g_Pseudomonas*, *g_Acetobacter*, *g_Streptococcus*, and *g_Pediococcus*. According to research, *g_Pseudomonas* secrete lipase, which breaks down lipids in feeds into free fatty acids (FFAs), promoting saturated fatty acid accumulation in meat and milk (47, 48). On the other hand, *g_Acetobacter* oxidizes sugars to produce acetate, the precursor of milk fat synthesis, thus facilitating milk fat synthesis in the mammary glands (49). Furthermore, Edward et al. (48) discovered that *g_Acetobacter* can promote milk fat synthesis. Based on these findings, we hypothesized that milk fat synthesis in A2A2 genotypes cows could be linked to *g_Acetobacter* and *g_Pseudomonas* content. Moreover, *g_Streptococcus* and *g_Pediococcus* are lactic acid bacteria that produce lactic acid, which can be used as a substrate for secondary fermentation to produce precursors for milk fat synthesis: acetate, propionate, and butyrate (50, 51). In addition to increasing energy conversion efficiency to milk fat through its involvement in amino acid biosynthesis and energy substrate metabolism, *g_Streptococcus* has also been positively associated with serum bile acid levels (52, 53). Furthermore, not only is *g_Pediococcus* positively correlated with bile acid content, but its *Pediococcus pentosaceus strain KID7* can regulate bile acid regulation via the bile salt hydrolase BSH (54–56). It is also noteworthy that bile acids promote fatty acid transport and absorption in the body (57). We also found that *g_Streptococcus* and *g_Pediococcus* were significantly positively correlated ($p < 0.01$) with primary bile acids (GCA and TCA). These findings collectively suggest that *g_Streptococcus* and *g_Pediococcus* may modulate the synthesis of GCA and TCA, among other bile acids, potentially impacting milk fat synthesis. However, additional research will be required to verify the specific contribution of these genera in milk fat synthesis.

Functional enrichment results in the KEGG analysis for the colony revealed that the JAK-ATAT pathway was enriched in cows containing β-casein A1. This pathway has been implicated in the occurrence of cardiovascular disease, diabetes mellitus, inflammation, and immune regulation (58, 59). Type A1 β-casein has negative effects on gastrointestinal, endocrine, neurological, and cardiovascular systems by promoting inflammation via the JAK-ATAT pathway (9, 10). A2A2 genotype cows The genes involved in inflammatory mediator-regulated and mTOR signaling pathways of the TRP channel, which inhibit inflammation and milk fat synthesis, respectively (60, 61). The high rate of milk fat in the A2A2 genotyped cows and the suppressive effect of "A2 milk" on the intestinal inflammation may contribute to this effect.

Taken together, these results suggest that the rumen fluid characteristic flora and metabolites of A2A2 genotyped dairy cows are involved in milk fat synthesis and inflammation inhibition. Studies have demonstrated that the mechanisms and pathways of milk fat synthesis are complex, and are not were understood. In this study, significant correlations were observed between the rumen characteristic flora (*g_Streptococcus*, *g_Pediococcus*, *g_Acetobacter*, and *g_Pseudomonas*) and metabolites involved in milk fat rate (AA, adrenic acid, taurocholic acid, and glycocholic acid) of the A2A2 genotypic dairy cows. Furthermore, we found that the characteristic metabolite AA enhances milk fat synthesis. However, this study only screened rumen characteristic flora and metabolites without conducting a joint analysis with serum and milk metabolites. In future, the characteristic metabolites in serum and milk of A2A2-type cows need to be investigated to identify biomarkers in rumen fluid and understand the mechanisms of milk lipid synthesis in A2A2-type cows.

Conclusion

In conclusion, cows with the A2A2 genotype in herds with similar body condition exhibited higher milk fat rates, with rumen signature metabolites including AA, adrenic acid, taurocholic acid and glycocholic acid, and signature genera including *g_Acetobacter*, *g_Pseudoxanthomonas*, *g_Streptococcus* and *g_Pediococcus*. Among them, the signature metabolite AA promotes the synthesis of milk lipids in BMECs, suggesting that they may serve as potential biomarkers in the A2A2 genotyped cows.

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 2021), China National Center for Bioinformation/Beijing institute of Genomics, Chinese Academy of Sciences (GSA: OMIX007432, GSA: OMIX007436) that are publicly accessible at https://ngdc.cncb.ac.cn/gsa.

Ethics statement

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

Author contributions

JiZ: Data curation, Software, Writing – original draft, Writing – review & editing. CW: Software, Writing – original draft, Writing – review & editing. JH: Writing – review & editing. RM: Writing – review & editing, Methodology. BY: Writing – review & editing, Data curation. WZ: Writing – review & editing, Formal analysis. HW: Writing – review & editing, Software. YG: Writing – review & editing, Supervision. JuZ: Software, Writing – review & editing, Writing – original draft.

Funding

The author(s) declare that financial support was received for the research, authorship, and/or publication of this article. This work was financially supported by the Key Research and Development Project

of Ningxia Hui Autonomous Region (2022BBF02017) and the Autonomous Region Youth Top Talent Training Project.

Acknowledgments

The authors wish to thank the laboratory staff for their ongoing assistance.

Conflict of interest

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

Publisher's note

Supplementary material

The Supplementary material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fvets.2024.1438717/full#supplementary-material

References

1. Jaiswal L, Worku M. Recent perspective on cow's milk allergy and dairy nutrition. *Crit Rev Food Sci Nutr*. (2022) 62:7503–17. doi: 10.1080/10408398.2021.1915241

2. Fernández-Rico S, Mondragón ADC, López-Santamarina A, Cardelle-Cobas A, Regal P, Lamas A, et al. A2 milk: new perspectives for food technology and human health. *Foods*. (2022) 11:2387. doi: 10.3390/foods11162387

3. De Poi R, De Dominicis E, Gritti E, Fiorese F, Saner S, de Laureto PP. Development of an LC-MS method for the identification of β-casein genetic variants in bovine milk. *Food Anal Methods*. (2020) 13:2177–87. doi: 10.1007/s12161-020-01817-0

4. Daniloski D, McCarthy NA, Markoska T, Auldist MJ, Vasiljevic T. Conformational and physicochemical characteristics of bovine skim milk obtained from cows with different genetic variants of β-casein. *Food Hydrocoll*. (2022) 124:107186. doi: 10.1016/j.foodhyd.2021.107186

5. Wang W, Zhang Y, Zhang X, Li C, Yuan L, Zhang D, et al. Heritability and recursive influence of host genetics on the rumen microbiota drive body weight variance in male Hu sheep lambs. *Microbiome*. (2023) 11:197. doi: 10.1186/s40168-023-01642-7

6. Marko R, Uros G, Branislav V, Milan M, Danijela K, Vlado T, et al. Beta-casein gene polymorphism in Serbian Holstein-Friesian cows and its relationship with milk production traits. *Acta Vet*. (2020) 70:497–510. doi: 10.2478/acve-2020-0037

7. Wada Y, Lönnerdal B. Bioactive peptides released from in vitro digestion of human milk with or without pasteurization. *Pediatr Res*. (2015) 77:546–53. doi: 10.1038/pr.2015.10

8. Cieślińska A, Kostyra E, Kostyra H, Oleński K, Fiedorowicz E, Kamiński S. Milk from cows of different β-casein genotypes as a source of β-casomorphin-7. *Int J Food Sci Nutr*. (2012) 63:426–30. doi: 10.3109/09637486.2011.634785

9. Chin-Dusting J, Shennan J, Jones E, Williams C, Kingwell B, Dart A. Effect of dietary supplementation with beta-casein A1 or A2 on markers of disease development in individuals at high risk of cardiovascular disease. *Br J Nutr*. (2006) 95:136–44. doi: 10.1079/BJN20051599

10. Sheng X, Li Z, Ni J, Yelland G. Effects of conventional milk versus milk containing only A2 β-casein on digestion in Chinese children: a randomized study. *J Pediatr Gastroenterol Nutr*. (2019) 69:375–82. doi: 10.1097/MPG.0000000000002437

11. Kay SS, Delgado S, Mittal J, Eshraghi RS, Mittal R, Eshraghi AA. Beneficial effects of milk having A2 β-casein protein: myth or reality? *J Nutr*. (2021) 151:1061–72. doi: 10.1093/jn/nxaa454

12. Jianqin S, Leiming X, Lu X, Yelland GW, Ni J, Clarke AJ. Effects of milk containing only A2 beta casein versus milk containing both A1 and A2 beta casein proteins on gastrointestinal physiology, symptoms of discomfort, and cognitive behavior of people with self-reported intolerance to traditional cows' milk. *Nutr J*. (2016) 15:35. doi: 10.1186/s12937-016-0147-z

13. Flint HJ, Bayer EA, Rincon MT, Lamed R, White BA. Polysaccharide utilization by gut bacteria: potential for new insights from genomic analysis. *Nat Rev Microbiol*. (2008) 6:121–31. doi: 10.1038/nrmicro1817

14. Jami E, White BA, Mizrahi I. Potential role of the bovine rumen microbiome in modulating milk composition and feed efficiency. *PLoS One*. (2014) 9:e85423. doi: 10.1371/journal.pone.0085423

15. Franzosa EA, Sirota-Madi A, Avila-Pacheco J, Fornelos N, Haiser HJ, Reinker S, et al. Gut microbiome structure and metabolic activity in inflammatory bowel disease. *Nat Microbiol*. (2019) 4:293–305. doi: 10.1038/s41564-018-0306-4

16. Tong J, Zhang H, Yang D, Zhang Y, Xiong B, Jiang L. Illumina sequencing analysis of the ruminal microbiota in high-yield and low-yield lactating dairy cows. *PLoS One*. (2018) 13:e198225:e0198225. doi: 10.1371/journal.pone.0198225

17. Wang Y, Nan X, Zhao Y, Jiang L, Wang M, Wang H, et al. Rumen microbiome structure and metabolites activity in dairy cows with clinical and subclinical mastitis. *J Anim Sci Biotechnol*. (2021) 12:36. doi: 10.1186/s40104-020-00543-1

18. Zhang H, Tong J, Zhang Y, Xiong B, Jiang L. Metabolomics reveals potential biomarkers in the rumen fluid of dairy cows with different levels of milk production. *Asian Australas J Anim Sci*. (2020) 33:79–90. doi: 10.5713/ajas.19.0214

19. Zhang L, Shen H, Zhang J, Mao S. Variety of rumen microbial populations involved in biohydrogenation related to individual milk fat percentage of dairy cows. *Front Vet Sci*. (2023) 10:1106834. doi: 10.3389/fvets.2023.1106834

20. Xiao S, Wang Q, Li C, Liu W, Zhang J, Fan Y, et al. Rapid identification of A1 and A2 milk based on the combination of mid-infrared spectroscopy and chemometrics. *Food Control*. (2022) 134:108659. doi: 10.1016/j.foodcont.2021.108659

21. Wu ZW, Gu YL, Zhang J, Liu LY, Wen W, Guo P, et al. The screening of A2 homozygous Holstein cows. *Heilongjiang Anim Husbandry Vet Med*. (2019) 8:44–7. doi: 10.13881/j.cnki.hljxmsy.2018.10.0106

22. Kind T, Wohlgemuth G, Lee DY, Lu Y, Palazoglu M, Shahbaz S, et al. FiehnLib: mass spectral and retention index libraries for metabolomics based on quadrupole and time-of-flight gas chromatography/mass spectrometry. *Anal Chem*. (2009) 81:10038–48. doi: 10.1021/ac9019522

23. Zhang B, Horvath S. A general framework for weighted gene co-expression network analysis. *Stat Appl Genet Mol Biol*. (2005) 4:17. doi: 10.2202/1544-6115.1128

24. Sing T, Sander O, Beerenwinkel N, Lengauer T. ROCR: visualizing classifier performance in R. *Bioinformatics*. (2005) 21:3940–1. doi: 10.1093/bioinformatics/bti623

25. Dozsa A, Dezso B, Toth BI, Bacsi A, Poliska S, Camera E, et al. PPARγ-mediated and arachidonic acid-dependent signaling is involved in differentiation and lipid production of human sebocytes. *J Invest Dermatol*. (2014) 134:910–20. doi: 10.1038/jid.2013.413

26. Wang M, Wang H, Zheng H, Dewhurst R, Roehe R. A knowledge-driven network-based analytical framework for the identification of rumen metabolites. *IEEE Trans Nanobioscience*. (2020) 19:518–26. doi: 10.1109/TNB.2020.2991577

27. Silva RCE, de Moura JFP, de Souza BB, Filho JMP, Bezerra LR, do Nascimento FS, et al. Polymorphism of the beta-casein gene and adaptability of Sindhi cows to semiarid conditions. *Trop Anim Health Prod*. (2021) 53:534. doi: 10.1007/s11250-021-02948-0

28. Perna A, Intaglietta I, Gambacorta E, Simonetti A. The influence of casein haplotype on quality, coagulation, and yield traits of milk from italian Holstein cows. *J Dairy Sci*. (2016) 99:3288–94. doi: 10.3168/jds.2015-10463

29. Jiang B, Qin C, Xu Y, Song X, Fu Y, Li R, et al. Multi-omics reveals the mechanism of rumen microbiome and its metabolome together with host metabolome participating in the regulation of milk production traits in dairy buffaloes. *Front Microbiol*. (2024) 15:1301292. doi: 10.3389/fmicb.2024.1301292

30. Nuomin BR, Tsuruta T, Nishino N. Modulatory effects of A1 milk, A2 milk, soy, and egg proteins on gut microbiota and fermentation. *Microorganisms*. (2023) 11:1194. doi: 10.3390/microorganisms11051194

31. Cagen LM, Baer PG. Adrenic acid inhibits prostaglandin synthesis. *Life Sci*. (1980) 26:765–70. doi: 10.1016/0024-3205(80)90281-7

32. Brouwers H, Jónasdóttir HS, Kuipers ME, Kwekkeboom JC, Auger JL, Gonzalez-Torres M, et al. Anti-inflammatory and proresolving effects of the omega-6 polyunsaturated fatty acid adrenic acid. *J Immunol*. (2020) 205:2840–9. doi: 10.4049/jimmunol.1801653

33. Chang YB, Ahn Y, Seo D, Bae S, Suh HJ, Hong YH, et al. *Centella asiatica* lowers body fat accumulation via regulating cholesterol homeostasis- and lipid metabolism-related genes in mice with high-fat, high-sugar diet-induced obesity. *Appl Biol Chem*. (2023) 66:88. doi: 10.1186/s13765-023-00846-7

34. Jia W, Wei M, Rajani C, Zheng X. Targeting the alternative bile acid synthetic pathway for metabolic diseases. *Protein Cell*. (2021) 12:411–25. doi: 10.1007/s13238-020-00804-9

35. Xue Y. Studies on the anti-inflammatory effect and its mechanism of taurocholic acid In: Dissertation: Inner Mongolia Agricultural University (2005)

36. Li HF. Studies on the anti-inflammatory effect and its mechanism of glycocholic acid In: Dissertation: Inner Mongolia Agricultural University (2006, 2006)

37. Albertolle ME, Peter GF. The relationships between cytochromes P450 and H_2O_2: production, reaction, and inhibition. *J Inorg Biochem*. (2018) 186:228–34. doi: 10.1016/j.jinorgbio.2018.05.014

38. Wang T, Fu X, Chen Q, Patra JK, Wang D, Wang Z, et al. Arachidonic acid metabolism and kidney inflammation. *Int J Mol Sci*. (2019) 20:3683. doi: 10.3390/ijms20153683

39. Calder PC, Grimble RF. Polyunsaturated fatty acids, inflammation and immunity. *Eur J Clin Nutr*. (2002) 56:S14–9. doi: 10.1038/sj.ejcn.1601478

40. Fan G, Li Y, Chen J, Zong Y, Yang X. DHA/AA alleviates LPS-induced Kupffer cells pyroptosis via GPR120 interaction with NLRP3 to inhibit inflammasome complexes assembly. *Cell Death Dis*. (2021) 12:73. doi: 10.1038/s41419-020-03347-3

41. Wan L. Molecular mechanism of GPR120 synergistically regulating of milk fat metabolism and inflammatory response in dairy cows breast epithelial cells. *Heilongjiang Bayi Nongken Univ*. (2024). doi: 10.27122/d.cnki.ghlnu.2023.000175

42. Mu T, Hu H, Ma Y, Feng X, Zhang J, Gu Y. Regulation of key genes for milk fat synthesis in ruminants. *Front Nutr*. (2021) 8:765147. doi: 10.3389/fnut.2021.765147

43. Tian Z, Zhang Y, Zhang H, Sun Y, Mao Y, Yang Z, et al. Transcriptional regulation of milk fat synthesis in dairy cattle. *J Funct Foods*. (2022) 96:105208. doi: 10.1016/j.jff.2022.105208

44. Le Hellard S, Mühleisen TW, Djurovic S, Fernø J, Ouriaghi Z, Mattheisen M, et al. Polymorphisms in SREBF1 and SREBF2, two antipsychotic-activated transcription factors controlling cellular lipogenesis, are associated with schizophrenia in German and Scandinavian samples. *Mol Psychiatry*. (2010) 15:463–72. doi: 10.1038/mp.2008.110

45. Wu X, Huang S, Huang J, Peng P, Liu Y, Han B, et al. Identification of the potential role of the rumen microbiome in milk protein and fat synthesis in dairy cows using metagenomic sequencing. *Animals*. (2021) 11:1247. doi: 10.3390/ani11051247

46. Xue MY, Sun HZ, Wu XH, Guan LL, Liu JX. Assessment of rumen bacteria in dairy cows with varied milk protein yield. *J Dairy Sci*. (2019) 102:5031–41. doi: 10.3168/jds.2018-15974

47. Qafary M, Khajeh K, Ramazzotti M, Moosavi-Movahedi AA, Chiti F. Urea titration of a lipase from *Pseudomonas* sp. reveals four different conformational states, with a stable partially folded state explaining its high aggregation propensity. *Int J Biol Macromol*. (2021) 174:32–41. doi: 10.1016/j.ijbiomac.2021.01.153

48. Edwards HD, Shelver WL, Choi S, Nisbet DJ, Krueger NA, Anderson RC, et al. Immunogenic inhibition of prominent ruminal bacteria as a means to reduce lipolysis and biohydrogenation activity in vitro. *Food Chem*. (2017) 218:372–7. doi: 10.1016/j.foodchem.2016.09.052

49. Lyons T, Bielak A, Doyle E, Kuhla B. Variations in methane yield and microbial community profiles in the rumen of dairy cows as they pass through stages of first lactation. *J Dairy Sci*. (2018) 101:5102–14. doi: 10.3168/jds.2017-14200

50. Caufield PW, Schön CN, Saraithong P, Li Y, Argimón S. Oral lactobacilli and dental caries: a model for niche adaptation in humans. *J Dent Res*. (2015) 94:110S–8S. doi: 10.1177/0022034515576052

51. Moye ZD, Zeng L, Burne RA. Fueling the caries process: carbohydrate metabolism and gene regulation by *Streptococcus mutans*. *J Oral Microbiol*. (2014) 6:24878. doi: 10.3402/jom.v6.24878

52. Wang D, Chen L, Tang G, Yu J, Chen J, Li Z, et al. Multi-omics revealed the long-term effect of ruminal keystone bacteria and the microbial metabolome on lactation performance in adult dairy goats. *Microbiome*. (2023) 11:215. doi: 10.1186/s40168-023-01652-5

53. Jin M, Cui J, Ning H, Wang M, Liu W, Yao K, et al. Alterations in gut microbiota and metabolite profiles in patients with infantile cholestasis. *BMC Microbiol*. (2023) 23:357. doi: 10.1186/s12866-023-03115-1

54. Fan X, Zhang Q, Guo W, Wu Q, Hu J, Cheng W, et al. The protective effects of *Levilactobacillus brevis* FZU0713 on lipid metabolism and intestinal microbiota in hyperlipidemic rats. *Food Sci Human Wellness*. (2023) 12:1646–59. doi: 10.1016/j.fshw.2023.02.021

55. Damodharan K, Lee YS, Palaniyandi SA, Yang SH, Suh J. Preliminary probiotic and technological characterization of *Pediococcus pentosaceus* strain kid 7 and in vivo assessment of its cholesterol-lowering activity. *Front Microbiol*. (2015) 6:768. doi: 10.3389/fmicb.2015.00768

56. Adebola OO, Corcoran O, Morgan WA. Prebiotics may alter bile salt hydrolase activity: possible implications for cholesterol metabolism. *Pharmanutrition*. (2020) 12:100182. doi: 10.1016/j.phanu.2020.100182

57. Zeng X, Zhang X, Su H, Gou H, Lau HC, Hu X, et al. Pien tze huang protects against non-alcoholic steatohepatitis by modulating the gut microbiota and metabolites in mice. *Engineering*. (2024) 35:257–69. doi: 10.1016/j.eng.2022.10.010

58. Huang I, Chung W, Wu P, Chen C. JAK-STAT signaling pathway in the pathogenesis of atopic dermatitis: an updated review. *Front Immunol*. (2022) 13:1068260. doi: 10.3389/fimmu.2022.1068260

59. Shen-Orr SS, Furman D, Kidd BA, Hadad F, Lovelace P, Huang Y, et al. Defective signaling in the JAK-STAT pathway tracks with chronic inflammation and cardiovascular risk in aging humans. *Cell Syst*. (2016) 3:374–384.e4. doi: 10.1016/j.cels.2016.09.009

60. Wang J, Cao Y, Long X, Li F, Jiang N, Sun M, et al. Acylated ghrelin activates PI3K and mTOR signaling pathway by promoting ThPOK acetylation to promote milk fat synthesis in bovine mammary epithelial cells. *J Agric Food Chem*. (2024) 72:390–404. doi: 10.1021/acs.jafc.3c06977

61. Trevisani M, Siemens J, Materazzi S, Bautista DM, Nassini R, Campi B, et al. 4-hydroxynonenal, an endogenous aldehyde, causes pain and neurogenic inflammation through activation of the irritant receptor TRPA 1. *Proc Natl Acad Sci USA*. (2007) 104:13519–24. doi: 10.1073/pnas.0705923104

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TYPE Correction
PUBLISHED 15 November 2024
DOI 10.3389/fvets.2024.1518338

OPEN ACCESS

APPROVED BY
Frontiers Editorial Office,
Frontiers Media SA, Switzerland

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RECEIVED 28 October 2024
ACCEPTED 29 October 2024
PUBLISHED 15 November 2024

CITATION
Zhao J, Wang C, Hu J, Ma R, Yu B, Zhao W, Wang H, Gu Y and Zhang J (2024) Corrigendum: Integrated metagenomics and metabolomics analyses revealed biomarkers in β-casein A2A2-type cows.
Front. Vet. Sci. 11:1518338.
doi: 10.3389/fvets.2024.1518338

Corrigendum: Integrated metagenomics and metabolomics analyses revealed biomarkers in β-casein A2A2-type cows

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KEYWORDS

Holstein dairy cows, beta-casein, A2A2, milk fat percentage, metabolomics, metagenomics

A Corrigendum on
Integrated metagenomics and metabolomics analyses revealed biomarkers in β-casein A2A2-type cows

by Zhao, J., Wang, C., Hu, J., Ma, R., Yu, B., Zhao, W., Wang, H., Gu, Y., and Zhang, J. (2024). *Front. Vet. Sci.* 11:1438717. doi: 10.3389/fvets.2024.1438717

In the published article, there was an error in the author list, and author Chuanchuan Wang's contribution as first author was omitted. The corrected author list appears below.

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The authors apologize for this error and state that this does not change the scientific conclusions of the article in any way. The original article has been updated.

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frontiers | Frontiers in Veterinary Science

TYPE Original Research
PUBLISHED 21 October 2024
DOI 10.3389/fvets.2024.1433876

OPEN ACCESS

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RECEIVED 16 May 2024
ACCEPTED 07 October 2024
PUBLISHED 21 October 2024

CITATION
La S, Li H, Zhang Y, Abaidullah M, Niu J, Gao Z, Liu B, Ma S, Cui Y, Li D and Shi Y (2024) Different sources of alfalfa hay alter the composition of rumen microbiota in mid-lactation Holstein cows without affecting production performance.
Front. Vet. Sci. 11:1433876.
doi: 10.3389/fvets.2024.1433876

Different sources of alfalfa hay alter the composition of rumen microbiota in mid-lactation Holstein cows without affecting production performance

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Alfalfa hay is a commonly used and important feed ingredient in dairy production. To better expand the alfalfa supply market, it is of great significance to explore the impact of alfalfa hay from different sources on dairy cow production performance. This study compared the effects of imported alfalfa hay from America (AAH) and Spain (SAH) on lactation performance and rumen microbiota of cows. Three hundred and sixty healthy mid-lactation Holstein cows with similar body weight, milk yield, and parity were randomly divided into two groups fed diets based on AAH or SAH for a 70-day experimental period. Each group was composed of four pens, with 45 cows in each pen. Daily records were kept for MY per cow and dry matter intake per pen. Twelve randomly selected cows per group were sampled to collect milk, feces, rumen fluid, and blood. The findings revealed no significant differences between the two groups in terms of production performance, nutrient apparent digestibility, serum biochemical indices, or rumen fermentation parameters. However, rumen microbial composition differed significantly between the two groups of cows based on β-diversity. On the genus level, the relative abundance of *Prevotella*, *Succinivibrionaceae_UCG-002* increased while that of *NK4A214_group*, *Ruminococcus*, *norank_f__F082* and *Lachnospiraceae_NK3A20_group* decreased in the SAH group compared with AAH group. There was no significant correlation between these core differential bacteria and the molar proportions of acetate and propionate, the concentration of total volatile fatty acids, and milk yield. In conclusion, the feeding effects of SAH were similar to those of AAH. These findings provided a reference for the application of alfalfa hay from different sources and for the improvement of the economic benefit of dairy farms.

KEYWORDS

alfalfa hay, lactation performance, ruminal fermentation, microbiota, dairy cow

1 Introduction

Alfalfa is widely used in animal husbandry due to its good palatability, low fiber content and high protein concentration (ranging from 17 to 22%) (1). Feeding alfalfa can effectively reduce the proportion of concentrates, especially that of high-protein ingredients in diets (2). Therefore, alfalfa has become an irreplaceable forage in dairy farming, and the beneficial impact of alfalfa hay on the milk performance of dairy cows has been widely recognized.

The demand for premium alfalfa hay has increased significantly in recent years due to a rise in the number of dairy cows and an improvement in the milk yield (MY) of dairy cows (3). Typically, dairy farms rely on multiple suppliers for their alfalfa, which contributes to the stable operation of the farms. Dairy farmers need to select alfalfa hay from different sources based on factors such as price to maximize their benefits. Especially since the outbreak of the COVID-19 pandemic, the price and supply stability of alfalfa hay were challenged due to transportation issues and other factors. As a result, the substitution of alfalfa hay from different sources had become more frequent. It is well known that the impacts of alfalfa on production performance in dairy cows are not fixed, as its nutrient composition is variable due to breed (4), storage methods (5) and harvest period. For example, Vagnoni and Broderick (6) found that more crude protein was degraded in the rumen in cows given alfalfa silage than in those given alfalfa hay, while cows given alfalfa hay had greater dry matter intake (DMI). Additionally, previous studies have conducted trials comparing alfalfa hay with other forages such as wheat straw and peanut seedlings (7–9). However, there have been few studies that compare how alfalfa hay from various sources affects dairy cows. Despite similar nutritional profiles, the frequent changes of alfalfa hay from various sources, especially those from different countries, have raised concerns among dairy farmers due to the potential threat to the stability of dairy cow production performance.

Ruminants possess a unique physiological structure that synergistically interacts with rumen bacteria to facilitate the fermentation and degradation of forage into volatile fatty acids (VFA). These VFA serve as a vital source of nutrition for ruminants (10). Indeed, the fermentation of fiber in the rumen produces two different lipogenic VFA: acetate and butyrate (11). A significant portion of butyrate is converted into β-hydroxybutyrate within the rumen wall tissue. This acetate and β-hydroxybutyrate serve as a substrate for about half of the fat found in milk. Alfalfa hay, as a high-protein forage, plays a crucial role in rumen microorganisms and thus affects dairy cow production performance (12).

Therefore, this experiment compared the effects of different sources of alfalfa hay imported from America (AAH) or Spain (SAH) with similar nutrient composition on lactation performance, nutrient apparent digestibility, serum biochemical indices, ruminal fermentation, and microbiota of dairy cows. We hypothesize that alfalfa hay from different sources with similar nutritional profiles will not impact dairy cow production performance, providing dairy farmers with more options to enhance the economic efficiency of the dairy farm.

2 Materials and methods

2.1 Alfalfa hay

The alfalfa hay used in this study, obtained from Spain and the US, respectively, was bought from Literana, LLC and Stone Wings II, LLC. We purchased 60 tons each of American and Spanish alfalfa hay, with 60 bales of each. We randomly selected 10 bales, and collected 500 g samples from the upper, middle and lower layers of each bundle, and mixed them for the determination of nutritional composition. Table 1 shows the nutrients present in alfalfa hay.

TABLE 1 Nutrient composition of alfalfa hay.

Item	AAH[a]	SAH[a]
Dry matter (DM)	90.52	91.92
Crude protein (% DM)	16.39	17.74
Neutral detergent fiber (% DM)	32.17	35.52
Acid detergent fiber (% DM)	24.35	26.53
Ether extract (% DM)	1.72	1.63
Calcium (% DM)	2.03	1.65
Phosphorus (% DM)	0.29	0.30
Relative feed value[b]	202.2	178.7

[a]AAH, American alfalfa hay; SAH, Spanish alfalfa hay.
[b]Relative feed value was calculated based on the contents of acid detergent fiber and neutral detergent fiber.

2.2 Cows, experimental design and diets

This study was approved by the Institutional Animal Care and Use Committee of Henan Agriculture University (Zhengzhou, China) (Approval Number: HNND2021062812). The trial was conducted in Xincai Ruiya Animal Husbandry Farm, Henan Province. Three hundred and sixty healthy mid-lactation Holstein dairy cows (MY = 21.98 ± 5.02 kg; days in milk = 208 ± 19.42 d; parity = 2.34 ± 0.47, mean ± SD) were randomly assigned to two groups (four replicates in each group and 45 cows in each replicate) fed diets based on AAH or SAH. The trial lasted 70 days, with 10 days for adaptation and 60 days for collecting data and samples. Dietary composition and nutrient levels are shown in Table 2. All cows were fed twice daily (07:00 and 19:00 h) and milked 3 times (06:30, 14:30, and 22:30 h) a day, and given access to water at all times. The barn was cleaned and disinfected once a week.

2.3 Collection of data and samples

During the collection period, the feed offered and rejected for each replication was noted daily to determine DMI. The MY of each of the 360 experimental cows was recorded daily. In addition, total mixed ration (TMR) samples were collected daily. After the experiment ended, the TMR samples were mixed in proportion for determining the nutritional composition of TMR. To determine whether there were differences in milk composition, apparent nutrient digestibility, and rumen microbiota between the two groups of cows, 12 cows were randomly selected from each group (three cows were in each repetition) for samples of milk, feces, blood, and rumen fluid. Milk samples were collected on day 0, 14, 28, 42, 56, and the last 4 days of the experiment. For each cow, 40 mL of milk samples was collected per collection day (in a 4:3:3 ratio in the morning, midday, and evening) and placed in a centrifuge tube containing potassium dichromate preservative. The milk samples were refrigerated at 4°C for milk composition analysis. Fecal samples were collected 4 times a day (06:00, 12:00, 18:00, and 24:00 h) during the last 4 days of the experiment. Approximately 250 g of feces were collected per cow each time using a rectal sampling method, mixed, and 120 g of the fecal samples were weighed and mixed with 30 mL of 10% tartaric acid. The feces samples collected over the 4 d were separately mixed at the end of the experiment for chemical analysis. Blood was collected through

TABLE 2 Ingredient and chemical composition of experimental diet.

Item	AAH[a]	SAH[a]
Ingredients (% DM)		
Corn silage	31.11	31.09
Brewer's grain	4.00	4.00
Oat hay	5.00	5.00
Corn grain, ground	7.30	7.29
Alfalfa hay	12.83	12.88
Soybean hull	4.45	4.45
Soybean meal	4.24	4.24
Cottonseed cake	4.34	4.33
Sodium bicarbonate	0.71	0.71
Concentrate supplement[b]	26.02	26.01
Chemical composition (% DM)		
Crude protein	14.91	14.93
Neutral detergent fiber	47.10	47.00
Acid detergent fiber	19.31	20.34
Calcium	0.82	0.89
Phosphorus	0.38	0.38
NEL[c] (MJ/kg)	6.66	6.65

[a]AAH, American alfalfa hay; SAH, Spanish alfalfa hay.
[b]Contained per kg premix dry matter: 31.3 mg Co, 343.5 mg Cu, 2, 258 mg Fe, 1, 160 mg Mn, 1, 534 mg Zn, 40.3 mg I, 17.7 mg Se, 317.4 KIU vitamin A, 80.8 KIU vitamin D, and 3, 030 IU vitamin E.
[c]NEL, net energy for lactating cow.

the caudal vein with 5 mL vacuum tubes before morning feeding on the last day of the trial. The blood was immediately sent to the laboratory and centrifuged at 3,000 × g for 15 min at 4°C. And then serum was gathered into 2 mL centrifuge tubes and kept at −20°C until assayed. Rumen fluid was collected on the last day of the trial using a gastric tube with an exterior diameter of 1 cm, an internal diameter of 0.8 cm, and a length of 300 cm, 2 h after morning feeding. To prevent saliva pollution, about 200 mL of rumen fluid from individual cows was removed and discarded. Next, 200 mL of rumen fluid was collected in a 500 mL beaker and immediately sent to the laboratory for pH analysis using a PHS-10 meter (Sartorius, Göttingen, Germany) and recording. After filtration through 4 layers of gauze, the rumen fluid was divided into 3 portions, each containing 10 mL. These samples were then frozen and stored at −80°C for subsequent determination of VFA and ammonia (NH_3-N), as well as for 16S rRNA sequencing.

2.4 Analytical methods

The concentrations of dry matter (DM), ether extract and acid detergent fiber in alfalfa hay, feces and TMR samples, as well as the acid insoluble ash in feces and TMR samples, were determined using the methods described in AOAC (13). The concentration of neutral detergent fiber in alfalfa hay, feces, and TMR samples was determined using the method of Van Soest et al. (14). The concentrations of crude protein and nitrogen were determined using an automatic Kjeldahl N analyzer (SKD-2000, Haineng Experimental Instrument Technology Co., Ltd., Shanghai, China). The concentrations of calcium and phosphorus in alfalfa hay and TMR were analyzed based on the method of Mattioli et al. (15). Acid insoluble ash was used as an endogenous indicator, according to the formula: the apparent digestibility of a nutrient (%) = 100 − 100 × the nutrient concentration in feces (%) × acid insoluble ash concentration in TMR (%)/the nutrient concentration in TMR (%)/acid insoluble ash concentration in feces (%), calculating the apparent digestibility of crude protein, ether extract, acid detergent fiber, and neutral detergent fiber.

An automated near-infrared milk analyzer (Seris300 CombiFOSS; Foss Electric, Hillerd, Denmark) was employed to measure the concentrations of milk fat, protein, lactose, milk solids, milk urea nitrogen (MUN), and somatic cell count in milk samples delivered to the Dairy Herd Improvement Testing Center in Henan Province. Serum concentrations of glucose, total protein, albumin, globulin, triglyceride, cholesterol, non-esterified fatty acids, β-hydroxybutyrate, urea, alanine aminotransferase and aspartate aminotransferase were assessed using commercially available assay kits obtained from Nanjing Jian Cheng Biological Technology Co. (Nanjing, Jiangsu, China).

For the rumen fluid samples used for the determination of VFA, after thawing at 4°C, they were treated with 25% metaphosphoric acid. Then, the concentrations of VFA (acetate, propionate, butyrate, isobutyrate, valerate, and isovalerate) were determined using a German Sykam ion chromatograph and a Dionex AS11-HC column (4 × 250 mm, Thermo Fisher Science, Waltham, MA, United States). The following chromatographic circumstances applied: a column temperature of 30°C, a flow rate of 1 mL/min, and an injection volume of 10 μL; the mobile phase was 0.1 mmol/L and 50 mmol/L NaOH solutions. The former was maintained for 28 min and the latter for 5 min. Finally, 0.1 mmol/L of NaOH solution was maintained for 10 min. For the rumen fluid samples used for the determination of NH_3-N, after thawing at 4°C, the NH_3-N concentration was determined using a UV-2100 spectrophotometer (UV2100, Shanghai Younike Instrument Co., Ltd., Shanghai, China) according to AOAC (13).

2.5 DNA extraction and 16S rRNA gene sequencing

Six rumen fluid samples were randomly selected from each group for DNA extraction and 16S rRNA gene sequencing. According to the manufacturer's instructions, total bacterial DNA from rumen fluid was obtained using the E.Z.N.A.® soil DNA Kit (Qiagen, United States). The NanoDrop-2000 UV–vis spectrophotometer (Thermo Scientific, Wilmington, United States) was used to detect DNA concentrations. Additionally, 1% agarose gel electrophoresis was used to evaluate DNA quality. The bacterial V3–V4 region of the 16S rRNA gene was amplified using primers for 338F (5′-ACTCCTACGGGAGGCAGCAG-3′) and 806R (5′-GGACTACHVGGGTWTCTAAT-3′) at a temperature of 55°C, as previously described (16). Following purification and quantification, PCR products were used by Majorbio Bio-Pharm Technology Co., Ltd. (Shanghai, China) to build libraries and sequence them on the Illumina MiSeq PE300 platform/NovaSeq PE250 platform (Illumina, San Diego, United States) in accordance with the established protocols. All results were based on sequenced reads and operational taxonomic

units. The BioProject accession number for the 16S rRNA sequencing data, which was uploaded to the NCBI database, is PRJNA898964.

2.6 Processing of sequencing data

The optimized sequences were obtained by double-end sequence quality control splicing of sequencing results (17) and using FLASH version 1.2.7 (18). The optimised sequences were based on the silva138/16s_bacteria species classification database and subjected to operational taxonomic unit (OTU) clustering analysis (confidence level 0.7) at 97% similarity using UPARSE version 7.1 (19, 20), and a table of OTU species classification statistics was generated by drawing parity at the minimum number of sample sequences. Species number analysis, Alpha diversity analysis (Chao1 and Simpson indices), Beta diversity analysis (principal co-ordinates analysis), community composition analysis and mapping were carried out on the online tool of Majorbio Cloud Platform.[1] Venn diagrams, which visually display the numbers of common and unique OTUs among groups, and the rarefaction curve (Shannon index) were drawn by R software (version 3.3.1). For the purpose of illustrating the variety and abundance of microbial communities, Chao1 and Simpson indices were calculated by Mothur (version 1.30.2) (21). Subsequently, the Bray-Curtis distance was calculated using Qiime software (version 1.9.1), and the principal co-ordinates analysis plot was generated using R software (version 3.3.1). The Vegan package based on the R software (Version 3.6.0) was used to test the differences in the microbial community structure among different treatments through ANOSIM. The relative abundance of phyla and genera was shown by barplot and heatmap, which were drawn by R software (version 3.3.1). The Wilcoxon rank-sum test method was used to analyze and identify the differences between the two groups. To calculate the impact of species abundance on the difference effect at the genus level, linear discriminant analysis effect size (LEfSe) and linear discriminant analysis were used. Spearman correlation coefficients were calculated between the top 20 species at the genus level and various ruminal fermentation parameters, as well as milk yield and milk composition using R language vegan package (version 3.3.1) and displayed on the heatmap. To predict functions of the ruminal microbiota, PICRUSt2 predictions of function were obtained based on the KEGG database.[2] The differences in KEGG pathways between two groups were assessed by two-sided Welch's t-test using STAMP software (version 2.1.3). Storey's false discovery rate method was used for multiple test corrections as recommended by the STAMP developers. The top 20 important biological information (functional) was selected by sorting based on effect sizes (22).

2.7 Statistical analysis

Daily MY of 360 cows and DMI per replicate throughout the experiment were used for statistical analysis. Data from 24 cows used for sample collection showed no outliers (data points outside ±3 standard deviations from the mean), and their MY, milk composition, blood biochemical parameters, and rumen fermentation parameters were used for statistical analysis. The measurements of milk composition, blood biochemical parameters, and rumen fermentation parameters were repeated at least 3 times. The normality test and the variance homogeneity test were carried out using the PROC UNIVARIATE model and the PROC DISCRIM model, respectively. According to the characteristics of the data, one - way ANOVA or nonparametric test was performed using the PROC MIXED model, and the results were expressed as means. p-values >0.05 and <0.10 were considered a significant trend, while p-values <0.05 were considered significant.

3 Results

3.1 Influence on production performance and nutrient digestibility

During the experiment, it was observed that alfalfa hay from different sources had no significant effect on the DMI (Figure 1A) and daily MY (Figure 1B) of the two groups of dairy cows ($p > 0.05$). This consistency was maintained throughout the entire collection period. Similarly, in the final 4 days of the experiment, there were no differences in MY among the cows used for sample collection ($p > 0.05$, Table 3). Furthermore, the analysis revealed no differences in milk components between the two dairy cow groups on specific days of the collection period, including days 14, 28, 42, 56, and the last 4 days ($p > 0.05$, Figure 2 and Table 3). This finding also extended to the apparent digestibility of nutrients, indicating that the source of alfalfa hay had no significant impact on this aspect as well ($p > 0.05$, Table 3).

3.2 Influence on blood biochemical indicators and rumen fermentation parameters

The serum concentrations of glucose, total protein, albumin, globulin, triglycerides, cholesterol, non-esterified fatty acids, β-hydroxybutyrate, and urea, as well as the activities of alanine aminotransferase and aspartate aminotransferase, showed no significant differences between the two groups of dairy cows ($p > 0.05$, Table 4). Similarly, rumen pH remained within the normal range and did not vary significantly between the groups ($p > 0.05$, Table 5). The rumen fermentation parameters were generally similar between the two groups, with the exception of isovalerate molar proportion, which was significantly higher in the SAH group compared to the AAH group ($p = 0.007$). Other fermentation parameters did not show statistically significant differences ($p > 0.05$).

3.3 Influence on the phylum-level diversity and composition of the rumen microbiota

The analysis of 12 rumen fluid samples resulted in 602,999 high-quality sequences after quality control, with an average sequence length of 417 bp and 251,558,244 bases. Taxonomic analysis at a 97% similarity threshold identified 1,981 OTUs across various taxonomic levels: Domain (1), Kingdom (1), Phylum (17), Class (34), Order (86), Family (149), Genus (304), and Species (589). Among these, the two

1 https://cloud.majorbio.com/page/tools/

2 http://www.genome.jp/kegg/

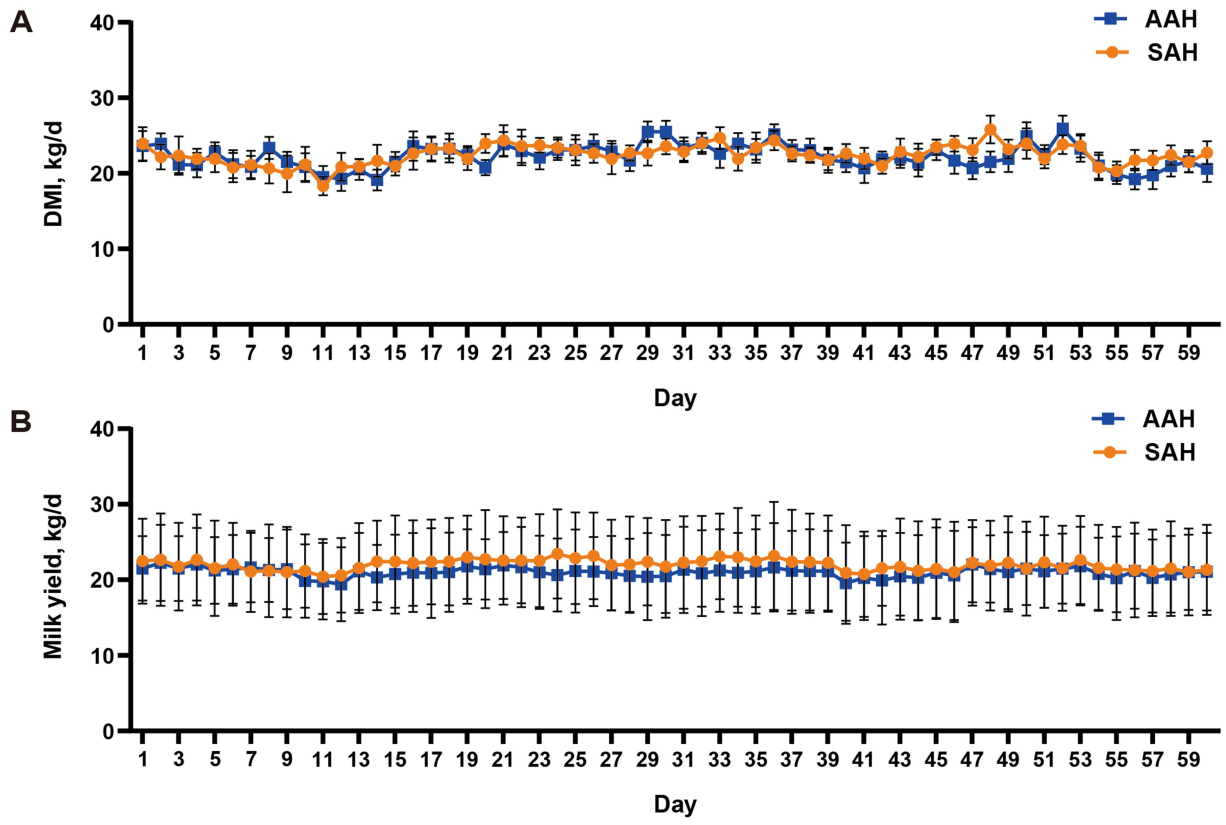

FIGURE 1
Comparison of different sources of alfalfa hay on dry matter intake [DMI, **(A)**] and milk yield [MY, **(B)**] in dairy cows. AAH, American alfalfa hay; SAH, Spanish alfalfa hay.

groups together had 1,745 OTUs, with 151 unique to the AAH group and 85 unique to the SAH group (Figure 3A). The alpha diversity, assessed using the Shannon index, approached a plateau, indicating sufficient sequencing depth (Figure 3B). The Chao1 index was significantly higher in the AAH group compared to the SAH group ($p < 0.05$, Figure 3C), while the Simpson index showed no significant difference between the groups ($p > 0.05$, Figure 3C). The principal co-ordinates analysis plot revealed distinct clustering of the AAH and SAH groups (Figure 3D). In terms of phylum composition, both groups were dominated by *Bacillota* and *Bacteroidota*, which together constituted over 86% of the microbiota in each group (Figure 3E). Significant differences were observed between the groups: the SAH group had higher relative abundances of *Bacteroidota* (Figure 3G), *Proteobacteria* (Figure 3H), and *Fibrobacterota* (Figure 3J) ($p < 0.05$), but lower relative abundances of *Bacillota* (Figure 3F) and *Actinobacteria* (Figure 3I) ($p > 0.05$) compared to the AAH group.

3.4 Influence on composition and difference at the genus level of rumen microbiota

The five most abundant genera in the rumen of AAH group and SAH group were *Prevotella* (12.65 and 25.76%), *Succiniclasticum* (6.31 and 6.18%), *Rikenellaceae_RC9_gut_group* (6.27 and 4.26%), *NK4A214_group* (6.46 and 3.30%) and *Ruminococcus* (5.77 and 3.29%), respectively (Figure 4A). Microbiota profiles in the AAH group and the SAH group showed distinct clustering patterns. Notably, most dominant genera in the AAH group were from the *Bacillota phylum*, whereas those in the SAH group predominantly belonged to the *Bacteroidota phylum* (Figure 4B). Further analysis using LEfSe and linear discriminant analysis revealed several genera with significantly different abundances between the two groups (Figures 4C,D). Specifically, cows in the SAH group showed a significant increase in the relative abundance of *Prevotella* (Figure 4E) and *Succinivibrionaceae_UCG-002* (Figure 4J) ($p < 0.05$), while the abundance of *NK4A214_group* (Figure 4F), *Ruminococcus* (Figure 4G), *norank_f__F082* (Figure 4H), and *Lachnospiraceae_NK3A20_group* (Figure 4I) ($p < 0.05$) was significantly reduced compared to the AAH group.

3.5 Influence on the correlation between ruminal microbes and DMI, milk yield, and milk composition

The Spearman correlation analysis revealed that *Prevotella* and *Succinivibrionaceae_UCG-002* were significantly positively correlated with isobutyrate, while *Christensenellaceae_R-7_group*, *Lachnospiraceae_NK3A20_group*, *norank_f__F082*, *NK4A214_group*, and *Rikenellaceae_RC9_gut_group* were negatively correlated with isobutyrate ($p < 0.05$). Additionally, *Lachnospiraceae_NK3A20_group* showed a significant positive correlation with A/P ($p < 0.05$), whereas

TABLE 3 Comparison of different sources alfalfa hay on lactation performance and nutrient digestibility in dairy cows.

Item	Treatments[a]		SEM[b]	*p*-value
	AAH	SAH		
Milk yield (kg/d)	21.02	21.26	0.916	0.899
Milk composition (%)				
Milk protein (MP)	3.77	3.67	0.761	0.537
Milk fat (MF)	3.84	3.78	0.154	0.839
Lactose	4.70	4.50	0.126	0.428
Total solids	13.05	12.66	0.192	0.327
MUN[c] (mg/dL)	13.40	13.68	0.410	0.745
SCC[d] ($\times 10^4$/mL)	22.84	25.10	3.986	0.785
MF:MP[e]	1.03	1.03	0.044	0.959
Nutrient digestibility (%)				
Dry matter	70.24	68.28	1.899	0.270
Organic matter	72.78	70.75	1.959	0.302
Crude protein	76.25	74.20	1.420	0.421
Ether extract	80.49	78.97	2.291	0.757
Neutral detergent fiber	71.56	69.58	1.469	0.190
Acid detergent fiber	55.33	48.70	2.258	0.150

[a]AAH, American alfalfa hay; SAH, Spanish alfalfa hay.
[b]SEM, standard error of mean.
[c]MUN, milk urea nitrogen.
[d]SCC, somatic cell count.
[e]MF:MP, the ratio of milk fat to milk protein.

Prevotellaceae_UCG-003 and *Prevotellaceae_UCG-001* were positively correlated with propionate ($p < 0.05$) but negatively correlated with A/P ($p < 0.05$, Figure 5A). Moreover, the analysis indicated that milk fat was significantly positively associated with *norank_f__UCG-011*, *Christensenellaceae_R-7_group*, *norank_f_Muribaculaceae*, and *Rikenellaceae_RC9_gut_group* ($p < 0.05$). In contrast, *Prevotella* exhibited a negative correlation with milk protein ($p < 0.05$), while *Lachnospiraceae_NK3A20_group* showed a positive correlation with milk protein ($p < 0.05$). *Ruminococcus* was found to have a significant positive correlation with total solids ($p < 0.05$). However, no significant correlation was observed between MY and rumen microorganisms ($p > 0.05$, Figure 5B).

3.6 PICRUSt predictions of ruminal microbial functions

Based on the KEGG database, functional predictions of rumen microorganisms were obtained using PICRUSt and analyzed with STAMP software. The top 20 metabolic pathways with the highest functional abundance were identified for comparison between the two groups (Figure 6). The analysis revealed that 7 metabolic pathways were significantly more abundant in the SAH group compared to the AAH group. These pathways included biosynthesis of secondary metabolites, amino sugar and nucleotide sugar metabolism, general metabolic pathways, purine metabolism, alanine, aspartate, and glutamate metabolism, and pyruvate metabolism. Conversely, eight metabolic pathways were notably reduced in the SAH group relative to the AAH group. These pathways included microbial metabolism in diverse environments, quorum sensing, carbon metabolism, two-component systems, aminoacyl-tRNA biosynthesis, pyrimidine metabolism, cysteine and methionine metabolism, and ABC transporters.

4 Discussion

Imported alfalfa hay from America is widely used in the Chinese dairy industry. Recently, SAH has become an important alternative source to stabilize the alfalfa hay supply market, particularly in the wake of the COVID-19 pandemic. Despite its utility, there are concerns over the potential impacts of SAH on MY and milk quality, which are crucial factors influencing dairy farmers' income. Thus, more research is needed to assess its dietary effects. This study aimed to compare the feeding effects of two types of imported alfalfa hay: SAH and AAH, by providing both hays to mid-lactation cows at a 12.88% dietary dry matter inclusion for a period of 60 days. Each hay was designed to provide the same nutritional level to ensure a fair comparison.

After 60 days of continuous monitoring on 360 dairy cows, the MY of AAH-cows and SAH-cows changed from 21.52 kg/d and 22.48 kg/d to 21.00 kg/d and 21.98 kg/d, respectively, which was in line with the expected MY fluctuations in mid-lactation cows (23). Similarly, there were no significant fluctuations in DMI between the two groups, with AAH-cows consuming approximately 22.24 kg/day and SAH-cows consuming 22.47 kg/day. In addition, milk samples were collected from 12 representative cows in each group to assess milk composition. The analysis showed no significant differences in milk fat percentage, milk protein percentage, or overall milk composition between the SAH and AAH groups. These results indicate that the effects of SAH on MY and milk composition are comparable to those of AAH. This conclusion is supported by the lack of significant differences in nutrient digestibility between the two groups.

To further explore the feeding effects of AAH and SAH, the representative indicators reflecting energy, protein and lipid metabolism in the serum were detected in this study, which were not affected by the different sources of alfalfa hay. In addition, there were no significantly differences in the activity of alanine aminotransferase and aspartate aminotransferase, which are sensitive indicators of liver metabolism and heart health in animals. Previous research has reported serum concentrations of glucose and urea in mid-lactation cows to be around 3.2 and 4.6 mmol/L, respectively (23). The results in this study align with these findings, despite variations that can be influenced by the animal diet (24). These results suggest that different sources of alfalfa hay do not affect the normal physiological condition of dairy cows during mid-lactation, further supporting the similarity in feeding effects between AAH and SAH.

To further compare the effects of SAH and AAH on dairy cows, rumen fermentation parameters were determined in this experiment. The rumen, a specialized digestive organ in ruminants, plays a critical role in the digestion process due to its large population of microorganisms (25). In the rumen, carbohydrates are degraded to produce VFA, such as propionic acid, acetate, and butyric acid, which are the primary productive substances and make up 70–80% of the total energy (26). In this study, the ruminal pH values for both groups

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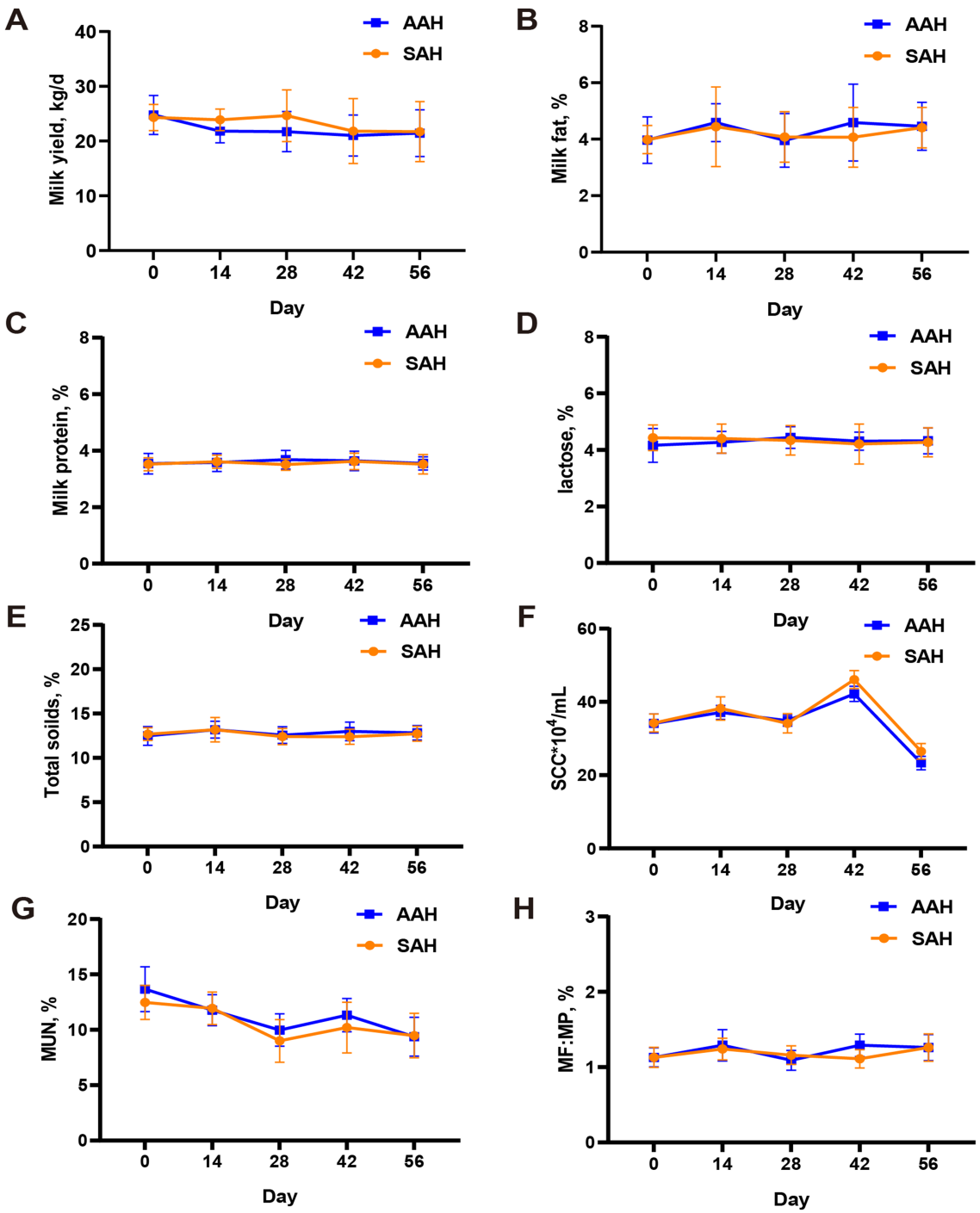

FIGURE 2
Comparison of different sources of alfalfa hay on milk yield and milk composition in dairy cows. **(A)** Milk yield. **(B)** Milk fat. **(C)** Milk protein. **(D)** Lactose. **(E)** Total soilds. **(F)** Somatic cell count (SCC). **(G)** Milk urea nitrogen (MUN). **(H)** The ratio of milk fat to milk protein (MF:MP). AAH, American alfalfa hay; SAH, Spanish alfalfa hay. Milk composition analysis in milk simples of AAH and SAH cows on days 0, 14, 28, 42 and 56 of the trial.

of cows ranged from 6.51 to 6.56, which are conducive to the growth of fibrolytic bacteria and effective fiber digestion (27). The results showed that there were no significant differences in pH or total VFA concentrations between the two groups. The stability of the rumen micro-ecology in adult ruminants may be the reason for this finding (28, 29). Additionally, the ratio of acetic acid to propionic acid, an important indicator of rumen fermentation efficiency (30), was found to be similar between the two groups of dairy cows in the current study, indicating that rumen fermentation was not affected by either alfalfa hay type. Furthermore, ruminal NH_3-N, a marker of protein breakdown and microbial protein synthesis (31), showed no significant differences between the groups. This result aligns with the digestibility of crude protein. In summary, SAH did not cause changes in rumen fermentation parameters.

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TABLE 4 Comparison of different sources alfalfa hay on serum biochemical indices in dairy cows.

Item	Treatments[a]		SEM[b]	*p*-value
	AAH	SAH		
Glucose (mmol/L)	3.17	3.25	0.103	0.726
Total protein (g/L)	77.4	82.0	1.738	0.227
Albumin (g/L)	40.0	36.1	0.979	0.078
Globulin (g/L)	37.4	45.8	2.587	0.139
Albumin/Globulin	1.08	0.82	0.064	0.068
Triglyceride (mmol/L)	0.198	0.160	0.011	0.124
Cholesterol (mmol/L)	4.96	4.50	0.314	0.477
Non-esterified fatty acid (mmol/L)	0.110	0.103	0.010	0.755
β-hydroxybutyrate (mmol/L)	0.708	0.633	0.033	0.291
Urea (mmol/L)	5.40	5.07	0.217	0.474
Alanine transaminases (U/L)	28.4	27.7	1.714	0.835
Aspartate transaminase (U/L)	77.6	97	7.577	0.233
AST/ALT[c]	2.82	3.33	0.184	0.198

[a]AAH, American alfalfa hay; SAH, Spanish alfalfa hay.
[b]SEM, standard error of mean.
[c]AST/ALT, alanine transaminase/aspartate transaminase.

TABLE 5 Comparison of different sources alfalfa hay on ruminal fermentation in dairy cows.

Item	Treatments[1]		SEM[2]	*p*-value
	AAH	SAH		
pH	6.51	6.56	0.068	0.731
Total VFA[3] (mM)	49.68	55.49	2.113	0.199
Mol/100 mol				
Acetate (A)	62.01	60.97	0.689	0.346
Propionate (P)	21.76	22.29	0.457	0.574
Butyrate	13.65	13.79	0.331	0.265
Valerate	1.63	1.71	0.084	0.194
Isobutyrate	0.291[b]	0.526[a]	0.034	0.007
Isovalerate	0.660	0.703	0.054	0.698
A:P[4]	2.88	2.74	0.090	0.467
Ammonia N (mg/100 mL)	9.33	11.27	1.217	0.460

[a,b]Means with different superscripts in each row differ significantly ($p < 0.05$).
[1]AAH, American alfalfa hay; SAH, Spanish alfalfa hay.
[2]SEM, standard error of mean.
[3]VFA, volatile fatty acids.
[4]A:P, acetate/propionate.

To demonstrate the abundance of microbial species in the rumen fluid samples, a rarefaction curve was constructed between the quantity of sequences obtained through random sampling and the Shannon indices of diversity. A flat curve indicated that the sequencing depth was sufficient and had captured most of the OTUs. Furthermore, we evaluated the alpha diversity to determine if different sources of alfalfa hay influenced microbial diversity in the rumen. The Chao1 index, which reflects the total number of species (32), showed significant differences between the two groups. The rumen microbial communities in the AAH and SAH groups were significantly different according to the β-diversity analysis. These findings indicate that the source of alfalfa hay affected the abundance, composition, and structure of the rumen microbial community.

In this study, 16S rRNA sequencing revealed that *Bacteroidota* and *Bacillota* had the greatest percentage of bacteria in the ruminal flora at the *phyla* taxonomic level (33, 34). *Bacteroidota* are primarily involved in breaking down intricate macromolecular organic materials, such as the conversion of carbohydrates into monosaccharides. Meanwhile, *Bacillota* produce extracellular enzymes, including proteases, lipases, and cellulases, which help hydrolyze proteins, lipids, amino acids, and hemicellulose (35). At the phylum level, *Bacillota* and *Bacteroidota* were the dominant bacterial groups in the rumen in this study. Specifically, the relative abundance of *Bacillota* was 56.35 and 40.88% in the AAH and SAH groups, respectively, while *Bacteroidota* constituted 35.99 and 46.64% in these groups. These findings are in line with findings from previous studies (16).

Ruminal microecological studies have proposed the existence of a core ruminal microbiome and have reported significant changes in the abundance of core bacterial genera between animals (36). Due to differences in diet, days in milk, parity, and sample size, the core rumen microbes can differ across studies to varying extents (37). *Prevotella* and *Succinivibri-onaceae_UCG-002*, which are more abundant in the SAH group at the genus level in the rumen, may be the core differential bacteria. *Prevotella*, belonging to the *Bacteroidota*, can decompose plant proteins, peptides, hemicellulose and pectin into acetic acid, succinic acid and a small amount of isobutyrate, which are directly utilized by dairy cows (16). According to Calabrò et al. (38), the higher *Provetella* content in the SAH group could be due to the higher hemicellulose content in this study. *Succinivibrionaceae_UCG-002*, belonging to the *Bacillota*, is a typical fiber-degrading bacterium that breaks down fiber and cellobiose into succinic acid, acetic acid and carbon dioxide (39). Correlation analysis revealed a significant positive relationship between the abundance of *Prevotella* and *Succinivibrionaceae_UCG-002* and the isobutyrate molar proportion in the rumen. The SAH group exhibited a higher isobutyrate molar proportion compared to the AAH group. In addition, *NK4A214_group*, *Ruminococcus*, *norank_f_F082* and *Lachnospiraceae_NK3A20_group* were more prevalent in the AAH group and might represent the core differential bacteria in this study. *Ruminococcus* and *NK4A214_group*, belonging to the *Bacillota*, are beneficial bacteria in the rumen, efficiently degrading starch and fiber, respectively, and producing VFAs to provide energy to the animal (40). *Norank_f__F082* and *Lachnospiraceae_NK3A20_group* are similarly the main components of rumen microbiota in ruminates and are closely related to VFA production (41, 42). In this study, *NK4A214_group*, *Ruminococcus*, *norank_f__F082* and *Lachnospiraceae_NK3A20_group* were significantly negatively correlated with isobutyrate molar proportion. However, these core differential microorganisms were not significantly correlated with total VFA, molar proportions of acetate and propionic acid, and MY. In fact, except for the isobutyrate molar proportion, there were no other significant differences between the two groups in the rumen fermentation parameters. This indicated that changes in these core microorganisms did not alter ruminal fermentation patterns, which is one of the reasons why different sources of alfalfa hay did not cause changes in cow performance. Future studies could explore combining alfalfa hay from various sources with other feed ingredients to enhance efficiency by altering rumen microbial

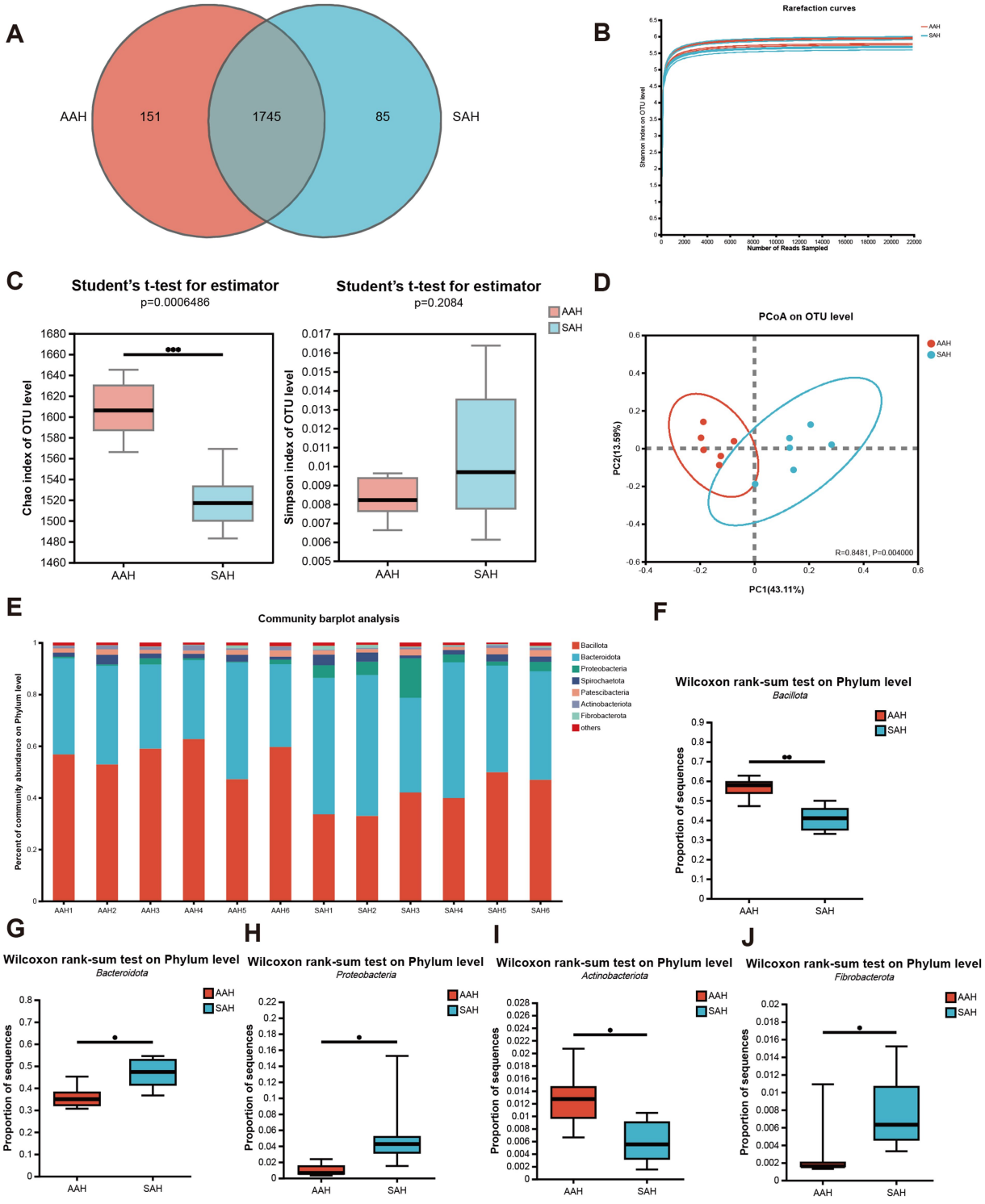

FIGURE 3
Comparison of different sources of alfalfa hay on ruminal microbiota in dairy cows. **(A)** Venn diagram of rumen microbiota on OTU level in the two groups. **(B)** Curve of rarefaction for sequencing data. **(C)** Alpha diversity. **(D)** Beta diversity. **(E)** Changes and differences in microbiome on phylum level. **(F–J)** Differential expression of ruminal microbes: **(F)** *Bacillota*; **(G)** *Bacteroidota*; **(H)** *Proteobacteria*; **(I)** *Actinobacteriota*; **(J)** *Fibrobacterota*. AAH, American alfalfa hay; SAH, Spanish alfalfa hay. * $0.01 < p \leq 0.05$; ** $0.001 < p \leq 0.01$.

composition and improving dairy cow feed efficiency, which is an approach to refined feeding.

We investigated the impact of different alfalfa hay sources on ruminal microbial metabolic pathways in dairy cows. Using STAMP software to analyze microbial functions, we found notable differences between the two groups. Feeding SAH significantly up-regulated functions related to the biosynthesis of secondary metabolites, amino sugar and nucleotide sugar metabolism, metabolic pathways, purine metabolism, alanine, aspartate, and glutamate metabolism, as well as pyruvate metabolism. Conversely, feeding AAH led to significant

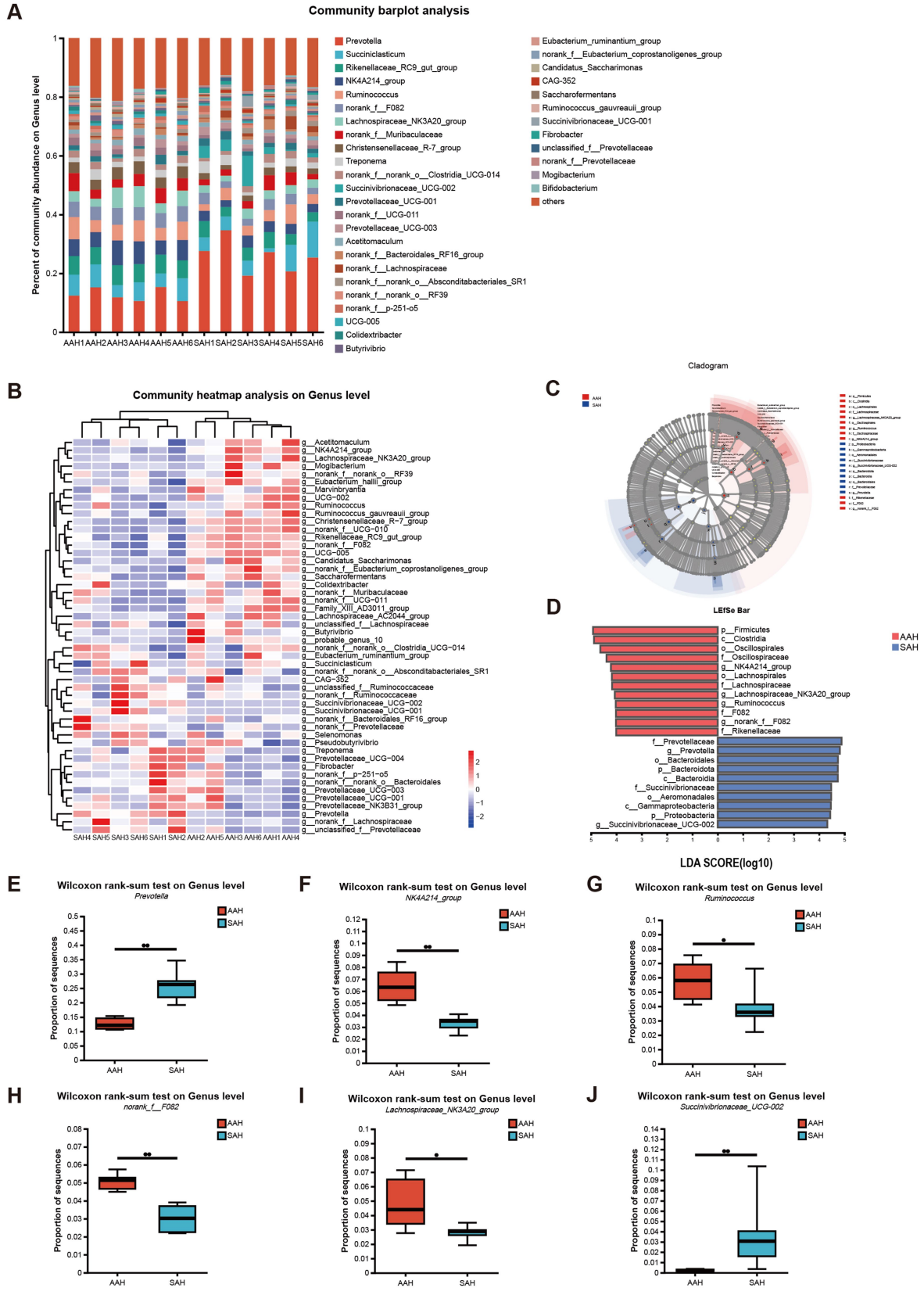

FIGURE 4
Analysis of the composition and difference of the rumen microbiota of dairy cows. AAH, American alfalfa hay; SAH, Spanish alfalfa hay. **(A)** Community bar plot analysis at the genus level. **(B)** Community heatmap analysis of 50 species at the genus level. **(C,D)** Linear discriminant analysis (LDA) effect size (LEfSe) analysis of differential enrichment of the ruminal microbes at the genus level: **(E)** *Prevotella*; **(F)** *NK4A214_group*; **(G)** *Ruminococcus*; **(H)** *norank_f__F082*; **(I)** *Lachnospiraceae_NK3A20_group*; **(J)** *Succinivibrionaceae_UCG-002*. AAH, American alfalfa hay; SAH, Spanish alfalfa hay. * $0.01 < p \leq 0.05$. ** $0.001 < p \leq 0.01$.

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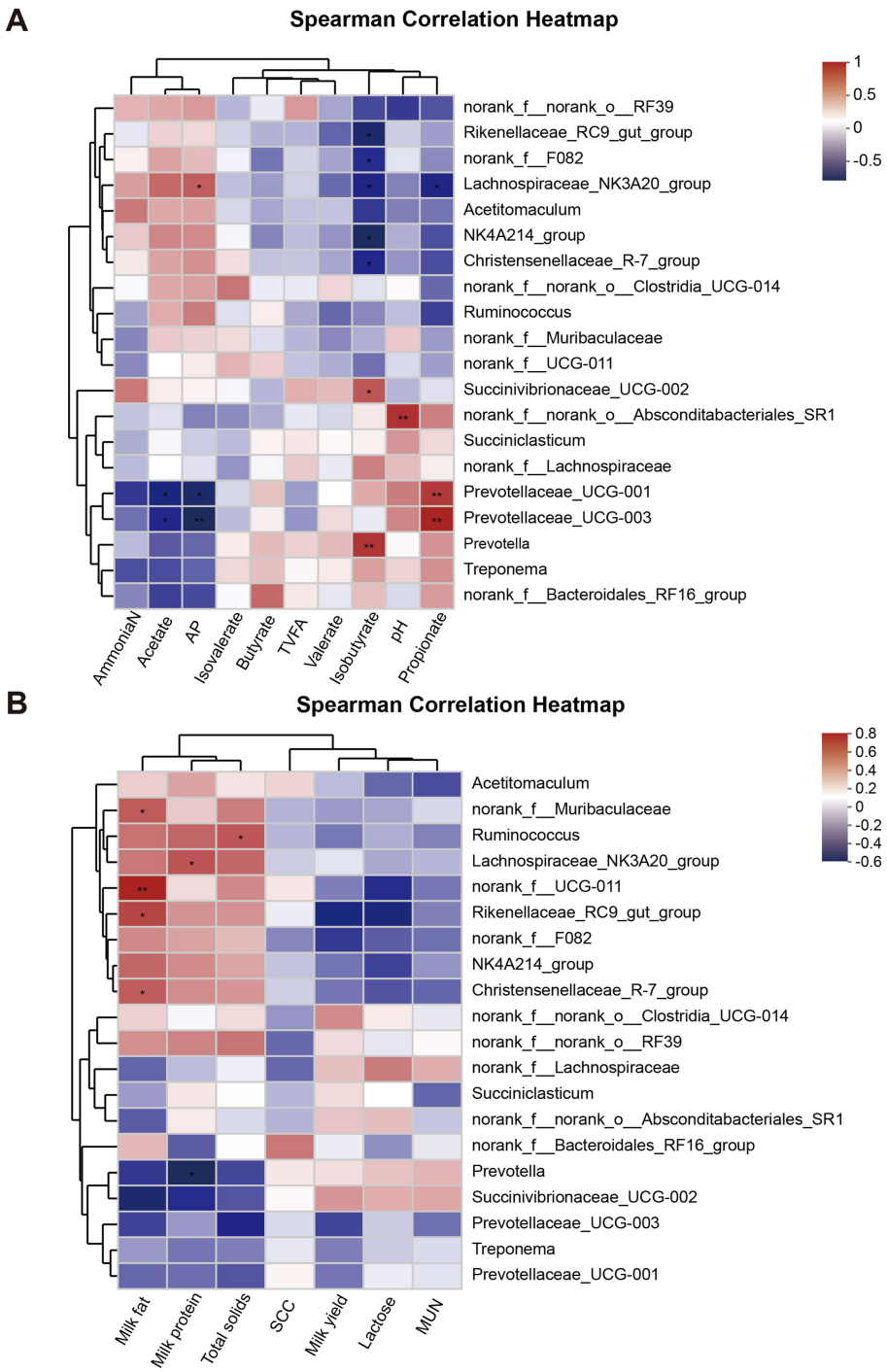

FIGURE 5
Analysis of the correlation between the rumen microbiota and milk yield, milk composition, and rumen fermentation parameters. **(A)** Correlation analysis of rumen microbiota with milk yield and milk composition. **(B)** Correlation analysis of rumen microbiota with rumen fermentation parameters. * $0.01 < p \leq 0.05$; ** $0.001 < p \leq 0.01$.

up-regulation of microbial metabolism in diverse environments, quorum sensing, carbon metabolism, two-component systems, aminoacyl-tRNA biosynthesis, pyrimidine metabolism, cysteine and methionine metabolism, and ABC transporters. Despite these differences in microbial composition and metabolic functions, there were no changes in MY, DMI, blood biochemical parameters, or ruminal fermentation patterns between the two groups. Further investigation is needed to understand and verify the reasons behind these findings.

5 Conclusion

We found that the SAH and AAH with the similar nutritional levels had the same feeding effects, as evidenced by no significant differences in MY, milk composition, blood biochemical parameters, or rumen fermentation parameters between the two groups of dairy cows. However, the relative abundances of *Prevotella*, *Succinivibrionaceae_UCG-002*, *NK4A214_group*, *Ruminococcus*,

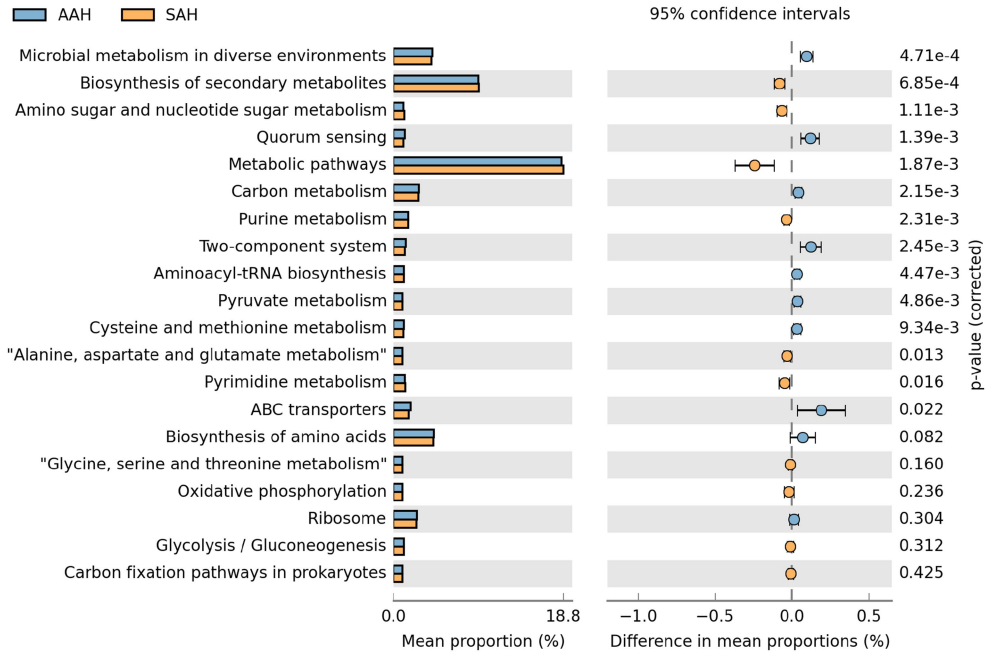

FIGURE 6
The top 20 metabolic pathways involved by rumen microbes in the two groups of dairy cows at KEGG level 3. AAH, American alfalfa hay; SAH, Spanish alfalfa hay.

norank_f_F082 and *Lachnospiraceae_NK3A20_group* were changed significantly between the two groups. Despite these changes, no significant correlation was found between these microbial variations and milk yield. These findings provided reference for the application of alfalfa hay from different sources, the improvement of economic benefit of dairy farm and expansion of the alfalfa supply market.

Data availability statement

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

Ethics statement

The animal study was approved by the Institutional Animal Care and Use Committee (IACUC) of Henan Agriculture University (Zhengzhou, China) (Approval Number: HNND2021062812). The study was conducted in accordance with the local legislation and institutional requirements.

Author contributions

SL: Investigation, Methodology, Validation, Writing – original draft. HL: Investigation, Methodology, Validation, Writing – original draft. YZ: Software, Writing – review & editing. MA: Writing – review & editing. JN: Software, Writing – review & editing. ZG: Formal analysis, Writing – review & editing. BL: Formal analysis, Writing – review & editing. SM: Data curation, Writing – review & editing. YC: Data curation, Writing – review & editing. DL: Data curation, Writing – review & editing. YS: Conceptualization, Funding acquisition, Project administration, Resources, Writing – review & editing.

Funding

The authors declare that financial support was received for the research, authorship, and/or publication of this article. This study was supported by the earmarked fund for China Forage and Grass Research System (CARS-34).

Acknowledgments

The authors would like to extend their gratitude to Xincai Ruiya Animal Husbandry Farm for providing the experimental animals and facilities, and to the staff for their dedicated care of the animals.

Conflict of interest

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

Publisher's note

References

1. Wang B, Tu Y, Jiang LS, Liu JX. Effect of cereal straw and alfalfa Hay diet on amino acid profile of gastrointestinal digesta in lactating dairy cows. *J Anim Physiol Anim Nutr*. (2018) 102:421–8. doi: 10.1111/jpn.12749

2. Woll FW. Alfalfa as a sole feed for dairy cattle. *J Dairy Sci*. (1918) 1:447–61. doi: 10.3168/jds.S0022-0302(18)94391-2

3. Wang Q, Zou Y. China's alfalfa market and imports: development, trends, and potential impacts of the US-China trade dispute and retaliations. *J Integr Agric*. (2020) 19:1149–58. doi: 10.1016/s2095-3119(19)62832-7

4. Wang X, Yan X, Mi F, Li H. Correlation analysis of alfalfa varieties based on production performances, winter survival rates and fall dormancies. *Legum Res*. (2021) 44:15–20. doi: 10.18805/lr-551

5. Xu W, Shi S, Qi J, Squires VR, Hua L, Zhang W, et al. Effect of different processing methods on the quality of alfalfa hay. *J Anim Vet Adv*. (2013) 12:689–93. doi: 10.3923/javaa.2013.689.693

6. Vagnoni DB, Broderick GA. Effects of supplementation of energy or ruminally undegraded protein to lactating cows fed alfalfa Hay or silage. *J Dairy Sci*. (1997) 80:1703–12. doi: 10.3168/jds.S0022-0302(97)76102-2

7. Christensen RG, Yang SY, Eun JS, Young AJ, Hall JO, MacAdam JW. Effects of feeding birdsfoot trefoil hay on neutral detergent Fiber digestion, nitrogen utilization efficiency, and lactational performance by dairy cows. *J Dairy Sci*. (2015) 98:7982–92. doi: 10.3168/jds.2015-9348

8. Dai Q, Hou Z, Gao S, Li Z, Wei Z, Wu D. Substitution of fresh forage ramie for alfalfa hay in diets affects production performance, milk composition, and serum parameters of dairy cows. *Trop Anim Health Prod*. (2019) 51:469–72. doi: 10.1007/s11250-018-1692-6

9. Obeidat BS, Subih HS, Taylor JB. Feeding alfalfa hay improves nursing performance of Awassi ewes and performance of growing lambs when used as a source of roughage compared with wheat straw. *J Anim Sci*. (2018) 96:32–3. doi: 10.1093/jas/sky027.061

10. Cammack KM, Austin KJ, Lamberson WR, Conant GC, Cunningham HC. Ruminnat nutrition symposium: tiny but mighty: the role of the rumen microbes in livestock production. *J Anim Sci*. (2018) 96:752–70. doi: 10.1093/jas/skx053

11. Cavaliere G, Trinchese G, Musco N, Infascelli F, De Filippo C, Mastellone V, et al. Milk from cows fed a diet with a high forage: concentrate ratio improves inflammatory state, oxidative stress, and mitochondrial function in rats. *J Dairy Sci*. (2018) 101:1843–51. doi: 10.3168/jds.2017-13550

12. Fustini M, Palmonari A, Canestrari G, Negri N, Formigoni A. Effect of alfalfa hay digestibility and inclusion rate in lactating dairy cows diets on feed intake, digestibility, ruminal Ph and productive performance. *Ital J Anim Sci*. (2015) 18:101066. doi: 10.1016/j.animal.2023.101066

13. AOAC. Official methods of analysis. *18th* ed. Arlington, VA: AOAC (2004).

14. Van Soest PJ, Robertson JB, Lewis BA. Methods for dietary fiber, neutral detergent fiber, and nonstarch polysaccharides in relation to animal nutrition. *J Dairy Sci*. (1991) 74:3583–97. doi: 10.3168/jds.S0022-0302(91)78551-2

15. Mattioli S, Dal Bosco A, Duarte JMM, D'Amato R, Castellini C, Beone GM, et al. Use of selenium-enriched olive leaves in the feed of growing rabbits: effect on oxidative status, mineral profile and selenium speciation of longissimus dorsi meat. *J Trace Elem Med Biol*. (2019) 51:98–105. doi: 10.1016/j.jtemb.2018.10.004

16. Cui Y, Liu H, Gao Z, Xu J, Liu B, Guo M, et al. Whole-plant corn silage improves rumen fermentation and growth performance of beef cattle by altering rumen microbiota. *Appl Microbiol Biotechnol*. (2022) 106:4187–98. doi: 10.1007/s00253-022-11956-5

17. Chen S, Zhou Y, Chen Y, Gu J. fastp: an ultra-fast all-in-one FASTQ preprocessor. *Bioinformatics*. (2018) 34:i884–90. doi: 10.1093/bioinformatics/bty560

18. Magoc T, Salzberg SL. FLASH: fast length adjustment of short reads to improve genome assemblies. *Bioinformatics*. (2011) 27:2957–63. doi: 10.1093/bioinformatics/btr507

19. Edgar RC. UPARSE: highly accurate OTU sequences from microbial amplicon reads. *Nat Methods*. (2013) 10:996–8. doi: 10.1038/nmeth.2604

20. Stackebrandt E, Goebel BM. 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*. (1994) 44:846–9. doi: 10.1099/00207713-44-4-846

21. Schloss PD, Westcott SL, Ryabin T, Hall JR, Hartmann M, Hollister EB, et al. Introducing mothur: open-source, platform-independent, community-supported software for describing and comparing microbial communities. *Appl Environ Microbiol*. (2009) 75:7537–41. doi: 10.1128/AEM.01541-09

22. Langille MG, Zaneveld J, Caporaso JG, McDonald D, Knights D, Reyes JA, et al. Predictive functional profiling of microbial communities using 16S rRNA marker gene sequences. *Nat Biotechnol*. (2013) 31:814–21. doi: 10.1038/nbt.2676

23. NRC. Nutrient requirements of dairy cattle. Washington, DC: National Academy Press (2001).

24. Musco N, Tudisco R, Grossi M, Mastellone V, Morittu V, Pero M, et al. Effect of a high forage: concentrate ratio on milk yield, blood parameters and oxidative status in lactating cows. *Anim Prod Sci*. (2020) 60:1531–8. doi: 10.1071/AN18041

25. Anantasook N, Wanapat M, Cherdthong A, Gunun P. Changes of microbial population in the rumen of dairy steers as influenced by plant containing tannins and saponins and roughage to concentrate ratio. *Asian Australas J Anim Sci*. (2013) 26:1583–91. doi: 10.5713/ajas.2013.13182

26. Sommer F, Baeckhed F. The gut microbiota—masters of host development and physiology. *Nat Rev Microbiol*. (2013) 11:227–38. doi: 10.1038/nrmicro2974

27. Russell JB, Wilson DB. Why are ruminal cellulolytic bacteria unable to digest cellulose at low Ph? *J Dairy Sci*. (1996) 79:1503–9. doi: 10.3168/jds.S0022-0302(96)76510-4

28. Chen XL, Wang JK, Wu YM, Liu JX. Effects of chemical treatments of rice straw on rumen fermentation characteristics, fibrolytic enzyme activities and populations of liquid- and solid-associated ruminal microbes in vitro. *Anim Feed Sci Technol*. (2008) 141:1–14. doi: 10.1016/j.anifeedsci.2007.04.006

29. Zhou YW, McSweeney CS, Wang JK, Liu JX. Effects of disodium fumarate on ruminal fermentation and microbial communities in sheep fed on high-forage diets. *Animal*. (2012) 6:815–23. doi: 10.1017/S1751731111002102

30. Wei X, Yin Q, Zhao H, He J, Cao Y, Yao J. Nicotinamide supplementation during postpartum and peripartum modulates hepatic energy and lipid metabolism, oxidative status, and metabolomics profile, as well as lipids in the adipose tissue of goats. *Anim Feed Sci Technol*. (2021) 274:114849. doi: 10.1016/j.anifeedsci.2021.114849

31. Annison EF, Bryden WL. Perspectives on ruminant nutrition and metabolism. II. Metabolism in ruminant tissues. *Nutr Res Rev*. (1999) 12:147–77. doi: 10.1079/095442299108728866

32. Barger K, Langsetmo L, Orwoll ES, Lustgarten MS. Investigation of the diet-gut-muscle axis in the osteoporotic fractures in men study. *J Nutr Health Aging*. (2020) 24:445–52. doi: 10.1007/s12603-020-1344-1

33. de Oliveira MNV, Jewell KA, Freitas FS, Benjamin LA, Tótola MR, Borges AC, et al. Characterizing the microbiota across the gastrointestinal tract of a Brazilian Nelore steer. *Vet Microbiol*. (2013) 164:307–14. doi: 10.1016/j.vetmic.2013.02.013

34. Zhang R, Ye H, Liu J, Mao S. High-grain diets altered rumen fermentation and epithelial bacterial community and resulted in rumen epithelial injuries of goats. *Appl Microbiol Biotechnol*. (2017) 101:6981–92. doi: 10.1007/s00253-017-8427-x

35. Li X, Yu YH. Biodiversity and screening of halophilic bacteria with hydrolytic and antimicrobial activities from Yuncheng Salt Lake, China. *Biologia*. (2015) 70:151–6. doi: 10.1515/biolog-2015-0033

36. Jami E, Mizrahi I. Similarity of the ruminal bacteria across individual lactating cows. *Anaerobe*. (2012) 18:338–43. doi: 10.1016/j.anaerobe.2012.04.003

37. Xue M, Sun H, Wu X, Guan LL, Liu J. Assessment of rumen microbiota from a large dairy cattle cohort reveals the pan and core bacteriomes contributing to varied phenotypes. *Appl Environ Microbiol*. (2018) 84:e00970. doi: 10.1128/aem.00970-18

38. Calabrò S, Infascelli F, Bovera F, Moniello G, Piccolo V. *In vitro* degradability of three forages: fermentation kinetics and gas production of NDF and neutral detergent-soluble fraction of forages. *J Sci Food Agric*. (2002) 82:222–9. doi: 10.1002/jsfa.1021

39. Wang Y, Jiang M, Zhang Z, Sun H. Effects of over-load iron on nutrient digestibility, haemato-biochemistry, rumen fermentation and bacterial communities in sheep. *J Anim Physiol Anim Nutr*. (2020) 104:32–43. doi: 10.1111/jpn.13225

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

41. Han H, Zhang L, Shang Y, Wang M, Phillips CJ, Wang Y, et al. Replacement of maize silage and soyabean meal with mulberry silage in the diet of Hu lambs on growth, gastrointestinal tissue morphology, rumen fermentation parameters and microbial diversity. *Animals*. (2022) 12:1406. doi: 10.3390/ani12111406

42. Yi S, Dai D, Wu H, Chai S, Liu S, Meng Q, et al. Dietary concentrate-to-forage ratio affects rumen bacterial community composition and metabolome of yaks. *Front Nutr*. (2022) 9:927206. doi: 10.3389/fnut.2022.927206

frontiers | Frontiers in Veterinary Science

TYPE Original Research
PUBLISHED 06 November 2024
DOI 10.3389/fvets.2024.1461391

OPEN ACCESS

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RECEIVED 08 July 2024
ACCEPTED 25 September 2024
PUBLISHED 06 November 2024

CITATION
Liu J, Chen J, Fang S, Sun B, Li Y, Guo Y, Deng M, Zhou D, Liu D and Liu G (2024) Effects of moringa polysaccharides on growth performance, immune function, rumen morphology, and microbial community structure in early-weaned goat kids. *Front. Vet. Sci.* 11:1461391. doi: 10.3389/fvets.2024.1461391

Effects of moringa polysaccharides on growth performance, immune function, rumen morphology, and microbial community structure in early-weaned goat kids

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The aim of this research was to investigate the effects of adding moringa polysaccharides (MOP) on the growth performance, immune function, rumen tissue morphology, and rumen microbial community in early-weaned goat kids. Twenty-one 7-day-old Leizhou male goat kids weighing (3.05 ± 0.63) kg, were randomly divided into a control group (CON group), a low-dose group (LOW group), and a high-dose group (HIG group). MOP was added to the goat kids' milk replacer (MR) at 0, 0.15, and 0.3% (on dry matter basis),fed until 60 days of age, and four goat kids in each group with body weights close to the mean of each group were selected for slaughter. The results showed that, compared to the CON group, the MOP groups significantly improved final body weight, body measurements, daily weight gain, and feed intake of the early weaned goat kids; significantly reduced the content of propionic acid, butyric acid, valeric acid, and ammoniacal nitrogen; and in addition, the addition of MOP could significantly increase the height of rumen nipple, the content of immunoglobulin G (IgG) in the serum. The HIG group significantly increased rumen pH, rumen muscularis layer thickness, rumen wall thickness, and serum immunoglobulin A (IgA), and immunoglobulin M (IgM). In conclusion, the addition of MOP positively impacted the growth performance, serum immune function, and rumen tissue morphology in early-weaned goat kids.

KEYWORDS

moringa polysaccharide, goat kid, immune function, rumen morphology, rumen microbiota

1 Introduction

As a unique digestive organ of ruminants, the development of rumen plays an important role in the health and digestive function of young ruminants. Early weaning of lambs can shorten the reproductive cycle of ewes and increase lambing frequency (1), and at the same time promote the development of rumen structure and function (2, 3). However, mother lamb

separation and changes in feed type can easily cause stress in early weaned lambs (4), which not only reduces the immunity and growth rate of lambs, but also results in the underutilization of nutrients and blockage of the immune system during the weaning period, which can easily lead to diseases such as intestinal pathogenic bacterial infections, intestinal homoeopathy imbalance and diarrhea, and even cause death (5–7). Antibiotics have been widely used to treat and prevent diarrhea in lambs (8), but the frequent use of antibiotics not only causes great damage to the balance of intestinal flora and immune system in lambs, but also the antibiotic residues in the intestinal tract and feces of the animals lead to the increased risk of bacterial microorganisms' resistance, and these antibiotic residues of fecal matter and animal products can cause great negative impacts on human beings and the ecological environment (9). Under the Chinese government's antibiotic ban, finding natural active substitutes for antibiotics to achieve healthy lamb farming is of great importance for the high-quality development of the goat industry.

Moringa is a perennial deciduous tree of the Moringaceae family, native to India, and nowadays it has been widely cultivated as a multipurpose plant in different countries and regions (10, 11). All parts of Moringa including leaves, rhizomes and seeds are rich in functional polysaccharides, which are not only of high nutritional value but also of medicinal value (10). The presence of ascorbic acid, carotenoids and flavonoids, phenols and other types of antioxidant compounds in the leaves of Moringa is a good source of natural antioxidants (12). Polysaccharides extracted from Moringa are highly bioactive and have anti-inflammatory, antioxidant, antimicrobial, immunomodulatory and gastrointestinal protective properties (13). Natural plant polysaccharides have strong immunomodulatory functions and are considered as ideal drugs to improve immunity and antitumor (14–16). The experimental results of Tian et al. (17) have showed that MOP enhanced immunity, improved intestinal flora and morphology, as well as modulated the metabolism in mice. Zhao et al. (18) demonstrated that MOP could improve calf diarrhea, improve immunity, improve intestinal flora as well as promote growth.

Therefore, we added different concentrations of MOP to explore its effects on growth performance, serum antioxidant capacity, immune capacity, gastrointestinal morphology and microbial composition of 7-day-old weaned goat kids. In order to evaluate the feeding value of MOP in early-weaned goat kids and determine the appropriate addition amount of MOP in the actual production of goat kids, it provides a theoretical basis for the more scientific and rational application of MOP in goat industry.

2 Materials and methods

2.1 Ethics statement

All experimental procedures in this study were approved by the Committee of Animal Experiments of South China Agricultural University (No. 2023g032).

2.2 Experimental design and treatments

The feeding experiment was conducted from November 2023 to January 2024 at a black goat farm in Qingyuan, Guangdong Province. During the trial period, the average temperature was 16.4°C (ranging from 5 to 28.5°C) and the average humidity was 67.1% (ranging from 22 to 93%).

MOP was purchased from Xi'an Clover Biotech Co., Ltd. (Xi'an, China), brown powder with 70% purity. Milk replacer (MR) was purchased from Beijing Precision Animal Nutrition Research Center (Beijing, China). Nutritional levels (dry matter basis): crude protein 27%, crude fat 18%, crude ash 10%, calcium 1.5%, phosphorus 1.2%. The starter diet was a granular compound feed formulated with reference to the nutritional requirements of NRC (1994), and the composition and nutritional levels of the basal ration are shown in Table 1.

Twenty-one 7-day-old Leizhou goat male goat kids of close weight and good health were selected with an average initial weight of (3.05 ± 0.63) kg. The goat kids were randomly divided into three groups of seven replicates of one goat kid.

The amount of MOP added was referred to the experimental results of Guo et al. (5). The control group (CON group) was fed milk replacer. The low dose group (LOW group) was fed milk replacer supplemented with 0.15% of their daily dry matter intake (DMI) of MOP. The high dose group (HIG group) was fed milk replacer supplemented with 0.3% of their daily dry matter intake (DMI) of MOP. The goat kids were fed from 7 days of age to 60 days of age, with a total experimental period of 54 days.

2.3 Feeding management

The test goat kids were suckled with their mothers until 7 days of age, and were forced to wean at 7 days of age. The goat kids were fed in separate pens (3.5 m long, 2.5 m wide and 1.5 m high), with partitions between the pens to prevent the groups from coming into contact with each other. Each pen is equipped with a drinking trough and trough for goat kids to freely eat open food, hay and sufficient water. The troughs were cleaned and replaced every morning. Straw is used as bedding for goat kids to lie on and is replaced every 2 days. All goat kids were provided with free access to starter feed and received an equal amount of milk replacer powder (2% of body weight) daily at 08:00, 12:00, and 16:00. The milk replacer powder was reconstituted with warm water at a 1:7 (weight/volume) ratio, thoroughly mixed until fully dissolved, and then dried at room temperature to 40°C. The reconstituted milk replacer was administered via a 250 mL bottle.

TABLE 1 Composition and nutritional levels of the starter feed (air-dry basis).

Ingredients	Composition (%)
Corn	46.40
Soybean meal	15.60
DDGS	10.35
Dried whey	20.25
$CaHPO_4$	1.00
Limestone	0.81
NaCl	4.09
Choline chloride	0.50
Premix	1.00
Total	100.00

The sanitation of the pens was carried out according to the management measures of the farm, and was cleaned and disinfected regularly. The goat pens were cleaned and disinfected regularly according to the farm management measures, and the goat were immunized according to the normal immunization procedures.

2.4 Experimental methods and parameter measurements

2.4.1 Feed intake measurement

Feed samples, including hay, milk replacer, and starter, were collected weekly during the trial and mixed at the end of the trial. The feed intake of each group of goat kids was recorded daily. The collected feed samples were oven dried at 65°C to constant weight, pulverized and then determined for dry matter, crude ash, crude protein, crude fat, acid detergent fiber, neutral detergent fiber, calcium and phosphorus with reference to the assay method of Horwitz et al. (19).

2.4.2 Growth performance measurement

Goat kid fasting weight and body measurements were determined at the beginning of the trial (i.e., when goat kids were 7 days old), and at the end of the trial (i.e., when goat kids were 60 days old) prior to morning feeding, and the average daily weight gain of the test goat kids was calculated.

2.4.3 Blood sample collection and analysis

Blood was collected from the jugular vein of the goat kids before morning feeding on the 60th day of the experiment using a procoagulant blood collection tube, centrifuging at 3,000*g* at 4°C for 15 min, and the serum was centrifuged and collected for the determination of biochemical, antioxidant, and immunological indices. Total protein (TP), albumin (ALB), globulin (GLB), urea nitrogen (BUN), glucose (GLU), triglyceride (TG), total cholesterol (TC), high-density lipoprotein (HDL) cholesterol, low-density lipoprotein (LDL) cholesterol, alanine aminotransferase (ALT), alanine oxalate aminotransferase (AST), and creatinine (CRE) were measured by using an automated biochemistry instrument (Zecheng CLS880); total antioxidant capacity (T-AOC) in serum was determined by 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) method (Item No.: A015-2-1); catalase (CAT) activity was determined by ammonium molybdate method (Item No.: A007-1-1); and superoxide dismutase (SOD) activity was determined by Water-Soluble Tetrazolium Salt-1(WST-1)method (Item No.: A001-3-2); Glutathione peroxidase (GSH-Px) activity (Cat. No.: A005-1-2); Malondialdehyde (MDA) content was determined by Thiobarbituric Acid (TBA) method (Cat. No.: A003-1-2); Serum immunoglobulin A (IgA), immunoglobulin G (IgG), immunoglobulin M (IgM) were determined by enzyme-linked immunosorbent assay (ELISA) kit; Serum The levels of pro-inflammatory factor interleukin-2 (IL-2), anti-inflammatory factor interleukin-6 (IL-6), tumor necrosis factor (TNF-α) and γ-interferon (IFN-γ) in serum were determined by using the kit of Nanjing Jianjian Institute of Biological Engineering, and the absorbance was measured by using an enzyme labeling instrument (RaytoRT-6100), and the specific procedures were carried out according to the instructions of the kit.

2.4.4 Collection and analysis of rumen tissue and rumen contents

Goat kids were euthanized and dissected for sampling within 15 min. Post-dissection rumen pH was determined using a pH meter (FE28, METTLER TOLEDO INSTRUMENTS CO., LTD., Shanghai, China). Goat kid rumen content samples were stored in liquid nitrogen for subsequent DNA extraction. Rumen fluid samples obtained by filtration using four layers of gauze were stored at −20°C for subsequent determination of fermentation parameters.

The rumen ventral sac tissue ($1.5 \times 1.5\,cm^2$) of goat kids was collected after autopsy with reference to the method of Li et al. (20), and after rinsing with phosphate buffer solution (PBS), the rumen tissue was fixed for 24 h in 4% paraformaldehyde solution, dehydrated, transparent, and wax impregnated to obtain paraffin-embedded wax blocks of rumen tissue.

2.5 Morphology and measurement of rumen tissue

The embedded wax block was cut into 4 μm thick slices with a paraffin slicer (RM2016), spread horizontally under 40°C warm water in a spreader, and the slide fished the tissue and baked the slices in a 60°C oven. After the water baking dry wax baked and removed, the slices were sequentially put into environmentally friendly dewaxing solution I 20 min—environmentally friendly dewaxing solution II 20 min—anhydrous ethanol I 5 min—anhydrous ethanol II 5 min—75% alcohol for 5 min, and washed with tap water. The slices were put into HD constant dye pretreatment solution for 1 min, hematoxylin dyeing solution for 3-5 min, washed with tap water, differentiation solution for differentiation, washed with tap water, return blue solution for return blue, rinsed with running water; dehydrated with 95% alcohol for 1 min, and stained into eosin dyeing solution for 15 s; anhydrous ethanol I2min—anhydrous ethanol II2min—anhydrous ethanol III2min—n-butanol I2min—n-butanol II2min-dimethyl I2min-xylene II2min transparent, neutral gum sealing, microscopic observation of histological morphology and scanning and photographing using panoramic scanner scanning software. The rumen papillae, papillae width, muscular layer thickness, rumen wall thickness, cuticle thickness and calculated papillae density, rumen papillae specific surface area using (Image-ProPlus6.0, United States) analysis software, one field of view was selected for each section, and histomorphology was counted in each field of view.

2.6 Measurement of rumen fermentation parameters

The concentrations of acetic acid (AA), propionic acid (PA), butyric acid (BA), valeric acid (VA), isobutyric acid (IBA), and lactic acid (LA) were detected by gas chromatography (Agilent 7890B, NYSE: A, Palo Alto, CL, United States) with reference to the method of Wang et al. (21). A total of 0.2 mL of 25% metaphosphoric acid was added to 1 mL of rumen fluid sample and mixed properly. Subsequently, the mixed samples were stored at −20°C for more than 24 h. The samples were then centrifuged at 10,000 rpm for 10 min at 4°C, and 1.0 mL of the supernatant was filtered through a 0.45 μm membrane. The filtrate was then injected into special gas phase vials where 0.4 μL of the sample was automatically injected into the HP-INNOWax gas phase

capillary column. The injector and detector temperatures were set to 250°C and 280°C, respectively, and the split ratio was set to 40:1. The column was heated from 120°C to 250°C at 10°C/min. Ammonia-nitrogen concentration (NH_3-N in rumen fluid) was determined by a colorimetric method as described by Ma et al. (22).

2.7 Determination of rumen microbial flora in rumen contents

Total genomic DNA samples were extracted using the OMEGA Soil DNA Kit (M5635-02) (Omega Bio-Tek, Norcross, GA, United States), following the manufacturer's instructions, and stored at −20°C prior to further analysis. The quantity and quality of extracted DNAs were measured using a NanoDrop NC2000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA) and agarose gel electrophoresis, respectively. PCR amplification of the bacterial 16S rRNA genes V3–V4 region was performed using the forward primer 338F (5'-ACTCCTACGGGAGGCAGCA-3′) and the reverse primer 806R (5'-GGACTACHVGGGTWTCTAAT-3′). Sample-specific 7-bp barcodes were incorporated into the primers for multiplex sequencing. The PCR components contained 5 μL of buffer (5×), 0.25 μL of Fast pfu DNA Polymerase (5 U/μL), 2 μL (2.5 mM) of dNTPs, 1 μL (10 uM) of each Forward and Reverse primer, 1 μL of DNA Template, and 14.75 μL of ddH2O. Thermal cycling consisted of initial denaturation at 98°C for 5 min, followed by 25 cycles consisting of denaturation at 98°C for 30 s, annealing at 53°C for 30 s, and extension at 72°C for 45 s, with a final extension of 5 min at 72°C. PCR amplicons were purified with Vazyme VAHTSTM DNA Clean Beads (Vazyme, Nanjing, China) and quantified using the Quant-iT PicoGreen dsDNA Assay Kit (Invitrogen, Carlsbad, CA, United States). After the individual quantification step, amplicons were pooled in equal amounts, and pair-end 2 × 250 bp sequencing was performed using the Illlumina NovaSeq platform with NovaSeq 6,000 SP Reagent Kit (500 cycles) at Shanghai Personal Biotechnology Co., Ltd. (Shanghai, China). The original 16S rRNA sequence data has been submitted to NCBI with the accession number PRJNA1159108.

2.8 Data processing and statistical analysis

Experimental data were organized and calculated using Excel 2019. Statistical analysis of the data was performed using SPSS 23.0 with one-way analysis of variance (ANOVA), and the results were expressed as means and standard error of the mean (SEM). Duncan's method of multiple comparisons was used for the test of variance, and $p < 0.05$ was used to indicate significant differences.

3 Results

3.1 Impact of moringa polysaccharides on the growth performance of goat kids

As shown in Table 2, initial body weight and milk replacer intake did not differ significantly among groups ($p > 0.05$); however, final body weight, average daily gain, openings, and green hay intake were significantly higher in the LOW and HIG groups compared to the CON group ($p < 0.05$).

TABLE 2 Effects of moringa oligosaccharides on the growth performance of early weaned goat kids.

Items	CON	LOW	HIG	SEM	*p*-Value
Initial BW (kg)	3.01	3.06	3.09	0.14	0.976
Final BW (kg)	5.40^{b}	6.95^{a}	7.36^{a}	0.30	0.009
ADG (g/d)	44.96^{b}	73.45^{a}	80.54^{a}	4.92	0.020
Milk replacer intake (g/d)	490.95	495.96	483.06	14.82	0.938
Starter intake (g/d)	284.11^{b}	519.24^{a}	582.39^{a}	49.11	0.032
Green hay intake (g/d)	90.79^{b}	165.29^{a}	166.32^{a}	10.68	0.003

CON, the milk replacer (n = 7); LOW, supplemented with 0.15% MOP in the milk replacer (n = 7); HIG, supplemented with 0.3% MOP in the milk replacer (n = 7); BW, body weight; ADG, average daily gain; $\bar{x}$ represents mean; SEM standard error of the mean. Same lowercase letters within the same row indicate no significant difference ($p > 0.05$), while different lowercase letters indicate a significant difference ($p < 0.05$). The same applies to the following tables.

3.2 Effect of moringa polysaccharides on serum biochemical parameters of goat kids

As shown in Table 3, the addition of MOP had no significant effect on GLU, TP, ALB, BUN, TG, TC, HDL, LDL, ALT, AST and CRE contents of goat kids ($p > 0.05$), in which the TG content showed a decreasing trend with the increase of MOP additions ($p = 0.088$); GLB content of the HIG group was significantly lower than that of the CON group ($p < 0.05$), the difference in GLB content between the LOW group and the CON and HIG groups was not significant ($p > 0.05$); the A/C ratio of the CON group was significantly lower than that of the LOW and HIG groups ($p < 0.05$).

3.3 The effect of moringa polysaccharides on serum antioxidant indices in goat kids

As shown in Table 4, the addition of MOP had no significant effect on T-AOC and CAT contents of goat kids ($p > 0.05$); the GSH-Px content of the LOW group was significantly lower than that of the CON group ($p < 0.05$), and the difference was not significant compared with that of the HIG group ($p > 0.05$); and the contents of SOD and MDA in the LOW group and the HIG group were significantly lower than that of the CON group ($p < 0.05$).

3.4 Effect of moringa polysaccharides on the content of serum immunoglobulins and cytokines in goat kids

As shown in Table 5, the effects of adding MOP on IFN-γ, IL-2 and IL-6 contents of goat kids were not significant among the groups

TABLE 3 Effect of moringa polysaccharides on serum biochemical parameters of goat kids.

Items	CON	LOW	HIG	SEM	*p*-Value
GLU (mmol/L)	4.11	5.34	4.78	0.28	0.199
TP (g/L)	51.11	47.72	47.16	0.88	0.131
GLB (g/L)	32.81^{a}	29.87^{ab}	26.74^{b}	1.05	0.028
ALB (g/L)	19.06	20.26	20.42	0.49	0.522
A/G	0.52^{b}	0.71^{a}	0.76^{a}	0.04	0.021
BUN (mmol/L)	7.31	7.88	6.25	0.34	0.125
TG (mmol/L)	0.37	0.13	0.17	0.05	0.088
TC (g/L)	1.37	1.74	1.26	0.11	0.175
HDL (mmol/L)	0.97	0.97	1.01	0.04	0.930
LDL (mmol/L)	1.17	1.41	1.10	0.07	0.180
ALT (U/L)	27.83	28.19	31.41	0.78	0.105
AST (U/L)	106.54	90.40	92.87	3.61	0.137
CRE (μmol/l)	13.41	6.20	7.24	1.68	0.166

CON, the milk replacer ($n = 3$); LOW, supplemented with 0.15% MOP in the milk replacer (n = 3); HIG, supplemented with 0.3% MOP in the milk replacer ($n = 3$).

TABLE 4 Effect of moringa polysaccharides on serum antioxidant indices in goat kids.

Items	CON	LOW	HIG	SEM	*p*-Value
T-AOC (U/L)	0.75	0.74	0.74	0.01	0.930
CAT (U/mL)	0.28	0.18	0.21	0.02	0.146
GSH-Px (mol/L)	105.67^{a}	79.40^{b}	97.91^{ab}	4.76	0.036
SOD (U/mL)	195.34^{a}	174.69^{b}	172.11^{b}	3.81	<0.001
MDA (nmol/mL)	2.55^{a}	1.98^{b}	2.02^{b}	0.12	0.049

CON, the milk replacer ($n = 3$); LOW, supplemented with 0.15% MOP in the milk replacer ($n = 3$); HIG, supplemented with 0.3% MOP in the milk replacer ($n = 3$).

($p > 0.05$); the IgA and IgM contents of the HIG group were significantly higher than those of the CON group ($p < 0.05$), and the IgA and IgM contents of the LOW group were not significantly different from those of the CON and HIG groups ($p > 0.05$); the IgG and TNF-α content was significantly higher than that of CON group ($p < 0.05$), but the difference between the two groups was not significant ($p > 0.05$).

3.5 The effect of moringa polysaccharides on rumen fermentation parameters in goat kids

Rumen fermentation parameters are summarized in Table 6. There were no significant differences among the Total VFA, AA, and IBA groups ($p > 0.05$). PA, BA, VA, and NH3-N in the CON group were significantly higher than those in the LOW and HIG groups ($p < 0.05$). The AA:PA ratio in the HIG group was significantly higher than in the LOW and CON groups ($p < 0.05$). The pH value of the HIG group was significantly higher than that of the CON group ($p < 0.05$), but there was no significant difference between the HIG and LOW groups ($p > 0.05$).

3.6 Effect of moringa polysaccharides on the morphology of goat kid rumen tissue

As shown in Figure 1, the CON group displayed short, thick, irregular, and untidy rumen papillae. In contrast, the rumen papillae in the LOW and HIG groups were elongated, neat, and relatively regular. The effect of moringa polysaccharides on the morphology of lamb rumen tissue is shown in Table 7. The differences among groups in papilla width, cuticle thickness and specific surface area of rumen papillae were not significant ($p > 0.05$); papilla height in the HIG and LOW groups was significantly higher than that in the CON group ($p < 0.05$); the thickness of the muscularis propria and the thickness of the rumen wall in the HIG group was significantly higher than that in the LOW and CON groups ($p < 0.05$); and the density of papillae in the CON group was significantly higher than that in the LOW and HIG groups ($p < 0.05$).

3.7 Effect of moringa polysaccharides on the microbial flora of goat kids

In this experiment, 12 samples of rumen contents from goat kids were sequenced and analyzed using IlluminaNovaseqMiseq platform, and a total of 944,860 original sequences were obtained, and quality control was performed on the obtained sequences, and a total of 878,895 valid sequences were obtained after removing low-quality, short-length, and chimerism. The valid sequences were clustered into OTUs according to 100% similarity, and a total of 7,670 OTUs were obtained, of which 247 OTUs were common to the three treatments, and 2,457, 2,433 and 2,215 OTUs were specific to the CON, LOW and HIG groups, respectively (Figure 2A). The observed curves of the species of the 12 samples are shown below, and the Rank Abundance curves reflect the groups' Differences in species uniformity and abundance, as shown in Figure 2B, each sample was similar in uniformity and abundance, and the above results indicate that the sequencing depth of this study is sufficient and reasonable, and the results are stable and reliable, and can be used for subsequent analysis.

3.8 Effect of moringa polysaccharides on the alpha diversity of rumen microbiota in goat kids

Alpha diversity indices are commonly used to assess the diversity and richness of microbial species, and the value of Goods coverage reflects the coverage of OTUs in the samples, and the larger the value is, the more sufficient the sequencing data are, in this experiment, the value of Goods coverage of all the samples was not less than 0.996, which indicated that the depth of this sequencing could basically cover all the species. The results of the indexes in this experiment are shown

TABLE 5 Effect of moringa polysaccharides on the content of serum immunoglobulins and cytokines in goat kids.

Items	CON	LOW	HIG	SEM	*p*-Value
IgA (μg/mL)	34.38^{b}	39.87^{ab}	44.70^{a}	1.70	0.013
IgG (μg/mL)	1568.44^{b}	1932.73^{a}	1987.27^{a}	76.07	0.016
IgM (μg/mL)	17.61^{b}	19.96^{ab}	22.27^{a}	0.84	0.046
IFN-γ (pg/mL)	42.85	40.70	41.21	1.02	0.728
IL-2 (pg/mL)	110.59	113.28	120.67	6.61	0.852
IL-6 (pg/mL)	249.33	240.37	244.36	5.20	0.825
TNF-α (pg/mL)	549.02^{b}	972.87^{a}	865.95^{a}	67.58	0.001

CON, the milk replacer ($n = 3$); LOW, supplemented with 0.15% MOP in the milk replacer ($n = 3$); HIG, supplemented with 0.3% MOP in the milk replacer ($n = 3$).

TABLE 6 Effect of moringa polysaccharides on rumen fermentation parameters in goat kids.

Items	CON	LOW	HIG	SEM	*p*-Value
pH	5.54^{b}	5.83^{ab}	6.01^{a}	0.10	0.020
NH_3-N (G/L)	214.69^{a}	70.02^{b}	75.02^{b}	25.67	0.003
Total VFA (mmol/L)	327.13	148.74	204.09	41.32	0.206
Acetic acid (mmol/L)	96.12	61.64	139.92	27.94	0.564
Propionic acid (mmol/L)	169.87^{a}	65.01^{b}	33.55^{b}	20.00	0.001
Acetate: Propionic	0.53^{b}	1.36^{b}	4.89^{a}	0.68	0.004
Butyric acid (mmol/L)	38.13^{a}	12.96^{b}	19.94^{b}	4.68	0.059
Valeric acid (mmol/L)	15.22^{a}	5.50^{b}	3.77^{b}	1.98	0.019
Isobutyric acid (mmol/L)	7.79	3.63	6.90	0.85	0.103

CON, the milk replacer ($n = 4$); LOW, supplemented with 0.15% MOP in the milk replacer ($n = 4$); HIG, supplemented with 0.3% MOP in the milk replacer ($n = 4$).

in Figures 2D–J, and the alpha diversity index did not produce significant differences among treatment groups ($p > 0.05$).

3.9 Effect of moringa polysaccharides on the beta diversity of rumen microbiota in goat kids

PCoA is an unconstrained data dimensionality reduction method that reflects the similarity between samples based on their distribution distances on the graph. The closer the samples are on the graph, the more similar they are. NMDS analysis focuses more on the ordinal relationships between values and reflects the differences between samples based on the distances between points. In this test the CON group was not clearly distinguished from the other groups and the three groups overlapped each other (Figure 2C).

PERMANOVA analysis was further used in this trial to explore the beta diversity of rumen flora. The results are shown in Table 8, and the differences between the groups were not significant ($p > 0.05$).

3.10 Effect of moringa polysaccharides on the composition of rumen microbiota in goat kids

At the phylum level, Bacteroidetes and Fimicutes were the two main phyla, accounting for more than 67% of the rumen flora of goat kids (Table 9). The HIG group increased the relative abundance of Bacteroidetes by 5.4% compared to the CON group, but there was no significant difference at the phylum level of the phyla between the groups' differences ($p > 0.05$) (Figure 3A).

At the genus level, the top 15 abundant genera were *Prevotella, Olsenella, Ruminococcaceae_Ruminococcus, Sharpea, Treponema, Succinivibrio, Sphaerochaeta, Butyrivibrio, Fibrobacter, Megasphaera, Succiniclasticum, Ruminobacter, YRC22, Anaerovibrio,* and *Schwartzia*.

The relative abundance results are shown in Table 10, the relative abundance of *Olsenella* genus. Increased in the high LOW group compared to the CON group, but there was no significant difference in the bacterial flora between the groups at the genus level ($p > 0.05$) (Figure 3B).

3.11 Stomach microbiota LEfSe analysis

LEfSe analysis is a statistical method commonly used to analyze differences between groups, which not only enables comparisons between different subgroups, but also finds biomarkers that are statistically different. In this study, LDA > 2 was used as the threshold value, and four differential bacteria were screened, which were three families and one genus. Among them, the differential bacteria with large LDA values mainly include the *Ruminococcaceae* family, *Bacillus* genus, *Staphylococcus* genus, and *Staphylococcaceae* family (Figure 4).

4 Discussion

The rumen of ruminant animals plays a crucial role in nutrient digestion and absorption, immune response, and host metabolism (23). Ruminal development in ruminants at a young age is crucial and may have long-term effects on their health and later growth and development. According to Wang et al. (24) the addition of fermented wheat bran polysaccharides (FWBPs) to milk replacer increased daily weight gain and decreased feed to weight ratio in early weaned lambs. Chen et al. (25) added Chinese medicinal polysaccharides (CMPs) to increase the average daily weight gain of lambs and improved the lamb

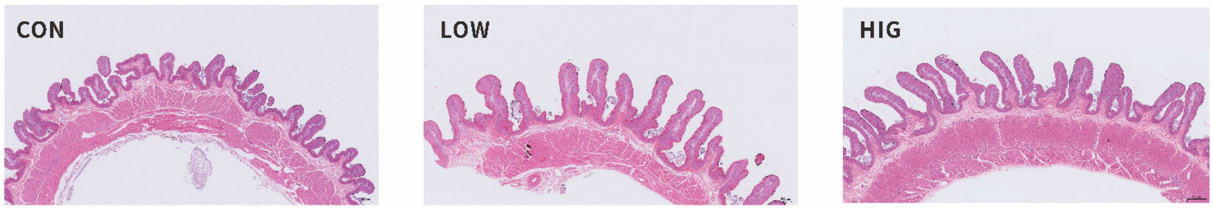

FIGURE 1
The histological sections of the rumen tissue in goat kids.

TABLE 7 Effect of moringa polysaccharides on the morphology of goat kid rumen tissue.

Items	CON	LOW	HIG	SEM	*p*-Value
Rumen papillae height (mm)	0.91^{b}	1.39^{a}	1.46^{a}	0.05	0.000
Rumen papillae width (mm)	0.49	0.48	0.48	0.01	0.819
Rumen muscular layer thickness (mm)	1.05^{b}	0.97^{b}	1.38^{a}	0.06	0.004
Rumen wall thickness (mm)	2.42^{b}	2.70^{b}	3.14^{a}	0.09	0.003
Stratum corneum thickness (mm)	0.0248	0.0237	0.0281	0.00	0.285
Rumen papillae density (items/mm^{2})	2.12^{a}	1.53^{b}	1.48^{b}	0.09	0.004
Rumen papillae surface area ratio	2.14	2.02	2.04	0.08	0.825

CON, the milk replacer ($n = 4$); LOW, supplemented with 0.15% MOP in the milk replacer ($n = 4$); HIG, supplemented with 0.3% MOP in the milk replacer ($n = 4$).

performance. Zhao et al. (18) added MOP to significantly increase the body weights of newborn calves. This is consistent with the significant increase in daily gain, body weight, and body size observed with MOP supplementation in this study. As the growth rate increases, nutritional demand also rises correspondingly. Additionally, in the MOP-added group, intake of green hay and other feeds increased significantly. This suggests that the improvement in growth performance is attributed to enhanced absorption and utilization of nutrients. Therefore, adding MOP can improve nutrient absorption and has a significant impact on promoting the growth and development of goat kids.

Changes in blood biochemical indicators can reflect alterations in the metabolic capacity of the organism. The serum A/G ratio and ALB content can reflect the protein synthesis of the organism, and the decrease of its ratio and content may be related to chronic inflammation caused by the accumulation of inflammatory cytokines (26, 27). The significant increase in the A/G ratio in this experiment indicated that the addition of MOP could reduce weaning stress in lambs and avoid stress-induced oxidative damage to proteins and lipids. Serum TG is a lipid metabolite (27). Addition of MOP in the present study reduced serum triglyceride levels in lambs. This is similar to the findings of Kwon et al. (28), which showed that plant polysaccharides such as MOP can affect lipid metabolism, leading to a decrease in serum TG content in goat kids. T-AOC, GSH-Px, SOD and other enzymes have important antioxidant functions, and T-AOC content can reflect the metabolic status of antioxidant free radicals, while MDA content can reflect the degree of oxidative stress, when the body undergoes oxidative stress, a large number of free radicals will be generated, and free radicals will react with lipids to produce MDA, which is the end product of lipid peroxidation, and the increase of MDA content represents the decrease of TG content. MDA is the end product of lipid peroxidation, and an increase in MDA content represents an increase in the level of oxidative stress in the body (29–31). In this study, there was no significant difference in the T-AOC content in the serum of goat kids among the groups, whereas the addition of MOP decreased the GSH-Px, SOD and MDA content in the serum of goat kids, which may be attributed to the binding of polysaccharides to the surfaces of cell specific surface molecules and thus inhibiting the excess oxygen radicals (7) thus acting to alleviate the level of oxidative stress in the organism. Similarly, Su et al. (32) fed capsaicin also reduced MDA levels, which is consistent with the results of the present study, suggesting that the addition of capsaicin and MOP, a substance with a better biological function, can inhibit the serum levels of excess oxygen radicals in goat kids and protect the organism from oxidative damage.

Immune cells in animal bodies produce cytokines such as interleukins and anti-tumor factors, which have anti-disease functions. In this study, IL-2 and IL-6 had no significant effect among the groups, while TNF-α increased significantly with the increase of MOP addition. TNF-α is a protein produced by a variety of cells, and its main role is to regulate the immune response, promote inflammatory response, and regulate apoptosis (cell death) (33). When the organism is subjected to infection, tissue damage, or other pathological conditions, cells may release more TNF-α. Therefore, elevated levels of TNF-α in the blood or tissues are often considered as one of the indicators of inflammatory states. Elevated levels of TNF-α may indicate that the organism is experiencing an inflammatory response or immune system activation. However, high levels of TNF-α may also be associated with a number of other diseases and pathological conditions. Plant polysaccharides have been reported to improve phagocytosis by macrophages, leading to significantly higher levels of TNF-α (34, 35). It is possible that plant polysaccharides exert their antitumor effects by affecting tumor differentiation and apoptosis, altering intracellular signaling and immune regulation (36, 37). Immunoglobulins IgA, IgM, and IgG, as important immunoreactive molecules in animals, can specifically bind to the corresponding antigens and participate in the regulation

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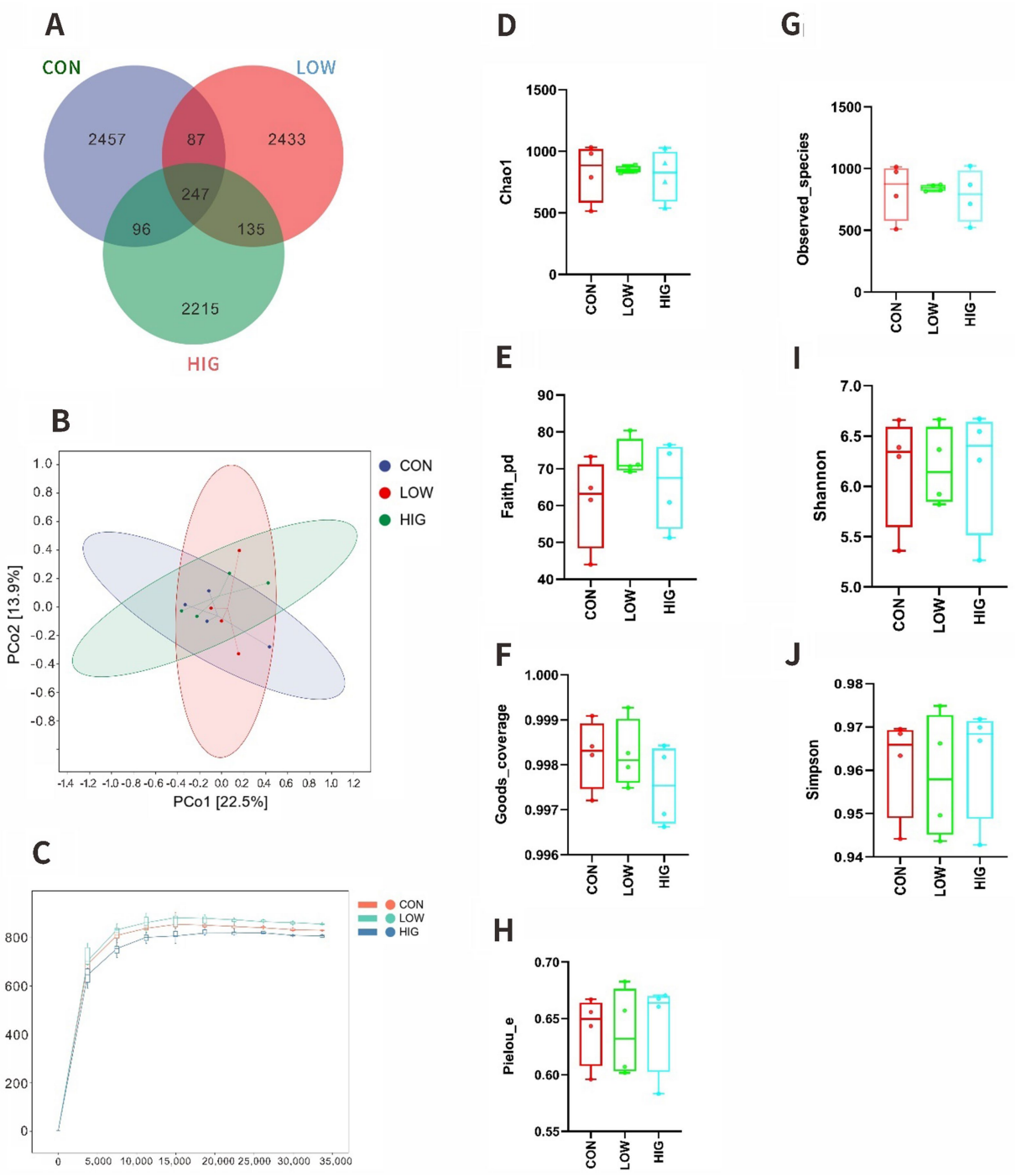

FIGURE 2
Effects of MOP on the alpha and beta diversity of the rumen microbiota in goat kids. Analysis of the alpha and beta diversity via **(A)** Venn figure of OTUs **(B)** Species observation curve in each group. **(C)** PCoA of weighted UniFrac distances, respectively **(D)** Chao1 index, **(E)** Faith_pd index, **(F)** Goods_coverage index, **(G)** Observed_species index, **(H)** Pielou_e index, and **(I)** Shannon index, **(J)** Simpson index; CON, the milk replacer ($n = 4$); LOW, supplemented with 0.15% MOP in the milk replacer ($n = 4$); HIG, supplemented with 0.3% MOP in the milk replacer ($n = 4$).

of body immunity, so that the elevation of the levels of IgA, IgM, and IgG indicates the improvement of immune function. The addition of MOP significantly increased the immunoglobulin level in serum in this study, which is consistent with the results of Chen et al. (25), indicating that plant polysaccharides such as MOP have the effect of increasing immunoglobulin level to enhance the immunity of goat kids.

Chen et al. showed that pH in rumen fluid is closely related to VFA, and that an increase in rumen VFA concentration can lead to a decrease in rumen pH (25). This is similar to the results of the present

TABLE 8 PERMANOVA analysis results.

Items	PERMANOVA		
	F	P	q
CON vs LOW	1.165	0.190	0.467
CON vs HIG	1.128	0.311	0.467
LOW vs HIG	0.795	0.705	0.705

CON, the milk replacer ($n = 4$); LOW, supplemented with 0.15% MOP in the milk replacer ($n = 4$); HIG, supplemented with 0.3% MOP in the milk replacer ($n = 4$).

TABLE 9 Effect of MOP on the relative abundance of rumen microbiota at the phylum level (%).

Items	CON	LOW	HIG	SEM	*p*-Value
Bacteroidetes	39.44	38.10	44.84	3.04	0.676
Firmicutes	34.28	29.45	32.69	1.93	0.627
Actinobacteria	7.04	13.24	9.56	1.74	0.375
Spirochaetes	7.24	9.04	8.36	0.80	0.695
Proteobacteria	8.17	6.11	1.75	1.78	0.356
Fibrobacteres	3.02	2.04	1.03	0.80	0.638
Tenericutes	0.16	0.40	0.34	0.11	0.697
Verrucomicrobia	0.06	0.14	0.28	0.09	0.645
Cyanobacteria	0.02	0.02	0.04	0.00	0.457
Synergistetes	0.02	0.03	0.02	0.00	0.344
Others	0.54	1.43	1.02	0.33	0.595

CON, the milk replacer ($n = 4$); LOW, supplemented with 0.15% MOP in the milk replacer ($n = 4$); HIG, supplemented with 0.3% MOP in the milk replacer ($n = 4$).

study, where the VFA content was higher in the CON group than in the test group, while the pH was lower in the CON group than in the test group. In this study, according to the ratio of acetic acid to propionic acid, the majority of acetic acid fermentation could be judged. Meanwhile, the high level of green hay in the MOP group also proved that the cellulose in the rumen of goat kids was better decomposed to produce acetic acid. The energy produced by VFA in the rumen of lambs during its metabolism can directly stimulate the development of the rumen (38), with BA playing the most prominent role, followed by PA and AA. BA has been reported to have a promotional effect on the growth and development of rumen epithelial cells, and at the same time, it can reduce the apoptosis of rumen epithelial cells (39, 40). And the rumen epithelial tissue, as an important component of rumen function, has the function of transporting nutrients from the rumen to the bloodstream (40). IBA belongs to branched-chain fatty acids, which originate from the fermentation of protein feeds, and the fermentation process is accompanied by hazardous substances such as hydrogen sulfide and cresol, and a low amount of IBA can help to maintain the health of the rumen (24). VFA in the rumen is absorbed through the rumen epithelial cells, with an absorption rate depending on VFA concentration, rumen surface area and availability of transporter proteins (41). Another study showed that decreased serum GLU levels in young ruminants may be associated with incomplete development of the gastrointestinal tract (42). In this experiment, the serum GLU level, rumen papilla length density, muscularis propria thickness, and rumen wall thickness in the CON group were lower than those in the test group with MOP addition. Additionally, the IBA content in the test group was lower than in the CON group, suggesting that the rumen of MOP-added goat kids was more developed, the rumen environment was healthier, and VFA absorption was more efficient. The relative abundance of Succinivibrio and Megasphaera was higher in the CON group than in the MOP-added group. Since Succinivibrio is involved in the synthesis of short-chain fatty acids (43), this may explain why the PA, BA, and VA contents were higher in the CON group compared to the test group. This was further supported by the lack of significant difference in total protein content in serum among the groups. NH_3-N in the rumen is mainly produced by fermentation of ingested protein feeds and is also an important source of microbial protein synthesis. Lv et al. demonstrated that the addition of fermented wheat bran polysaccharides selectively increased rumen microbial populations, which led to a decrease in NH_3-N content and its uptake by the rumen epithelial cells (44). The results of the present study showed that the addition of MOP reduced the NH_3-N content in the rumen of goat kids, and the lower NH_3-N content may be a result of the efficient transport of NH_3-N into the bloodstream by the better developed rumen epithelial tissues to participate in microbial protein synthesis, thus promoting the growth and development of goat kids.

The degree of rumen development can be measured by the development status of rumen epithelial cells, such as rumen papilla length and width (44). The rumen epithelium consists of rumen, muscularis propria, and epithelial cells, which have the functions of nutrient absorption, transportation, and metabolism (24). The fibrous component in the early primary goat kid diet is considered a key factor influencing the rumen development of goat kids. The fiber-rich diet provides the necessary physical stimulation for the physiological development of the rumen of young ruminants (45–47). At the same time, a large number of studies have shown that only after the rumen nipple start to grow and the thickness of the rumen wall begins to increase. Bian et al. (48) show that feeding fiber-rich alfalfa hay promotes the rumen peristalsis, accelerates the fermentation of rumen microorganisms, promotes the development of the rumen muscle layer, and further improves the digestive capacity of goat kids. In this study, the intake of goat kid mouth and green hay in the MOP test group was significantly higher than that in the CON group, and the addition of high-dose MOP significantly increased the height, muscle layer thickness and rumen wall thickness of the goat kids, indicating that the improvement of hay and open feed promoted the development of rumen in lactating ruminants. The proliferation of ruminal epithelial cells can also promote the growth of ruminal papilla and width and improve the muscle layer thickness. Other studies showed that increasing the height and width of the rumen nipple increased the absorption area of the rumen epithelium, but decreased the rumen nipple density (49). This is consistent with the results that adding MOP significantly reduced the density of rumen nipple in this study, but the addition of MOP had no effect on the width, cuticle thickness and specific surface area, which is inconsistent with Wang (24) and other studies, which may have different effects of different types of polysaccharide on the development of goat kids, and its regulation mechanism needs further study.

The rumen, as a specialized digestive organ in ruminants, contains a large number of microbial communities, such as bacteria, fungi, and archaea, which can help the host digest cellulose and other carbohydrates (50, 51). Microbial community colonization is also relevant and important for rumen development in young animals (52).

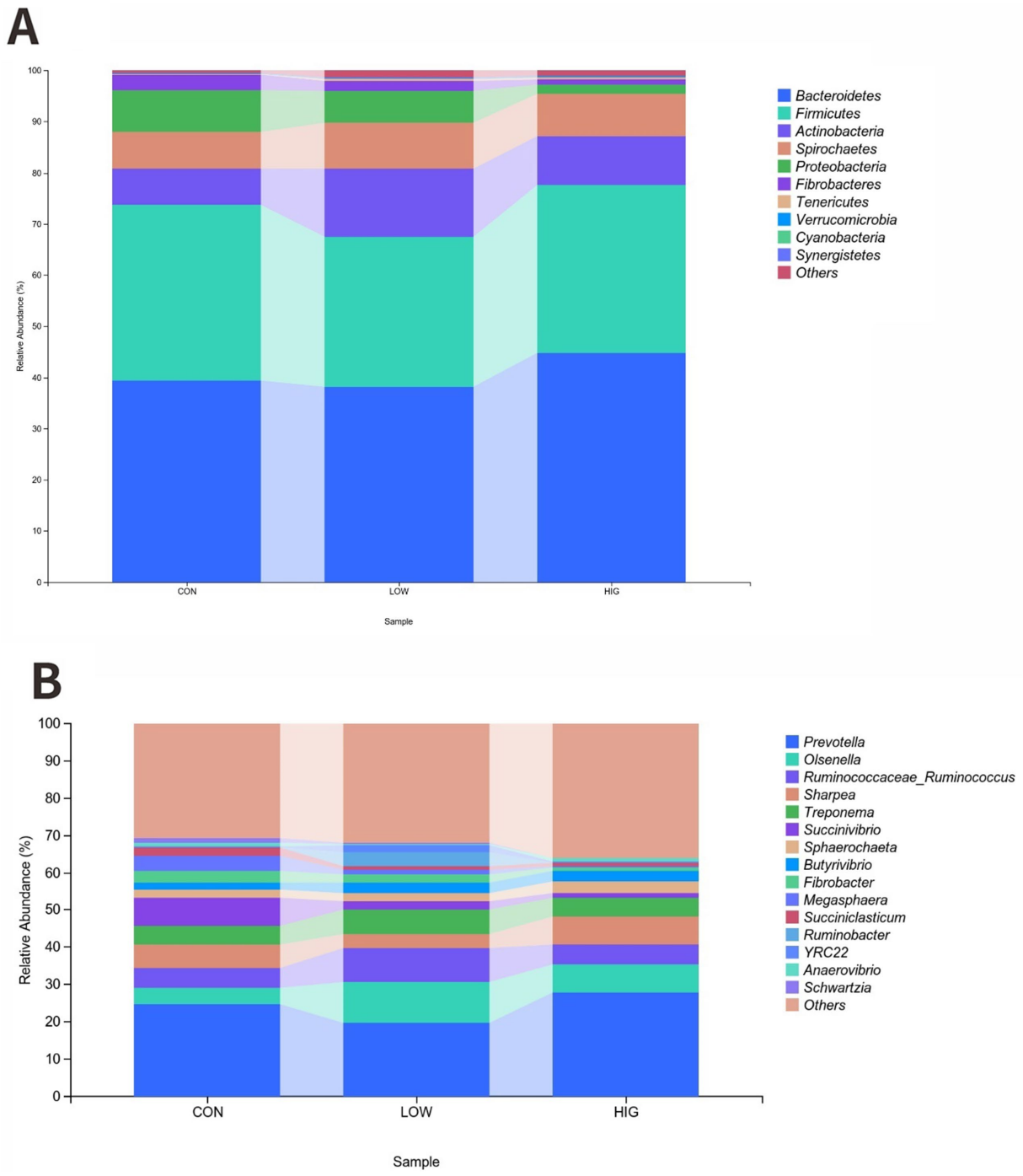

FIGURE 3
Effect of MOP on the composition of the rumen microbial community. **(A)** Phylum level **(B)** Genus level.

Therefore, the effect of MOP addition on the structure and composition of rumen microbiota in goat kids was analyzed by 16SrRNA sequencing. In the present study, there was no significant difference between the CON group and the MOP test group in terms of flora alpha and beta diversity. Wang et al. (24). found that the addition of fermented bran polysaccharides did not have a significant effect on the alpha and beta diversity of the goat kid flora; Chen et al. (25) found that the addition of herbal polysaccharides did not have a significant effect on the alpha and beta diversity of the goat kid flora, which was in agreement with the present study. The current study showed that *Anaplasma* phylum, Thick-walled phylum and *Actinobacteria* phylum are the core phyla of the rumen with the highest relative abundance (1, 53). In this study, MOP had no effect on the structure and composition of the rumen microbiota. This

TABLE 10 Effect of MOP on the relative abundance of rumen microbiota at the genus level (%).

Items	CON	LOW	HIG	SEM	*p*-Value
Prevotella	24.88	19.71	27.82	1.91	0.228
Olsenella	4.09	11.05	7.58	1.44	0.140
Ruminococcaceae_Ruminococcus	5.39	9.06	5.24	1.20	0.372
Sharpea	6.39	3.73	7.71	1.52	0.600
Treponema	5.03	6.71	4.98	1.05	0.780
Succinivibrio	7.50	2.08	1.11	1.52	0.188
Sphaerochaeta	2.21	2.32	3.38	0.73	0.802
Butyrivibrio	1.93	2.70	2.60	0.67	0.900
Fibrobacter	3.02	2.04	1.03	0.80	0.638
Megasphaera	4.02	1.31	0.32	1.34	0.552
Succiniclasticum	2.18	1.13	0.99	0.28	0.159
Ruminobacter	0.05	3.61	0.00	1.20	0.408
YRC22	0.38	1.96	0.29	0.51	0.356
Anaerovibrio	0.91	0.32	0.74	0.15	0.303
Schwartzia	1.22	0.13	0.19	0.34	0.365
Others	30.81	32.14	36.01	2.41	0.701

CON, the milk replacer ($n = 4$); LOW, supplemented with 0.15% MOP in the milk replacer ($n = 4$); HIG, supplemented with 0.3% MOP in the milk replacer ($n = 4$).

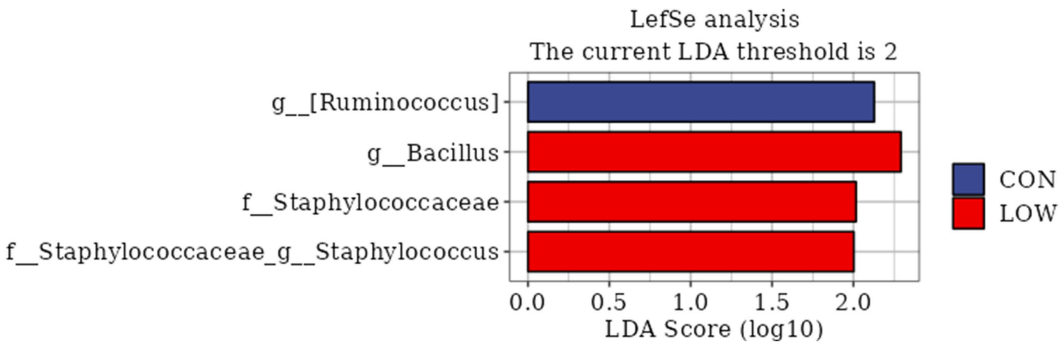

FIGURE 4
Bacterial taxa differences between control and LOW groups. Linear discriminant analysis (LDA) coupled with effect size (LEfSe) analysis was performed to identify the bacterial taxa differentially represented in control and LOW groups at different taxonomy levels. CON, the milk replacer (n = 4); LOW, supplemented with 0.15% MOP in the milk replacer (n = 4).

might be due to the fact that the amount of milk replacer administered or its passage through the esophageal groove into the abomasum and intestines resulted in minimal impact on rumen fermentation and microbiota composition in goat kids (1). In addition, LEfSe analysis showed that goat kids in the LOW group were enriched with differential OTUs; most of these OTUs belonged to the genus Bacillus, and the Bacillus group includes many polysaccharide-degrading bacteria that contribute to the production of VFA in the gut. The cellulose secreted by Bacillus helps the animals to digest fiber, and produces various antimicrobial peptides that maintain the normal microbiota of the animals (54). Based on the present results and references, we hypothesize that MOP supplementation with MR stimulates the proliferation of fibrinolytic bacteria, such as Bacillus spp., which are producers of VFAs. This, in turn, increases the production of VFAs and microbial proteins, accelerates rumen development, and consequently improves the growth performance of early-weaned goat kids.

5 Conclusion

The addition of MOP to milk replacer powder in early-weaned goat kids increased daily weight gain, feed intake, immunoglobulin G, tumor necrosis factor α levels, and rumen height, while decreasing the levels of propionic acid, butyric acid, valeric acid, ammonia nitrogen, and density. Specifically, high doses of MOP (0.3%) significantly

increased serum immunoglobulin A, immunoglobulin M, rumen muscle thickness, rumen wall thickness, and rumen contents pH, and also increased the relative abundance of Actinobacteria and Butyrivibrio species in the goat kid rumen. In summary, the addition of MOP to milk replacer powder for early-weaned goat kids can promote rumen growth and development, and improve immune function and growth performance.

Data availability statement

The data presented in the study are deposited in the Sequence Read Archive (SRA) repository, accession number PRJNA1159108.

Ethics statement

The animal study was approved by Committee of Animal Experiments of South China Agricultural University. The study was conducted in accordance with the local legislation and institutional requirements.

Author contributions

JL: Writing – original draft, Writing – review & editing, Conceptualization, Data curation, Formal analysis, Software, Validation, Visualization. JC: Writing – review & editing, Data curation, Formal analysis, Visualization. SF: Investigation, Writing – review & editing. BS: Formal analysis, Methodology, Writing – review & editing. YL: Formal analysis, Software, Writing – review & editing. YG: Methodology, Supervision, Writing – review & editing. MD: Formal analysis, Supervision, Writing – review & editing. DZ: Investigation, Writing – review & editing. DL: Conceptualization, Funding acquisition, Methodology, Supervision, Writing – review & editing. GL: Conceptualization, Methodology, Project administration, Supervision, Writing – review & editing.

Funding

The author(s) declare that financial support was received for the research, authorship, and/or publication of this article. This work was supported by Science and Technology Program from Forestry Administration of Guangdong Province (2023KJCX004); National Natural Science Foundation of China (32102557); Guangdong Provincial Department of Agriculture and Rural Affairs Project (2022-XPY-00-009).

Conflict of interest

DZ was employed by Guangdong Leader Intelligent Agriculture Co., LTD.

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

Publisher's note

Supplementary material

The Supplementary material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fvets.2024.1461391/full#supplementary-material

References

1. Zhang Q, Li C, Niu X, Zhang Z, Li F, Li F. The effects of milk replacer allowance and weaning age on the performance, nutrients digestibility, and ruminal microbiota communities of lambs. *Anim Feed Sci Technol*. (2019) 257:114263. doi: 10.1016/j.anifeedsci.2019.114263

2. Carballo OC, Khan MA, Knol FW, Lewis SJ, Stevens DR, Laven RA, et al. Impact of weaning age on rumen development in artificially reared lambs1. *J Anim Sci*. (2019) 97:3498–510. doi: 10.1093/jas/skz148

3. Wang S, Chai J, Zhao G, Zhang N, Cui K, Bi Y, et al. The temporal dynamics of rumen microbiota in early weaned lambs. *Microorganisms*. (2022) 10. doi: 10.3390/microorganisms10010144

4. Li C, Wang W, Liu T, Zhang Q, Wang G, Li F, et al. Effect of early weaning on the intestinal microbiota and expression of genes related to barrier function in lambs. *Front Microbiol*. (2018) 9:1431. doi: 10.3389/fmicb.2018.01431

5. Guo G, Yang W, Fan C, Lan R, Gao Z, Gan S, et al. The effects of fucoidan as a dairy substitute on diarrhea rate and intestinal barrier function of the large intestine in weaned lambs. *Front Vet Sci*. (2022) 9:1007346. doi: 10.3389/fvets.2022.1007346

6. Zhang K, Xu Y, Yang Y, Guo M, Zhang T, Zong B, et al. Gut microbiota-derived metabolites contribute negatively to hindgut barrier function development at the early weaning goat model. *Anim Nutr*. (2022) 10:111–23. doi: 10.1016/j.aninu.2022.04.004

7. Liu M, Liu W, Zhang W, Niu J, Yao J. Effect of marine red yeast Rhodosporidium paludigenum on diarrhea rate, serum antioxidant competence, intestinal immune capacity, and microflora structure in early-weaned lambs. *Biomed Res Int*. (2022) 2022:1–7. doi: 10.1155/2022/2228632

8. Guo H, Cui J, Li Q, Liang X, Li J, Yang B, et al. A multi-omic assessment of the mechanisms of intestinal microbes used to treat diarrhea in early-weaned lambs. *mSystems*. (2024) 9:e00923-53. doi: 10.1128/msystems.00953-23

9. Bollen C, Louwagie E, Verstraeten N, Michiels J, Ruelens P. Environmental, mechanistic and evolutionary landscape of antibiotic persistence. *EMBO Rep*. (2023) 24:e57309. doi: 10.15252/embr.202357309

10. Sharma K, Kumar M, Waghmare R, Suhag R, Gupta OP, Lorenzo JM, et al. Moringa (*Moringa oleifera* lam.) polysaccharides: extraction, characterization, bioactivities, and industrial application. *Int J Biol Macromol*. (2022) 209:763–78. doi: 10.1016/j.ijbiomac.2022.04.047

11. Al Husnan LA, Alkahtani MDF. Impact of moringa aqueous extract on pathogenic bacteria and fungi in vitro. *Ann Agric Sci*. (2016) 61:247–50. doi: 10.1016/j.aoas.2016.06.003

12. Özcan MM. Moringa spp: composition and bioactive properties. *S Afr J Bot*. (2020) 129:25–31. doi: 10.1016/j.sajb.2018.11.017

13. Li C, Dong Z, Zhang B, Huang Q, Liu G, Fu X. Structural characterization and immune enhancement activity of a novel polysaccharide from *Moringa oleifera* leaves. *Carbohydr Polym*. (2020) 234:115897. doi: 10.1016/j.carbpol.2020.115897

14. Li C, You L, Fu X, Huang Q, Yu S, Liu RH. Structural characterization and immunomodulatory activity of a new heteropolysaccharide from *Prunella vulgaris*. *Food Funct*. (2015) 6:1557–67. doi: 10.1039/C4FO01039F

15. Schepetkin IA, Quinn MT. Botanical polysaccharides: macrophage immunomodulation and therapeutic potential. *Int Immunopharmacol*. (2006) 6:317–33. doi: 10.1016/j.intimp.2005.10.005

16. Guo M, Li Z. Polysaccharides isolated from *Nostoc commune* Vaucher inhibit colitis-associated colon tumorigenesis in mice and modulate gut microbiota. *Food Funct*. (2019) 10:6873–81. doi: 10.1039/c9fo00296k

17. Tian H, Liang Y, Liu G, Li Y, Deng M, Liu D, et al. *Moringa oleifera* polysaccharides regulates caecal microbiota and small intestinal metabolic profile in C57BL/6 mice. *Int J Biol Macromol*. (2021) 182:595–611. doi: 10.1016/j.ijbiomac.2021.03.144

18. Zhao C, Li H, Gao C, Tian H, Guo Y, Liu G, et al. *Moringa oleifera* leaf polysaccharide regulates fecal microbiota and colonic transcriptome in calves. *Int J Biol Macromol*. (2023) 253:127108. doi: 10.1016/j.ijbiomac.2023.127108

19. Horwitz W, Latimer G. AOAC official methods of analysis of AOAC international. Gaithersburg, MD, USA: AOAC International (2005).

20. Li Y, Guo YL, Zhang CX, Cai XF, Liu P, Li CL. Effects of physical forms of starter feed on growth, nutrient digestibility, gastrointestinal enzyme activity, and morphology of pre- and post-weaning lambs. *Animal*. (2021) 15:100044. doi: 10.1016/j.animal.2020.100044

21. Wang Q, Wang Y, Wang X, Dai C, Tang W, Li J, et al. Effects of dietary energy levels on rumen fermentation, microbiota, and gastrointestinal morphology in growing ewes. *Food Sci Nutr*. (2020) 8:6621–32. doi: 10.1002/fsn3.1955

22. Ma T, Tu Y, Zhang NF, Deng KD, Diao QY. Effect of the ratio of non-fibrous carbohydrates to neutral detergent Fiber and protein structure on intake, digestibility, rumen fermentation, and nitrogen metabolism in lambs. *Asian Australas J Anim Sci*. (2015) 28:1419–26. doi: 10.5713/ajas.15.0025

23. Lin L, Xie F, Sun D, Liu J, Zhu W, Mao S. Ruminal microbiome-host crosstalk stimulates the development of the ruminal epithelium in a lamb model. *Microbiome*. (2019) 7:83. doi: 10.1186/s40168-019-0701-y

24. Wang W, Wang Y, Cui Z, Yang Y, An X, Qi J. Fermented wheat bran polysaccharides intervention alters rumen bacterial community and promotes rumen development and growth performance in lambs. *Front Vet Sci*. (2022) 9:841406. doi: 10.3389/fvets.2022.841406

25. Chen H, Guo B, Yang M, Luo J, Hu Y, Qu M, et al. Response of growth performance, blood biochemistry indices, and rumen bacterial diversity in lambs to diets containing supplemental probiotics and Chinese medicine polysaccharides. *Front Vet Sci*. (2021) 8:681389. doi: 10.3389/fvets.2021.681389

26. Abdelsattar M, Vargas-Bello-Pérez E, Zhang N. Age-related changes in blood biochemical composition of Hu sheep. *Ital J Anim Sci*. (2022) 21:1297–306. doi: 10.1080/1828051X.2022.2108730

27. Abdel-Salam AM, Zeitoun MM, Abdelsalam MM. Effect of synbiotic supplementation on growth performance, blood metabolites, insulin and testosterone and wool traits of growing lambs. *J Biol Sci*. (2014) 14:292–8. doi: 10.3923/jbs.2014.292.298

28. Kwon HJ, Kim Y, Choung SY. Effects of natural product extract on the fatty liver induced by alcohol diet in rats. *J Health Sci*. (2004) 50:466–73. doi: 10.1248/jhs.50.466

29. Nielsen F, Mikkelsen BB, Nielsen JB, Andersen HR, Grandjean P. Plasma malondialdehyde as biomarker for oxidative stress: reference interval and effects of life-style factors. *Clin Chem*. (1997) 43:1209–14. doi: 10.1093/clinchem/43.7.1209

30. Cheng Y, Chen Y, Li J, Qu H, Zhao Y, Wen C, et al. Dietary beta-sitosterol regulates serum lipid level and improves immune function, antioxidant status, and intestinal morphology in broilers. *Poult Sci*. (2020) 99:1400–8. doi: 10.1016/j.psj.2019.10.025

31. Yang W, Chen J, Guo G, Wang S, Peng S, Gao Z, et al. The effects of Fucoidan dietary supplementation on growth performance, serum antioxidant capacity, immune function indices and intestinal morphology in weaned kids. *Animals (Basel)*. (2022) 12:574. doi: 10.3390/ani12050574

32. Su M, She Y, Deng M, Guo Y, Li Y, Liu G, et al. The effect of capsaicin on growth performance, antioxidant capacity, immunity and gut Micro-organisms of calves. *Animals (Basel)*. (2023) 13. doi: 10.3390/ani13142309

33. Kushibiki S. Tumor necrosis factor-alpha-induced inflammatory responses in cattle. *Anim Sci J*. (2011) 82:504–11. doi: 10.1111/j.1740-0929.2011.00931.x

34. Yin M, Zhang Y, Li H. Advances in research on immunoregulation of macrophages by plant polysaccharides. *Front Immunol*. (2019) 10:10. doi: 10.3389/fimmu.2019.00145

35. Xie J, Wu D, Li W, Ning C, Tang Y, Zhao J, et al. Effects of polysaccharides in *Lycium Barbarum* berries from different regions of China on macrophages function and their correlation to the Glycosidic linkages. *J Food Sci*. (2017) 82:2411–20. doi: 10.1111/1750-3841.13813

36. Peng Y, Zhu X, Yang G, Zhang J, Wang R, Shen Y, et al. Ultrasonic extraction of *Moringa oleifera* seeds polysaccharides: optimization, purification, and anti-inflammatory activities. *Int J Biol Macromol*. (2024) 258:128833. doi: 10.1016/j.ijbiomac.2023.128833

37. Du X, Zhao Y, Ma Y, Xing H, Li X. Effect of Radix Hedysari polysaccharide on glioma by cell cycle arrest and TNF-α signaling pathway regulation. *Int J Polym Sci*. (2019) 2019:1–7. doi: 10.1155/2019/2725084

38. Sun D, Yin Y, Guo C, Liu L, Mao S, Zhu W, et al. Transcriptomic analysis reveals the molecular mechanisms of rumen wall morphological and functional development induced by different solid diet introduction in a lamb model. *J Anim Sci Biotechnol*. (2021) 12:33. doi: 10.1186/s40104-021-00556-4

39. Mentschel J, Leiser R, Mulling C, Pfarrer C, Claus R. Butyric acid stimulates rumen mucosa development in the calf mainly by a reduction of apoptosis. *Arch Tierernahr*. (2001) 55:85–102. doi: 10.1080/17450390109386185

40. Malhi M, Gui H, Yao L, Aschenbach JR, Gabel G, Shen Z. 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*. (2013) 96:7603–16. doi: 10.3168/jds.2013-6700

41. Petrič D, Mravčáková D, Kucková K, Kišidayová S, Cieslak A, Szumacher-Strabel M, et al. Impact of zinc and/or herbal mixture on ruminal fermentation, microbiota, and histopathology in lambs. *Front Vet Sci*. (2021) 8. doi: 10.3389/fvets.2021.630971

42. Belanche A, Palma-Hidalgo JM, Nejjam I, Jiménez E, Martín-García AI, Yáñez-Ruiz DR. Inoculation with rumen fluid in early life as a strategy to optimize the weaning process in intensive dairy goat systems. *J Dairy Sci*. (2020) 103:5047–60. doi: 10.3168/jds.2019-18002

43. Shao P, Sha Y, Liu X, He Y, Guo X, Hu J, et al. Astragalus additive in feed improved serum immune function, rumen fermentation and the microbiota structure of early-weaned lambs. *J Appl Microbiol*. (2023) 134:1–14. doi: 10.1093/jambio/lxad278

44. Lv F, Wang X, Pang X, Liu G. Effects of supplementary feeding on the rumen morphology and bacterial diversity in lambs. *PeerJ*. (2020) 8:e9353. doi: 10.7717/peerj.9353

45. Norouzian MA, Valizadeh R, Vahmani P. Rumen development and growth of Balouchi lambs offered alfalfa hay pre- and post-weaning. *Trop Anim Health Prod*. (2011) 43:1169–74. doi: 10.1007/s11250-011-9819-z

46. Xie B, Huang W, Zhang C, Diao Q, Cui K, Chai J, et al. Influences of starter NDF level on growth performance and rumen development in lambs fed isocaloric and isonitrogenous diets. *J Anim Sci*. (2020) 98:1–8. doi: 10.1093/jas/skaa093

47. Van Soest PJ, Robertson JB, Lewis BA. Methods for dietary fiber, neutral detergent fiber, and nonstarch polysaccharides in relation to animal nutrition. *J Dairy Sci*. (1991) 74:3583–97. doi: 10.3168/jds.S0022-0302(91)78551-2

48. Bian G, Yu S, Cheng C, Huang H, Liu J. Ruminal microbiota-host crosstalks promote ruminal epithelial development in neonatal lambs with alfalfa hay introduction. *mSystems*. (2024) 9:e0103423. doi: 10.1128/msystems.01034-23

49. Itnan R, Voigt J, Schönhusen U, Wegner J, Kokardova M, Hagemeister H, et al. Influence of dietary concentrate to forage ratio on the development of rumen mucosa in calves. *Arch Anim Nutr*. (1998) 51:279–91. doi: 10.1080/17450399809381926

50. Edwards JE, McEwan NR, Travis AJ, Wallace RJ. 16S rDNA library-based analysis of ruminal bacterial diversity. *Antonie Van Leeuwenhoek*. (2004) 86:263–81. doi: 10.1023/B:ANTO.0000047942.69033.24

51. Mizrahi I, Wallace RJ, Morais S. The rumen microbiome: balancing food security and environmental impacts. *Nat Rev Microbiol*. (2021) 19:553–66. doi: 10.1038/s41579-021-00543-6

52. Arshad MA, Hassan FU, Rehman MS, Huws SA, Cheng Y, Din AU. Gut microbiome colonization and development in neonatal ruminants: strategies, prospects, and opportunities. *Anim Nutr*. (2021) 7:883–95. doi: 10.1016/j.aninu.2021.03.004

53. Yin X, Ji S, Duan C, Tian P, Ju S, Yan H, et al. Age-related changes in the ruminal microbiota and their relationship with rumen fermentation in lambs. *Front Microbiol*. (2021) 12:679135. doi: 10.3389/fmicb.2021.679135

54. Zhang K, Li B, Guo M, Liu G, Yang Y, Wang X, et al. Maturation of the goat rumen microbiota involves three stages of microbial colonization. *Animals (Basel)*. (2019) 9:1028. doi: 10.3390/ani9121028

frontiers | Frontiers in Veterinary Science

TYPE Original Research
PUBLISHED 12 November 2024
DOI 10.3389/fvets.2024.1482800

OPEN ACCESS

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RECEIVED 18 August 2024
ACCEPTED 25 October 2024
PUBLISHED 12 November 2024

CITATION
Zhang L, Qin G, Guo J, Zhang M, E G, Huang Y and Han Y (2024) Effect of dietary supplementation of yeast culture *Saccharomyces cerevisiae* in lactating female goats. *Front. Vet. Sci.* 11:1482800. doi: 10.3389/fvets.2024.1482800

Effect of dietary supplementation of yeast culture *Saccharomyces cerevisiae* in lactating female goats

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This study was designed to investigate the effects of adding a novel yeast culture, *Saccharomyces cerevisiae* refermented sorghum distiller's dried grains with solubles (SSDDGS), to the diets of lactating female goats on lactation performance and lamb growth performance. We divided 10 lactating Dazu black goats of similar age, weight, and offspring into two groups: one fed a pelleted diet with 50 g/day SSDDGS (ET), and the other without SSDDGS as a control (EC) for 7 weeks. We monitor the weight changes of each goat and collect blood and milk samples from experimental ewes at specific times for hormone and milk composition determination. We use ultra-performance liquid chromatography tandem mass spectrometry (UPLC-MS/MS) to detect metabolites in the serum of lactating ewes. Our results showed that SSDDGS supplementation significantly reduced female goats' average daily weight loss during weeks 2–4 of lactation and increased serum IGF-1 and prolactin levels at week 4 ($p < 0.05$). SSDDGS supplementation in early lactation significantly increased milk protein, lactose, and ash content ($p < 0.05$). UPLC-MS/MS analysis showed that SSDDGS changed the levels of 58 metabolites in the serum of lactating goats. These metabolites were mainly involved in the sohingolipid signaling pathway, and cysteine, methionine, and sphingolipid metabolism. In summary, Yeast culture SSDDGS reduced weight loss, enhanced milk quality, and modified metabolic profiles in early lactation goats, providing insight into the potential regulatory role and mechanism of yeast culture in lactation female goats.

KEYWORDS

yeast culture, lactation, weight, metabolism, female goats

1 Introduction

The low fertility rate of goats poses a major challenge to the development of the goat industry. In addition to the number of lambs, the survival rate of lambs is also an extremely important reproductive trait, while the breast milk is the main source of nutrition for lambs. The digestive and circulatory system of female goats are weak in the early stages of lactation, and undergo significant metabolic shifts and energy allocation changes including weight loss, increased glucose intake, and compromised immunity (1). Insufficient or unbalanced intake of nutrients from feed will easily lead to a decline in the health status of female goats, which cannot meet the needs of rapid growth and development of lambs,

TABLE 1 Nutrient level of full-value pelleted diet fed to lactating female goats (dry matter basis).

Nutrient level	Content
Digestible energy, MJ/kg	11.1
Crude protein, %	12.5
Crude fiber, %	15.7
Crude ash, %	10.0
Ca, %	1.0
P, %	0.6

causing slow growth and even death of lamb (2). Therefore, it is critical to design a feed suitable for lactating female goats to improve lactation performance in early lactation.

Yeast culture (YC) mainly consists of yeast extracellular metabolites, modified medium after fermentation and a small amount of inactive yeast cells. Supplementing YC in ruminant diet can improve the rumen pH environment, the efficiency of crude fiber digestibility and growth performance (3, 4). YC can increase body score, milk production and quality in the early lactation stage of dairy cows and sows (5–8). *Saccharomyces cerevisiae* refermented sorghum distiller's dried grains with solubles (SSDDGS) is a novel YC that is produced by re-fermentation of the distiller's grains substrate, which greatly increased the yield and reduced the cost of yeast culture production. The supplementation of SSDDGS in pig diet improved the lactation performance of sows (9). However, the study on the effect of the novel YC SSDDGS on the goat lactation performance remains uncertain.

Therefore, this study aims to determine the effect of supplementing a novel yeast culture SSDDGS in lactating goat diet on the weight loss of female goat, lactation performance, hormones and metabolites.

2 Materials and methods

2.1 Animal handling and sample collection

All animal tests and handling adhered to the regulations set forth by the Southwestern University Institutional Animal Care and Use Committee (IACUC-20210515-05). The feeding experiment was conducted at Tengda Animal Husbandry, Inc. in Chongqing, China. Ten lactating female goats who had just given birth with comparable weights were randomly divided them into two groups, and each female goats with twin lambs. One group served as the EC group and was fed a full-value pelleted diet (906) procured from Pizhou Xiaohe Technology Development Ltd, and the nutritional level of the diets are shown in Table 1. The other group constituted the ET group received a full-value pelleted diet (906) supplemented with 50 g/d novel yeast culture of *Saccharomyces cerevisiae* refermented sorghum distiller's dried grains with solubles (SSDDGS). Both groups of lactating female goats were given the same weight of feed twice daily at 7 a.m. and 3 p.m.

The experimental period started after 1 week of prefeeding, and the formal feeding period was 7 weeks, from weeks 2 to 8 of lactation. Peripheral blood and milk samples of lactating female goats were collected at week 4 and 8 of lactation. Blood samples were placed overnight at 4°C and then centrifuged at 3,000 rpm for 10 min, and the serum sample was collected. The weight of lactating female goats and offspring lambs was recorded at 4 and 8 weeks of lactation, and the ADL (average daily weight loss) of female goats and ADG of offspring lambs at 2–4 and 2–8 weeks of lactation.

2.2 Determination of hormones and milk composition

These concentrations of serum growth hormone releasing hormone (GHRH), somatostatin (SS), insulin-like growth factor-1 (IGF-1), prolactin (PRL), growth hormone (GH) in lactating female goats were quantified at week 4 and 8 of lactation via radioimmunoassay at the Sino-British Institute of Biotechnology in Beijing, China. Additionally, these milk components, including milk fat, protein, lactose, ash content and dry matter, were tested using an automatic milk composition analyzer (LACTOSCAN MCC 50, Milkotronic, Bulgaria).

2.3 Widely targeted metabolomics analysis of goat serum

Metabolites from the serum of lactating goat were determined by UPLC-MS/MS, according to the previous studies (10–12). Briefly, 50 μL of the sample and 300 μL of an extraction solution (comprising CAN and Methanol in a 1:4 ratio; V/V) containing internal standards was centrifuged at 12,000 rpm at 4°C and then the supernatant were used for UPLC-MS analysis. The samples were analyzed using a UPLC system that featured a UPLC column (2.1 mm × 100 mm, 1.8 μm; Waters ACQUITY UPLC HSS T3 C18). The column temperature was maintained at 40°C and the flow rate was set at 0.4 mL/min. The solvent system consisted of A (water with 0.1% formic acid) and B (acetonitrile with 0.1% formic acid). The gradient program of the system was as follows: 95:5 V/V at 0 min; 10:90 V/V at 11.0 min, 10:90 V/V at 12.0 min; 95:5 V/V at 12.1 min; and 95:5 V/V at 14.0 min. The injection volume was 2 μL. To ensure system stability, one QC sample was introduced every 10 test samples.

Analysis of the samples was conducted using an MS/MS system equipped with an ESI Turbo Ion-Spray interface, which operated in both positive and negative ion modes. The operational parameters of the ESI source were as follows: source temperature, 500°C; ion spray voltage (IS), 5,500 V (positive) and −4,500 V (negative); and ion source gases I, II, and curtain gas were maintained at 55, 60, and 25.0 psi, respectively.

2.4 Statistical analysis

ADL of female goat, ADG of lambs, hormone concentrations, milk composition data were analyzed using a general linear model in GraphPad Prism8.0.1 software (San Diego, CA, USA), with treatment and time as the fixed factors, and the individual samples as a random factor. The interaction of treatment and time was

TABLE 2 Effects of SSDDGS on the ADL of the lactation female goats and the ADG of offspring lambs.

Item	Week 2–4 of lactation		Week 2–8 of lactation		SEM	*p*-value		
	ET	EC	ET	EC		Treatment	Time	Treatment × Time
ADL of female goats, kg	0.08[a]	0.22[b]	0.08[a]	0.10[ab]	0.02	0.016	0.048	0.070
ADG of lambs, kg	0.10[a]	0.08[a]	0.09[a]	0.10[a]	0.01[a]	0.447	0.732	0.035

[a,b]Different superscript letters represent significant differences in the same row ($p < 0.05$). SSDDGS, Saccharomyces cerevisiae refermented sorghum distiller's dried grains with solubles; ADG, Average daily weight gain; ADL, Average daily weight loss; ET, SSDDGS treatment group; EC, control group.

TABLE 3 Effects of SSDDGS on the hormone concentration of female goats.

Hormone concentration	Week 4 of lactation		Week 8 of lactation		SEM	*p*-value		
	ET	EC	ET	EC		Treatment	Time	Treatment × time
GHRH, ng/mL	42.90	40.70	47.58	42.29	1.18	0.114	0.180	0.500
SS, pg/mL	20.78	21.96	21.63	21.83	0.47	0.500	0.723	0.633
IGF-1, ng/mL	185.78[a]	154.67[b]	156.89[b]	144.10[b]	4.56	0.003	0.006	0.161
PRL, μIU/mL	245.97[a]	223.17[b]	255.53[ab]	240.10[ab]	3.92	0.007	0.048	0.561
GH, ng/ml	4.65	4.62	5.58	4.72	0.23	0.353	0.283	0.382

[a,b]Different superscript letters represent significant differences in the same row ($p < 0.05$). GHRH, growth hormone releasing hormone; SS, somatostatin; IGF-1: insulin-like growth factor-1; PRL, prolactin; GH, growth hormone.

TABLE 4 Effects of SSDDGS on the milk composition in lactating female goats.

Milk composition	Week 4 of lactation		Week 8 of lactation		SEM	*p*-value		
	ET	EC	ET	EC		Treatment	Time	Treatment × time
Fat, %	4.92	4.80	6.35	5.88	0.32	0.648	0.061	0.786
Protein, %	3.60[a]	3.34[b]	3.50[ab]	3.30[ab]	0.05	0.026	0.477	0.768
Lactose, %	5.43[a]	5.04[b]	5.30[ab]	5.00[ab]	0.07	0.020	0.531	0.762
Ash content, %	0.33[a]	0.30[b]	0.32[ab]	0.30[ab]	0.00	0.018	0.539	0.712
Dry matter, %	14.29	13.51	15.02	14.97	0.28	0.443	0.054	0.494

[a,b]Different superscript letters represent significant differences in the same row ($p < 0.05$).

included in the model. These differences between the means were analyzed using Duncan's multiple comparisons.

Multivariate statistical analysis included unsupervised principal component analysis (PCA) and supervised orthogonal partial least squares-discriminant analysis (OPLS-DA). The variable importance in $p \leq 0.05$, projection (VIP) ≥ 1 and log2FC (fold-change) ≤ 0.67 or log2FC ≥ 1.5 represented a significant difference in metabolites between the ET group and the EC group. The identified metabolites were annotated using the KEGG compound database, and KEGG pathways were used for metabolite set enrichment analysis. A *p*-value obtained from the hypergeometric test of <0.05 indicated biological significance. $P < 0.05$ was considered to be statistically significant. Results are expressed as the mean ± the standard error (SEM).

3 Results

3.1 Changes in weight of lactating female goats and offspring lambs

The average daily weight loss (ADL) of lactating female goats in the experimental treatment (ET) group was significantly lower during week 2–4 of lactation than that in the experimental control (EC) group (Table 2, $p < 0.05$). There was no significant difference in the average daily weight gain (ADG) of offspring lambs between the ET and EC groups during week 2–4 or 2–8 of lactation (Table 2, $p > 0.05$).

3.2 Changes in serum hormones in lactating female goats

Serum insulin-like growth factor-1 (IGF-1) or prolactin (PRL) concentration in the ET group was significantly higher than that in the EC group at week 4 of lactation (Table 3, $p < 0.05$). In addition, in the ET group, IGF-1 concentration at week 8 of lactation was significantly lower than that at week 4 of lactation (Table 3, $p < 0.05$).

3.3 Effects of SSDDGS on milk quality in lactation female goats

Milk composition was more abundant in the ET group compared to the EC group at week 4 of lactation. The content of milk protein, milk lactose or ash in the ET group was significantly

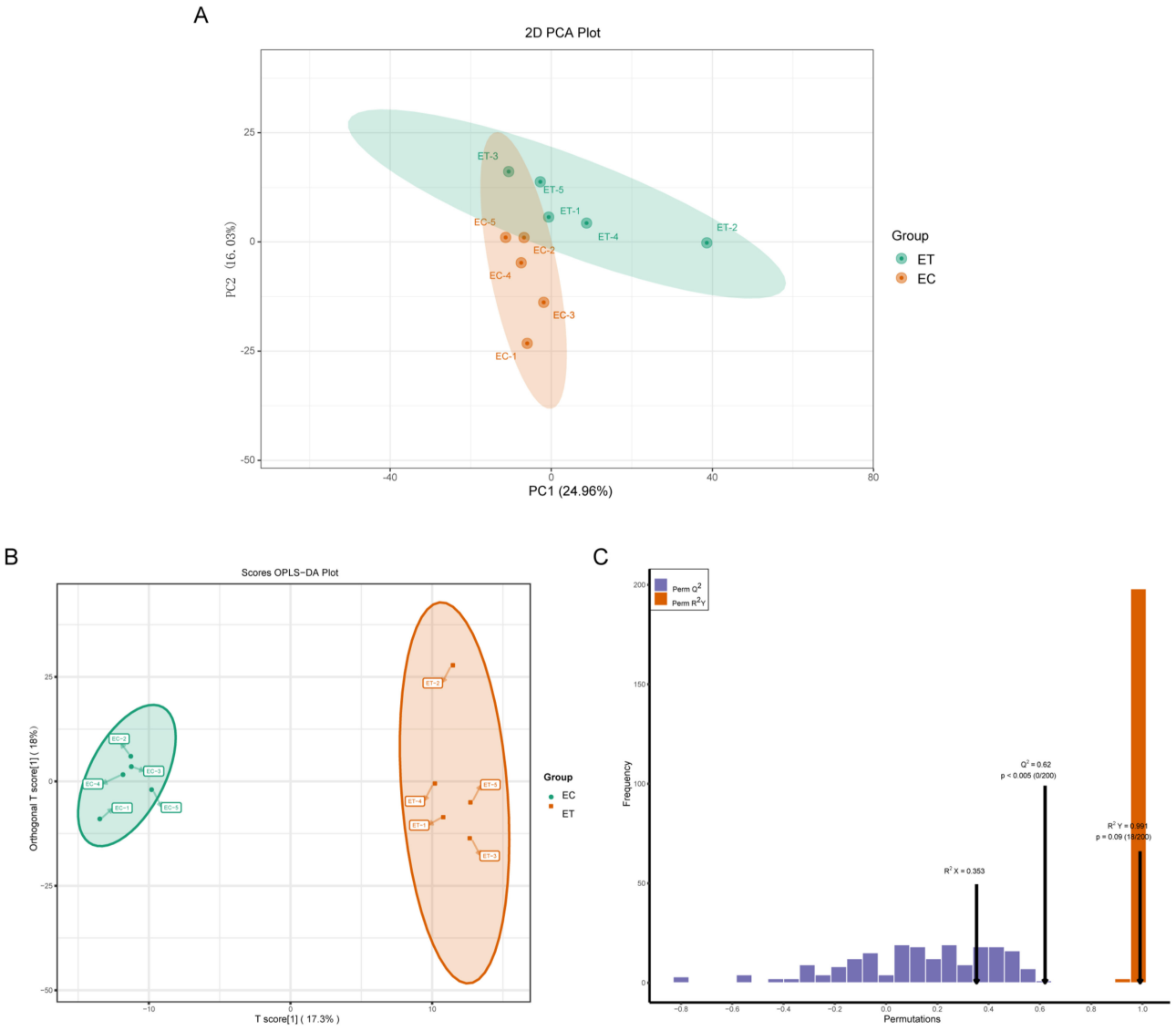

FIGURE 1
Effects of SSDDGS on goat serum metabolites were analyzed by principal component analysis (PCA) and orthogonal partial least squares-discriminant analysis (OPLS-DA). **(A)** PCA diagram of ET and EC groups. PC1 is the first principal component, and PC2 is the second principal component. each dot represents a sample, and the same color represents the same group; **(B)** OPLS-DA score plot of ET and EC groups, and the percentage represents the interpretation rate of the component to the data set; **(C)** OPLS-DA verification diagram, R^2X, R^2Y and Q^2 are the prediction parameters for evaluating the OPLS-DA model, and when $1 > R^2$ Y and $Q^2 > 0.4$, the models were determined to be stable and reliable.

higher than that in EC group at week 4 of lactation (Table 4, $p < 0.05$).

3.4 Effects of SSDDGS on metabolites of goats

Effects of SSDDGS on metabolites of goats was analyzed by ultra-performance liquid chromatography tandem mass spectrometry (UPLC-MS/MS). The principal component analysis (PCA) score plot (Figure 1A) revealed that the model interpretation rates for PC1 and PC2 were 24.96% and 16.03%, respectively, with a clear separation of samples between the two groups. The OPLS-DA results mirrored those obtained from PCA, with a principal component of prediction in the ET and EC groups accounting for 17.3% of the model interpretation rate (Figure 1B). Furthermore, the OPLS-DA model displayed the predictive parameters of $Q^2 = 0.62$ and $R^2Y = 0.991$, indicating the stability and reliability of the model (Figure 1C).

The identification of 60 significantly different metabolites in the ET group compared with the EC group (Table 5, Figure 2A). Among these, 31 metabolites were downregulated, whereas 29 were upregulated (Figure 2B). The various types of metabolites include L-leucylglycine, aspartic acid, sphingosine 1-phosphate, cinnamyl glycine, serine and hexanoyl glycine.

The metabolic pathways of these 60 different metabolites were analyzed by KEGG enrichment analyses. The results revealed the involvement of 25 metabolic pathways, among which the with the *p*-value closest to 0 and the highest number of differentially significant metabolites included sohingolipid sianaling pathway,

TABLE 5 Different metabolites of ET group compared with EC group.

Compounds	Class	VIP	*P*-value	log1.5FC	Type
1,6-Di-O-phosphono-D-fructose	Organic acid and its derivatives	1.44	0.05	−20.76	Down
12,13-DiHOME	FA	1.49	0.05	−2.72	Down
12-HHT	FA	1.55	0.05	−2.19	Down
2-(4-hydroxyphenyl) propionate	Benzene and substituted derivatives	1.59	0.04	−2.16	Down
2-Butyl-3-(4-hydroxybenzoyl)benzofuran	Benzene and substituted derivatives	1.60	0.04	−2.10	Down
2-Hydroxy-3-Methyl Butanoic Acid	Organic acid and its derivatives	1.60	0.04	−2.04	Down
2-Hydroxymelatonin	Hormones and hormone related compounds	1.60	0.04	−1.82	Down
2-hydroxyphenylacetic acid	Organic acid and its derivatives	1.60	0.04	−1.45	Down
3-(4-Hydroxyphenyl)-1-propanol	Organic acid and its derivatives	1.61	0.04	−1.34	Down
3-Methylcrotonyl Glycine	Organic acid and its derivatives	1.61	0.04	−1.31	Down
4-Hydroxy-3-methylbenzoic acid	Organic acid and its derivatives	1.62	0.04	−1.25	Down
4-Hydroxyquinoline	Benzene and substituted derivatives	1.62	0.03	−1.23	Down
4-Methoxysalicylic Acid	Benzene and substituted derivatives	1.68	0.03	−1.22	Down
6-Methylnicotinamide	Heterocyclic compounds	1.69	0.03	−1.18	Down
8,8a-deoxy-oleane	Others	1.71	0.03	−1.18	Down
Acetylcholine	Alcohol and amines	1.72	0.03	−1.18	Down
Acetylvaline	Amino acid and its metabolites	1.72	0.03	−1.17	Down
Carnitine C11:1	FA	1.73	0.03	−1.17	Down
Carnitine C16:0	FA	1.73	0.03	−1.12	Down
Carnitine C18:0	FA	1.74	0.03	−1.11	Down
Carnitine C18:1:2DC	FA	1.74	0.03	−1.08	Down
Carnitine C6:0	FA	1.75	0.03	−1.07	Down
Carnitine C8-OH	FA	1.75	0.02	−1.05	Down
Cyclo(Ala-Pro)	Amino acid and its metabolites	1.76	0.02	−1.03	Down
Cytidine 5′-diphosphate	Nucleotide and its metabolites	1.78	0.02	−1.03	Down
Deoxyguanosine	Nucleotide and its metabolites	1.78	0.02	−1.02	Down
Gln-Gly	Amino acid and its metabolites	1.79	0.02	−1.01	Down
Glu-Val	Amino acid and its metabolites	1.81	0.02	−1.00	Down
Gly-Gln	Amino acid and its metabolites	1.82	0.02	−1.00	Down
Guanosine	Nucleotide and its metabolites	1.87	0.02	1.02	Up
Hexadecanedioic acid	FA	1.90	0.02	1.06	Up
Hexanoyl Glycine	Amino acid and its metabolites	1.90	0.02	1.09	Up
Ile-Pro-Ile	Amino acid and its metabolites	1.90	0.02	1.11	Up
Imidazole-4-methanol	Heterocyclic compounds	1.90	0.01	1.12	Up
Indole-2-Carboxylic Acid	Heterocyclic compounds	1.91	0.01	1.20	Up
L-Aspartic Acid	Amino acid and its metabolites	1.92	0.01	1.23	Up
L-Isserine	Amino acid and its metabolites	1.94	0.01	1.30	Up
L-Serine	Amino acid and its metabolites	1.96	0.01	1.35	Up
L-tyrosine methyl ester 4-sulfate	Amino acid and its metabolites	1.97	0.01	1.41	Up
LPE(15:0/0:0)	GP	2.00	0.01	1.42	Up

(Continued)

TABLE 5 (Continued)

Compounds	Class	VIP	*P*-value	log1.5FC	Type
Leu-Gly	Amino acid and its metabolites	2.01	0.01	1.51	Up
Leu-Met	Amino acid and its metabolites	2.01	0.01	1.57	Up
MG(18:2/0:0/0:0)	GL	2.02	0.01	1.57	Up
Methyldopa	Amino acid and its metabolites	2.04	0.00	1.63	Up
N-Acetyl-L-alanine	Amino acid and its metabolites	2.05	0.00	1.68	Up
N-Acetylglycine	Amino acid and its metabolites	2.10	0.00	1.70	Up
N-Acetylhistamine	Alcohol and amines	2.11	0.00	1.73	Up
N-Cinnamylglycine	Organic acid and its derivatives	2.15	0.00	1.87	Up
N-Formylmethionine	Amino acid and its metabolites	2.17	0.00	1.88	Up
N-Propionylglycine	Amino acid and its metabolites	2.20	0.00	1.90	Up
Octadecanedioic acid	Organic acid and its derivatives	2.20	0.00	1.94	Up
Pantothenol	CoEnzyme and vitamins	2.21	0.00	2.07	Up
Phe-Thr	Amino acid and its metabolites	2.22	0.00	2.63	Up
Salicyluric acid	Benzene and substituted derivatives	2.22	0.00	2.64	Up
Sphingosine 1-phosphate	SL	2.27	0.00	2.91	Up
Thr-Phe	Amino acid and its metabolites	2.27	0.00	3.00	Up
Tricarballylic acid	Organic acid and its derivatives	2.33	0.00	4.35	Up
Cyclo(pro-pro)	Amino acid and its metabolites	2.39	0.00	4.89	Up

cysteine and methionine metabolism and sphingolipid metabolism (Figure 2C).

Meanwhile, to further analyzed the potential correlations between female goat phenotypic data and differential metabolites in serum, we performed Pearson correlation analysis (Figure 3). L-leucylglycine, aspartic acid, sphingosine and 1-phosphate exhibited positive correlations with serum biochemical index (IGF-1, PRL) and milk composition (protein, lactose, and ash content) of female goats ($P < 0.05$). In addition, monoglyceride, monoglyceride and amino acid (AA) such as cinnamyl glycine, serine and hexanoyl glycine had negative significant correlations with serum biochemical index (IGF-1, PRL) and milk composition (protein, lactose, and ash content) of female goats ($P < 0.05$).

4 Discussion

Yeast culture contains a large number of beneficial microorganisms and easily digestible small molecules, which is very beneficial to improve animal health and production performance. During early lactation, female goats experience increased energy expenditure, heightened production of volatile fatty acids, and decreased body weight (1). Supplementation of YC in diet can enhance nutrient digestibility and promoting animal growth by increasing the diversity and abundance of rumen microorganisms (13–15).

In this study, the supplementation of SSDDGS in diet significantly reduced the ADL of lactating female goats during early lactation. Similarly, Song et al. reported that incorporating YC into the diet improved the rumen microbial community and increased the ADG of lambs (16). Additionally, in this study, the supplementation of SSDDGS in diet significantly increased the serum IGF-1 concentration during early lactation. IGF-1 is an endocrine hormone primarily produced by the liver and promotes animal growth and development, which is involved in lipid metabolism, insulin secretion, and glucose uptake (17). These results indicate that the dietary supplementation of SSDDGS reduced weight loss of lactating female goats during early lactation through the role of IGF-1.

Supplementation of YC in diet can effectively improve animal lactation performance. In this study, the supplementation of SSDDGS in diet significantly increased the milk protein, milk lactose and ash content during early lactation, although SSDDGS supplementation in diet did not increase ADG of offspring lambs in lactation, which was an important indicator of milk production in female goats. These studies are consistent with previous studies. YC supplementation increased milk protein content and reduced the incidence of mastitis in cows (18). Similarly, the milk protein and fat content in cows increased after YC supplementation, particularly during early lactation, without affecting overall milk production (19). Supplementation of YC or *Saccharomyces cerevisiae* in diet increased milk yield and fat production in lactating female goats (20, 21). Additionally, in this study, the supplementation of SSDDGS in diet significantly increased the serum PRL levels during early lactation. PRL is vital for sustaining mammary gland function, milk yield and milk quality in ruminants. Chen et al. found that PRL increased milk protein synthesis by stimulating mammary epithelial cell metabolism (22). These results suggest that supplementation of YC

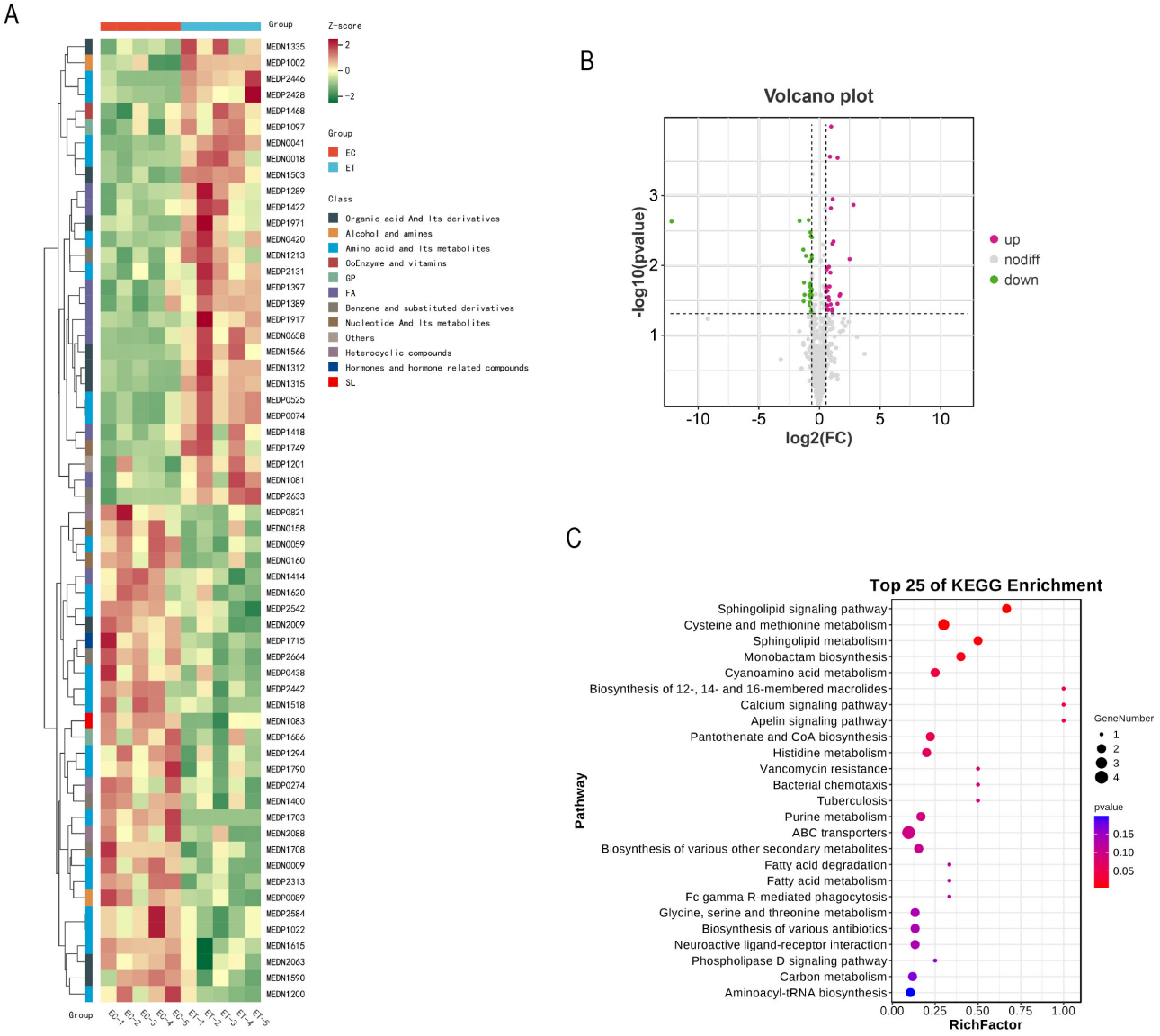

FIGURE 2
Effects of SSDDGS on metabolites of goats. **(A)** Differential metabolite clustering heat map. **(B)** Volcanic diagram of differential metabolites. **(C)** KEGG enrichment map of differential metabolites. The color of the dot represents the size of *P*-value, and the deeper the red color on behalf of the enrichment of the more significant.

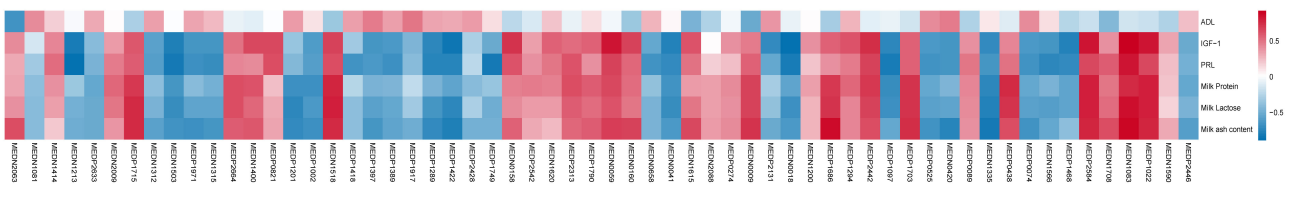

FIGURE 3
Pearson correlation analysis between phenotypic data and serum differential metabolites in female goats. Phenotypic data were obtained from the 4th week of lactation, Pearson correlation coefficient >0.7 with a $P < 0.05$ were considered significant.

in diet improved the milk quality of lactating female goats during early lactation through the role of PRL.

The supplementation of SSDDGS in diet played a crucial role in the inflammation and the immune system of organisms based on metabolome analysis. Supplementing with *Saccharomyces cerevisiae* fermentation postbiotics in calves increased resistance to bovine respiratory disease through the systemic and mucosal immune responses (23). *Saccharomyces cerevisiae* culture can

reduce the number of somatic cells in milk and enhance the antioxidant capacity in cow under heat stress (24). During lactation, there is a high demand for glucose for milk synthesis and fatty acid production. However, excessive energy consumption can lead to mastitis, and excess production of free fatty acids results in the formation of ketone bodies, which can reduce the immunity of female goats (25). From the results of this study, we observed a significant upregulation of Carnitine C11:1 and Carnitine C18:1:2DCs in the SSDDGS group, which are involved in fatty acid metabolism and degradation pathways. The expression abundance of Sphingosine 1-phosphate was significantly downregulated and involved in the Fc gamma R-mediated phagocytosis and tuberculosis pathway. Previous reports have shown that carnitine supplements increase the flux of metabolites through pyruvate carboxylase, thereby increasing insulin secretion and liver glucose output(26). In the mitochondria isolated from pig liver fed carnitine, it was found that the amount of mitochondrial pyruvate carboxylase increased threefold. Pigs fed carnitine are better able to utilize fat to obtain energy, transfer carbon to amino acid synthesis, and save branched chain amino acids used for protein synthesis (27). Similarly, another study found that high levels of carnitine can enhance lipid metabolism in sheep, thereby altering lactation performance (28). However, there has been no research on supplementing carnitine in the diet of goats, and these reports suggest that carnitine may be the main substance that improves goat lactation performance.

In addition, deoxyguanosine, Gly Leu, Phe Thr, and sphingosine 1-phosphate were significantly positively correlated with IGF-1 and PRL levels. Related to this, Purine metabolism, ABC transporters, Calcium signaling pathway and Apelin signaling pathway were significantly enriched. Luo et al. investigated the effects of fermented soybean meal (FSBM) rich in isoflavone glycosidic ligands at different levels on ewes from late pregnancy to early lactation. Feeding ewes with FSBM6 reduced the concentrations of hydrogen peroxide and deoxyguanosine in the placenta, improved the antioxidant capacity of both the mother and placenta, and improved serum hormones and milk quality (29). Sphingosine kinase (SK) catalyzes the formation of sphingosine-1-phosphate (S1P), which plays an essential role in cell growth and survival (30). Döll et al. found that prolactin (PRL) activates SK-1 but not SK-2 isoforms in a human breast cancer cell line (MCF7). The delayed activation of SK-1 results from up-regulation of mRNA and protein expression and is due to increased activity of the SK-1 promoter by a mechanism involving STAT5 activation as well as protein kinase C and classical mitogen-activated protein kinases. The delayed activation of SK-1 results from up-regulation of mRNA and protein expression due to increased SK-1 promoter activity and involves STAT5 activation as well as protein kinase C and classical mitogen-activated protein kinase (31). This suggests that sphingosine-1-phosphate is likely to play an important role in lactation performance.

5 Conclusion

This study demonstrates that the dietary supplementation of yeast culture SSDDGS reduced weight loss and improved milk quality of female goats in the early stage of lactation. Furthermore, SSDDGS may play a vital role in energy metabolism and immune responses primarily through modulation of bile acid and caffeine metabolic pathways. This study will help us better understand the effects and mechanism a novel yeast culture SSDDGS in lactating female goats.

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

Author contributions

LZ: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Writing – original draft, Writing – review & editing. GQ: Conceptualization, Formal analysis, Supervision, Writing – review & editing. JG: Formal analysis, Supervision, Writing – review & editing. MZ: Supervision, Writing – review & editing. GE: Supervision, Writing – review & editing. YHa: Supervision, Writing – review & editing. YHu: Conceptualization, Formal analysis, Funding acquisition, Supervision, Writing – review & editing.

Funding

The author(s) declare financial support was received for the research, authorship, and/or publication of this article. This research was supported by the Chongqing Science and Technology Innovation Special Project (No. CSTB2022TIAD-CUX0013; cstc2019jscx-gksbX0135) and the Strategic Collaboration Project of Chongqing Municipal People's Government and Chinese Academy of Agricultural Sciences, and Fundamental Research Funds for the Central Universities (SWU-XDJH202301).

Acknowledgments

The research team would like to thank Tengda Animal Husbandry, Inc. for animal feeding and sample collection during this research.

Conflict of interest

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

Publisher's note

References

1. Zamuner F, Cameron AWN, Carpenter EK, Leury BJ, DiGiacomo K. Endocrine and metabolic responses to glucose, insulin, and adrenocorticotropin infusions in early-lactation dairy goats of high and low milk yield. *J Dairy Sci.* (2020) 103:12045–58. doi: 10.3168/jds.2020-18625

2. Ataollahi F, McGrath S, Friend M, Dutton G, Peters A, Bhanugopan M. Evaluating the effect of calcium, magnesium and sodium supplementation of Merino ewes on their lambs' growth. *Aust Vet J.* (2023) 101:391–96. doi: 10.1111/avj.13274

3. Razzaghi A, Malekkhahi M, Brito AF. Lactation performance, milk fat output, and nutrient digestibility responses to the addition of liquid molasses or yeast culture in dairy cows fed super-conditioned corn. *J Dairy Sci.* (2023) 106:6080–93. doi: 10.3168/jds.2022-22768

4. Zhang X, Dong X, Wanapat M, Shah AM, Luo X, Peng Q, et al. Ruminal pH pattern, fermentation characteristics and related bacteria in response to dietary live yeast (Saccharomyces cerevisiae) supplementation in beef cattle. *Anim Biosci.* (2022) 35:184–95. doi: 10.5713/ab.21.0200

5. Chen H, Liu S, Li S, Li D, Li X, Xu Z, et al. Effects of yeast culture on growth performance, immune function, antioxidant capacity and hormonal profile in Mongolian ram lambs. *Front Vet Sci.* (2024) 11:1424073. doi: 10.3389/fvets.2024.1424073

6. Kim SW, Duarte ME. Saccharomyces yeast postbiotics supplemented in feeds for sows and growing pigs for its impact on growth performance of offspring and growing pigs in commercial farm environments. *Anim Biosci.* (2024) 37:1463–73. doi: 10.5713/ab.23.0467

7. Verdier-Metz I, Delbès C, Bouchon M, Rifa E, Theil S, Chaucheyras-Durand F, et al. Dietary Live Yeast Supplementation Influence on Cow's Milk, Teat and Bedding Microbiota in a Grass-Diet Dairy System. *Microorganisms*. (2023) 11:673. doi: 10.3390/microorganisms11030673

8. Zhao Y, Wang Q, Zhou P, Li Z, Zhong W, Zhuo Y, et al. Effects of yeast culture supplementation from late gestation to weaning on performance of lactating sows and growth of nursing piglets. *Animal.* (2022) 16:100526. doi: 10.1016/j.animal.2022.100526

9. Song D, Li X, Cheng Y, Wu G, Xiao X, Wang F, et al. Effects of supplementing sow diets with Saccharomyces cerevisiae refermented sorghum dried distiller's grains with solubles from late gestation to weaning on the performance of sows and progeny. *J Anim Sci.* (2017) 95:2025–31. doi: 10.2527/jas.2017.1438

10. Qin G, Zhang L, Guo J, Fang S, Zeng Y, Huang Y, et al. Combined proteomic and metabolomic analysis reveals comprehensive regulation of somatostatin DNA vaccine in goats. *Int J Mol Sci*. (2024) 25:6888. doi: 10.3390/ijms25136888

11. Wang H, Li B, Li A, An C, Liu S, Zhuang Z. Integrative metabolomics, enzymatic activity, and gene expression analysis provide insights into the metabolic profile differences between the slow-twitch muscle and fast-twitch muscle of *Pseudocaranx dentex*. *Int J Mol Sci.* (2024) 25:6131. doi: 10.3390/ijms25116131

12. Xiao Y, Li X, Zeng X, Wang H, Mai Q, Cheng Y, et al. Low ω-6/ω-3 ratio high-fat diet improves rat metabolism via purine and tryptophan metabolism in the intestinal tract, while reversed by inulin. *J Agric Food Chem.* (2019) 67:7315–24. doi: 10.1021/acs.jafc.9b02110

13. Liu S, Yang L, Zhang Y, Chen H, Li X, Xu Z, et al. Review of yeast culture concerning the interactions between gut microbiota and young ruminant animals. *Front Vet Sci.* (2024) 11:1335765. doi: 10.3389/fvets.2024.1335765

14. Ogbuewu IP, Mbajiorgu CA. Meta-analysis of Saccharomyces cerevisiae on enhancement of growth performance, rumen fermentation and haemato-biochemical characteristics of growing goats. *Heliyon.* (2023) 9:e14178. doi: 10.1016/j.heliyon.2023.e14178

15. Zhang X., Liu T., Hou X., Hu C., Zhang L., Wang S., et al. Multi-channel metabolomics analysis identifies novel metabolite biomarkers for the early detection of fatty liver disease in dairy cows. *Cells*. (2022) 11:2883. doi: 10.3390/cells11182883

16. Song B, Wu T, You P, Wang H, Burke JL, Kang K, et al. Dietary supplementation of yeast culture into pelleted total mixed rations improves the growth performance of fattening lambs. *Front Vet Sci.* (2021) 8:657816. doi: 10.3389/fvets.2021.657816

17. Kasprzak A. Insulin-like growth factor 1 (IGF-1) signaling in glucose metabolism in colorectal cancer. *Int J Mol Sci.* (2021) 22:6434. doi: 10.3390/ijms22126434

18. Nocek JE, Holt MG, Oppy J. Effects of supplementation with yeast culture and enzymatically hydrolyzed yeast on performance of early lactation dairy cattle. *J Dairy Sci.* (2011) 94:4046–56. doi: 10.3168/jds.2011-4277

19. Kalmus P, Orro T, Waldmann A, Lindjärv R, Kask K. Effect of yeast culture on milk production and metabolic and reproductive performance of early lactation dairy cows. *Acta Vet Scand.* (2009) 51:32. doi: 10.1186/1751-0147-51-32

20. Mavrommatis A, Mitsiopoulou C, Christodoulou C, Karabinas D, Nenov V, Zervas G., et al. Dietary supplementation of a live yeast product on dairy sheep milk performance, oxidative and immune status in peripartum period. *J Fungi (Basel).* (2020) 6:334. doi: 10.3390/jof6040334

21. Ogbuewu IP, Mbajiorgu CA. Corrigendum: Meta-analytic effect of Saccharomyces cerevisiae on dry matter intake, milk yield and components of lactating goats. *Front Vet Sci.* (2023) 10:1144334. doi: 10.3389/fvets.2023.1144334

22. Chen Z, Luo J, Zhang C, Ma Y, Sun S, Zhang T, et al. Mechanism of prolactin inhibition of miR-135b via methylation in goat mammary epithelial cells. *J Cell Physiol.* (2018) 233:651–62. doi: 10.1002/jcp.25925

23. Maina TW, McDonald PO, Rani Samuel BE, Sardi MI, Yoon I, Rogers A, et al. Feeding Saccharomyces cerevisiae fermentation postbiotic products alters immune function and the lung transcriptome of preweaning calves with an experimental viral-bacterial coinfection. *J Dairy Sci.* (2024) 107:2253–67. doi: 10.3168/jds.2023-23866

24. Du D, Feng L, Chen P, Jiang W, Zhang Y, Liu W, et al. Effects of saccharomyces cerevisiae cultures on performance and immune performance of dairy cows during heat stress. *Front Vet Sci.* (2022) 9:851184. doi: 10.3389/fvets.2022.851184

25. Bouvier-Muller J, Allain C, Enjalbert F, Tabouret G, Portes D, Caubet C, et al. Response to dietary-induced energy restriction in dairy sheep divergently selected for resistance or susceptibility to mastitis. *J Dairy Sci.* (2016) 99:480–92. doi: 10.3168/jds.2015-9785

26. Carlson DB, McFadden JW, D'Angelo A, Woodworth JC, Drackley JK. Dietary L-carnitine affects periparturient nutrient metabolism and lactation in multiparous cows. *J Dairy Sci.* (2007) 90:3422–41. doi: 10.3168/jds.2006-811

27. Owen KQ, Ji H, Maxwell CV, Nelssen JL, Goodband RD, Tokach MD, et al. Dietary L-carnitine suppresses mitochondrial branched-chain keto acid dehydrogenase activity and enhances protein accretion and carcass characteristics of swine. *J Anim Sci.* (2001) 79:3104–12. doi: 10.2527/2001.79123104x

28. Bai U, Su X, Zheng Z, Zhang L, Ma Y, Dou Y, et al. Comparative metabolomics analysis of small-tailed han and DairyMeade ovine milk. *Eur Food Res Technol.* (2021) 247:2869–76. doi: 10.1007/s00217-021-03840-9

29. Luo Z, Yu S, Zhu Y, Zhang J, Xu W, Xu J. Effect of various levels of isoflavone aglycone-enriched fermented soybean meal on redox status, serum hormones and milk quality in ewes. *S Afr J Anim Sci.* (2018) 48:673–82. doi: 10.4314/sajas.v48i4.9

30. Glueck M, Lucaciu A, Subburayalu J, Kestner RI, Pfeilschifter W, Vutukuri R, et al. Atypical sphingosine-1-phosphate metabolites-biological implications of alkyl chain length. *Pflugers Archiv-Eur J Physiol.* (2024). doi: 10.1007/s00424-024-03018-8

31. Döll F, Pfeilschifter J, Huwiler A. Prolactin upregulates sphingosine kinase-1 expression and activity in the human breast cancer cell line MCF7 and triggers enhanced proliferation and migration. *Endocr Relat Cancer.* (2007) 14:325–35. doi: 10.1677/ERC-06-0050

frontiers | Frontiers in Veterinary Science

TYPE Original Research
PUBLISHED 13 November 2024
DOI 10.3389/fvets.2024.1498639

OPEN ACCESS

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RECEIVED 19 September 2024
ACCEPTED 24 October 2024
PUBLISHED 13 November 2024

CITATION
Zhang J, Guan F, Huang S, Ma Y, Wen S, Jin W and Mao S (2024) Fermented soybean meal modified the rumen microbiota and increased the serum prolactin level in lactating Holstein cows.
Front. Vet. Sci. 11:1498639.
doi: 10.3389/fvets.2024.1498639

Fermented soybean meal modified the rumen microbiota and increased the serum prolactin level in lactating Holstein cows

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This study aimed to investigate the effects of fermented soybean meal (FSM) on milk production, blood parameters, and rumen fermentation and microbial community in dairy cows. In this study, 48 healthy Holstein cows (parity, 3.0 ± 0.6; days in milk, 86.0 ± 6.7) were used. Cows were randomly assigned into four groups (CON, T-200, T-400, and T-600) with 12 cows per group. Cows in CON were not supplemented with FSM. Cows in T-200, T-400, and T-600 were supplemented with 200, 400, and 600 g/head/day FSM, respectively. This study lasted 5 weeks (1-week adaptation and 4-week treatment). The results showed that FSM did not affect milk yield and milk components ($p > 0.05$). In the serum, FSM greatly increased prolactin ($p < 0.01$), and a dosage effect was observed. Aspartate aminotransferase and total protein were the highest in the T-400 ($p < 0.05$), and triglycerides was the lowest in T-200 ($p < 0.05$), and there was no difference for the 3 measurements between the other 3 groups ($p > 0.05$). In the rumen, FSM did not affect pH, microbial crude protein, acetate, propionate, butyrate, valerate, total volatile fatty acids and the ratio of acetate:propionate ($p > 0.05$), only changed NH_3-N, isobutyrate and isovalerate ($p < 0.05$). The results of the rumen bacterial 16S rRNA genes sequencing showed that FSM decreased the richness ($p < 0.05$) and evenness ($p < 0.05$) of the bacterial communities. PCoA analysis showed that FSH altered the rumen bacterial community (ANOSIM, $R = 0.108$, $p = 0.002$). In the relative abundance of phyla, FSM increased Firmicutes ($p = 0.015$) and Actinobacteriota ($p < 0.01$) and Patescibacteria ($p = 0.012$), decreased Bacteroidota ($p = 0.024$). In the relative abundance of genera, FSM increased Christensenellaceae R-7 group ($p = 0.011$), *Lactococcus* ($p < 0.01$), *Candidatus Saccharimonas* ($p < 0.01$), *Olsenella* ($p < 0.01$), decreased Muribaculaceae_norank ($p < 0.01$). Conclusively, supplemented FSM altered the rumen fermentation parameters and bacterial community, and increased serum prolactin level in lactating Holstein cows. These findings may provide an approach to keep the peak of lactation in dairy cows.

KEYWORDS

fermented soybean meal, cows, milk performance, serum biochemical indices, rumen fermentation, bacterial community

1 Introduction

Fermented soybean meal (FSM) is a high-quality plant protein source for animals, containing probiotics, digestive enzymes, bioactive peptides, antioxidants and providing immunomodulatory effects (1). Many studies reported that feeding FSM to animals (pigs, chicken and calves) showed positive effects with improved nutrient digestibility and intestinal health and production performance (2–4). Due to the ban on the use of antimicrobial growth promoters in animal production, the use of FSM in ruminants has attracted a great interest.

In the study of Kim et al. (5), FSM had been used in a calf starter and showed positive effects on the health and growth of calves. As demonstrated by Feizi et al. (4), FSM improved the starter intake in calves, and altered the rumen fermentation and microbiota. In another study of Rezazadeh et al. (6), feeding FSM helped calves adapt to weaning stress during cold weather. One study in lactating cows reported that feeding FSM increased milk protein yield, milk fat yield and fat corrected milk, and decreased milk somatic cell count (7). Studies also showed that feeding FSM alter the rumen fermentation parameters and rumen microbiota in lactating Holstein cows (7, 8). However, the results were not consistent. As reported by Wang et al. (8), FSM reduced total volatile fatty acid concentration, acetate to propionate ratio and increased propionate percentage. According to Amin et al. (7), FSM increased rumen pH, acetate percentage and acetate to propionate ratio. Most studies regarding the use of FSM in ruminants have been focused on calves, few studies investigated the lactating cows, especially for cows in early stage of lactation (1).

We hypothesized that feeding FSM could cause changes in the rumen fermentation and microbiota and blood parameters which could lead to improve in the milk performance of dairy cows. In this study, we aimed to explore the effects of feeding FSM on the milk performance, blood parameters, and rumen fermentation and bacterial community in dairy cows in the early stage of lactation. The results would offer a reference for the application of FSM in the dairy cow industry.

2 Materials and methods

2.1 Animals, diets, and management

This experiment was conducted from November 2020 to December 2020 at Shanghai Jinshan Yinan Dairy Farm (Shanghai, China). In this study, 48 healthy Holstein cows in similar parity (3.0 ± 0.6) and lactation stages (86.0 ± 6.7 day in milk) and milk yield (41.0 ± 2.8 kg) were used. Cows were randomly assigned into 4 groups (CON, T-200, T-400, and T-600) with 12 cows per group. Cows in CON were not supplemented with FSM. Cows in T-200, T-400, and T-600 were supplemented with 200, 400, and 600 g/head/day FSM, respectively. This study lasted 5 weeks (1-week adaptation and 4-week treatment). FSM (yellow granular substance, fermented by *Saccharomyces cerevisiae* and *Bacillus subtilis*) used in this study was purchased from Shanghai Yuanyao Agriculture Co., Ltd. The basic diet used in this study was formulated based on NRC (2001) guidelines for lactating cows. The nutritional composition of FSM was shown in Supplementary Table S1. The ingredients and chemical composition of the basic diet were shown in Supplementary Table S2. All cows were housed in a tie stall barn, milked three times daily using a fully automated pipeline milking machine (02,30, 10,30, 16:30), and fed with total mixed ration three times daily (03,30, 10,30, 16:30), ensuring that cows had at least 20 h of free access to feed per day and free access to fresh water.

2.2 Sampling

Milk yield was determined by a Tunisian flow-meter (JHF-G17, Sichuan Jinhaifeng Animal Husbandry Technology Co., Ltd., Sichuan, China). Milk samples were collected at the last 2 days in each week, and preserved with potassium dichromate, at 4°C. Milk samples collected from the morning, afternoon, and evening milking daily were mixed at a ratio of 4:3:3 before determining the milk composition using a near-infrared analyzer (MilkoScanTM 7 RM, Foss Electric, Denmark).

The blood samples were collected via the tail vein of the cows before morning feeding on the last day of the trial. The collected blood samples were immersed in warm water (37°C) for 10 min immediately before centrifuging at 3,500 r/min for 15 min. The supernatant was collected and stored at −20°C for the determination of serum biochemical indices.

The rumen content samples were collected at 4 h after morning feeding using an oral ruminal tube (Wuhan Kelibao Co., Ltd., Wuhan, China) on the last day of the trial. In order to avoid saliva contamination, the first 200 mL rumen fluid was discarded. A portion of the rumen content was stored in liquid nitrogen for the measuring the microbial community. Another portion was filtered through four layers of sterilized cheesecloth, and stored at −20°C for the determination of microbial crude protein, NH_3-N, and volatile fatty acids.

2.3 Chemical analysis

The serum biochemical indices were measured using a fully automated biochemical analyzer (Vital Scientific NV, The Netherlands) following the standard procedure. Prolactin (PRL) is a milk-production hormone, was measured using an enzyme-linked immunosorbent assay kit (ELISA kit, Shanghai, China).

The pH value of rumen fluid was measured using a portable pH meter (HI 9024C; HANNA Instruments, Woonsock, RI). The concentration of NH_3-N in rumen fluid was determined using a phenol sodium hypochlorite colorimetric method according to Weatherburn (9). The microbial crude protein (MCP) content in rumen fluid was determined using a Coomassie Brilliant Blue colorimetric method according to Makkar et al. (10). The volatile fatty acids (VFA) concentration in rumen fluid was determined by a gas chromatography (GC-2014B, Shimadzu, Japan) equipped with a capillary column (column temperature: 110°C, film thickness: 30 m × 0.32 mm × 0.25 μm) (11).

2.4 Rumen microbial genomic DNA extraction

Rumen microbial genomic DNA was extracted using a phenol-chloroform extraction and cell lysis methods (12). The concentration

of DNA was measured by a Nanodrop spectrophotometer (Nyxor Biotech; Paris, France) and stored at −80°C for further sequencing.

2.5 MiSeq sequencing

A pair of PCR primers was used to amplify the V3-V4 region of the rumen bacterial 16S rRNA genes (13). The primers were 341F (5-CCTAYGGGGRBGCASCAG-3) and 806R (5-GGACTACNNGGGTATCTAAT-3). The amplicons were sequenced on an Illumina MiSeq PE 300 platform (Illumina Inc., San Diego, California, United States) in a commercial laboratory (Shanghai Biozeron Technology Co., Ltd., Shanghai, China). The raw data were stored in the Sequence Read Archive (SRA) database, the accession number is PRJNA1162692.

2.6 Data analysis

Trimmomatic (v.0.33) software was used to trim adapters and low-quality sequences. FLASH (1.2.7) software was utilized to concatenate paired segments into a sequence (14). A software (QIIME2 v1.9.0) was used to process the raw Illumina fastq files (15). Bases with an average quality value below 20 were filtered. UPARSE software was used to classify sequences with a similarity level ≥ 97% into OTUs (16). The SILVA database was used to perform the taxonomic assignment of the representative OTU sequences (17). Principal coordinate analysis (PCoA) was conducted based on the Bray–Curtis metrics (18). The differences among groups was evaluated by ANOSIM using the vegan package in R.

2.7 Statistical analysis

A SPSS 20.0 software (SPSS Inc., Chicago, IL, United States) was used to analyze the data in this study.

The data (milk yield, and components) were analyzed with repeated measurements using a MIXED procedure, and adjusted with the data of adaption period as a covariate factor. The model included the fixed effects of treatment (CON, T-200, T-400, and T-600), time (week 1 to 4), treatment × time, and covariate. Time (week) was used as a repeated measurement with cows as the subject.

Data (rumen fermentation parameters, serum biochemical indices) were analyzed using the one-way ANOVA test. Significant difference between treatments was evaluated using Duncan's test. Data on bacterial communities were analyzed using the nonparametric test (Kruskal-Wallis). Significance was declared at $p < 0.05$. All results were expressed as mean ± standard error.

3 Results

3.1 Milk yield and milk composition

As shown in Table 1, there were no effects of treatment ($p > 0.05$), time ($p > 0.05$), and treatment by time ($p > 0.05$) for milk yield, milk fat percentage, total milk solids, and somatic cell count. There were effects of time ($p < 0.05$), but not treatment ($p > 0.05$) and treatment by time ($p > 0.05$) for milk protein percentage, milk lactose percentage or milk urea nitrogen concentration.

3.2 Serum biochemical indices

As shown in Table 2, there were treatment effects for PRL (prolactin), AST (Aspartate aminotransferase), TP (Total Protein), TRIG (Triglycerides) in the serum ($p < 0.05$). PRL showed a dosage effect, and increased with the dosage increase of FSM (394.67, 493.81, 536.16, and 608.13 mIU/L, $p < 0.01$). AST was higher in T-400 ($p < 0.05$), and did not differ between the other 3 groups ($p > 0.05$). TP was higher in T-400 than that in CON and T-200 ($p < 0.05$), and T-400 did not differ with T-600 ($p > 0.05$). TRIG was lower in T-200 than that in CON and T-600 ($p < 0.05$), and T-200 did not differ with T-400 ($p > 0.05$). There were no treatment effects for T-SOD (superoxide dismutase), ALT (Alanine aminotransferase), ALB (Albumin), ALP (Alkaline Phosphatase), GLOB (Globulin), A/G (Albumin/ Globulin), ALP (Alkaline Phosphatase), CK (Creatine Kinase), LDH (Lactic Acid Dehydrogenase), HDL-C (High-density lipoprotein), LDL-C (Low-density lipoprotein), CREAT (Creatinine), TCHO (Total cholesterol), GLU (Glucose), and UA (uric acid) in the serum ($p > 0.05$).

3.3 Rumen fermentation parameters

As shown in Table 3, there were no treatment effects for rumen pH, microbial crude protein, acetate, propionate, butyrate, valerate, total volatile fatty acids and the ratio of acetate to propionate ($p > 0.05$). There were treatment effects for the concentration of NH_3-N, isobutyrate, and isovalerate ($p < 0.05$). The concentration of NH_3-N was higher in T-400 than the other 3 groups ($p < 0.05$). Isobutyrate was lower in T-200 and T-400 than that in CON ($p < 0.05$), but not differ with that in T-600 ($p > 0.05$). Isovalerate was lower in T-400 than that in CON and T-600 ($p < 0.05$), but not differ with that in T-200 ($p > 0.05$).

3.4 Rumen bacterial community

There were total of 2,128,625 high-quality sequences were obtained from 48 samples, with an average of 44,346 sequences for each sample. The rarefaction curve tended to flatten out, indicating that the sequencing depth were sufficient for analyzing the rumen bacterial communities (Supplementary Figure S1). PCoA based on the Bray Curtis metric algorithm showed that FSM altered the rumen bacterial community structure (ANOSIM: $R = 0.108$, $p = 0.002$) (Figure 1). Significant differences were observed between CON and T-400 ($R = 0.074$, $p = 0.032$); CON and T-600 ($R = 0.109$, $p = 0.004$); T-200 and T-400 ($R = 0.076$, $p = 0.018$); T-200 and T-600 ($R = 0.103$, $p = 0.003$). There were no differences between CON and T-200 ($R = 0.042$, $p = 0.441$); T-400 and T-600 ($R = 0.039$, $p = 0.539$).

As shown in Table 4, there were treatment effects for the number of OTUs ($p < 0.01$), Chao 1 index ($p = 0.043$), and Shannon index ($p = 0.028$). The number of OTUs and Chao 1 and Shannon were lower in T-400 and T-600 than that in CON ($p < 0.05$). There were no treatment effects for Simpson ($p = 0.051$).

TABLE 1 Effects of feeding FSM on milk yield and milk composition in lactating cows.

Items	Treatment				SEM	*p*-value		
	CON	T-200	T-400	T-600		Treatment	Time	Treatment × Time
Milk yield (kg)	40.85	40.90	42.00	41.45	0.42	0.498	0.638	0.217
Milk fat (%)	3.83	3.91	3.56	3.69	0.06	0.569	0.170	0.310
Milk protein (%)	3.13	3.02	3.19	3.23	0.03	0.146	0.001	0.950
Milk lactose (%)	5.28	5.23	5.29	5.33	0.02	0.546	<0.001	0.085
Total milk solids (%)	12.55	12.51	12.36	12.55	0.08	0.471	0.253	0.470
Somatic cell count (10^3/mL)	59.08	105.50	158.46	70.83	36.18	0.528	0.826	0.384
Milk urea nitrogen (g/L)	15.65	16.04	16.06	17.85	0.26	0.572	<0.001	0.421

TABLE 2 Effects of feeding FSM on serum biochemical indices in lactating cows.

Items	Treatment				SEM	*P*-value
	CON	T-200	T-400	T-600		
T-SOD(U/mL)	100.17	105.75	107.33	109.92	6.57	0.758
AST (U/L)	78.08^{b}	79.67^{b}	94.83^{a}	74.58^{b}	4.83	0.025
ALT (U/L)	32.08	33.08	35.75	33.42	1.59	0.425
TP (g/L)	73.19^{b}	73.08^{b}	76.66^{a}	75.74^{ab}	0.99	0.027
ALB(g/L)	37.33	37.88	37.98	37.87	0.44	0.710
GLOB(g/L)	35.86	35.21	38.68	37.87	1.02	0.064
A/G	1.04	1.08	0.98	1.02	0.04	0.184
ALP(U/L)	60.83	69.42	70.58	76.75	4.94	0.168
CK(U/L)	204.50	155.67	318.25	164.67	70.74	0.936
LDH (U/L)	923.75	918.00	952.17	915.58	32.92	0.852
UREA (mmol/L)	5.10	4.73	4.87	5.03	0.18	0.482
CREA (μmol/L)	58.98	61.58	57.07	60.19	1.65	0.273
GLU (mmol/L)	2.45	2.35	2.42	2.71	0.11	0.114
UA (μmol/L)	58.50	58.50	60.00	54.00	2.76	0.339
TCHO (mmol/L)	6.52	6.35	6.90	6.30	0.30	0.481
TRIG (mmol/L)	0.24^{a}	0.15^{b}	0.20^{ab}	0.23^{a}	0.02	0.012
HDL-C (mmol/L)	1.78	1.89	1.64	1.44	0.13	0.085
LDL-C (mmol/L)	2.48	2.23	2.49	2.30	0.15	0.524
PRL(mIU/L)	394.67^{c}	493.81^{b}	536.16^{ab}	608.13^{a}	26.68	<0.01

AST, Aspartate aminotransferase; ALT, Alanine aminotransferase; TP, Total Protein; ALB, Albumin; ALP, Alkaline Phosphatase; GLOB, Globulin; A/G, Albumin/ Globulin; ALP, Alkaline Phosphatase; CK, Creatine Kinase; LDH, Lactic Acid Dehydrogenase; HDL-C, High-density lipoprotein; LDL-C, Low-density lipoprotein; CREAT, Creatinine; TCHO, Total cholesterol; TRIG, Triglycerides; GLU, Glucose; PRL, prolactin; T-SOD, superoxide dismutase; UA, uric acid. ^{a,b,c}Within a row, mean values with different superscript letters indicate a significant difference ($P < 0.05$).

As shown the relative abundance of phyla in Table 5, there were treatment effects for Firmicutes ($p = 0.015$), Bacteroidota ($p = 0.024$), Actinobacterota ($p < 0.01$), and Patescibacteria ($p = 0.012$). Firmicutes was higher in T-400 and T-600 than that in CON and T-200 ($p < 0.05$). Bacteroidota was lower in T-400 and T-600 than that in CON and T-200 ($p < 0.05$).

Actinobacterota was higher in T-400 and T-600 than that in CON ($p < 0.05$). Patescibacteria was higher in T-600 than that in the other 3 groups ($p < 0.05$).

As shown the relative abundance of genera in Table 6, there were treatment effects for *Muribaculaceae_norank* ($p < 0.01$), *Christensenellaceae R-7 group* ($p = 0.011$), *Lactococcus* ($p < 0.01$), *Candidatus Saccharimonas* ($p < 0.01$), and *Olsenella* ($p < 0.01$). *Muribaculaceae_norank* was lower in T-400 and T-600 than that in CON ($p < 0.05$). *Christensenellaceae R-7 group* was higher in T-600 than that in CON and T-200 ($p < 0.05$). *Lactococcus* and *Candidatus Saccharimonas* were higher in T-600 than that in the other 3 groups ($p < 0.05$). *Olsenella* was higher in T-400 and T-600 than that in CON ($p < 0.05$).

4 Discussion

FSM is a high-quality protein, containing probiotics, digestive enzymes, bioactive peptides, antioxidants, and low-antinutritional-factors. Feeding FSM would provide positive effects on dairy cows,

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TABLE 3 Effects of feeding FSM on rumen fermentation parameters in lactating cows.

	Treatment					
Items	CON	T-200	T-400	T-600	SEM	*P*-value
Ruminal pH	6.28	6.33	6.21	6.33	0.09	0.727
NH_3-N (mg/dL)	12.05^{b}	13.11^{b}	16.86^{a}	13.57^{b}	1.01	0.011
Microbial crude protein (mg/dL)	39.69	39.74	34.39	33.89	2.99	0.335
Acetate (mmol/L)	77.17	73.81	76.25	78.84	2.61	0.590
Propionate (mmol/L)	29.84	26.06	27.55	26.43	1.66	0.378
Isobutyrate (mmol/L)	1.08^{a}	0.87^{b}	0.85^{b}	0.97^{ab}	0.06	0.026
Butyrate (mmol/L)	14.20	13.93	14.76	15.42	0.60	0.310
Isovalerate (mmol/L)	1.61^{a}	1.39^{ab}	1.30^{b}	1.62^{a}	0.09	0.036
Valerate (mmol/L)	1.81	1.66	1.71	1.83	0.13	0.769
Total volatile fatty acids (mmol/L)	125.70	117.72	122.42	125.11	4.30	0.546
Acetate: Propionate	2.64	2.92	2.86	3.05	0.13	0.188

$^{a, b, c}$Within a row, mean values with different superscript letters indicate a significant difference ($P < 0.05$).

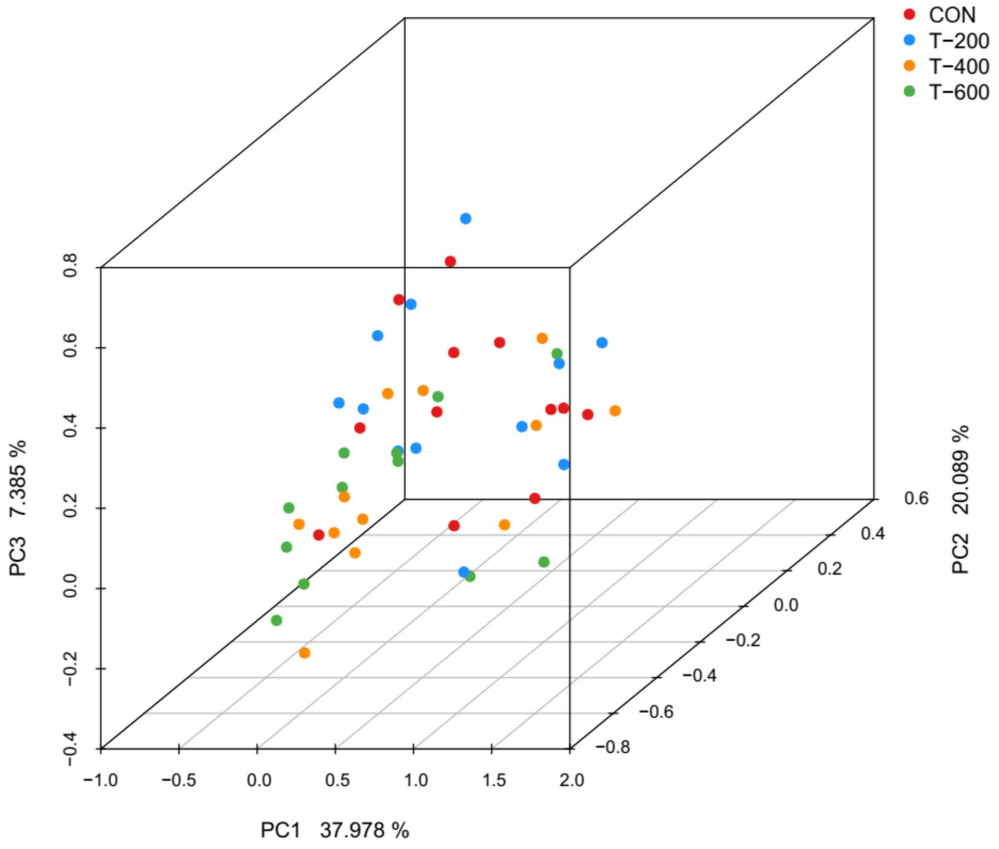

FIGURE 1
Principal coordinate analysis (PCoA) plot of ruminal bacterial communities based on the Bray Curtis metrics.

especially for the cows in the early stage of lactation, during which cows suffer multiple stress. In this study, feeding FSM did not affect the milk yield and milk composition, just observed a numerical increase in milk yield, milk protein percentage and milk urea nitrogen. It is not consistent with a previous study. Amin et al. (7) reported that feeding FSM increased the milk protein yield, milk fat yield and fat corrected milk, and decreased milk somatic cell count in cows in early lactation stage (54 days in milk). The different results observed may be attributed to the varying dosages of FSM supplementation, the inoculum used, the composition of the basic diets, and the lactation stages of cows involved in the different studies (1). Studies regarding FSM on lactating cows are very few, thus more works are needed to elucidate the action mode of FSM in lactating cows.

Serum biochemical indices are indirect indicators of the health and metabolic status of livestock. Feeding FSM caused a little change in a few blood measurements, but caused a great increase in the serum prolactin concentration. These changes were not observed or not measured in the previous studies of Amin et al. (7) and Wang et al. (8). Prolactin is an important lactation hormone that plays a crucial role in promoting mammary gland development, milk synthesis, milk yield, and maintaining lactation (19). During milk synthesis, prolactin facilitates the absorption of glucose and amino acids, as well as the synthesis of milk lactose, fat, casein, and lactoglobulin (20). The mechanism underlying the increase of serum prolactin by feeding FSM is still unclear. In this study, FSM treatment only last 4 weeks, the effect of maintenance of lactation did not exhibited. Further studies would last 8 weeks or longer to explore the effect of FSM on the lactation maintenance in lactating cows. Nevertheless, the new finding might provide a new strategy for the utilization of FSM on lactating cows, especially in the early lactation stage.

Feeding FSM did not affect the concentration of acetate, propionate, butyrate, total volatile fatty acids in the rumen, only caused a little change in concentration of NH_3-N, isobutyrate and isovalerate. Two previous studies reported that FSM changed the rumen fermentation parameters in lactating Holstein cows (7, 8). Wang et al. (8) reported that FSM reduced total volatile fatty acid concentration, acetate to propionate ratio and increased propionate percentage. Amin et al. (7) reported that FSM increased rumen pH, acetate percentage and acetate to propionate ratio. The results from these studies were not consistent. The underlying reasons remain to elucidate.

Feeding FSM caused changes in the rumen bacterial community. The previous studies also reported that feeding FSM modified the rumen bacterial communities in lactating Holstein cows (7, 8). However, the changes in these studies were not consistent. In the current study, feeding FSM decreased the number of OTUs, Chao 1 and Shannon indexes, and increased the relative abundance of Firmicutes and decreased the relative abundance of Bacteroidota in the phylum levels, which were not observed in the two previous studies. Amin et al. (7) reported that FSM enriched the genus of *Muribaculaceae_norank*, which were reduced in the current study. Both the current study and the study of Amin et al. (7) observed the enrichment of the genus of *Christensenellaceae R-7 group* by feeding FSM. The enrichment of the genera of *Lactococcus*, *Candidatus Saccharimonas* and *Olsenella* was not observed in the two previous studies. Fernando et al. (21) reported that *Firmicutes* were more adapted to fiber fermentation, while *Bacteroidota* was more effective in degrading starch. Wang et al. (22) reported that *Actinobacteriota* had the ability to degrade polysaccharides. It suggested that FSM enhanced the fiber fermentation ability in the rumen. Lagkouvardos et al. (23) reported that the genomes of *Muribaculaceae* contained a substantial and versatile set of carbohydrate-active enzymes, suggesting that the members in this family had the ability to degrade complex carbohydrates, the authors also stated that the fitness of *Muribaculaceae* species in degrading dietary carbohydrates most likely explains the decreased occurrence in the feeding trials using high-calories or carbohydrate-enriched diets. *Lactococcus* are homofermentative and are used for the production of L(+) lactic acid from glucose. In dairy industry, *Lactococcus* species are used majorly in the production of lactic acid from lactose, hydrolysis of casein, fat lipolysis by weak esterase activities, and citric acid fermentation (24). The enrichment of *Lactococcus* might be due to the enhancement of the fiber degradation or due to the nutrients provided by FSM. The enrichment of *Candidatus Saccharimonas* in the rumen was observed by feeding a *Saccharomyces cerevisiae* fermentation product in lactating Holstein cows (25). Tong et al. (26) reported that the abundance of the *Candidatus Saccharimonas* was positively correlated with the concentration of propionate in the rumen of lactating cows. Ranilla et al. (27) observed that an antioxidant (carvacrol) enriched *Candidatus Saccharimonas* in an *in vitro* trial. It suggested that the enrichment of *Candidatus Saccharimonas* might be associated with the antioxidant provided by FSM. The members of *Olsenella* could utilize starch and glycogen, producing lactate, acetate, and formate (28). Kim et al. (29) reported that the relative abundance of *Olsenella* was higher in the rumen of Holstein cows fed a high-grain diet. McLoughlin et al. (30) reported that the relative abundance of *Olsenella* in the rumen was positively associated with feed efficiency in sheep. Elolimy et al. (31) observed a higher relative abundance of *Olsenella* in the hindgut of Holstein heifer calves with high feed efficiency. However, Ellison et al. (32) found a higher abundance of *Olsenella* in the rumen of low feed efficient lambs fed a concentrate diet. It suggested that FSM increased the

TABLE 4 Effects of feeding FSM on alpha diversity of rumen bacterial community in lactating cows.

	Treatment					
Items	CON	T-200	T-400	T-600	SEM	*P*-value
OTU	3389[a]	3179[ab]	2882[b]	2931[b]	96	<0.01
Chao 1	4779[a]	4400[ab]	4145[b]	4186[b]	145	0.043
Shannon	6.682[a]	6.64[ab]	6.47[b]	6.48[b]	0.06	0.028
Simpson	0.0046	0.0044	0.0059	0.0066	0.001	0.051

[a, b, c]Within a row, mean values with different superscript letters indicate a significant difference ($P < 0.05$).

TABLE 5 Effects of feeding FSM on the relative abundance of phyla of rumen bacterial community in lactating cows.

	Relative abundance, %					
Phylum	CON	T-200	T-400	T-600	SEM	*P*-value
Firmicutes	42.19[b]	40.93[b]	49.13[a]	51.36[a]	1.27	0.015
Bacteroidota	48.34[a]	48.88[a]	40.54[b]	39.01[b]	1.38	0.024
Proteobacteria	1.01	1.62	1.72	2.05	0.22	0.290
Euryarchaeota	3.24	3.29	2.27	1.87	0.23	0.081
Actinobacteriota	0.97[c]	1.25[bc]	2.26[a]	1.74[ab]	0.12	<0.01
Spirochaetota	1.58	1.25	1.12	0.90	0.09	0.103
Patescibacteria	1.37[b]	1.51[b]	1.59[b]	2.00[a]	0.06	0.012

[a, b, c]Within a row, mean values with different superscript letters indicate a significant difference ($P < 0.05$).

TABLE 6 Effects of feeding FSM on the relative abundance of genera of rumen bacterial community in lactating cows*.

		Treatment					
Phylum	Genus	CON	T-200	T-400	T-600	SEM	*P*-value
Bacteroidota	*Prevotella*	24.86	25.97	21.18	19.27	1.44	0.233
	Rikenellaceae RC9 gut group	5.73	6.15	5.36	6.51	0.30	0.586
	Muribaculaceae_norank	5.69 ^{a}	4.72 ^{ab}	3.50 ^{b}	3.39 ^{b}	0.31	<0.01
	Prevotella_7	1.85	1.06	1.12	0.35	0.29	0.131
	F082_norank	3.42	4.2	3.84	4.81	0.20	0.076
	Bacteroidales RF16 group_norank	1.46	1.39	1.12	1.30	0.10	0.735
	Prevotellaceae YAB2003 group	0.37	0.65	0.33	0.16	0.066	0.301
	Prevotellaceae UCG-003	1.13	1.29	0.95	0.87	0.062	0.154
Firmicutes	*Succiniclasticum*	6.61	5.83	7.19	8.23	0.54	0.626
	NK4A214 group	6.67	5.91	7.93	7.93	0.38	0.143
	Ruminococcus	3.54	4.26	4.35	5.08	0.31	0.405
	Christensenellaceae R-7 group	3.99 ^{bc}	3.75 ^{c}	5.42 ^{ab}	6.35 ^{a}	0.31	0.011
	Clostridia UCG-014_norank	2.52	2.52	2.74	2.18	0.20	0.254
	Lachnospiraceae NK3A20 group	2.62	2.13	3.49	2.96	0.21	0.066
	[Ruminococcus] gauvreauii group	0.54	0.53	0.88	0.60	0.10	0.613
	UCG-005	0.91	0.61	0.39	0.51	0.089	0.084
	Butyrivibrio	0.60	1.06	0.86	1.20	0.094	0.355
	Acetitomaculum	1.30	1.32	1.80	2.05	0.097	0.210
	Lactococcus	0.005 ^{b}	0.017 ^{b}	0.18 ^{b}	0.54 ^{a}	0.065	<0.01
Proteobacteria	*Acinetobacter*	0.012	0.025	0.39	0.88	0.180	0.056
Spirochaetota	*Treponema*	1.56	1.24	1.10	0.89	0.093	0.107
Patescibacteria	*Candidatus Saccharimonas*	1.19 ^{c}	1.34 ^{bc}	1.53 ^{b}	1.96 ^{a}	0.067	<0.01
Actinobacteriota	*Bifidobacterium*	0.23	0.40	0.76	0.53	0.087	0.108
	Olsenella	0.43 ^{c}	0.52 ^{bc}	0.97 ^{a}	0.65 ^{ab}	0.062	<0.01

*Genera whose relative abundance >0.5% in at least one group were shown. ^{a,b,c}Within a row, mean values with different superscript letters indicate a significant difference ($P < 0.05$).

feed efficiency in the current study. Unfortunately, the feed efficiency was not determined in this study. It should be measured in the further study.

FSM is a high-quality plant protein source containing more than 50% crude protein. The study supplemented FSM directly into the diets without modifying the dietary protein levels across the various treatment groups. As a result, the dietary crude protein levels in the treatment groups increased by approximately 0.7 to 1.4% compared to the control group. A slight rise was observed in the numeric value of the milk protein percentage and milk urea nitrogen, but this increase was statistically insignificant. The further studies would adjust the dietary protein levels to be the same across all treatments.

5 Conclusion

Feeding FSM to lactating cows did not affect the milk performance, but increased the serum prolactin levels which would help cows maintain the lactation. Moreover, feeding FSM only caused a minor change in rumen fermentation parameters, but greatly alter the rumen microbiota, with the increase of Firmicutes, and decrease of Bacteroidota in the relative abundance. Though more work should be done to demonstrate the effects of FSM, these findings may provide an approach to keep the peak of lactation in dairy 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 in the article/Supplementary material.

Ethics statement

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

Author contributions

JZ: Data curation, Investigation, Visualization, Writing – original draft. FG: Writing – original draft, Investigation. SH: Investigation,

Writing – original draft. YM: Writing – review & editing, Investigation. SW: Writing – review & editing, Conceptualization. WJ: Writing – original draft, Writing – review & editing. SM: Conceptualization, Funding acquisition, Project administration, Supervision, Writing – review & editing.

Funding

The author(s) declare that financial support was received for the research, authorship, and/or publication of this article. This research was supported by the National Natural Science Foundation of China (32072755).

Conflict of interest

YM was employed by the Jiangsu Jiahui Biotechnology Co., Ltd. and SW was employed by the Shanghai Menon Animal Nutrition Technology Co., Ltd.

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

Publisher's note

Supplementary material

The Supplementary material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fvets.2024.1498639/full#supplementary-material

References

1. Lambo MT, Ma H, Zhang H, Song P, Mao H, Cui G, et al. Mechanism of action, benefits, and research gap in fermented soybean meal utilization as a high-quality protein source for livestock and poultry. *Anim Nutr.* (2024) 16:130–46. doi: 10.1016/j.aninu.2023.10.003

2. Yuan L, Chang J, Yin Q, Lu M, Di Y, Wang P, et al. Fermented soybean meal improves the growth performance, nutrient digestibility, and microbial flora in piglets. *Anim Nutr.* (2017) 3:19–24. doi: 10.1016/j.aninu.2016.11.003

3. Jazi V, Mohebodini H, Ashayerizadeh A, Shabani A, Barekatain R. Fermented soybean meal ameliorates *Salmonella Typhimurium* infection in young broiler chickens. *Poultry Sci.* (2019) 98:5648–60. doi: 10.3382/ps/pez338

4. Feizi LK, Zad SS, Jalali SAH, Rafiee H, Jazi MB, Sadeghi K, et al. Fermented soybean meal affects the ruminal fermentation and the abundance of selected bacterial species in Holstein calves: a multilevel analysis. *Sci Rep*. (2020) 10:12062. doi: 10.1038/s41598-020-68778-6

5. Kim MH, Yun CH, Lee CH, Ha JK. The effects of fermented soybean meal on immunophysiological and stress-related parameters in Holstein calves after weaning. *J Dairy Sci*. (2012) 95:5203–12. doi: 10.3168/jds.2012-5317

6. Rezazadeh F, Kowsar R, Rafiee H, Riasi A. Fermentation of soybean meal improves growth performance and immune response of abruptly weaned Holstein calves during cold weather. *Anim Feed Sci Technol*. (2019) 254:114206. doi: 10.1016/j.anifeedsci.2019.114206

7. Amin AB, Zhang L, Zhang J, Mao S. Fermented soybean meal modified the rumen microbiome to enhance the yield of milk components in Holstein cows. *Appl Microbiol Biotechnol*. (2022) 106:7627–42. doi: 10.1007/s00253-022-12240-2

8. Wang Z, Yu Y, Li X, Xiao H, Zhang P, Shen W, et al. Fermented soybean meal replacement in the diet of lactating Holstein dairy cows: modulated rumen fermentation and ruminal microflora. *Front Microbiol*. (2021) 12:625857. doi: 10.3389/fmicb.2021.625857

9. Weatherburn MW. Phenol-hypochlorite reaction for determination of ammonia. *Anal Chem*. (1967) 39:971–4. doi: 10.1021/ac60252a045

10. Makkar H, Sharma O, Dawra R, Negi S. Simple determination of microbial protein in rumen liquor. *J Dairy Sci*. (1982) 65:2170–3. doi: 10.3168/jds.S0022-0302(82)82477-6

11. Wang Y, Xu L, Liu J, Zhu W, Mao S. A high grain diet dynamically shifted the composition of mucosa-associated microbiota and induced mucosal injuries in the colon of sheep. *Front Microbiol*. (2017) 8:2080. doi: 10.3389/fmicb.2017.02080

12. Mao S, Zhang R, Wang D, Zhu W. 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*. (2012) 8:237–13. doi: 10.1186/1746-6148-8-237

13. Behrendt L, Larkum AW, Trampe E, Norman A, Sørensen SJ, Kühl M. Microbial diversity of biofilm communities in microniches associated with the didemnid ascidian Lissoclinum patella. *ISME J*. (2012) 6:1222–37. doi: 10.1038/ismej.2011.181

14. Magoč T, Salzberg SL. FLASH: fast length adjustment of short reads to improve genome assemblies. *Bioinformatics*. (2011) 27:2957–63. doi: 10.1093/bioinformatics/btr507

15. Campbell BJ, Polson SW, Hanson TE, Mack MC, Schuur EA. The effect of nutrient deposition on bacterial communities in Arctic tundra soil. *Environ Microbiol*. (2010) 12:1842–54. doi: 10.1111/j.1462-2920.2010.02189.x

16. Edgar RC. Search and clustering orders of magnitude faster than BLAST. *Bioinformatics*. (2010) 26:2460–1. doi: 10.1093/bioinformatics/btq461

17. Quast C, Pruesse E, Yilmaz P, Gerken J, Schweer T, Yarza P, et al. The SILVA ribosomal RNA gene database project: improved data processing and web-based tools. *Nucleic Acids Res*. (2013) 41:D590–6. doi: 10.1093/nar/gks1219

18. Mitter EK, de Freitas JR, Germida JJ. Bacterial root microbiome of plants growing in Oil Sands reclamation covers. *Front Microbiol*. (2017) 8:849. doi: 10.3389/fmicb.2017.00849

19. Lacasse P, Ollier S, Lollivier V, Boutinaud M. New insights into the importance of prolactin in dairy ruminants. *J Dairy Sci*. (2016) 99:864–74. doi: 10.3168/jds.2015-10035

20. Kim YJ. Pivotal roles of prolactin and other hormones in lactogenesis and the nutritional composition of human milk. *Clin Exp Pediatr*. (2020) 63:312–3. doi: 10.3345/cep.2020.00311

21. Fernando SC, Purvis H, Najar F, Sukharnikov L, Krehbiel C, Nagaraja T, et al. Rumen microbial population dynamics during adaptation to a high-grain diet. *Appl Environ Microbiol*. (2010) 76:7482–90. doi: 10.1128/AEM.00388-10

22. Wang C, Dong D, Wang H, Müller K, Qin Y, Wang H, et al. Metagenomic analysis of microbial consortia enriched from compost: new insights into the role of Actinobacteria in lignocellulose decomposition. *Biotechnol Biofuels*. (2016) 9:1–17. doi: 10.1186/s13068-016-0440-2

23. Lagkouvardos I, Lesker TR, Hitch TCA, Gálvez EJC, Smit N, Neuhaus K, et al. Sequence and cultivation study of Muribaculaceae reveals novel species, host preference, and functional potential of this yet undescribed family. *Microbiome*. (2019) 7:28. doi: 10.1186/s40168-019-0637-2

24. Kim W. The genus *Lactococcus* In: WH Holzapfel and BJB Wood, editors. Lactic acid bacteria: Biodiversity and Taxonomy John Wiley & Sons, Ltd. (2014) doi: 10.1002/9781118655252

25. Jiang Q, Sherlock DN, Elolimy AA, Yoon I, Loor JJ. Feeding a *Saccharomyces cerevisiae* fermentation product during a gut barrier challenge in lactating Holstein cows impacts the ruminal microbiota and metabolome. *J Dairy Sci*. (2024) 107:4476–94. doi: 10.3168/jds.2023-24147

26. Tong J, Zhang H, Yang D, Zhang Y, Xiong B, Jiang L. Illumina sequencing analysis of the ruminal microbiota in high-yield and low-yield lactating dairy cows. *PLoS One*. (2018) 13:e0198225. doi: 10.1371/journal.pone.0198225

27. Ranilla MJ, Andrés S, Gini C, Biscarini F, Saro C, Martín A, et al. Effects of Thymbra capitata essential oil on in vitro fermentation end-products and ruminal bacterial communities. *Sci Rep*. (2023) 13:4153. doi: 10.1038/s41598-023-31370-9

28. Göker M, Held B, Lucas S, Nolan M, Yasawong M, Glavina Del Rio T, et al. Complete genome sequence of *Olsenella uli* type strain (VPI D76D-27C). *Stand Genomic Sci*. (2010) 3:76–84. doi: 10.4056/sigs.1082860

29. Kim YH, Nagata R, Ohkubo A, Ohtani N, Kushibiki S, Ichijo T, et al. Changes in ruminal and reticular pH and bacterial communities in Holstein cattle fed a high-grain diet. *BMC Vet Res*. (2018) 14:310. doi: 10.1186/s12917-018-1637-3

30. McLoughlin S, Spillane C, Claffey N, Smith PE, O'Rourke T, Diskin MG, et al. Rumen microbiome composition is altered in sheep divergent in feed efficiency. *Front Microbiol*. (2020) 11:1981. doi: 10.3389/fmicb.2020.01981

31. Elolimy A, Alharthi A, Zeineldin M, Parys C, Loor JJ. Residual feed intake divergence during the preweaning period is associated with unique hindgut microbiome and metabolome profiles in neonatal Holstein heifer calves. *J Anim Sci Biotechnol*. (2020) 11:13. doi: 10.1186/s40104-019-0406-x

32. Ellison MJ, Conant GC, Lamberson WR, Cockrum RR, Austin KJ, Rule DC, et al. Diet and feed efficiency status affect rumen microbial profiles of sheep. *Small Rumin Res*. (2017) 156:12–9. doi: 10.1016/j.smallrumres.2017.08.009

frontiers | Frontiers in Veterinary Science

TYPE Original Research
PUBLISHED 27 November 2024
DOI 10.3389/fvets.2024.1503172

OPEN ACCESS

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RECEIVED 28 September 2024
ACCEPTED 30 October 2024
PUBLISHED 27 November 2024

CITATION
Cheng Y, Zhang H, Zhang J, Duan H, Yin Y, Li Y and Mao S (2024) Effects of fermented rice husk powder on growth performance, rumen fermentation, and rumen microbial communities in fattening Hu sheep. *Front. Vet. Sci.* 11:1503172. doi: 10.3389/fvets.2024.1503172

Effects of fermented rice husk powder on growth performance, rumen fermentation, and rumen microbial communities in fattening Hu sheep

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Introduction: This study aimed to examine the effects of fermented rice husk powder feed on growth performance, apparent nutrient digestibility, and rumen microbial communities in fattening Hu sheep.

Methods: Twenty-one male Hu sheep with similar body weights (32.68 ± 1.59 kg) were randomly assigned to three groups: a control group (CON) receiving a TMR with soybean straw, a rice husk powder group (RH), and a fermented rice husk powder group (FHR).

Results: The results indicated that the FHR group exhibited a significant increase in ADG and FBW of Hu sheep compared to the other two groups ($p < 0.05$). The digestibility of CP and EE was significantly higher in the CON and FHR groups than in the RH group ($p < 0.01$). Furthermore, the digestibility of DM in the CON group was higher than in the FHR and RH groups ($p < 0.01$). The FHR group showed lower NDF and ADF digestibility compared to the CON group, but higher than the RH group ($p < 0.05$). Additionally, serum ALB and ALT levels in the CON group were elevated compared to those in the two groups ($p < 0.05$). The rumen concentrations of TVFA, butyrate, and valerate in the FHR group were significantly elevated compared to the other two groups ($p < 0.05$). At the genus level, the relative abundances of *Rikenellaceae RC9 gut group*, *Succinimonas*, *UCG-010_norank*, *UCG-005*, *p-251-o5_norank*, and *Lachnospiraceae AC2044 group* were significantly diminished in the FHR group compared to the CON group ($p < 0.05$). In contrast, the relative abundance of *Succinivibrio* was significantly higher ($p < 0.05$), while the abundances of *Eubacterium coprostanoligenes group_norank* and *Quinella* were significantly lower ($p < 0.05$) in the RH group compared to the CON group. Spearman correlation analysis revealed negative correlations between the *Rikenellaceae RC9 gut group* and propionate, butyrate, and TVFA, as well as between *Prevotellaceae UCG-003* and both propionate and TVFA. Conversely, *Ruminococcus* showed a positive correlation with propionate and TVFA.

Discussion: In conclusion, replacing 15% of soybean straw with fermented rice husk powder feed modified the rumen microbiota and improved the growth performance of fattening Hu sheep.

KEYWORDS

fermented rice husk, Hu sheep, growth performance, rumen fermentation, rumen microbiota

1 Introduction

Roughage is an essential feed ingredient for ruminants, not only providing substantial nutrients but also playing a critical role in maintaining rumen health (1). In China, due to the scarcity of high-quality forages, large quantities of forage such as alfalfa and oat grass are imported annually to meet ruminant production needs (2). However, while high-quality roughage is limited, a large amounts of agricultural by-products, such as straw and rice husks, remain underutilized (3). Rice husk, a major by-product of rice milling, is abundant and inexpensive, with an annual production of approximately 40 million tons in China (4). Research indicates that the cellulose content in rice husks is high and has potential to partially replace traditional roughage for ruminants (5). However, the coarse and hard texture of rice husks results in poor palatability, low intake, and low digestibility when fed directly (6). Current methods for processing agricultural by-products, including physical and chemical approaches, have not been widely adopted due to high energy consumption, environmental pollution, and immature technology (7). How to use rice husk powder to fill the gap in roughage resources has become a new challenge.

In recent years, biological fermentation methods have shown promising results in reducing the fiber content of agricultural by-products and enhancing their feed value (8). Microbial fermentation, a common method for improving feed palatability and nutritional value, works by decomposing macromolecules into a range of metabolites that promote digestion and health while reducing harmful substances in the feed (9). Previous studies have demonstrated that microbial fermentation can improve feed palatability and significantly increase nutrient content, leading to improved growth indicators in animals (10, 11). For instance, adding fermented by-products to lamb starter feeds has been shown to increase average daily gain and enhance the richness and diversity of rumen microbial communities (12). Similarly, incorporating fermented mushroom residues into total mixed rations (TMR) has significantly improved production performance, meat quality, and the diversity and abundance of rumen bacterial communities in Hu sheep (13). Our initial experiments determined the optimal strains and fermentation conditions for probiotic fermentation of rice husk powder (14). Post-fermentation, a significant reduction the pH of the rice husk powder was observed, alongside a notable increase in the viable microbial count. Additionally, the contents of acid-soluble protein and crude fat exhibited substantial increases of 118.05 and 60.44%, respectively (14). Given the observed enhancements in fermentation indices and nutritional content, we hypothesized that compound fermented rice husk powder may positively impact the growth of meat sheep. Therefore, this study aimed to evaluate the effects of partially substituting soybean straw with fermented rice husk powder on sheep growth performance, nutrient digestibility, and rumen microbiota. The findings were intended to offer theoretical guidance for the safe and efficient utilization of fermented rice husk powder.

2 Materials and methods

2.1 Animals, diets, and experimental design

The experimental protocol was approved by the Animal Care and Use Committee of Nanjing Agricultural University (protocol number: SYXK2017-0007).

A total of 21 healthy male Hu sheep in the fattening stage with an average body weight (BW) of 34.19 ± 1.40 kg were selected for the study and randomly allocated into three groups, each consisting of seven sheep. The experimental groups included a control group (CON), a rice husk powder group (RH), and a fermented rice husk powder group (FHR). The control group was fed a total mixed ration (TMR) containing soybean straw powder, while the rice husk powder group replaced 15% of the soybean straw powder in the TMR with rice husk powder feed (composed of 80% rice husk powder, 15% corn grain, and 5% soybean meal). The fermented rice husk powder group substituted 15% of the soybean straw powder in the TMR with fermented rice husk powder feed. The diets were formulated according to nutrient requirements of meat-type sheep and goat (NY/T 816–2021) (15). All raw materials were procured from Da Bei Nong Technology Co., Ltd. in Anhui Province. Ingredients and chemical composition of the experimental diets are presented in Table 1. Each sheep was housed in an individual pen

TABLE 1 Ingredients and chemical composition of the experimental diets.

Items	Groups		
	CON	RH	FHR
Ingredients, % DM			
Corn grain	26.00	28.25	26.00
Cottonseed meal	4.00	4.00	4.00
Soybean meal	3.00	3.75	3.00
Malt sprouts	5.00	5.00	5.00
Wheat bran	7.00	7.00	7.00
Corn bran	10.00	10.00	10.00
Soybean hull	10.00	10.00	10.00
Soybean straw powder	25.50	10.50	10.50
Rice husk powder	—	12.00	—
Fermented rice husk powder feed[1]	—	—	15.00
Calcium carbonate	0.80	0.80	0.80
Salt	0.80	0.80	0.80
Calcium monophosphate	0.80	0.80	0.80
Distillers' Grains	5.00	5.00	5.00
Premix[2]	2.10	2.10	2.10
Nutrient composition, % DM			
CP	16.32	16.45	16.83
EE	4.77	4.40	4.57
NDF	48.30	45.25	44.60
ADF	26.00	25.59	26.00
ME, MJ/kg DM	9.32	9.40	9.30
Ca	0.76	0.79	0.78
P	0.60	0.67	0.64

[1]The fermented rice husk powder feed consists of a fermented substrate consisting of 80% rice husk meal, 15% corn grain and 5% soybean meal. [2]Formulated to provide (per kilogram of premix): 150000KIU of vitamin A, 100000 KIU of vitamin D_3, 600 IU of vitamin E, 0.3 g of Cu, 1.2 g of Fe, 1.8 g of Mn, 25 mg of I, 10 mg of Se, 15 mg of Co.

(1.5 m x 2 m) with wooden slatted floors and had free access to drinking water. All the sheep were fed twice daily at 07:00 and 16:00, ensuring a surplus of 5–10%. The pre-feeding period lasted for 7 days, followed by a formal experimental period of 35 days. All sheep were uniformly dewormed prior to the experiment.

The probiotics incorporated in the fermented rice husk powder feed, such as *Lactobacillus plantarum* L1, *Bacillus subtilis* B6, and *Saccharomyces cerevisiae* Y2, were sourced from Nanjing Zhirun Biotechnology Group Co., Ltd. The inoculation process employed a ratio of 1:2:2 for *Lactobacillus plantarum* L1, *Bacillus subtilis* B6, and *Saccharomyces cerevisiae* Y2, respectively, with the probiotic solution constituting 7% of the total feed volume. To facilitate optimal fermentation, the moisture content of the feed was maintained at 35%. The fermentation process occurred under controlled conditions at 25°C for 96 h, following the method outlined by Cheng et al. (14). The nutritional compositions of both the rice husk powder feed and the fermented rice husk powder feed are provided in Appendix 1.

2.2 Sampling and measurement

2.2.1 Growth performance

On the first day prior to the formal experimental period and on the last day of the experimental period, the sheep were weighed before the morning feeding to calculate the average daily gain (ADG). Additionally, the diet offered and the orts were measured daily for each group of sheep to assess dry matter intake (DMI) and the feed-to-gain ratio (F/G) of the Hu sheep.

2.2.2 Apparent nutrient digestibility

During the digestibility assessment, each sheep was individually housed in a pen. On the last day of each week of the formal experimental period, fecal samples were collected directly from the rectum. Collections occurred twice daily, once in the morning and once in the evening. The samples were thoroughly homogenized, a portion was combined with an equal volume of 10% dilute sulfuric acid for nitrogen fixation. All fecal samples were stored at −20°C.

During the concluding 3 days of the experiment, feed samples were collected, thoroughly mixed, and subjected to the quartering method for sampling. After the experiment, both fecal and feed samples were dried in an oven at 65°C for 48 h. Subsequently, the samples were ground using a Cyclotec mill (Tecator 1,093; Tecator AB, Höganäs, Sweden) with a 40-mesh sieve for conventional nutrient analysis. A portion of the air-dried feed was further dried at 105°C for 3 h to determine the dry matter content. The methods used to measure neutral detergent fiber (NDF) and acid detergent fiber (ADF) were based on Van Soest et al. (16), while the crude protein (CP), crude fat (EE), and ash content in feed and feces were measured according to AOAC (17). The apparent digestibility was calculated using acid-insoluble ash (AIA) as a marker, following the methods of Van Keulen and Chaney (1977). The calculation formula is as follows:

$$\text{Nutrient digestibility}(\%) = \left[1 - \left(\frac{\text{AIA concentration in feed}}{\text{AIA concentration in feces}}\right) \times \left(\frac{\text{Nutrient concentration in feces}}{\text{Nutrient concentration in feed}}\right)\right] \times 100\%$$

2.2.3 Serum biochemical indices

On the 34th day of the formal experimental period, blood samples were collected from the jugular vein using a vacuum tube 2.5 h post-morning feeding. Following clotting, the blood samples were centrifuged at 3500 × g for 15 min at 4°C, and the serum was stored in liquid nitrogen for subsequent analysis of aspartate aminotransferase (AST), alanine aminotransferase (ALT), and alkaline phosphatase (ALP), total protein (TP), albumin (ALB), Urea, glucose (GLU), total cholesterol (TCHO), and triglycerides (TG). Serum biochemical indices were measured using the Beckman Coulter AU5800 automatic biochemical analyzer (United States). The globulin concentration (GLB) was calculated by subtracting the albumin concentration from the total protein concentration.

2.2.4 Rumen fermentation parameters

On the 35th day of the formal experimental period, rumen fluid was collected 2.5 h post-morning feeding using an oral stomach tube. The first 50 mL of the sample was discarded to minimize contamination from saliva. Following immediate filtration through four layers of cheesecloth, the pH of the rumen fluid was measured using a portable pH meter (HI-9024C, HANNA Instruments, United States). The remaining samples were subpackaged and stored at −20°C for subsequent analysis. 1 mL of rumen fluid was mixed with 0.2 mL of 25% (w/v) orthophosphoric acid and analyzed for VFA concentrations using gas chromatography (GC-14B, Shimadzu, Japan) (18). The ammonia nitrogen (NH_3-N) concentration in the rumen fluid was measured using a colorimetric method (19). Microbial crude protein (MCP) concentration was determined using a Bradford protein concentration assay kit (Beijing Solarbio Science & Technology Co., Ltd).

2.2.5 DNA extraction, 16S rRNA amplicon sequencing, and bacterial composition analysis

Total microbial genomic DNA was extracted from rumen content samples using the E.Z.N.A.® soil DNA Kit (Omega Bio-Tek, Norcross, GA, United States) according to the 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 was amplified with primer pairs 338F: ACTCCTACG GGAGGCAGCAG and 806R: GGACTACHVGGGTWTCTAAT (20) by an ABI Gene Amp® 9,700 PCR thermocycler (ABI, CA, United States). Purified amplicons were pooled in equimolar amounts and paired-end sequenced on an Illumina Mi Seq PE300 platform/ Nova Seq PE250 platform (Illumina, San Diego, USA) according to the standard protocols by Majorbio Bio-Pharm Technology Co. Ltd. (Shanghai, China).

Raw FASTQ files were de-multiplexed using an in-house Perl script, then quality-filtered by fastp version 0.19.6 (21) and merged by FLASH version 1.2.7 (22) with the following criteria: 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. 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. Then the optimized sequences were clustered into

TABLE 2 Effects of fermented rice husk powder on growth performance in fattening Hu sheep.

Items	Groups			SEM	*P*-value
	CON	RH	FHR		
DMI, g/d	1453.1	1438.6	1608.2	35.03	0.084
IBW, kg	32.63	32.69	32.71	0.36	0.996
FBW, kg	38.54^{b}	37.86^{b}	39.76^{a}	0.40	0.039
ADG, g/d	166.5^{ab}	147.8^{b}	204.5^{a}	9.70	0.043
F/G	9.04	9.97	8.21	0.38	0.166

CON (Control diet), RH (Containing 15% rice husk powder feed), FHR (Containing 15% fermented rice husk powder feed).

TABLE 3 Effects of fermented rice husk powder on nutrient apparent digestibility in fattening Hu sheep.

Items	Groups			SEM	*P*-value
	CON	RH	FHR		
DM, %	62.40^{a}	58.86^{b}	59.64^{b}	0.54	<0.001
CP, %	75.28^{a}	69.87^{b}	75.42^{a}	0.59	<0.001
EE, %	67.36^{a}	60.42^{b}	68.69^{a}	1.21	<0.001
NDF, %	47.23^{a}	29.49^{c}	34.47^{b}	1.34	<0.001
ADF, %	33.76^{a}	16.47^{c}	22.80^{b}	1.41	<0.001

CON (Control diet), RH (Containing 15% rice husk powder feed), FHR (Containing 15% fermented rice husk powder feed).

operational taxonomic units (OTUs) using UPARSE 7.1 (23) with a 97% sequence similarity level. The most abundant sequence for each OTU was selected as a representative sequence. On the basis of the above analyses, a series of in-depth statistical and visual analyses, such as multivariate analysis and difference significance test, were conducted on the community composition of multiple samples. Alpha diversity was calculated using Qiime software (Version 1.9.1), and differences between groups were analyzed using R software (Version 2.15.3). Bray-Curtis distances were computed using the default script from the Phyloseq package to measure beta diversity. Principal Component Analysis (PCoA) was conducted using the ade4 and ggplot2 packages of R software.

2.3 Statistical analyses

The growth performance, apparent digestibility, rumen fermentation parameters, and serum biochemical indicators of Hu sheep were analyzed using one-way ANOVA in SPSS 26.0, and multiple comparison tests (SNK method) were performed. Covariance analysis was used for the FBW, with the covariate being the IBW. The differences in alpha diversity indicators and relative abundance of microbial communities were analyzed using non parametric tests (Kruskal Wallis). Spearman's rank correlation coefficients were calculated between the relative abundances of all pairs of genera using the Hmisc package in R (version 4.0.3). Only significant correlations ($p < 0.05$) with a Spearman's correlation coefficient $|R| > 0.6$ were retained for further analysis. An adjacency matrix based on significant Spearman correlations was created to represent the network. The network was visualized using Gephi. Nodes in the network represented genera, and edges represented significant correlations between them. Node size was proportional to the genus's relative abundance, and edge thickness was proportional to the strength of the correlation. The significance level $p < 0.05$ indicates significant differences, while $p < 0.01$ indicates extremely significant differences in the data.

3 Results

3.1 Growth performance

No significant differences in IBW, DMI, and F/G were observed among the three groups of Hu sheep ($p > 0.05$). However, there was a significant improvement in FBW ($p < 0.05$). Compared to the RH group, the ADG in the FHR group was significantly higher ($p < 0.05$), while no significant differences were noted between the FHR and control groups ($p > 0.05$) (Table 2).

3.2 Apparent digestibility of nutrients

The apparent digestibility of CP and EE was significantly higher in the FHR and control groups compared to the RH group ($p < 0.001$), with no significant difference observed between the FHR and CON groups ($p > 0.05$). The apparent digestibility of DM, NDF, and ADF was significantly lower in the RH and FHR groups compared to the CON ($p < 0.001$). No significant difference in DM digestibility was found between the RH and FHR groups. However, the digestibility NDF and ADF was significantly higher in the FHR group compared to the RH group ($p < 0.05$) (Table 3).

3.3 Serum biochemical indices

In comparison to the CON group, the levels of ALB and ALT were significantly decreased in both the RH and FHR groups ($p < 0.05$). However, no significant differences were observed among the three groups for other indices, including AST, TP, GLOB, ALP, Urea, GLU, TCHO, and TG ($p > 0.05$) (Table 4).

3.4 Rumen fermentation parameters

There were no significant differences in rumen pH among the three groups ($p > 0.05$), but the FHR group had the lowest pH. Concentrations of TVFA, butyrate, and valerate were significantly elevated in the FHR group compared to the other two groups ($p < 0.05$). Both the RH and FHR groups exhibited a significant decrease in the A/P compared to the CON group ($p < 0.05$). No significant differences were observed in the concentrations of acetate, propionate, NH_3-N, and MCP among the three groups ($p > 0.05$; Figure 1).

3.5 Rumen microbial diversity

In terms of alpha diversity, no significant differences were observed in the ACE and Chao1 indices among the CON, RH, and FHR groups ($p > 0.05$). However, the FRH and RH groups significantly increased the Simpson index ($p < 0.05$), while the Shannon index of the FHR group was

TABLE 4 Effects of fermented rice husk powder on serum biochemical parameters in fattening Hu sheep.

Items	Groups			SEM	*p*-value
	CON	RH	FHR		
TP, g/L	60.33	56.26	56.55	1.03	0.203
ALB, g/L	27.11[a]	24.11[b]	24.87[b]	0.53	0.046
GLB, g/L	33.21	32.14	31.69	0.81	0.750
A/G	0.83	0.79	0.80	0.02	0.697
UREA, mmol/L	9.08	8.47	9.84	0.31	0.209
TCHO, mmol/L	1.39	1.38	1.51	0.05	0.550
TG, mmol/L	0.30	0.37	0.42	0.03	0.244
GLU, mmol/L	4.43	5.64	5.53	0.29	0.168
ALP, U/L	546.7	415.6	457.4	26.49	0.116
ALT, U/L	19.71[a]	15.14[b]	15.71[b]	0.81	0.034
AST, U/L	102.7	89.86	100.00	3.29	0.253

CON (Control diet), RH (Containing 15% rice husk powder feed), FHR (Containing 15% fermented rice husk powder feed).

lower ($p < 0.05$). The PCoA results based on the Unweighted-UniFrac metric demonstrated a distinct separation of the FHR group from both the CON and RH groups (R = 0.388, $p = 0.001$) (Figure 2).

3.6 Rumen fermentation parameters

A total of 29 phyla were identified, with Bacteroidota (63.97%), Firmicutes (23.10%), Proteobacteria (8.87%), and Cyanobacteria (1.15%) having a relative abundance greater than 1% (Figure 3A). The relative abundances of Acidobacteriota and Chloroflexi in the FHR group were lower than in the RH group, although no differences were noted when compared with the CON group (Figure 3B).

At the genus level, 13 genera had a relative abundance greater than 1%. The FHR group exhibited a significantly lower relative abundance of *Rikenellaceae RC9 gut group*, *Succinimonas*, *UCG-010_norank*, *UCG-005*, *p-251-o5_norank*, and *Lachnospiraceae AC2044 group* compared to the CON group ($p < 0.05$). Conversely, the relative abundance of *Succinivibrio* in the RH group was significantly higher than that in the CON group ($p < 0.05$), while the relative abundances of *Eubacterium coprostanoligenes group_norank* and *Quinella* were lower than in the CON group ($p < 0.05$). However, no differences were found in the relative abundances of these genera between the FHR and RH groups ($p > 0.05$; Figures 3C,D).

3.7 Relationships between major rumen bacteria, fermentation parameters, and growth performance

Spearman correlation analysis was conducted to investigate the relationships between rumen VFA and the top 15 ranked bacterial genera based on relative abundance. The results revealed significant negative correlations between *Rikenellaceae RC9 gut group* and propionate, butyrate, and total VFA. Additionally, *Prevotellaceae UCG-003* exhibited negative correlations with propionate and TVFA, whereas *Ruminococcus* showed a positive correlation with propionate, and, total VFA. The correlation between the isovalerate and *Ruminococcus*, Muribaculaceae_norank, and F082_norank had a significantly positive correlation. *UCG-002* is positively correlated with propionate and negatively correlated with A/P (Figure 4A). These findings suggest a potential role of rumen microorganisms in regulating rumen fermentation.

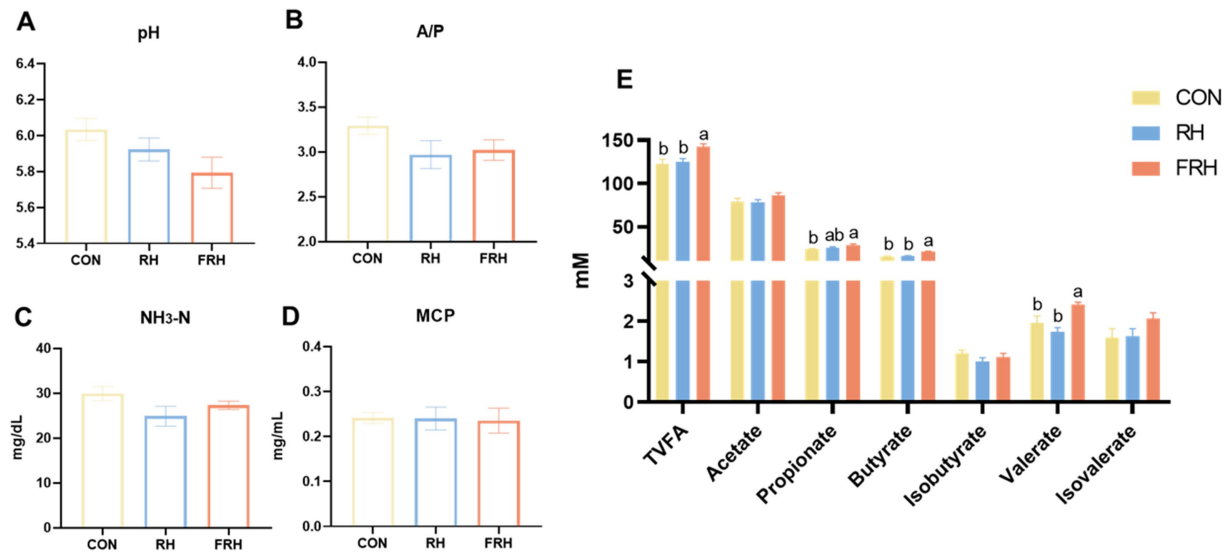

FIGURE 1
Effects of fermented rice husk powder on rumen fermentation parameters in fattening Hu sheep. (A) rumen pH, (B) A/P = acetate to propionate ratio, (C) NH3-N = ammonia nitrogen, (D) MCP = microbial protein, (E) rumen VFA.

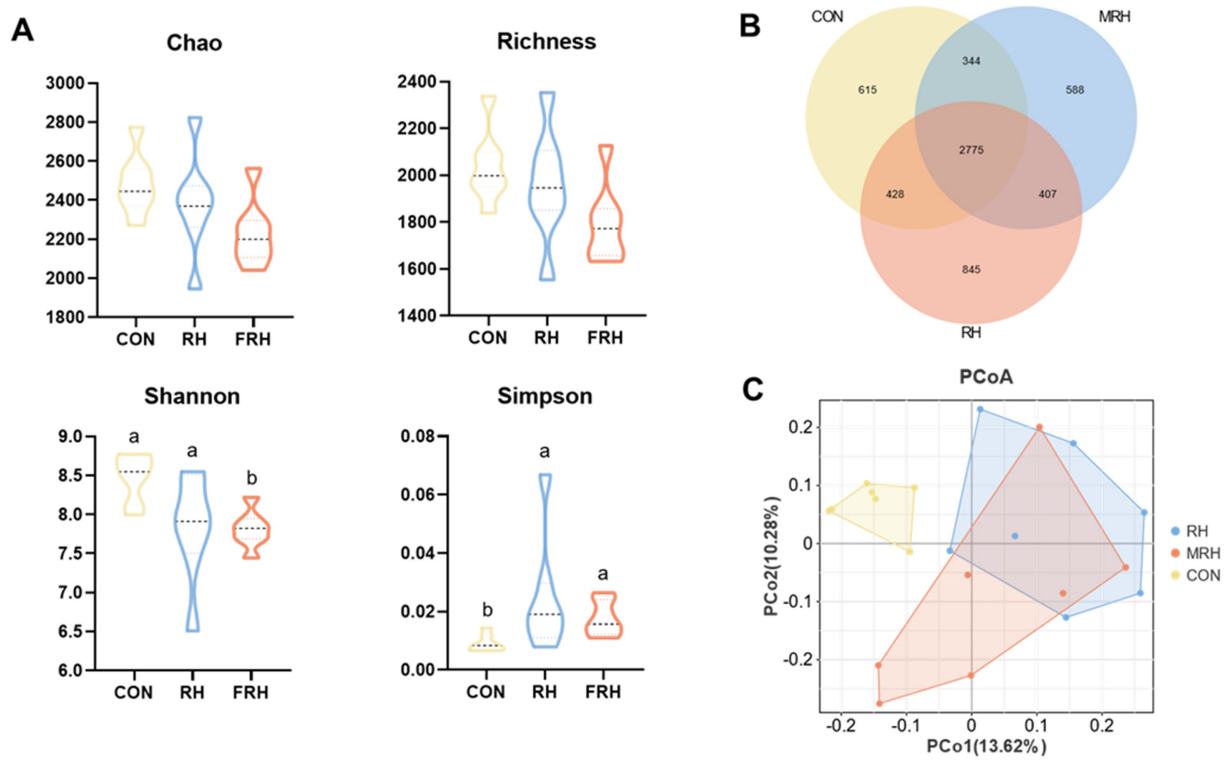

FIGURE 2
Effects of fermented rice husk powder on rumen microbial diversity in fattening Hu sheep. (A) Alpha diversity analysis, (B) Venn diagram and microbial operational taxonomic unit (ASV), (C) Bray-Curtis PCoA by rumen bacteria.

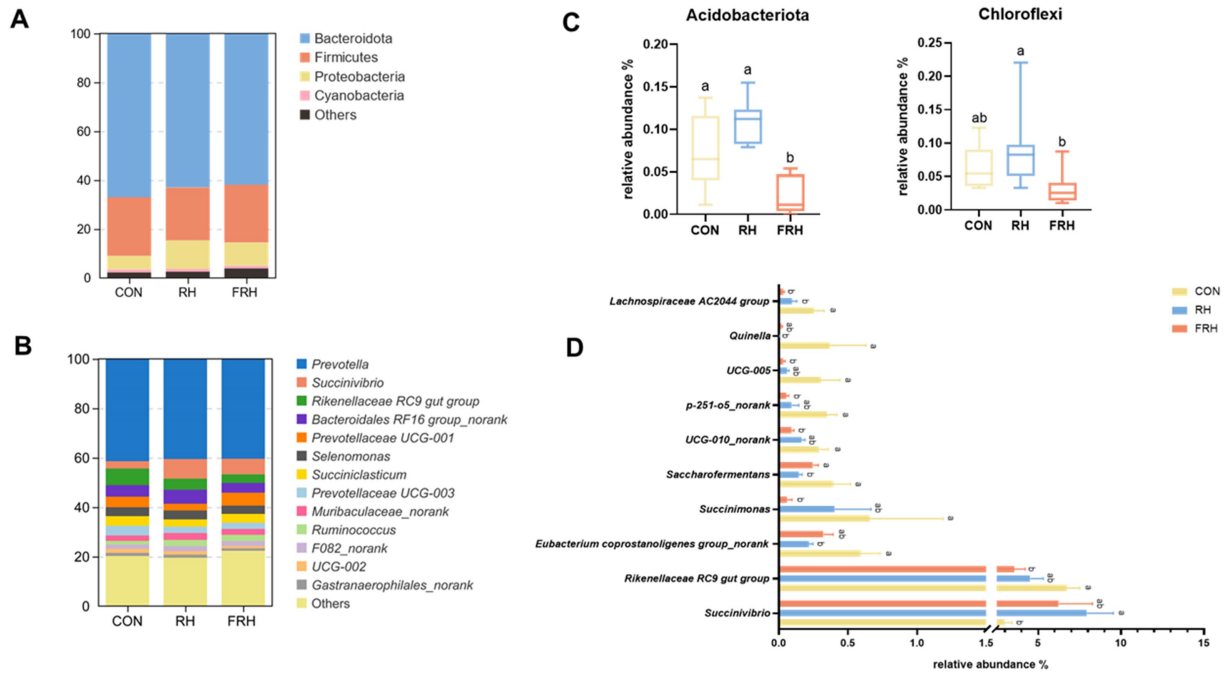

FIGURE 3
Effect of fermented rice husk powder on the relative abundance (relative abundance >1% in at least one treatment) of rumen bacteria at phylum (A) and genus (C) levels in fattening Hu sheep. (B) Differential rumen bacteria at the phylum level. (D) Differential rumen bacteria at the genus level.

Correlation network analyses were further performed to investigate the relationships between rumen microorganisms and sheep growth performance. A correlation was defined as having a strong interactive relationship if it had a *p*-value <0.05 and an R value >0.4 (Figure 4B). The results suggest that *Veillonellaceae UCG-001*, *Bacteroidales RF16 group_norank*, *Anarovibrio*, and *Gastroanaerophilales_norank* have the potential to influence DMI. Additionally, *Prevotellaceae UCG-001* and *Clostridia UCG-014_norank* are correlated with FBW. While *Lachnospiraceae Unclassified* and *Veillonellaceae UCG-001* has a strong correlation with ADG.

4 Discussion

4.1 Growth performance

Enzymes secreted by microorganisms during the fermentation process can degrade complex compounds in plant fibers, thereby imparting a distinctive aroma to fermented feed and subsequently enhancing animal intake (24). Research found that feeding fermented rice straw containing probiotics to fattening cattle improved growth performance compared to the Control (25). Similarly, fermented TMR was found to notably increase the feed intake and growth performance of Hanwoo steers (26). However, the findings of the current experiment revealed no significant differences in feed intake among the three groups of Hu sheep, suggesting that the fermented feed did not enhance animal feed intake. This outcome may be attributed to variations in the fermentation strains utilized in different studies, leading to negligible changes in the flavor compounds of the fermented rice husk powder.

Research indicates that the incorporation of fermented feed can positively influence animal growth. For instance, the study conducted in the same area (27) demonstrated that supplementing with 10% fermented mulberry leaves significantly improved the average daily gain of Hu sheep. Furthermore, the fermentation of rice straw using a combination of calcium oxide, *Bacillus* spp., and coconut water markedly increased the digestibility of OM, CP, and DM (28). Additionally, the inoculation of corn silage with either *Lactobacillus buchneri* or *Lactobacillus plantarum* separately improved the intake of dry matter (DM), crude protein (CP), and neutral detergent fiber (NDF) in lambs by Basso et al. (29). In this experiment, the ADG and FBW of the FHR group were significantly higher than those of the other two groups. This finding indicates that fermented rice husk powder increased the ADG and final weight of the sheep without a corresponding increase in feed intake.

4.2 Apparent digestibility of nutrients

It is widely acknowledged that microbial fermentation can improve the quality of substandard feed materials (30). Research has demonstrated that treating rice husks through biodegradation by *Pleurotus ostreatus* results in an increase in CP content from 2.15 to 9.3%, alongside a 14.76% rise in digestibility. Additionally, there is a notable reduction in crude fiber and lignin content by 35.4 and 40.9%, respectively (31). The enhancement of nutrient digestibility in animals fed fermented feed is likely attributable to modifications in the physical properties of fibers induced by the fermentation process, which facilitate microbial breakdown in the rumen (32).

The experiment shows that the FHR group's digestibility is higher than the RH group but lower than the CON group. This implies that fermenting rice husk powder improves its digestibility, though not to the level of the CON group. The difference may be due to the higher lignocellulose content in rice husk compared to soybean stalk powder. The digestibility of CP and EE in

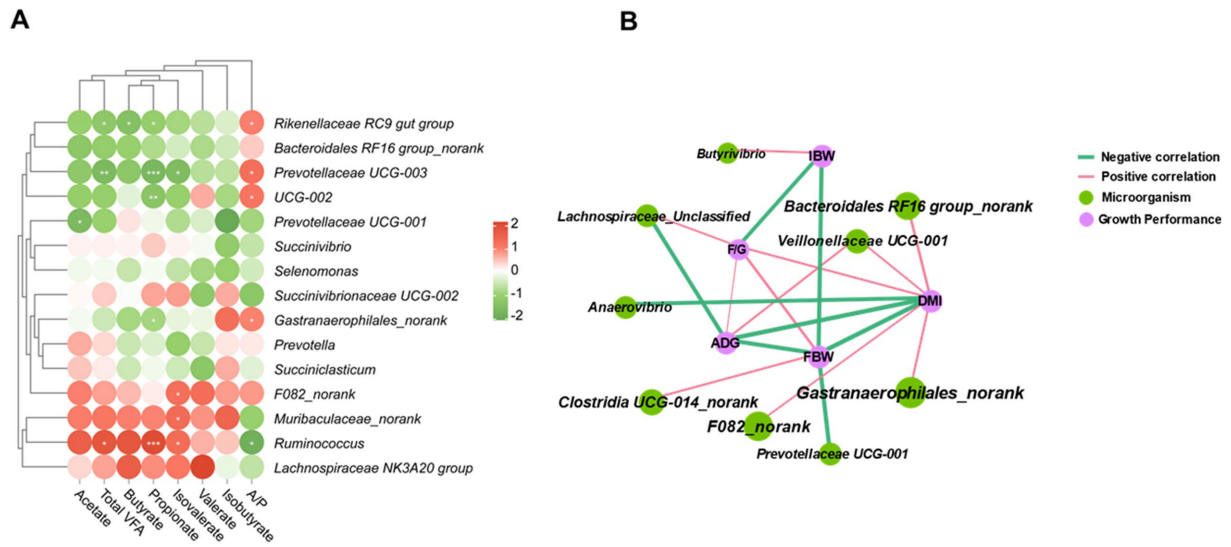

FIGURE 4
Relationships of bacterial communities, VFA fermentation parameters and growth performance. The heatmap shows that the correlation between the predominant rumen bacteria (relative abundance of the top 15) and VFA fermentation parameters (A), the networks are constructed based on the adjacency matrix of microbial abundance and growth performance from the correlation analysis (B) ($p < 0.05$, $R > 0.6$).

fermented rice husk powder was similar to the CON group but significantly higher than the RH group. Fiber digestibility varied among the three groups, suggesting that rice husk powder's lignocellulose is harder to degrade than soybean stalk powder. However, probiotic fermentation significantly improved its digestibility. Similar results were observed in a study where feeding fermented cottonseed hulls to calves significantly improved their ADG, even though the DM digestibility of the fermented cottonseed hulls was markedly lower than that of the CON group (33). These findings suggest that fermented feeds with high lignocellulose content can enhance digestibility to some extent and promote growth in animals.

4.3 Serum biochemical indices

The results of the serum biochemical indicators revealed that the ALB and ALT levels were significantly decreased in the RH and FHR group compared to the CON group, while no significant differences were observed for other indicators, such as GLB. Previous research indicates that increased protein metabolism can result in elevated ALT levels. However, disruptions in hepatocytes cellular membrane may also contribute to higher serum ALT levels (34). Variations in ALB levels partially reflect the nutritional quality of dietary protein and the status of protein digestion, absorption, and metabolism in the animal (35). It is possible that the higher protein content in soybean stalk powder compared to rice husk powder led to the increased ALT and ALB levels in the serum of the Hu sheep in the control group.

4.4 Rumen fermentation parameters

Feeding fermented feeds led to a decrease in rumen pH, likely due to microbial facilitation of carbohydrate breakdown during the fermentation process, which subsequently increases VFA production (36). The fermentation process modified the fiber composition of rice husk powder, thereby influencing the rumen fermentation patterns. Notably, the butyrate level in the rumen of the FHR group was significantly elevated compared to the other two groups, while the valerate level was significantly higher than in the RH group. Numerous studies have demonstrated that butyrate plays a crucial role in the development of the rumen epithelium in lambs (37). The marked increase in butyrate levels in the rumen of sheep fed fermented rice husk powder suggests that this dietary intervention may promote rumen development. Furthermore, the A/P in the rumen fluid of the FHR group was significantly lower compared to the control group, indicating that the inclusion of fermented rice husk powder in the diet altered the rumen fermentation pattern.

4.5 Rumen microbiota

Rumen microorganisms play a crucial role in fermentation process and overall health. The present study's diversity analysis results reveal significant alterations in rumen microbial populations due to the different dietary formulations. Previous research indicates that fermented feeds generally do not lead to dramatic shifts in dominant microbial phyla (38). However, in this study, the relative abundances of Acidobacteriota and Chloroflexi were significantly reduced in the FHR group compared to the RH group. At present, the functional roles of Acidobacteriota and Chloroflexi within the rumen remain inadequately understood. It has been observed that feeding a blend of cinnamaldehyde, eugenol, and capsicum oleoresin significantly reduced the relative abundance of Acidobacteriota and Chloroflexi in the rumen, suggesting potential associations with decreased inflammation and apoptosis in the rumen (39).

The FHR group reduced the relative abundances of the Rikenellaceae RC9 gut group, *UCG-010_norank*, *p-251-o5_norank*, *UCG-005*, *Quinella*, and *Lachnospiraceae AC2044 group*. The *Rikenellaceae RC9 gut group* is commonly found in the digestive tracts of various animals and is closely associated with the digestion of carbohydrates and celluloses (40). Additionally, it plays a role in starch degradation (41). Correlation analysis revealed a negative correlation between the *Rikenellaceae RC9 gut group* and various VFA, with a positive correlation observed solely with the A/P. This finding diverges from prior studies, possibly due to differences in the fiber composition of soybean straw powder and fermented rice husk powder. Such differences may result in shifts in the dominant microorganisms involved in degradation and VFA production.

Ruminococcus and *Quinella* are primarily associated with the degradation of cellulose and carbohydrates and can produce succinate, a precursor of propionate (42). A strong positive correlation was found between *Ruminococcus* and propionate concentration, suggesting that the dominant microorganisms involved in carbohydrate degradation in the FHR group may be influenced by *Ruminococcus*. The correlation network indicated that Veillonellaceae UCG-001, Bacteroidales *RF16 group_norank*, *Anaerovibrio*, and *Gastranaerophilales_norank* have the potential to regulate DMI in the rumen. *Anaerovibrio* has been reported to generate propionate through lipid metabolism, suggesting its potential role in modulating DMI in lambs (43). Furthermore, the Veillonellaceae family is linked to the remaining feed intake in beef cattle, indicating that *Veillonellaceae UCG-001* may collectively regulate DMI in sheep. The functional ecological niches of different microbial communities present in the rumen interact synergistically, affecting rumen microbial function. Feeding fermented rice husk powder may enrich the microbial network that promotes DMI in sheep, thereby enhancing their growth and development.

5 Conclusion

The incorporation of 15% fermented rice husk powder feed has been demonstrated to enhance the growth performance and nutrient digestion of Hu sheep, as well as to increase the concentrations of propionate, butyrate, and valerate in the rumen. This improvement in rumen fermentation and growth performance in sheep is likely mediated by rumen microorganisms, including *Veillonellaceae UCG-001*, *Bacteroidales RF16 group_norank*, *Anaerovibrio*, and *Gastranaerophilales_norank*. Therefore, fermented rice husk powder feed represents a promising alternative to traditional roughage in the diet to enhance animal performance.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Ethics statement

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

Author contributions

YC: Data curation, Visualization, Writing – original draft. HZ: Data curation, Visualization, Writing – original draft. JZ: Visualization, Writing – review & editing. HD: Visualization, Writing – review & editing. YY: Formal analysis, Funding acquisition, Project administration, Writing – review & editing. YL: Formal analysis, Funding acquisition, Project administration, Resources, Writing – review & editing. SM: Conceptualization, Formal analysis, Investigation, Project administration, Resources, Supervision, Writing – review & editing.

Funding

The author(s) declare that financial support was received for the research, authorship, and/or publication of this article. This work was financially supported by Zhejiang Provincial Department of Agriculture and Rural Affairs (no. zjxmcytd20241415).

Conflict of interest

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Supplementary material

The Supplementary material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fvets.2024.1503172/full#supplementary-material

References

1. Zhu X, Liu B, Xiao J, Guo M, Zhao S, Hu M, et al. Effects of different roughage diets on fattening performance, meat quality, fatty acid composition, and rumen microbe in steers. *Front Nutr*. (2022) 9:885069. doi: 10.3389/fnut.2022.885069

2. Wang Y, Zhao Y, Xue F, Nan X, Wang H, Hua D, et al. Nutritional value, bioactivity, and application potential of Jerusalem artichoke (*Helianthus tuberosus* L.) as a Neotype feed resource. *Anim Nutr*. (2020) 6:429–37. doi: 10.1016/j.aninu.2020.09.001

3. Sun N, Gao C, Ding Y, Bi Y, Seglah PA, Wang Y. Five-dimensional straw utilization model and its impact on carbon emission reduction in China. *Sustainability*. (2022) 14:16722. doi: 10.3390/su142416722

4. Zhang Y, Wang L, Li T, Shen Y, Luo J. Acid soaking followed by steam Flash-explosion pretreatment to enhance saccharification of Rice husk for poly (3-Hydroxybutyrate) production. *Int J Biol Macromol*. (2020) 160:446–55. doi: 10.1016/j.ijbiomac.2020.05.218

5. Amata I. The use of non-conventional feed resources (Ncfr) for livestock feeding in the tropics: a review. *J Glob Biosci*. (2014) 3:604–13.

6. Kumar S, Sangwan P, Dhankhar RMV, Bidra S. Utilization of Rice husk and their ash: a review. *Res J Chem Env Sci*. (2013) 1:126–9.

7. Salim AA, Grbavčić S, Šekuljica N, Stefanović A, Tanasković SJ, Luković N, et al. Production of enzymes by a newly isolated Bacillus Sp. Tmf-1 in solid state fermentation on agricultural by-products: the evaluation of substrate pretreatment methods. *Bioresour Technol*. (2017) 228:193–200. doi: 10.1016/j.biortech.2016.12.081

8. Capanoglu E, Nemli E, Tomas-Barberan F. Novel approaches in the valorization of agricultural wastes and their applications. *J Agric Food Chem*. (2022) 70:6787–804. doi: 10.1021/acs.jafc.1c07104

9. Van Soest PJ. Nutritional ecology of the ruminant. New York: Cornell University Press (2018).

10. Muck R, Nadeau E, McAllister T, Contreras-Govea F, Santos M, Kung L Jr. Silage review: recent advances and future uses of silage additives. *J Dairy Sci*. (2018) 101:3980–4000. doi: 10.3168/jds.2017-13839

11. Shi C, Zhang Y, Lu Z, Wang Y. Solid-state fermentation of corn-soybean meal mixed feed with *Bacillus subtilis* and *Enterococcus faecium* for degrading Antinutritional factors and enhancing nutritional value. *J Anim Sci Biotechnol*. (2017) 8:50–9. doi: 10.1186/s40104-017-0184-2

12. Zhou R, Wang L, Li Y, Wu H, Lu L, Zang R, et al. Effects of tail vegetable fermented feed on the growth and rumen microbiota of lambs. *Animals*. (2024) 14:303. doi: 10.3390/ani14020303

13. Huang X, Zhou L, You X, Han H, Chen X, Huang X. Production performance and rumen bacterial community structure of Hu sheep fed fermented spent mushroom substrate from *Pleurotus eryngii*. *Sci Rep*. (2023) 13:8696. doi: 10.1038/s41598-023-35828-8

14. Cheng Y, Fan Y, Mao S, Ding L. Optimization of fermentation parameters for fermented feed production based on rice husk meal and variation in nutrients of the feed. *Anim Husbandry Vet Med*. (2023) 55:30–6.

15. Bureau F. *Ministry of Agriculture and rural Affairs of the People's republic of China*. China Fishery Statistical Yearbook No. 25. (2021).

16. Van Soest PV, Robertson JB, Lewis BA. Methods for dietary Fiber, neutral detergent Fiber, and nonstarch polysaccharides in relation to animal nutrition. *J Dairy Sci*. (1991) 74:3583–97. doi: 10.3168/jds.S0022-0302(91)78551-2

17. Williams S. Official methods of analysis of the Association of Official Analytical Chemists. Rockville, MA: Association of Official Analytical Chemists (1984).

18. Mao S, Zhang G, Zhu W. Effect of disodium fumarate on ruminal metabolism and rumen bacterial communities as revealed by denaturing gradient gel electrophoresis analysis of 16s ribosomal. *DNA*. (2008) 140:293–306. doi: 10.1016/j.anifeedsci.2007.04.001

19. Chaney AL, Marbach EP. Modified reagents for determination of Urea and Ammonia. *Clin Chem*. (1962) 8:130–2. doi: 10.1093/clinchem/8.2.130

20. Xu N, Tan GC, Wang HY, Gai XP. Effect of biochar additions to soil on nitrogen leaching, microbial biomass and bacterial community structure. *Eur J Soil Biol*. (2016) 74:1–8. doi: 10.1016/j.ejsobi.2016.02.004

21. Chen S, Zhou Y, Chen Y, Gu J. Fastp: An ultra-fast all-in-one Fastq preprocessor. *Bioinformatics*. (2018) 34:i884–90. doi: 10.1093/bioinformatics/bty560

22. Magoc T, Salzberg SL. Flash: fast length adjustment of short reads to improve genome assemblies. *Bioinformatics*. (2011) 27:2957–63. doi: 10.1093/bioinformatics/btr507

23. Wei PT, Xi C, Laczkovich I, Alonzo F. *Staphylococcus aureus* adapts to the host nutritional landscape to overcome tissue-specific branched-chain fatty acid requirement. *Proc Nat Acad Sci*. (2021) 118:e2022720118. doi: 10.1073/pnas.2022720118

24. Senanayake D, Torley PJ, Chandrapala J, Terefe NS. Microbial fermentation for improving the sensory, nutritional and functional attributes of legumes. *Fermentation*. (2023) 9:635. doi: 10.3390/fermentation9070635

25. Kabir M, Alam M, Hossain M, Ferdaushi Z. Effect of feeding probiotic fermented Rice straw-based Total mixed ration on production, blood parameters and Faecal microbiota of fattening cattle. *J Anim Health Prod*. (2022) 10:190–7. doi: 10.17582/journal.jahp/2022/10.2.190.197

26. Kim TI, Mayakrishnan V, Lim DH, Yeon JH, Baek KS. Effect of fermented Total mixed rations on the growth performance, carcass and meat quality characteristics of Hanwoo steers. *Anim Sci J*. (2018) 89:606–15. doi: 10.1111/asj.12958

27. Su Y, Chen G, Cai Y, Gao B, Zhi X, Chang F. Effects of *Broussonetia papyrifera*-fermented feed on the growth performance and muscle quality of Hu sheep. *Can J Anim Sci*. (2020) 100:771–80. doi: 10.1139/cjas-2018-0167

28. Yanuarianto O, Amin M, Iqbal M, Hasan SD. Kecernaan bahan kering dan bahan organik jerami padi yang difermentasi dengan Kombinasi Kapur Tohor, *Bacillus* s., dan Air Kelapa pada Waktu yang Berbeda. *Indonesian J Anim Sci Technol*. (2015) 1:55–61. doi: 10.29303/jitpi.v1i1.10

29. Basso F, Adesogan A, Lara E, Rabelo C, Berchielli TT, Teixeira I, et al. Effects of feeding corn silage inoculated with microbial additives on the ruminal fermentation, microbial protein yield, and growth performance of lambs. *J Anim Sci*. (2014) 92:5640–50. doi: 10.2527/jas.2014-8258

30. Choi Y, Rim JS, Na Y, Lee SR. Effects of dietary fermented spent coffee ground on nutrient digestibility and nitrogen utilization in sheep. *Asian Australas J Anim Sci*. (2018) 31:363–8. doi: 10.5713/ajas.17.0654

31. Beg S, Zafar SI, Shah F. Rice husk biodegradation by *Pleurotus ostreatus* to produce a ruminant feed. *Agric Wastes*. (1986) 17:15–21. doi: 10.1016/0141-4607(86)90145-9

32. Xu Y, Aung M, Sun ZY, Zhou YQ, Xue TH, Cheng XM, et al. Ensiling of Rice straw enhances the nutritive quality, improves average daily gain, reduces methane production and increases ruminal bacterial diversity in growing Hu lambs. *Anim Feed Sci Technol*. (2023) 295:115513. doi: 10.1016/j.anifeedsci.2022.115513

33. Shivakumar M, Pugashetti B, Naik D, Hosamani S, Kulkarni V. Effect of feeding treated and untreated cotton seed Hull diet on growth and digestibility in calves. *J Agric Sci*. (2005) 18:121–3.

34. Hamdi M. The effects of Amofer palm oil waste-based complete feed to blood profiles and liver function on local sheep. *Int J Sci Eng*. (2012) 3:17–21. doi: 10.12777/ijse.3.1.17-21

35. Cheng G, Chen F, Liu G, Fu G, Wang C, Zhou Q, et al. Effects of *Lactobacillus plantarum* and it mixed with Urea added in whole corn silage on growth performance, slaughter performance and muscle quality of Dorper sheep. *Chin J Anim Nutr*. (2019) 31:5857–65.

36. Goad D, Goad C, Nagaraja T. Ruminal microbial and fermentative changes associated with experimentally induced subacute acidosis in steers. *J Anim Sci*. (1998) 76:234–41. doi: 10.2527/1998.761234x

37. Świerk S, Przybyło M, Flaga J, Szczepanik K, Garus-Piętak A, Biernat W, et al. Effect of increased intake of concentrates and sodium butyrate supplementation on ruminal epithelium structure and function in growing rams. *Animal*. (2023) 17:100898. doi: 10.1016/j.animal.2023.100898

38. Wang Z, Yu Y, Li X, Xiao H, Zhang P, Shen W, et al. Fermented soybean meal replacement in the diet of lactating Holstein dairy cows: modulated rumen fermentation and ruminal microflora. *Front Microbiol*. (2021) 12:625857. doi: 10.3389/fmicb.2021.625857

39. Wang WW, Wang Y, Guo T, Gao C, Yang Y, Yang L, et al. Blend of Cinnamaldehyde, eugenol, and Capsicum oleoresin improved rumen health of lambs fed high-concentrate diet as revealed by fermentation characteristics, epithelial gene expression, and bacterial community. *Animals*. (2023) 13:1663. doi: 10.3390/ani13101663

40. Tian H, Chen Y, Zhu N, Guo Y, Deng M, Liu G, et al. Effect of *Broussonetia papyrifera* silage on the serum indicators, hindgut parameters and fecal bacterial Community of Holstein Heifers. *AMB Express*. (2020) 10:197–16. doi: 10.1186/s13568-020-01135-y

41. Li Z, Wright ADG, Liu H, Fan Z, Yang F, Zhang Z, et al. Response of the rumen microbiota of sika deer (*Cervus nippon*) fed different concentrations of tannin rich plants. *PLoS One*. (2015) 10:e0123481. doi: 10.1371/journal.pone.0123481

42. Sijpesteijn AK. On *Ruminococcus flavefaciens*, a cellulose-decomposing: bacterium from the rumen of sheep and cattle. *Microbiology*. (1951) 5:869–79. doi: 10.1099/00221287-5-5-869

43. Li FY, Guan LL. Metatranscriptomic profiling reveals linkages between the active rumen microbiome and feed efficiency in beef cattle. *Appl Environ Microb*. (2017) 83:17. doi: 10.1128/AEM.00061-17

frontiers | Frontiers in Veterinary Science

TYPE Original Research
PUBLISHED 06 January 2025
DOI 10.3389/fvets.2024.1523062

OPEN ACCESS

EDITED BY
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RECEIVED 05 November 2024
ACCEPTED 11 December 2024
PUBLISHED 06 January 2025

CITATION
Wang H, Liu H, Pan S, Ma Z, Wang Y, Liu J, Wang C and An Z (2025) Effects of lipopolysaccharide infusion on feed intake, apparent digestibility, rumen fermentation and microorganisms of young Holstein bulls fed diets with different ratios of lysine and methionine.
Front. Vet. Sci. 11:1523062.
doi: 10.3389/fvets.2024.1523062

Effects of lipopolysaccharide infusion on feed intake, apparent digestibility, rumen fermentation and microorganisms of young Holstein bulls fed diets with different ratios of lysine and methionine

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The aim of this experiment was to investigate the effects of intravenous infusion of lipopolysaccharide (LPS) and feeding different ratios of lysine (Lys) and methionine (Met) on feed intake, apparent digestibility, rumen fermentation and microorganisms in young Holstein bulls. Five seven-month-old Holstein bulls with similar body weights (279 ± 42 kg) were selected and subjected to a 5 × 5 Latin square experiment. The control group (CON) was fed with basal diet and the ratio of Lys to Met in the diet was adjusted to 3.0: 1. The experimental groups were received LPS infusion while being fed the basal diet (TRT1), along with LPS infusion and the addition of rumen-protected lysine (RPL) and rumen-protected methionine (RPM) to make the ratio of Lys to Met to 2.5:1 (TRT2), 3.0:1 (TRT3) and 3.5: 1 (TRT4), respectively. The LPS jugular infusion dose was set at 0.01 μg/kg body weight on days 1–3 and 0.05 μg/kg body weight on days 4–7. The trial was conducted over five periods, consisting of a 7-day trial period and a 6-day interval. The results indicated that there were no significant effects of LPS infusion on feed intake and apparent digestibility in young Holstein bulls fed different ratios of Lys and Met ($p > 0.05$). The treatment had no significant effects on the pH and total volatile fatty acids ($p > 0.05$). Compared with CON, the acetate content in the experimental groups exhibited an increasing trend ($p = 0.066$), while the content of NH_3-N decreased significantly ($p < 0.05$). LPS infusion had no significant effect on rumen microorganisms at either the species or phylum level ($p > 0.05$). However, feeding different ratios of Lys and Met could significantly increasing the abundance of *Oribacterium* ($p < 0.05$) and tended to increase the abundance of *norank_f__norank_o__RF_39* at the genus level ($p = 0.087$). These findings suggest that adding RPL and RPM into the diet may enhance the rumen environment in young Holstein bulls. Under the conditions of this experiment, adding RPL and RPM can mitigate the negative effects associated with LPS infusion, with an optimal ratio of Lys and Met is 3.0:1.

KEYWORDS

lipopolysaccharide, lysine, methionine, young Holstein bulls, rumen microorganism

1 Introduction

Lipopolysaccharide (LPS) is a common substance that induces inflammation, which can lead to weakened immunity, decreased feed intake, and substandard product quality in livestock and poultry. In the process of protein digestion and utilization of dairy cattle, the rumen characteristics lead to low protein utilization efficiency (1). Amino acids (AA) are the fundamental building blocks of proteins, and the essence of protein nutrition lies in amino acid nutrition. By reducing the protein level in the diet, an appropriate amount of AA can be provided to effectively meet the growth requirements of dairy cattle (2). The overall concentration of AA in the body is strictly regulated through transport, metabolism, and protein biosynthesis/degradation. Organs work together to achieve systemic amino acid homeostasis, and carried out through intestinal absorption and peptides and renal reabsorption. The liver metabolizes AA and muscles participate in protein biosynthesis and degradation, and the nervous and endocrine systems regulate this transport and metabolism (3). Host-rumen microbiota interaction is an important component and complex ecosystem of many physiological processes. Ruminant can consume large amounts of forage and convert it into products, mainly relying on some bacteria in the rumen to secrete cellulase, which could decompose partly cellulose as an energy source for the body (4).

Lysine (Lys) and methionine (Met) are the two primary limiting essential AA for ruminant growth, playing a crucial role in supporting gastrointestinal function and the gut-related immune system. Met promotes the growth and development of immune organs, enhances the proliferation of immune cells, and facilitates the synthesis of immune-related compounds, thereby regulating the body's immune response (5). Lys induces antioxidant enzymes to prevent oxidative stress through the Nrf_2 pathway, improves the repair capabilities of proteins and DNA, and enhances the cellular redox state of intestinal cells, thus bolstering the body's antioxidant capacity (6). Junior et al. (7) found the supplementation of rumen-protected methionine (RPM) could enhance the performance of dairy cows during peak lactation by increasing amino acid metabolism. It has been reported that RPM supplementation could alleviate heat stress (8). Moreover, the addition of Lys significantly improved immune function in dairy cows (9). Although RPM has been rumen protected, adding RPM could enhance the rumen environment of cows (10). Therefore, the purpose of this study is to evaluate the effects of lipopolysaccharide (LPS) infusion on feed intake, apparent digestibility, rumen fermentation and microorganisms in young Holstein bulls fed diets with different ratios of Lys and Met. This experiment can provide theoretical support for adjusting the amino acid structure of the diet to alleviate stress in dairy cows, which is of great significance for practical production.

2 Materials and methods

2.1 Animal, diet and design

This study was conducted from October to December 2023 in a dairy farm and approved by the Experimental Animal Ethics Committee of Zhejiang A&F University (ZAFUAC202448). Five seven-month-old Holstein bulls with similar body weights (279 ± 42 kg) were selected for the study. The dietary composition and nutritional components are shown in Table 1. A 5 × 5 Latin square design was employed for the experiment. The control group (CON) was fed a basal diet with Lys to Met ratio of 3.0:1, and the experimental groups received the with basal diet and infused LPS (TRT1), infused LPS and added rumen-protected lysine (RPL) and RPM in the diet, resulting in ratios of 2.5:1 (TRT2), 3.0:1 (TRT3), and 3.5:1 (TRT4), respectively. LPS (*E. coli* O111:B4, L2630, 297–473-0) was sourced from Sigma-Aldrich. RPL and RPM were obtained from Kemin Industries, Inc. The rumen absorption rates of RPL and RPM are 65% and 50%, respectively, while the small intestine absorption rate is greater than 80%. To avoid excessive drug stimulation, the infusion was adjusted from a low dose to a high dose. The concentration of LPS solution infused into the jugular vein was 0.01 μg/kg BW on days 1–3 and 0.05 μg/kg BW on days 4–7. The trial was conducted over five periods, each lasting 13 days, which included a 7-day experimental period followed by a 6-day rest period. During both the rest and experimental periods, the bulls were fed twice daily and had unrestricted access to water.

TABLE 1 Dietary and nutrition composition levels.

Dietary components%		Nutrient level%	
Wheat straw	13.04	DM	92.91
Peanut seedlings	26.08	CP	14.09
Corn silage	34.78	Ash	10.17
Corn	4.57	EE	4.75
Soybean meal	10.43	NDF	34.14
Brewer's grains	6.52	ADF	29.73
Bran	3.26		
[1]Premix	0.44		
Stone powder	0.44		
NaCl	0.22		
Dicalcium phosphate	0.22		
Total	100		

[1]Premix/kg: 580 kIU vitamin A; 220 kIU vitamin d3; 5.76 kIU vitamin e; 3,200 mg zinc; 2,560 mg manganese; 2,400 mg iron; 1,600 mg copper; 55 mg iodine; 20 mg cobalt; 50 mg selenium.

On the day before the start of each period, 1 L of infusion solution was prepared according to their weight and stored at 4°C. The solution was allowed to return to room temperature before infusion. A constant flow pump was used for the infusion, which lasted approximately 6 h. The bulls were arranged in order, and a central venous catheter was inserted into the jugular vein. The catheters were flushed with saline before infusion and promptly sealed with heparin sodium to prevent blockage due to blood coagulation. The surgical site for each trial was not on the same side as the previous one, allowing sufficient time for wound recovery.

2.2 Sample collection and determination

2.2.1 Sample collection

Each bull was independently fed twice a day, the remaining feed was weighed, the daily feed intake of each bull was calculated, and the feed intake of each bull was calculated according to the treatment. On the last 3 days of each period, spot collection of feces every 6 h. The first collection

on the second day would be collected 2 h in advance and 4 h in advance on the third day. Fecal samples would be pooled by periods and bulls. The feces were then mixed according to the cattle at the end of each period and stored at −80°C. After a 7-day experimental phase, 50 mL of rumen fluid was collected before morning feeding, discarding the first 200 mL to avoid saliva contamination. The rumen fluid was filtered through four layers of sterile gauze, and the pH was immediately measured. The sample was divided into 2 mL centrifuge tubes and stored at −80°C for microbiological testing. The rumen microbiota was analyzed by Shanghai Meiji Biomedical Technology Co., Ltd.

2.2.2 DNA extraction, PCR amplification and Illumina MiSeq sequencing

Total microbial genomic DNA was extracted using the E.Z.N.A.® soil DNA Kit (Omega Bio-tek, Norcross, GA, U.S.) according to manufacturer's instructions. The quality and concentration of DNA were determined by 1.0% agarose gel electrophoresis and a NanoDrop2000 spectrophotometer (Thermo Scientific Inc., USA). The hypervariable region V3-V4 of the bacterial 16S rRNA gene were amplified with primer pairs338F (5'-ACTCCTACGGGAG GCAGCAG-3′) and 806R (5'-GGACTACHVGGGTWTCTAAT-3′), using the following conditions: 3 min of denaturation at 95°C, 27 cycles of 30 s at 95°C, 30 s of annealing at 55°C, and 45 s of elongation at 72°C, and a final extension for 10 min at 72°C (11, 12). Each PCR experiment used a 20-μL reaction mixture containing 4 μL of 5 × FastPfu buffer, 2 μL of 2.5 mM deoxynucleoside triphosphates, 0.8 μL of each primer (5 M), 0.4 μL of FastPfu polymerase, and 10 ng of template DNA. The PCR product was extracted from 2% agarose gel and purified using the PCR Clean-Up Kit (YuHua, Shanghai, China) according to manufacturer's instructions and quantified using Qubit 4.0 (Thermo Fisher Scientific, USA). Purified amplicons were pooled in equimolar amounts and paired-end sequenced on an Illumina Nextseq2000 platform (Illumina, San Diego,USA) according to the standard protocols by Majorbio Bio-Pharm Technology Co. Ltd. (Shanghai, China).

2.2.3 Sample determination

Rumen fluid was centrifuged at 500 × g at 4°C for 15 min. Subsequently, 3 mL of the supernatant was mixed with 1 mL of 77.6% trichloroacetic acid solution, vortex-mixed, and divided into two portions. One placed in an ice bath for 45 min and the other centrifuged at 27,000 × g for 15 min. Microbial protein (MCP) was measured using the Coomassie brilliant blue method with a multifunctional enzyme-labeled instrument (Tecan Infinite 200Pro, Tecan Group, Switzerland) at a wavelength of 595 nm. The collected rumen fluid was centrifuged at 1,000 × g at 4°C for 10 min. 1 mL of the supernatant was transferred into a centrifuge tube, and 0.1 mL of 25% metaphosphate was added. The mixture was then placed in an ice bath for 30 min, followed by centrifugation at 1,000 × g at 4°C for 15 min. The supernatant was analyzed for acetate, propionate, butyrate, isobutyrate, valerate, and isovalerate using a gas chromatograph (6,890 N, Agilent, USA). The concentration of NH_3-N in rumen fluid was determined using indophenol colorimetry (13).

The contents of dry matter (DM) and crude protein (CP) in both feed and feces were determined using the AOAC method (1990) (14). Additionally, the levels of acid detergent fiber (ADF) and neutral detergent fiber (NDF) were assessed following the method established by Van Soest et al. (15).

2.3 Statistical analysis

The feed intake, apparent digestibility, rumen fermentation and microbial composition were all sorted by Excel 2022. Feed intake, apparent digestibility, rumen fermentation indexes and microbial composition were analyzed by PROC GLM program in SAS9.4 statistical software. Treatment and period were fixed effects in the model, while bulls were random effects. Test results are presented as mean and mean standard error (SEM). $p < 0.05$ indicated significant difference, and $0.05 \leq p < 0.10$ indicated trend of difference.

3 Results

3.1 Feed intake

The effects of LPS infusion on feed intake of young Holstein bulls fed diets with different ratios of Lys and Met is shown in Table 2. During the whole period, treatment had no significant effect on feed intake of young Holstein bulls ($p > 0.05$).

3.2 Apparent digestibility

Effect of LPS infusion on apparent digestibility of nutrients in young Holstein bulls fed diets with different ratios of Lys and Met are shown in Table 3. Treatment had no significant effect on the apparent digestibility of DM, CP, NDF and ADF among groups ($p > 0.05$).

3.3 Rumen fermentation

Effects of LPS infusion on rumen fermentation in young Holstein bulls fed diets with different ratios of Lys and Met are shown in Table 4. The NH_3-N content of CON and TRT1 was significantly higher than that of TRT3 and TRT4 ($p < 0.05$), and it is the lowest in

TABLE 2 Effect of lipopolysaccharide infusion on feed intake of young Holstein bulls fed diets with different ratios of Lys and Met.

Items	Treatments					SEM	*p*-value
	CON	TRT1	TRT2	TRT3	TRT4		
Mean daily feed intake kg/d DM	14.91	13.97	15.19	14.96	14.95	0.709	0.423

CON = basal diet, the ratio of Lys to Met is 3.0:1; TRT1 = LPS infusion and basal diet, the ratio of Lys to Met is 3.0:1; TRT2 = LPS infusion, basal diet added rumen-protected amino acids, the ratio of Lys to Met is 2.5:1; TRT3 = LPS infusion, basal diet added rumen-protected amino acids, the ratio of Lys to Met is 3.0:1; TRT4 = LPS infusion, basal diet added rumen-protected amino acids, the ratio of Lys to Met is 3.5:1; SEM = standard error of the mean.

TABLE 3 Effects of lipopolysaccharide infusion on apparent digestibility of young Holstein bulls fed diets with different ratios of Lys and Met.

Items	Treatments					SEM	*p*-value
	CON	TRT1	TRT2	TRT3	TRT4		
DM	64.02	61.44	62.70	66.08	64.99	2.939	0.946
CP	71.72	68.42	70.51	73.28	71.90	1.655	0.840
NDF	50.86	47.31	58.51	58.90	54.38	7.680	0.855
ADF	42.89	38.25	54.01	56.00	53.58	5.637	0.107

CON = basal diet, the ratio of Lys to Met is 3.0:1; TRT1 = LPS infusion and basal diet, the ratio of Lys to Met is 3.0:1; TRT2 = LPS infusion, basal diet added rumen-protected amino acids, the ratio of Lys to Met is 2.5:1; TRT3 = LPS infusion, basal diet added rumen-protected amino acids, the ratio of Lys to Met is 3.0:1; TRT4 = LPS infusion, basal diet added rumen-protected amino acids, the ratio of Lys to Met is 3.5:1; SEM, standard error of the mean.

TABLE 4 Effects of lipopolysaccharide infusion on rumen fermentation of young Holstein bulls fed diets with different ratios of Lys and Met.

Items	Treatments					SEM	*p*-value
	CON	TRT1	TRT2	TRT3	TRT4		
pH	6.39	6.47	6.51	6.49	6.52	0.204	0.992
NH_3-N, mg/L	2.36^{a}	2.38^{a}	1.47^{ab}	0.92^{b}	1.27^{b}	0.349	0.029
MCP, mg/mL	1.26	1.32	1.24	1.42	1.51	0.167	0.791
TVFAs, mmol/L	85.63	96.10	87.20	103.50	100.88	5.926	0.201
Acetate, mmol/L	52.13	59.30	52.10	64.63	63.28	4.613	0.066
Propionate, mmol/L	13.81	15.55	13.85	17.24	16.67	1.392	0.340
Isobutyrate, mmol/L	5.00	5.68	5.13	6.12	5.93	0.560	0.573
Butyrate, mmol/L	6.61	7.10	6.83	7.04	6.81	0.403	0.910
Isovalerate, mmol/L	3.14	3.40	3.28	3.35	3.14	0.287	0.945
Valerate, mmol/L	4.94	5.07	5.00	5.11	5.06	0.154	0.943
Acetate: Propionate	3.85	3.83	3.92	3.75	3.80	0.236	0.989

CON = basic diet, the ratio of Lys to Met is 3.0:1; TRT1 = LPS infusion and basic diet, the ratio of Lys to Met is 3.0:1; TRT2 = LPS infusion, basic diet added rumen-protected amino acids, the ratio of Lys to Met is 2.5:1; TRT3 = LPS infusion, basic diet added rumen-protected amino acids, the ratio of Lys to Met is 3.0:1; TRT4 = LPS infusion, basic diet added rumen-protected amino acids, the ratio of Lys to Met is 3.5:1; SEM, standard error of the mean. ab means in a row that do not have a common superscript letter differ significantly ($p < 0.05$); ab means in a row that have a common superscript letter no differ significantly ($p > 0.05$).

TRT3. During the whole experimental period, the acetate content in the experimental group showed an increasing trend ($p = 0.066$) with the highest content in the TRT3, and the treatment had no significant effect on the pH, MCP, total volatile fatty acids (TVFAs), propionate, isobutyrate, butyrate, isovalerate, valerate and the ratio of acetate and propionate of rumen fluid ($p > 0.05$).

3.4 Rumen microorganisms

As shown in Figure 1, rumen microbial dilution correlation curve tended to be stable when the measured effective sequence number reached 22,000, indicating that the data amount was reasonable.

A total of 25 rumen samples were collected for DNA extraction and 16S rRNA sequencing. As shown in Figure 2, Venn diagram showed the number of common and specific ASVs in the rumen of bulls in different groups: the five groups had 417 ASVs in common, including 38 specific ASVs in the CON, 41 specific ASVs in the TRT1, 37 specific ASVs in the TRT2, 66 specific ASVs in the TRT3 and 38 specific ASVs in the TRT4.

Alpha diversity mainly reflects the diversity of species. As shown in Table 5, goods-coverage indexes were all above 0.9, which indicated that species diversity and community structure could be well reflected. There was no significant effect of LPS and feeding with different ratios of Lys and Met on the alpha diversity of rumen microorganisms in bulls ($p > 0.05$).

As shown in Table 6, there was no significant effect of LPS infusion on the species difference of rumen microorganisms at the phylum level in bulls which fed diets with different ratios of Lys and Met ($p > 0.05$). Table 7 showed that feeding diets with different proportions of Lys and Met could significantly increase the content of *Oribacterium* in the rumen ($p < 0.05$), with that highest *Oribacterium* content in bulls treated with TRT3. Additionly, *norank_f__norank_o__RF39* showed an increasing trend ($p = 0.078$) at genus level.

4 Discussion

The aim of this experiment is to explore effects of lipopolysaccharide infusion on feed intake, apparent digestibility, rumen fermentation and microorganisms of young Holstein bulls fed diets with different ratios of Lys to Met. The experimental results can provide theoretical support for exploring the amino acid structure of dairy cows' diets under chronic stress.

Feed intake is the primary factor of determining efficiency in production, and whether it is normal or not significantly impacts animal production (16). Stress is one of the main contributors to decreased feed intake in animals. Low feed intake adversely affects the rumen condition

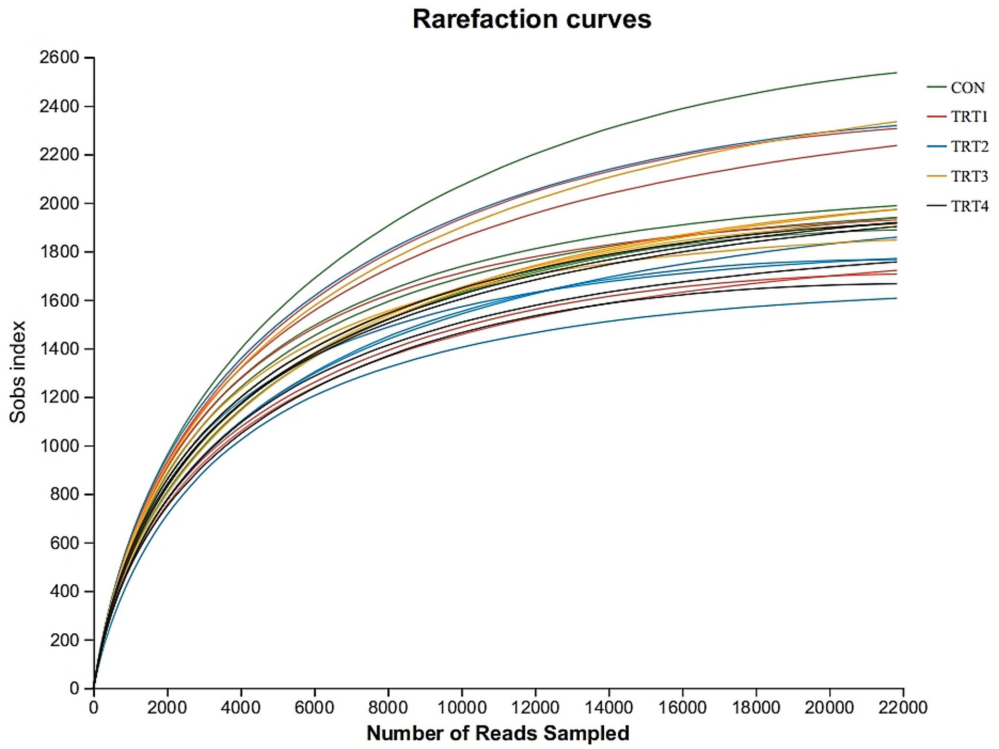

FIGURE 1
Sample dilution curve in different groups. CON = basal diet, the ratio of Lys to Met is 3.0:1; TRT1 = LPS infusion and basal diet, the ratio of Lys to Met is 3.0:1; TRT2 = LPS infusion, basal diet added rumen-protected amino acids, the ratio of Lys to Met is 2.5:1; TRT3 = LPS infusion, basal diet added rumen-protected amino acids, the ratio of Lys to Met is 3.0:1; TRT4 = LPS infusion, basal diet added rumen-protected amino acids, the ratio of Lys to Met is 3.5:1.

of ruminants (17–19). It has been discovered that a state of stress could lead to a significant reduction in feed intake among mice (20). It has been demonstrated that a variety of stresses including oxidative stress can lead to low feed intake in ruminants (21). Meanwhile, Kang et al. (22) reported that intraperitoneal injection of a high dose of LPS inhibited feed intake. Similar results have been found in studies of dairy cows (23). However, no difference in feed intake obtained by LPS infusion may causing by the dose in this study, which lead to varying doses of RPL and RPM did not significantly effect of young Holstein bulls. The LPS dose selected in this experiment was relatively low, as well as in order to avoid acute stress on the bulls. And a concentration gradient was used to gradually increase the infusion dose, giving the bulls enough time to adapt. This may be the reason why the feed intake decreased to a certain extent while no difference among groups.

Apparent digestibility reflects the efficiency with animals utilize feed. It has been found that incorporating RPL and RPM into the diet of yaks increased nutrient digestibility (24). Dietary addition of RPL and RPM have also been shown to promote the digestibility of CP and NDF in other ruminants (25, 26), likely because methionine or its analogs increased the abundance of cellulose-decomposing bacteria in the rumen (27), there are differences from our experimental results, which also may be related to the rumen passage rate of AA and the level of feeding management. However, the same trend has not been shown in studies of dairy cows (28, 29), which aligns with the findings of this study. Zhao et al. (30) and Lee et al. (31) found that RPM and other essential amino AA did not influence the apparent digestibility of nutrients in dairy cows' diets, consistent with our research findings. Similarly, Odedra et al. (32) reported that supplementing RPL into the basal diet of goats did not significantly affect the apparent digestibility of DM, ADF, and NDF, consistent with the results of this study. The discrepancies in these research findings may be attributed to the use of different types of rumen-protected AA across studies, which exhibit distinct rumen protection rates, rumen microbial metabolism rates, and apparent digestibility rates.

In the normal physiological range, a lower rumen pH value is beneficial for the rumen development of growing cattle (33). However, when the pH exceeds this range, whether it becomes too acidic or too alkaline, it can lead to a decline in animal production performance, directly negatively impacting rumen fermentation. In severe cases, subacute acidosis may occur, potentially resulting in the death of cattle (34, 35). In this study, the treatments had no significant effect on the rumen pH of young Holstein bulls. Ruminants cannot directly utilize ammonia nitrogen, however, rumen bacteria possess the ability to capture free ammonia nitrogen in the rumen for MCP synthesis (36). The rumen and intestinal microflora serve as intermediaries in nutrient absorption. Through a symbiotic relationship with rumen microorganisms, all forms of dietary nitrogen sources are ultimately degraded into peptides, AA, and ammonia in the rumen. Increasing MCP yield is an effective strategy for enhancing ruminant performance and reducing protein feed waste (37). Macelline et al. (38) reported that broilers fed a low-protein diet supplemented with synthetic AA could sustain growth performance and intestinal integrity while reducing nitrogen excretion. In ruminant, low-protein diet supplemented with RPM promoted rumen digestibility and the

10.3389/fvets.2024.1523062

production of volatile fatty acids (39). Additionally, Van et al. (40) discovered that supplementing RPM and RPL in a low-protein diet can improve production performance, maintain nitrogen balance, and enhance nitrogen utilization efficiency in dairy cows. However, some studies also showed that the addition of Lys and Met had no effect on rumen fermentation parameters and only improved nitrogen use efficiency (29, 41). Under these experimental conditions, feeding different proportions of RPL and RPM decreased the concentration of NH_3-N in the rumen, corroborating the aforementioned findings. Löest et al. (42) found that cattle exposed to LPS exhibited reduced recyclable nitrogen, leading to increased AA oxidation. The observed rise in ruminal NH_3-N concentration 24 h after LPS infusion may be attributed to an imbalance between tissue AA supply and immune system AA demand, or due to increased AA oxidation to support other metabolic functions (43). It indicated supplementation of ruminal AA helps maintain this balance and improves nitrogen utilization (44, 45). This suggests that under LPS infusion conditions, incorporating RPL and RPM into the diet of young Holstein bulls can enhance nitrogen utilization efficiency, reduce nitrogen excretion, improve NH_3-N utilization efficiency, and optimize their rumen fermentation status, and the optimal ratio of Lys and Met in diet is 3.0: 1.

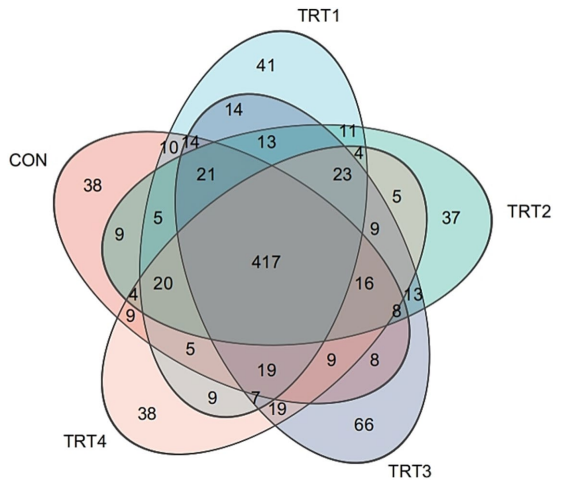

FIGURE 2
ASV level venn diagramin different groups. CON = basal diet, the ratio of Lys to Met is 3.0:1; TRT1 = LPS infusion and basal diet, the ratio of Lys to Met is 3.0:1; TRT2 = LPS infusion, basal diet added rumen-protected amino acids, the ratio of Lys to Met is 2.5:1; TRT3 = LPS infusion, basal diet added rumen-protected amino acids, the ratio of Lys to Met is 3.0:1; TRT4 = LPS infusion, basal diet added rumen-protected amino acids, the ratio of Lys to Met is 3.5:1.

LPS can cause rumen microbial dysbiosis in cows (46). One of the purposes of this study is to evaluate the effects of LPS infusion on rumen microorganisms in young Holstein bulls fed diets with different ratios of Lys and Met. In this study, feeding diets with different proportions of Lys and Met had no significant effect on rumen microbial diversity at the phylum level and the alpha diversity of rumen microbiota, which aligns with the results reported by the previous studies (47, 48). At the genus level, different proportions of Lys and Met were found to increase the abundance of *norank_f__norank_o__RF39* and *Oribacterium*, maintained rumen homeostasis. The abundance of *norank_f__norank_o__RF39* showed a significant positive correlation with NDF digestibility (49). Therefore, the higher NDF in TRT3 compared to other groups may be related to the increase in *norank_f__norank_o__RF39*. *Oribacterium* is a strict anaerobic bacterium that plays a crucial role in synthesizing acetate, propionate, and butyrate for its host (50, 51).

TABLE 5 Effects of lipopolysaccharide infusion on alpha diversity of young Holstein bulls fed diets with different ratios of Lys and Met.

Items	Treatments					SEM	*p*-value
	CON	TRT1	TRT2	TRT3	TRT4		
Chao1	2,140	2067	1926	2,115	1923	89.768	0.203
Simpson's index	2058	1980	1863	2007	1832	77.045	0.109
Shannon index	6.79	6.72	6.51	6.73	6.60	0.105	0.153
Goods-coverage	0.99	0.99	0.99	0.99	0.99	0.001	0.570

CON = basal diet, the ratio of Lys to Met is 3.0:1; TRT1 = LPS infusion and basal diet, the ratio of Lys to Met is 3.0:1; TRT2 = LPS infusion, basal diet added rumen-protected amino acids, the ratio of Lys to Met is 2.5:1; TRT3 = LPS infusion, basal diet added rumen-protected amino acids, the ratio of Lys to Met is 3.0:1; TRT4 = LPS infusion, basal diet added rumen-protected amino acids, the ratio of Lys to Met is 3.5:1; SEM, standard error of the mean.

TABLE 6 Comparison of species differences in rumen microorganisms at phylum level in young Holstein bulls infused lipopolysaccharide and fed diets with different Lys and Met ratios (relative abundance >0.5%).

Items	Groups					SEM	*p*-value
Species name	CON	TRT1	TRT2	TRT3	TRT4		
Bacteroidota	50.29	48.79	48.25	41.45	55.66	5.267	0.359
Firmicutes	42.43	41.19	43.57	50.58	38.14	4.910	0.423
Patescibacteria	3.60	5.89	3.73	3.51	2.46	1.178	0.255
Spirochaetota	1.28	1.39	1.49	0.80	1.32	0.219	0.426
Actinobacteriota	0.56	0.70	0.73	1.11	1.12	0.213	0.148

CON = basal diet, the ratio of Lys to Met is 3.0:1; TRT1 = LPS infusion and basal diet, the ratio of Lys to Met is 3.0:1; TRT2 = LPS infusion, basal diet added rumen-protected amino acids, the ratio of Lys to Met is 2.5:1; TRT3 = LPS infusion, basal diet added rumen-protected amino acids, the ratio of Lys to Met is 3.0:1; TRT4 = LPS infusion, basal diet added rumen-protected amino acids, the ratio of Lys to Met is 3.5:1; SEM, standard error of the mean.

10.3389/fvets.2024.1523062

TABLE 7 Comparison of species differences in rumen microorganisms at genus level in young Holstein bulls fed diets with different ratios of Lys and Met by lipopolysaccharide infusion.

Items	Treatments					SEM	*p*-value
	CON	TRT1	TRT2	TRT3	TRT4		
Prevotella	26.12	21.87	23.95	15.20	30.18	4.723	0.293
Rikenellaceae_RC9_gut_group	7.39	9.70	7.35	8.85	8.23	1.034	0.465
norank_f__F082	6.20	7.41	6.29	8.39	6.21	1.060	0.526
Christensenellaceae_R-7_group	5.04	6.36	5.34	6.39	5.29	0.992	0.661
NK4A214_group	4.67	5.42	4.62	5.40	4.81	0.651	0.693
Candidatus_Saccharimonasw	3.18	5.73	3.41	3.21	2.30	1.150	0.225
unclassified_c__Clostridia	2.65	2.11	2.78	3.72	2.66	0.482	0.252
Prevotellaceae_UCG-003	2.76	1.92	3.45	1.26	2.94	0.777	0.341
norank_f__norank_o__ Clostridia_UCG-014	3.26	1.91	2.50	2.49	1.71	0.477	0.156
unclassified_f__Lachnospiraceae	2.03	2.25	2.28	3.39	1.66	0.500	0.209
norank_f__Bacteroidales_RF16_ group	1.62	1.69	1.06	3.18	2.07	0.864	0.374
norank_f__norank_o__RF39	2.10	1.47	2.01	2.31	1.26	0.283	0.078
Lachnospiraceae_NK3A20_group	1.52	1.35	1.41	2.12	1.75	0.528	0.723
Ruminococcus	1.95	1.03	1.28	1.82	1.27	0.422	0.483
norank_f__UCG-010	1.41	1.41	1.36	1.43	1.30	0.219	0.989
Butyrivibrio	0.91	0.93	1.68	1.54	1.15	0.441	0.655
Prevotellaceae_UCG-001	1.20	1.11	1.29	0.81	1.49	0.317	0.609
Saccharofermentans	0.96	1.09	1.31	1.20	0.99	0.182	0.613
norank_f_Eubacterium_ coprostanoligenes_group	1.06	0.87	1.50	1.12	0.89	0.257	0.425
UCG-005	1.12	1.07	0.91	1.07	1.13	0.255	0.977
Anaeroplasma	1.18	1.02	1.05	1.10	0.92	0.270	0.955
Succiniclasticum	1.14	1.39	1.24	0.57	0.89	0.394	0.629
Treponema	1.05	1.23	1.20	0.62	1.12	0.189	0.202
norank_f__Muribaculaceae	1.02	1.02	1.02	0.73	0.82	0.156	0.564
Oribacterium	0.50^{b}	1.10^{a}	0.89^{ab}	1.28^{a}	0.42^{b}	0.191	0.010
unclassified_f__Prevotellaceae	0.78	0.76	0.96	0.45	0.93	0.245	0.449
Acetitomaculum	0.53	0.61	0.58	0.88	0.58	0.181	0.551

CON = basal diet, the ratio of Lys to Met is 3.0:1; TRT1 = LPS infusion and basal diet, the ratio of Lys to Met is 3.0:1; TRT2 = LPS infusion, basal diet added rumen-protected amino acids, the ratio of Lys to Met is 2.5:1; TRT3 = LPS infusion, basal diet added rumen-protected amino acids, the ratio of Lys to Met is 3.0:1; TRT4 = LPS infusion, basal diet added rumen-protected amino acids, the ratio of Lys to Met is 3.5:1; SEM = standard error of the mean. ab means in a row that do not have a common superscript letter differ significantly ($p < 0.05$); ab means in a row that have a common superscript letter no differ significantly ($p > 0.05$).

Additionally, enhanced fiber degradation by the action of *Oribacterium* was reported in the previous study (52). These TVFAs can be secreted and absorbed by the intestinal tract as nutrients and are also essential for maintaining the host's health. These changes may lead to the degradation of nutrients and improve utilization efficiency in the rumen. The increase in rumen acetic acid concentration in TRT3 may be related to the increase in *Oribacteria* abundance.

5 Conclusion

In conclusion, adding RPL and RPM in diets for young Holstein bulls which infused by LPS has no effect on feed intake and apparent digestibility, but can reduce the concentration of NH_3-N in the rumen, the lowest one is when Lys to Met is 3.0:1. Meanwhile, it can increase the abundance of *norank_f__norank_o__RF39* and *Oribacterium* at genus level, the highest one is when Lys to Met is 3.0:1, helping to maintain a stable state of rumen microflora. Thus, it has the best improvement effect on the rumen of young Holstein bulls infused by LPS whenthe ratio of Lys to Met in the diet was 3.0:1.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Ethics statement

The animal study was approved by Experimental Animal Ethics Committee of Zhejiang A&F University. The study was conducted in accordance with the local legislation and institutional requirements.

Author contributions

HW: Data curation, Writing – original draft. HL: Resources, Supervision, Visualization, Writing – review & editing, Methodology. SP: Data curation, Methodology, Writing – original draft. ZM: Investigation, Writing – original draft. YW: Investigation, Resources, Writing – original draft. JL: Project administration, Supervision, Writing – review & editing. CW: Funding acquisition, Writing – review & editing, Project administration, Supervision, Validation. ZA: Writing – review & editing, Data curation, Project administration, Software, Visualization.

Funding

The author(s) declare that financial support was received for the research, authorship, and/or publication of this article. This research was funded by National Natural Science Foundation of China (32172742), Zhejiang Provincial Science and Technology Plan Project (2022C04038), Zhejiang Animal Husbandry Industry Technology Project, Zhejiang Support Xinjiang Science and Technology Project, and Naqu Science and Technology Plan Project (NQKJ-2023-08), the modern agricultural industry technology system (CARS-36).

Conflict of interest

YW was employed by Kemin (China) Technologies Co. Ltd.

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

Generative AI statement

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

Publisher's note

References

1. Schuba J, Südekum K-H, Pfeffer E, Jayanegara A. Excretion of faecal, urinary urea and urinary non-urea nitrogen by four ruminant species as influenced by dietary nitrogen intake: a meta-analysis. *Livest Sci*. (2017) 198:82–8. doi: 10.1016/j.livsci.2017.01.017

2. Sinclair KD, Garnsworthy PC, Mann GE, Sinclair LA. Reducing dietary protein in dairy cow diets: implications for nitrogen utilization, milk production, welfare and fertility. *Animal*. (2014) 8:262–74. doi: 10.1017/S1751731113002139

3. Coleman DN, Lopreiato V, Alharthi A, Loor JJ. Amino acids and the regulation of oxidative stress and immune function in dairy cattle. *J Anim Sci*. (2020) 98:S175–93. doi: 10.1093/jas/skaa138

4. Zeineldin M, Barakat R, Elolimy A, Salem AZM, Elghandour MMY, Monroy JC. Synergetic action between the rumen microbiota and bovine health. *Microb Pathog*. (2018) 124:106–15. doi: 10.1016/j.micpath.2018.08.038

5. Long X, Liu Q, Chan M, Wang Q, Sun SSM. Metabolic engineering and profiling of rice with increased lysine. *Plant Biotechnol J*. (2013) 11:490–501. doi: 10.1111/pbi.12037

6. Gresner N, Rodehutscord M, Südekum KH. Amino acid pattern of rumen microorganisms in cattle fed mixed diets-an update. *J Anim Physiol Anim Nutr*. (2022) 106:752–71. doi: 10.1111/jpn.13676

7. Junior VC, Lopes F, Schwab CG, Toledo MZ, Collao-Saenz EA. Effects of rumen-protected methionine supplementation on the performance of high production dairy cows in the tropics. *PLoS One*. (2021) 16:e0243953. doi: 10.1371/journal.pone.0243953

8. Coleman DN, Totakul P, Onjai-Uea N, Aboragah A, Jiang Q, Vailati-Riboni M, et al. Rumen-protected methionine during heat stress alters mTOR, insulin signaling, and 1-carbon metabolism protein abundance in liver, and whole-blood transsulfuration pathway genes in Holstein cows. *J Dairy Sci*. (2022) 105:7787–804. doi: 10.3168/jds.2021-21379

9. Guadagnin AR, Fehlberg LK, Thomas B, Sugimoto Y, Shinzato I, Cardoso FC. Effect of feeding rumen-protected lysine through the transition period on postpartum uterine health of dairy cows. *J Dairy Sci*. (2022) 105:7805–19. doi: 10.3168/jds.2022-21934

10. Gu F, Liang S, Zhu S, Liu J, Sun HZ. Multi-omics revealed the effects of rumen-protected methionine on the nutrient profile of milk in dairy cows. *Food Res Int*. (2021) 149:110682. doi: 10.1016/j.foodres.2021.110682

11. Liu C, Zhao D, Ma W, Guo Y, Wang A, Wang Q, et al. Denitrifying sulfide removal process on high-salinity wastewaters in the presence of Halomonas sp. *Appl Microbiol Biotechnol*. (2016) 100:1421–6. doi: 10.1007/s00253-015-7039-6

12. Zhang F, Xie Y, Peng R, Ji X, Bai L. Heavy metals and nutrients mediate the distribution of soil microbial community in a typical contaminated farmland of South China. *Sci Total Environ*. (2024) 947:174322. doi: 10.1016/j.scitotenv.2024.174322

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

14. AOAC. Official methods of analysis. Arlington, VA: Association of Official Analytical Chemists (1990).

15. Van Soest PJ, Robertson JB, Lewis BA. Methods for dietary fiber, neutral detergent fiber, and nonstarch polysaccharides in relation to animal nutrition. *J Dairy Sci*. (1991) 74:3583–97. doi: 10.3168/jds.S0022-0302(91)78551-2

16. Assan D, Huang Y, Mustapha UF, Addah MN, Li G, Chen H. Fish feed intake, feeding behavior, and the physiological response of apelin to fasting and refeeding. *Front Endocrinol*. (2021) 12:798903. doi: 10.3389/fendo.2021.798903

17. Goopy JP, Korir D, Pelster D, Ali AIM, Wassie SE, Schlecht E, et al. Severe below-maintenance feed intake increases methane yield from enteric fermentation in cattle. *Br J Nutr*. (2020) 123:1239–46. doi: 10.1017/S0007114519003350

18. Ali DN, Hennessy DR. The effect of feed intake on the rate of flow of digesta and the disposition and activity of oxfendazole in sheep. *Int J Parasitol*. (1993) 23:477–84. doi: 10.1016/0020-7519(93)90036-X

19. Adams-Progar AL, Friend TH, Holub GA, Krenek AJ, Garey SM, Terrill CL. Effects of repeated transport on Holstein calf post-transport behavior and feed intake. *J Anim Sci*. (2015) 93:731–6. doi: 10.2527/jas.2014-7724

20. Xiao Y, Kronenfeld JM, Renquist BJ. Feed intake-dependent and -independent effects of heat stress on lactation and mammary gland development. *J Dairy Sci*. (2020) 103:12003–14. doi: 10.3168/jds.2020-18675

21. Bühler S, Frahm J, Tienken R, Kersten S, Meyer U, Huber K, et al. Effects of energy supply and nicotinic acid supplementation on serum anti-oxidative capacity and on expression of oxidative stress-related genes in blood leucocytes of periparturient primi- and pluriparous dairy cows. *J Anim Physiol Anim Nutr*. (2018) 102:e87–98. doi: 10.1111/jpn.12705

22. Kang A, Xie T, Zhu D, Shan J, Di L, Zheng X. Suppressive effect of ginsenoside Rg3 against lipopolysaccharide-induced depression-like behavior and neuroinflammation in mice. *J Agric Food Chem*. (2017) 65:6861–9. doi: 10.1021/acs.jafc.7b02386

23. Lippolis KD, Cooke RF, Schubach KM, Marques RS, Bohnert DW. Effects of intravenous lipopolysaccharide administration on feed intake, ruminal forage degradability, and liquid parameters and physiological responses in beef cattle. *J Anim Sci*. (2017) 95:2859–70. doi: 10.2527/jas.2017.1502

24. Wang H, Fu J, Wu X, Wang Y, Li W, Huang Y, et al. Effects of dietary protein level and rumen-protected methionine and lysine on growth performance, rumen fermentation and serum indexes for yaks. *Animals*. (2024) 14:1751. doi: 10.3390/ani14121751

25. Liu YQ, Wang C, Liu C, Zhang J, Liu Q. Effects of coated folic acid and coated methionine on growth performance, nutrient digestibility and rumen fermentation in Simmental bulls. *Anim Feed Sci Tech*. (2023) 298:115596. doi: 10.1016/j.anifeedsci.2023.115596

26. Chen J, Niu X, Li FD, Guo L. Replacing soybean meal with distillers dried grains with solubles plus rumen-protected lysine and methionine: effects on growth performance, nutrients digestion, rumen fermentation, and serum parameters in Hu sheep. *Animals*. (2021) 11:2428. doi: 10.3390/ani11082428

27. Soto RC, Muhammed SA, Newbold CJ, Stewart CS, Wallace RJ. Influence of peptides, amino acids and urea on microbial activity in the rumen of sheep receiving grass hay and on the growth of rumen bacteria in vitro. *Anim Feed Sci Tech*. (1994) 49:151–61. doi: 10.1016/0377-8401(94)90088-4

28. Li Y, Wei J, Dou M, Liu S, Yan B, Li C, et al. Effects of rumen-protected methionine supplementation on production performance, apparent digestibility, blood parameters, and ruminal fermentation of lactating Holstein dairy cows. *Front Vet Sci*. (2022) 9:981757. doi: 10.3389/fvets.2022.981757

29. Wei J, Cao Z, Li Y. 294 effect of supplementing rumen-protected methionine on production, apparent digestibility and economic effectiveness in lactating Holstein dairy cows. *J Anim Sci*. (2021) 99:159–9. doi: 10.1093/jas/skab235.292

30. Zhao K, Liu W, Lin XY, Hu ZY, Yan ZG, Wang Y, et al. Effects of rumen-protected methionine and other essential amino acid supplementation on milk and milk component yields in lactating Holstein cows. *J Dairy Sci*. (2019) 102:7936–47. doi: 10.3168/jds.2018-15703

31. Lee C, Giallongo F, Hristov AN, Lapierre H, Cassidy TW, Heyler KS, et al. Effect of dietary protein level and rumen-protected amino acid supplementation on amino acid utilization for milk protein in lactating dairy cows. *J Dairy Sci*. (2015) 98:1885–902. doi: 10.3168/jds.2014-8496

32. Odedra MD, Ravikala K, Bhadaniya AR, Gajera AP. Effect of supplemental rumen bypass lysine and methionine on digestibility and efficiency of nutrients in growing jaffrabadi heifers. *Indian J Vet Sci Biotechnol*. (2015) 10:54–9.

33. Villot C, Meunier B, Bodin J, Martin C, Silberberg M. Relative reticulo-rumen pH indicators for subacute ruminal acidosis detection in dairy cows. *Animal*. (2018) 12:481–90. doi: 10.1017/S1751731117001677

34. Cerrato-Sánchez M, Calsamiglia S, Ferret A. Effects of time at suboptimal pH on rumen fermentation in a dual-flow continuous culture system. *J Dairy Sci*. (2007) 90:1486–92. doi: 10.3168/jds.S0022-0302(07)71634-X

35. Mensching A, Hummel J, Sharifi AR. Statistical modeling of ruminal pH parameters from dairy cows based on a meta-analysis. *J Dairy Sci*. (2020) 103:750–67. doi: 10.3168/jds.2019-16802

36. Pengpeng W, Tan Z. Ammonia assimilation in rumen bacteria: a review. *Anim Biotechnol*. (2013) 24:107–28. doi: 10.1080/10495398.2012.756402

37. Lu ZY, Xu ZH, Shen ZM, Tian YC. Dietary energy level promotes rumen microbial protein synthesis by improving the energy productivity of the ruminal microbiome. *Front Microbiol*. (2019) 10:847. doi: 10.3389/fmicb.2019.00847

38. Macelline SP, Wickramasuriya SS, Cho HM, Kim E, Shin TK, Hong JS, et al. Broilers fed a low protein diet supplemented with synthetic amino acids maintained growth performance and retained intestinal integrity while reducing nitrogen excretion when raised under poor sanitary conditions. *Poultry Sci*. (2020) 99:949–58. doi: 10.1016/j.psj.2019.10.035

39. Abbasi IHR, Abbasi F, Abd El-Hack ME, Swelum AA, Yao J, Cao Y. Post-ruminal effects of rumen-protected methionine supplementation with low protein diet using long-term simulation and in vitro digestibility technique. *AMB Express*. (2018) 8:36. doi: 10.1186/s13568-018-0566-7

40. Van den Bossche T, Goossens K, Ampe B, Haesaert G, De Sutter J, De Boever JL, et al. Effect of supplementing rumen-protected methionine, lysine, and histidine to low-protein diets on the performance and nitrogen balance of dairy cows. *J Dairy Sci*. (2023) 106:1790–802. doi: 10.3168/jds.2022-22041

41. Ao R, Liang C, Zhiwei K, Bin Z, ChuanShe Z, Zhiliang T, et al. Effects of dietary methionine and lysine supplementation on growth performance, meat composition and rumen fermentation pattern in goats. *Indian J Anim Res*. (2019) 53:1629–34. doi: 10.18805/ijar.B-1073

42. Löest CA, Gilliam GG, Waggoner JW, Turner JL. Post-ruminal branched-chain amino acid supplementation and intravenous lipopolysaccharide infusion alter blood metabolites, rumen fermentation, and nitrogen balance of beef steers. *J Anim Sci*. (2018) 96:2886–906. doi: 10.1093/jas/sky168

43. Reeds PJ, Jahoor F. The amino acid requirements of disease. *Clin Nutr*. (2001) 20:15–22. doi: 10.1054/clnu.2001.0402

44. Reeds PJ, Fjeld CR, Jahoor F. Do the differences between the amino acid compositions of acute-phase and muscle proteins have a bearing on nitrogen loss in traumatic states? *J Nutr*. (1994) 124:906–10. doi: 10.1093/jn/124.6.906

45. Calder PC. Branched-chain amino acids and immunity. *J Nutr*. (2006) 136:288S–93S. doi: 10.1093/jn/136.1.288S

46. Hu X, Li S, Mu R, Guo J, Zhao C, Cao Y, et al. The rumen microbiota contributes to the development of mastitis in dairy cows. *Microbiol Spectr*. (2022) 10:e0251221. doi: 10.1128/spectrum.02512-21

47. Guo Y, Fan Z, Li M, Xie H, Peng L, Yang C. Effects of sodium nitrate and coated methionine on lactation performance, rumen fermentation characteristics, amino acid metabolism, and microbial communities in lactating buffaloes. *Microorganisms*. (2023) 11:675. doi: 10.3390/microorganisms11030675

48. Hassan FU, Guo Y, Li M, Tang Z, Peng L, Liang X, et al. Effect of methionine supplementation on rumen microbiota, fermentation, and amino acid metabolism in in vitro cultures containing nitrate. *Microorganisms*. (2021) 9:1717. doi: 10.3390/microorganisms9081717

49. Jiang F, Gao Y, Peng Z, Ma X, You Y, Hu Z, et al. Isoacids supplementation improves growth performance and feed fiber digestibility associated with ruminal bacterial community in yaks. *Front Microbiol*. (2023) 14:1175880. doi: 10.3389/fmicb.2023.1175880

50. Tian Y, Gu C, Yan F, Gu Y, Feng Y, Chen J, et al. Alteration of skin microbiome in CKD patients is associated with pruritus and renal function. *Front Cell Infect Microbiol*. (2022) 12:923581. doi: 10.3389/fcimb.2022.923581

51. Carlier JP, Bedora-Faure M, K'ouas G, Alauzet C, Mory F. Proposal to unify *Clostridium orbiscindens* winter et al. 1991 and *Eubacterium plautii* (Séguin 1928) Hofstad and Aasjord 1982, with description of *Flavonifractor plautii* gen. Nov., comb. nov., and reassignment of *Bacteroides capillosus* to *Pseudoflavonifractor capillosus* gen. Nov., comb. Nov. *Int J Syst Evol Microbiol*. (2010) 60:585–90. doi: 10.1099/ijs.0.016725-0

52. Uddin MK, Mahmud MR, Hasan S, Peltoniemi O, Oliviero C. Dietary micro-fibrillated cellulose improves growth, reduces diarrhea, modulates gut microbiota, and increases butyrate production in post-weaning piglets. *Sci Rep*. (2023) 13:6194. doi: 10.1038/s41598-023-33291-z

frontiers | Frontiers in Veterinary Science

TYPE Original Research
PUBLISHED 10 January 2025
DOI 10.3389/fvets.2024.1528126

OPEN ACCESS

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RECEIVED 14 November 2024
ACCEPTED 27 December 2024
PUBLISHED 10 January 2025

CITATION
Hou L, Duan P, Yang Y, Shah AM, Li J, Xu C and Guo T (2025) Effects of residual black *wolfberry* fruit on growth performance, rumen fermentation parameters, microflora and economic benefits of fattening sheep. *Front. Vet. Sci.* 11:1528126. doi: 10.3389/fvets.2024.1528126

Effects of residual black *wolfberry* fruit on growth performance, rumen fermentation parameters, microflora and economic benefits of fattening sheep

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Introduction: The residual black wolfberry fruit (RBWF) is rich in nutrients and contains a diverse range of active substances, which may offer a viable alternative to antibiotics. This experiment was conducted to investigate the impact of varying levels of RBWF on the growth performance and rumen microorganisms of fattening sheep, and to quantify its economic benefits.

Methods: In this experiment, 40 three-month-old and male Duolang sheep with an average weight of 29.85 kg, selected for their propensity to gain weight, were randomly assigned to one of four groups, with ten sheep in each group. To this end, each group was fed with a different proportion of RBWF (0%, 2%, 5%, 8%), and rumen fluid samples were collected to detect differences in fermentation parameters and microbial structure.

Results: The findings indicate that the dry matter intake, OM and NDF apparent digestibility of Duolang sheep in the H2 group were found to be significantly higher than those observed in the other groups ($P < 0.05$). The concentration of volatile fatty acids (VFAs), including acetate, propionate, iso-butyrate, butyrate and iso-valerate, in rumen fluid demonstrated a linear increase with the supplementation of RBWF in the diet ($P < 0.05$). The dominant bacteria in the rumen of Duolang sheep were identified as Prevotella, Christensenellaceae R7 group, NK4A214 group, Ruminococcus, and Rikenellaceae RC9 gut group. Compared with the CK group, the relative abundance of Prevotella, NK4A214 group, unclassified Prevotellaceae and Lachnospiraceae NK3A20 group in the rumen of sheep in each experimental group increased to varying degrees. The gross profit of the H2 group was significantly higher than that of the other groups.

Conclusion: In conclusion, the supplementation of RBWF has been demonstrated to enhance the growth performance of Duolang sheep, optimise rumen fermentation parameters, and ultimately increase gross profit, of which 5 % is the best.

KEYWORDS

residual black *wolfberry* fruit, *Duolang* sheep, growth performance, rumen microorganisms, economic benefits

1 Introduction

Lycium barbarum is a deciduous shrub belonging to the Solanaceae family (1). It is a widely utilized ingredient in the food and medical industries, due to the presence of bioactive metabolites in the branches, leaves and fruits (2). From a global perspective, China is the world's foremost producer of *wolfberries*, accounting for over 80% of global cultivation. The majority of China's *wolfberry* cultivation is concentrated in the provinces of Ningxia, Qinghai, Gansu and Xinjiang. It is reported that in 2023, the total area devoted to *wolfberry* cultivation in China will reach 1216.67 km^2, with an estimated output of 1.4 Mt. of fresh fruit and 0.24 Mt. of dry fruit. In the production of fresh *wolfberry*, a residual quantity of 5 to 10% of the fruit is inevitable. This is typically discarded as waste, resulting in a considerable amount of wastage. The contents of crude protein (CP), crude fat (EE), crude ash (Ash), total carbohydrates and dietary fiber were found to be 9.2, 3.0, 5.6, 61.6 and 12.1%, respectively (3). Furthermore, *Lycium ruthenicum Murray* is a rich source of polyphenols, polysaccharides, alkaloids, anthocyanins and other biochemical components. These component confer a range of beneficial effects, including immune regulation, antioxidant activity, anti-aging, anti-tumor, anti-fatigue and anti-inflammatory actions (4). Zhao et al. (5) demonstrated that the inclusion of 0.6% *Lycium barbarum* polysaccharide (LBP) in the diet of dairy cows resulted in a notable enhancement in their production performance, accompanied by an augmentation in the body's antioxidant capacity and immunity. Zhang et al. (6) discovered that fermented *wolfberry* residue not only enhances carcass weight in fattening sheep but also stimulates the expression of chemokines and immune-related pathways in sheep. Gan et al. (7) demonstrated that LBP can induce an immune response, thereby regulating the immune response to diseases such as cancer.

In comparison to red *wolfberry*, black *wolfberry* exhibited a higher concentration of phenols, concentrated tannins and monomeric anthocyanins, and also demonstrated enhanced anti-inflammatory and antioxidant activities (8, 9). Currently, there is a plethora of research exploring the utilization of by-products derived from red fruit *wolfberry*. However, despite the unique functional components present in black fruit *wolfberry*, it has not yet been subjected to the same degree of investigation. A substantial body of evidence attests to the potential value of incorporating red *wolfberry* by-products into livestock and poultry production (10). It may therefore be hypothesized that the inclusion of black *wolfberry* fruit in the diet of animals could have a beneficial effect on their production performance. The objective of this experiment was to investigate the impact of varying levels of residual black *wolfberry* fruit (RBWF) on the growth performance, nutrient apparent digestibility, rumen fermentation parameters, microflora and gross profit of *Duolang* sheep. This was done in order to establish a theoretical basis for the application of residual black *wolfberry* fruit in sheep production.

2 Materials and methods

2.1 Ethics committee approval

The study was carried out in accordance with the procedures sanctioned for this research, which have been approved by the Science and Technology Ethics Committee of Xinjiang Academy of Animal Sciences, China (ethics number 20230508). These procedures adhere to the principles and regulations for ethical protection in human and animal biological science and technology in China.

TABLE 1 The main nutritional components of RBWF (DM basis, %).

Items	CP	EE	Ash	CF	ADF	NDF	Ca	Pi
RBWF	14.29	11.46	9.20	8.86	16.60	28.00	0.24	0.30

2.2 Experimental animals and group design

In this experiment, following the collection of the black *wolfberry* fruit in October 2022, the fruit that did not meet the quality standard was identified as RBWF. The nutritional components of RBWF were then determined, as illustrated in Table 1. Based on the principle of equal energy and nitrogen, four diets containing different proportions (0, 2, 5, and 8%) of RBWF were formulated (NRC 2007).

Forty male lambs, 3 months aged and with a mean body weight of 29.85 ± 2.00 kg, were used in this experiment. Following deworming, a single-factor completely randomized experimental design was implemented, with the lambs randomly divided into four groups, each with 10 replicates. Four distinct proportions of RBWF diets were provided, comprising the following: a control group (CK, 0% RBWF), experimental group 1 (H1, 2% LBL), experimental group 2 (H2, 5% LBL) and experimental group 3 (H3, 8%LBL). The specific diet composition and nutrient composition are presented in Table 2. The experiment spanned 70 days, comprising a 10-day pre-experimental period and a 60-day experimental period.

2.3 Feeding management

Prior to the commencement of the experiment, the sheep house was thoroughly cleaned and disinfected, and the test sheep were subjected to a series of preparatory procedures, including cutting, deworming, and medicated bathing. During the course of the experiment, the sheep were maintained in separate columns and provided with food twice daily, at 10:00 and 18:00, respectively. The *Duolang* sheep were permitted to feed and drink freely throughout the duration of the experiment.

2.4 Sample collection and measurements

2.4.1 Growth performance and apparent digestibility

The quantity of feed provided and the quantity of residual feed were recorded on a daily basis. Prior to the morning feeding on the 1st, 30th and 60th days of the experiment, each sheep was weighed. Thereafter, the average daily feed intake (ADFI), average daily gain (ADG) and feed-to-weight ratio (F:G) were calculated. A digestion test was conducted over the final 10 days of the formal test period, comprising a 5-day adaptation period. Fecal samples were collected using the total fecal collection method over 5 consecutive days. The total fecal matter of each sheep was weighed prior to morning feeding on a daily basis, with 10% of the total fecal matter collected from each sheep. Following the conclusion of the experiment, the collected fecal samples were combined in a uniform manner, with 10% of the samples obtained through the quartering method and stored at −20°C for subsequent analysis.

TABLE 2 Composition and nutrient levels of the basal diet (DM basis, %).

Items	CK	H1	H2	H3
Ingredients				
Corn	33.80	35.00	32.50	31.00
Wheat bran	9.00	6.40	6.28	5.15
cottonseed meal	13.70	13.30	12.92	12.65
RBWF	0.00	2.00	5.00	8.00
Corn stalk	18.00	18.00	18.00	17.90
Alfalfa	20.50	20.30	20.30	20.30
Premix[1]	5.00	5.00	5.00	5.00
Total	100.00	100.00	100.00	100.00
Nutritional level[2]				
ME (MJ/kg)	11.06	11.02	10.98	10.93
CP	13.96	13.97	13.99	13.99
NDF	31.12	31.14	31.24	31.45
ADF	16.80	16.85	17.07	17.30
Ca	0.70	0.93	1.16	1.10
P	0.49	0.56	0.64	0.41

[1]The premix provided the following per kg of the diet: VA 150,000 IU, VD3 56,500 IU, VE 8,000 IU, Se (as sodium selenite) 14 mg, I (as potassium iodide) 58 mg, Cu (as copper sulfate) 290 mg, Mn (as manganese sulfate) 1,925 mg, Zn (as zinc oxide) 2,050 mg, Co (as cobalt sulfate) 24 mg.
[2]Nutritional level were measured values.

An analysis was conducted on samples of diet, ingredients, and feces for dry matter (method 930.15), CP (method 990.03), EE (method 920.39), Ca (method 978.02), and P (method 946.06) using the AOAC procedures (11). NDF and ADF content were determined following Van Soest's (12) method. ME was calculated based on the measured nutritional value:

$$ME = 0.046 + 0.820 \times (17.211 - 0.135 \times NDF)$$

2.4.2 Rumen fermentation parameters and microorganisms

Immediately following the formal test, the test sheep were slaughtered after 16 h of fasting and rumen fluid was collected. The pH value was immediately measured following filtration through four layers of gauze using a portable pH meter (PHS-3C, Shanghai, China), and the measurement was repeated 3 times. To assess rumen fermentation parameters and rumen microflora, the remaining samples were transferred to 15 mL and 5 mL freezing tubes and stored in −20°C and − 80°C freezers, respectively. The concentration of NH_3-N was determined by phenol sodium hypochlorite colorimetry; the concentration of volatile fatty acids (VFAs) was determined by gas chromatography.

2.4.3 Extraction of DNA and sequencing of 16S rDNA

The TGuide S96 Magnetic Bead Method Soil/Fecal Genomic DNA Extraction Kit (Tiangen, Beijing, China) was used to extract DNA from the rumen specimens for 16S rDNA sequencing analysis. The concentration of the extracted nucleic acids was determined using an enzyme marker (GeneCompany Limited, Hong Kong, China, model Synergy HTX), and their integrity was assessed through agarose electrophoresis at a concentration of 1.8% (Beijing Bomei Fuxin Technology Co., Ltd., Beijing, China).

The highly variable V3-V4 regions of bacterial 16S rDNA were amplified by PCR using universal bacterial primers 338F (5′-ACTCCTACGGGGAGGCAGCA-3′) and 806R (5′- GGACTACH VGGGGTWTCTAAT-3′) after extracting total DNA from the samples. The final products were cleaned, quantified, and mixed to form a sequencing collection. After passing quality assessments, the collection was analyzed on the Illumina NovaSeq 6,000 instrument (San Diego, CA, USA). Raw data files from various high-throughput sequencers, including the Illumina NovaSeq, were processed to generate sequenced reads. These reads include both the actual sequences and quality metrics. The initial reads were refined with the Trimmomatic v0.33 tool. To further refine the data, Cutadapt 1.9.1 was utilized to eliminate primer sequences and produce polished reads.

2.5 Statistical analysis

The preliminary sorting of the experimental data was conducted using Excel 2023 software, while the significance test was performed with the one-way ANOVA program in SPSS 26.0 statistical software. Subsequently, linear and quadratic analyses were carried out on rumen fermentation parameters, employing the Duncan method for multiple comparison differences. The level of significance was determined by $p < 0.05$, while $0.05 < p \leq 0.10$ indicated a trend. The taxonomy annotation of the OTUs was conducted by classifying representative organisms using a Bayesian classifier based on the SILVA database (version 138). Alpha diversity analyzes species diversity and complexity using ACE, Chao1, Simpson, and Shannon indices. Beta diversity analyses were conducted using principal coordinate analysis (PCoA) and nonmetric multidimensional scaling (NMDS) to evaluate distinctions between groups. LEfSe (Line Discriminant Analysis (LDA) Effect Size) was employed to facilitate a comparative analysis of the various treatment groups. The differences between the classification levels were then analyzed individually, from the lowest level (species) to the highest (phylum), with the objective of identifying biomarkers exhibiting statistically significant differences between the groups.

3 Results

3.1 Effects of varying levels of RBWF addition on the growth performance and nutrient apparent digestibility of *Duolang* sheep

Table 3 illustrates the impact of incorporating varying levels of RBWF into the diet on the growth performance of *Duolang* sheep. It can be observed that following a 60-day feeding period, the final weight gain of sheep in the H2 group was 14.89 kg, representing a 4.78, 12.97, and 13.32% increase compared to the CK, H1, and H3 groups (14.21 kg, 13.18 kg, and 13.14 kg, respectively). The H2 group exhibited the most favorable outcome, although no statistically significant difference was observed ($p > 0.05$). Concurrently, the dry matter intake of the H2 group was markedly higher than that of the CK group ($p < 0.05$).

Table 4 demonstrated that dietary RBWF supplementation had no statistically significant impact on the apparent digestibility of dry

TABLE 3 Effects of RBWF on growth performance of *Duolang* sheep (n = 10).

Items[1]	CK	H1	H2	H3	SEM	p-value[2]		
						Trt	L	Q
Initial body weights, kg	29.91	30.05	29.90	29.54	0.230	0.901	0.586	0.605
Final weight, kg	43.77	43.09	45.06	42.70	0.557	0.522	0.821	0.526
ADG, g	249.29	231.19	261.18	230.48	8.394	0.563	0.733	0.788
total gain weight, kg	14.21	13.18	14.89	13.14	0.478	0.563	0.733	0.788
ADFI, kg	1.79^{b}	1.73^{b}	1.84^{a}	1.65^{c}	0.043	0.021	<0.001	<0.001
F:G	7.52	7.78	7.23	7.67	0.271	0.925	0.976	0.909

[1]ADG, average daily gain; ADFI, average daily feed intake; F:G, ADFI/ADG.
[2]Trt, treatment effect; L, linear; Q, quadratic.
[a,b,c] Different letters indicate significant differences between different groups ($p < 0.05$). SEM is the pooled standard error between five groups; the p-value indicates significance.

TABLE 4 Effect of RBWF on apparent digestibility of nutrients in *Duolang* sheep (n = 10).

Items	CK	H1	H2	H3	SEM	p-value[1]		
						Trt	L	Q
DM	70.60	68.80	71.20	66.90	0.638	0.059	0.099	0.277
OM	59.76^{b}	58.64^{b}	61.61^{a}	56.46^{c}	0.577	0.047	0.091	0.144
CP	73.26	73.04	73.45	72.63	0.173	0.397	0.342	0.396
EE	55.67	56.24	56.43	56.57	0.418	0.896	0.478	0.809
NDF	43.72^{b}	44.76^{ab}	47.55^{a}	41.81^{b}	0.677	0.010	0.536	0.005
ADF	24.93	25.88	27.74	23.69	0.634	0.135	0.728	0.048

[1]Trt, treatment effect; L, linear; Q, quadratic.
[a,b,c] Different superscripts indicate significant differences within a row ($p < 0.05$). SEM is the pooled standard error between five groups; the p-value indicates significance.

TABLE 5 Effects of RBWF on rumen fermentation parameters in *Duolang* sheep (n = 5).

Items[1]	CK	H1	H2	H3	SEM	p-value[2]		
						Trt	L	Q
pH	5.72	5.50	5.42	5.27	0.078	0.288	0.080	0.392
NH_3-N, mg/100 mL	26.13	27.3	29.62	31.89	0.847	0.081	0.001	0.030
Acetate, mmol/L	88.49^{c}	100.81^{b}	115.63^{a}	109.56^{b}	3.964	<0.001	0.026	0.047
Propionate, mmol/L	27.34^{c}	30.67^{b}	33.54^{a}	31.82^{b}	1.104	<0.001	0.044	0.248
Iso-butyrate, mmol/L	0.87^{c}	1.09^{b}	1.43^{a}	1.49^{a}	0.082	0.002	0.045	0.272
Butyrate, mmol/L	20.43^{c}	25.78^{b}	31.59^{a}	29.29^{a}	1.608	0.005	0.046	0.064
Iso-valerate, mmol/L	1.34^{c}	2.01^{b}	2.83^{a}	2.57^{a}	0.186	0.001	0.035	0.032
Valerate, mmol/L	1.61	1.74	1.82	1.93	0.141	0.612	0.014	0.130
A:P	3.24	3.29	3.44	3.42	0.067	0.866	0.096	0.278
TVFAs, mmol/L	140.08^{d}	162.10^{c}	183.29^{a}	176.92^{ab}	6.485	<0.001	0.030	0.116

[1]NH_3-N, ammonia nitrogen; TVFA, total volatile fatty acids.
[2]Trt, treatment effect; L, linear; Q, quadratic.
[a, b, c, d] Different superscripts indicate significant differences within a row ($p < 0.05$). SEM is the pooled standard error between five groups; the p-value indicates significance.

matter (DM), crude protein (CP), ether extract (EE), acid detergent fiber (ADF), calcium (Ca) and phosphorus (P) in *Duolang* sheep when compared with the CK group ($p > 0.05$). However, the apparent digestibility of organic matter (OM) and NDF in the H2 group was found to be significantly higher than that observed in the H3 group ($p < 0.05$).

3.2 Effects of different levels of RBWF supplementation on rumen fermentation parameters in *Duolang* sheep

Table 5 illustrates the impact of dietary supplementation with RBWF on rumen fermentation parameters in *Duolang* sheep. It can

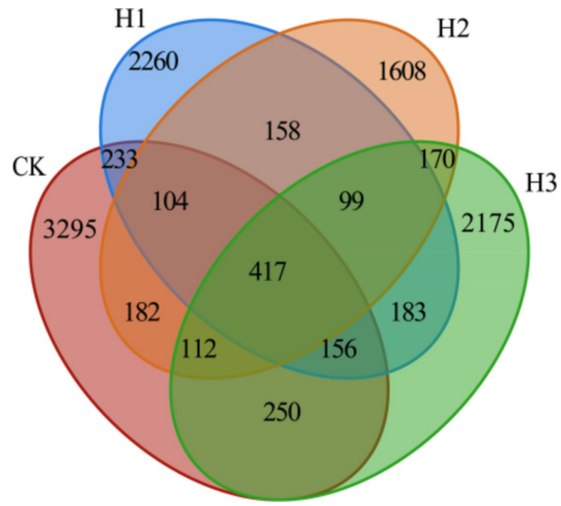

FIGURE 1
Venn diagram of ruminal fluid flora.

be observed that no statistically significant difference was evident in pH and NH_3-N concentration between the experimental groups ($p > 0.05$). In comparison to the CK group, the pH of the rumen in sheep exhibited a linear decline ($p = 0.080$), while the NH_3-N concentration demonstrated a notable linear increase ($p < 0.05$). The concentrations of acetate and propionate in the rumen of the H2 group were found to be significantly higher than those observed in the other experimental groups. The concentrations of iso-butyrate, butyrate, iso-valerate and total volatile fatty acids (TVFAs) in the rumen of the H2 and H3 groups were significantly higher than those of the CK group ($p < 0.05$). As the ratio of RBWF supplementation increased, the concentrations of acetate, propionate, iso-butyrate, butyrate, iso-valerate, n-valeric acid and total volatile fatty acids (VFAs) in the rumen exhibited a linear increase ($p < 0.05$). However, the quadratic effect of acetate and iso-valerate was statistically significant ($p < 0.05$).

3.3 Effects of different levels of RBWF supplementation on ruminal microbiota diversity analysis in *Duolang* sheep

A total of 1,348,210 readings were obtained from 16 samples in four groups (5 in CK, 4 in H1, 3 in H2 and 4 in H3). Following quality control and splicing, 1,269,853 clean readings were obtained, with an average of 79,365 clean readings obtained for each sample. The number of unique OUTs in each group was as follows: 3,295 in CK, 2,260 in H1, 1,608 in H2 and 2,175 in H3. The total number of OUTs across all four groups was 4,170 (Figure 1).

As illustrated in Figure 2, dietary RBWF supplementation had no discernible impact on the alpha diversity of rumen microbiota in sheep. With regard to beta diversity, the results of PCoA (Figure 3A) and NMDS (Figure 3B) analysis demonstrated that there was no notable separation between the distribution of rumen microbial representative points across different treatment groups, thereby substantiating that the addition of RBWF had no substantial influence on the beta diversity of rumen microorganisms in *Duolang* sheep.

3.4 Effects of different levels of RBWF supplementation on analysis of microbial composition and community structure in *Duolang* sheep

A LEfSe (linear discriminant analysis effect size) analysis was conducted to identify discrepancies in the taxonomic composition of bacterial species. The figure presents a representative branch diagram of the main microbiome structure (Figure 4), illustrating the most notable differences between the groups of different treatment components at the phylum, class, order, family, genus, and species levels. The data indicated that 12 branches were more abundant in the CK group, 3 branches were more abundant in the H1 group, 11 branches were more abundant in the H2 group, and 9 branches were more abundant in the H3 group. The abundance differences of different bacterial groups among CK, H1, H2, and H3 are shown in Figure 5. The figure illustrates the abundance differences of various bacterial groups between the CK, H1, H2, and H3 groups. Notably, the *Lachnospiraceae ND3007 group*, *Eubacterium coprostanoligenes group*, and *Bifidobacterium longum exhibited* the most pronounced differences in abundance within the CK group. In contrast, the *Prevotellaceae UCG-001* and *Parasutterella excrementihominis genera* demonstrated the most significant abundance differences within the HC group. The bacterial genera *Terrisporobacter*, *Dubosiella* and *Enterococcus* were more abundant in H2, while *Muribaculum* and *Pseudomonadales* were more abundant in H3.

The differences in rumen microbial species abundance between the various treatment groups were subjected to analysis. At the phylum level (Figure 6A), the dominant bacterial species in the rumen of *Duolang* sheep were identified as *Firmicutes*, *Bacteroidota*, *Proteobacteria*, *Fibrobacterota*, and *Patescibacteria*. Following the addition of RBWF, no significant overall effect on the abundance of phylum-level flora was observed ($p > 0.05$). However, the relative abundance of *Bacteroidota* and *Fibrobacterota* in H1 group increased (Supplementary Table S1). At the genus level (Figure 6B), the dominant bacteria in the rumen bacteria of *Duolang* sheep were *Prevotella*, *Christensenellaceae R7 group*, *NK4A214 group*, *Ruminococcus*, and *Rikenellaceae RC9 gut group*. After the addition of RBWF, the overall effect on the abundance of genus-level bacteria was not significant ($p > 0.05$). Nevertheless, there was a notable increase in the relative abundance of *Prevotella*, *NK4A214 group* and *Lachnospiraceae NK3A20 group*, while the relative abundance of *Prevotellaceae UCG 001* decreased (Supplementary Table S2).

3.5 Correlation analysis of growth performance, nutrient apparent digestibility and rumen fermentation parameters with main bacteria at genus level

As illustrated in the Figure 7, the relative abundance of rumen-dominant bacteria exhibited a correlation with growth performance, nutrient apparent digestibility, and rumen fermentation parameters. The abundances of *F082*, *Christensenellaceae R7 group*, *NK4A214 group* and *Ruminococcus* were found to be significantly and positively correlated with ADG ($p < 0.05$). In contrast, *Prevotellaceae UCG 001* was observed to be negatively correlated with FCR and pH, and positively correlated with other indexes. Of these, it was found to be significantly and positively correlated with OM apparent digestibility ($p < 0.05$).

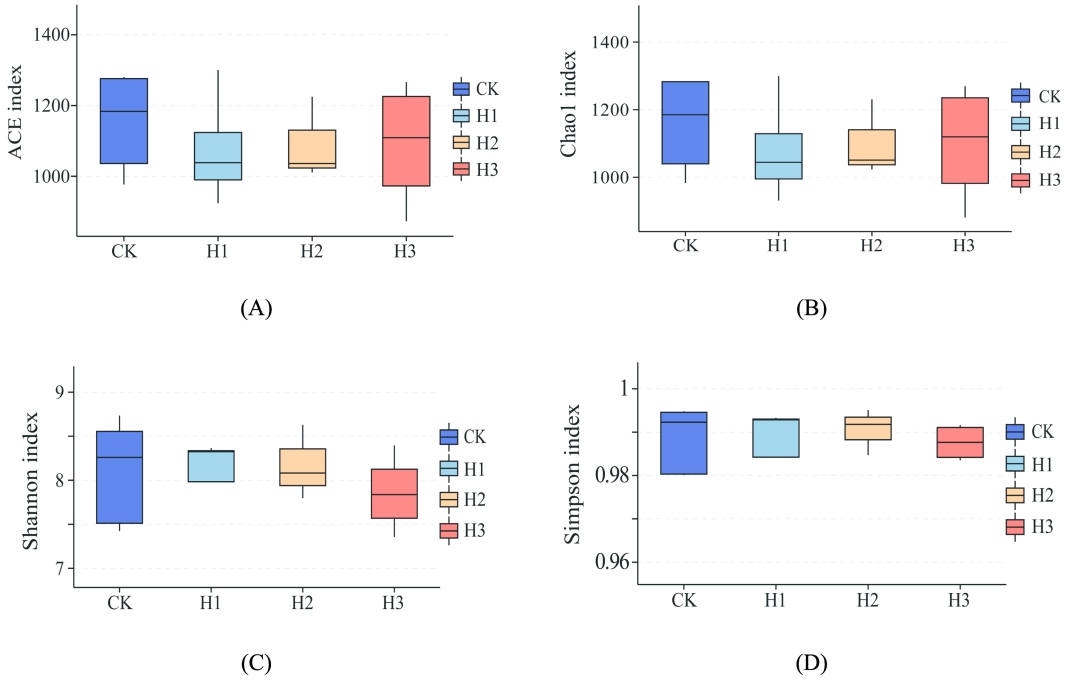

FIGURE 2
Alpha diversity analysis of rumen flora. **(A)** ACE index of species richness; **(B)** Chao1 index of species richness; **(C)** Shannon index of species diversity; **(D)** Simpson index of species diversity.

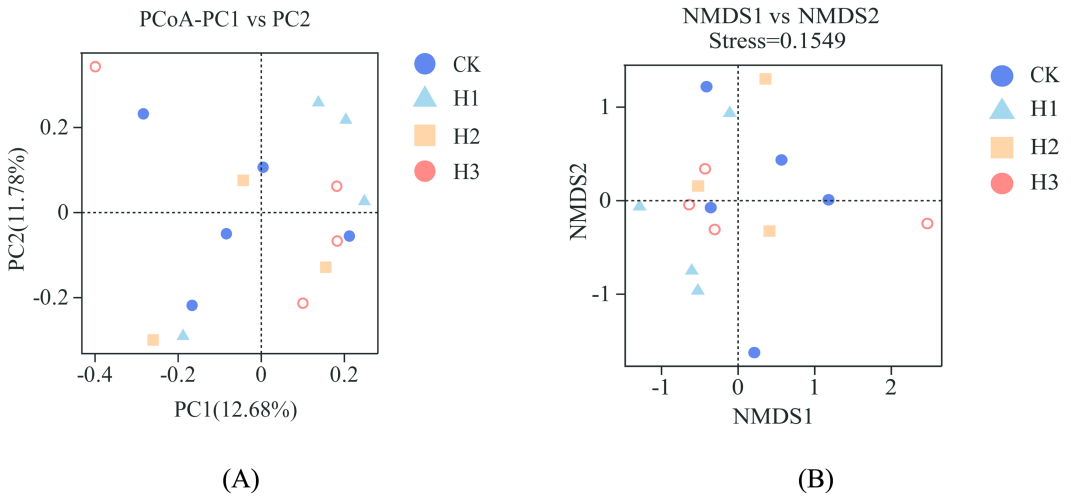

FIGURE 3
Beta diversity analysis of rumen flora. **(A)** PCoA principal axis analysis; **(B)** NMDS non-metric multidimensional scaling analysis.

3.6 Effects of different levels of RBWF addition on economic benefits of *Duolang* sheep

The feed unit price of each group was found to be between 2.25 and 2.39 yuan/kg. As illustrated in the Table 5, as increase in the proportion of RBWF added to the diet was associated with a gradual decrease in feed unit price. The gross profit of the H2 group was observed to have increased by 19.34% in comparison to the CK group (Table 6).

4 Discussion

The current global concern surrounding antibiotic resistance is well documented (13). The intensification of animal husbandry practices at home and abroad has resulted in the pervasive use of antibiotics in animal husbandry production, which has introduced significant risks to food safety and environmental safety. The utilization of antibiotics in human and livestock production will inevitably result in the emergence of drug resistance (14). In order to

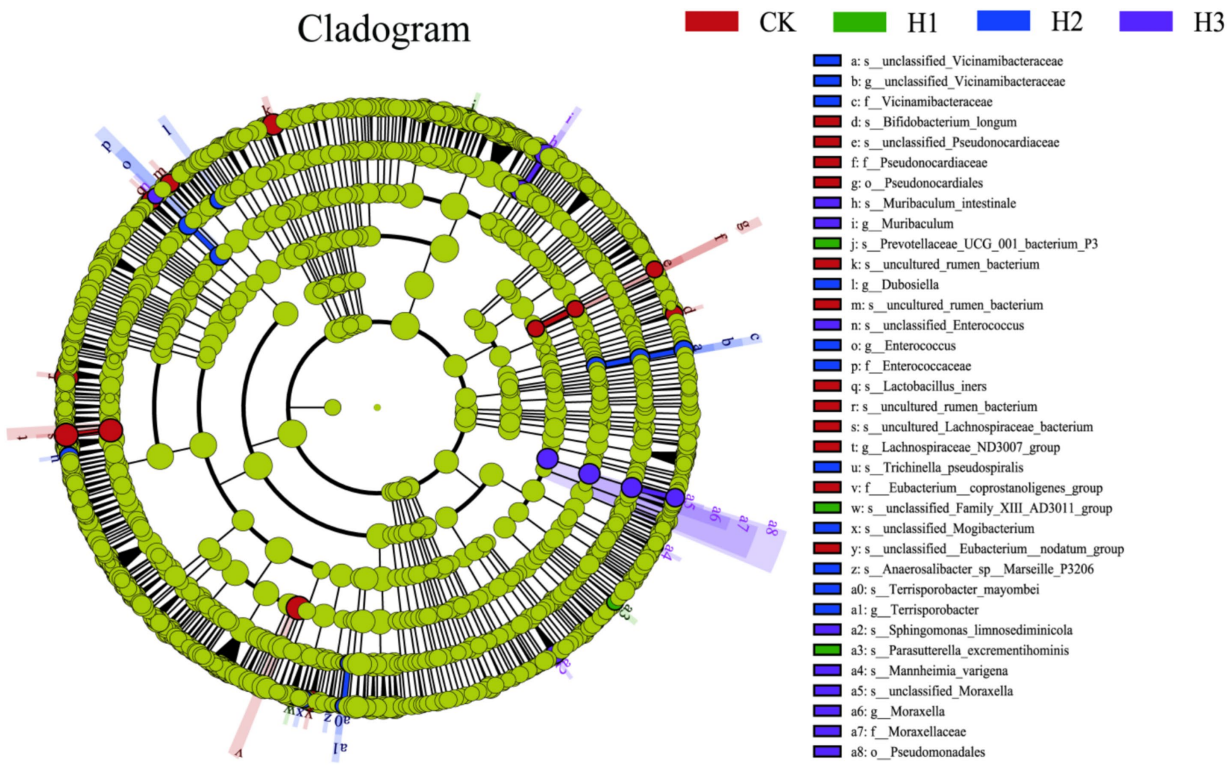

FIGURE 4
LEfSe (linear discriminant analysis effect size) cladogram comparing microbial communities among the 2.5 elevations.

mitigate the adverse effects of antibiotic resistance on human health, a number of healthcare institutions have introduced a series of policies aimed at promoting the rational use of antibiotics (15). *Lycium barbarum*, a traditional Chinese herbal medicine and health food, has been the subject of considerable interest due to the diversity of its functional components (16). Yin et al. (17) demonstrated that the inclusion of LBP in the diet of weaned piglets resulted in enhanced growth performance, antioxidant capacity, and immunity, as well as the regulation of intestinal microbial composition. Furthermore, they established that LBP can serve as an effective substitute for antibiotics in the feed of weaned piglets. Tian et al. (18) observed that *Lycium barbarum* partially alleviated the intestinal ecological imbalance caused by antibiotics by regulating the intestinal flora, and also produced short-chain fatty acids (SCFAs) to improve intestinal barrier function. Prior research has demonstrated that the bioactive components present in *Lycium barbarum* exhibit a range of biological functions that can prevent and treat chronic diseases, thereby positioning it as a potential alternative to antibiotics (19) The current research mainly focuses on the role of mining and extracting some active ingredients in *Lycium barbarum* as feed additives in livestock and poultry production and clinical practice (20, 21). However, the extraction method also affects the chemical properties of the active ingredients of *Lycium barbarum* (22). At the same time, there are few studies on the effects of *wolfberry* fruit on growth performance, nutrient apparent digestibility, rumen fermentation parameters and microbial flora structure of fattening sheep. Therefore, the objective of this study was to elucidate the impact of incorporating RBWF into the diet of fattening *Duolang* sheep and evaluate its economic benefits.

Prior research has demonstrated a significant correlation between the average daily feed intake of fattening sheep and feed conversion efficiency, with feed conversion efficiency being closely associated with growth performance (23). The administration of LBP to a diet regimen was observed to enhance the growth performance and digestive enzyme activity of broilers (24). Hao et al. demonstrated that the administration of Chinese *wolfberry* and *astragalus* extracts in lieu of a 1% basal diet can enhance the growth performance of Tibetan fragrant pigs (25). The results of this experiment demonstrated that the incorporation of 5% RBWF into the diet led to a notable increase in the dry matter intake of fattening sheep. This was accompanied by a similar trend in both the daily gain and total weight gain. However, no significant effect was observed, which may be attributed to the considerable inter-individual variability within the experimental group. In contrast to the aforementioned findings, Guo et al. (26) observed that the addition of *Lycium barbarum* to the basal diet resulted in a notable enhancement in the average body weight and growth performance of rats. This discrepancy may be attributed to the variation in nutrient digestion and absorption rates resulting from the disparate digestive tract structures of the experimental animals.

Apparent digestibility is a significant indicator of the rate of digestion and absorption of nutrients in the experimental diet. It provides insight into whether the diet meets the nutritional requirements for animal growth (27). The polysaccharide derived from *Lycium barbarum* is a complex mixture of active ingredients with a multitude of potential applications and a promising future in development (28). The study demonstrated that the intake of polysaccharides in the diet exerts a discernible influence on the feed intake and apparent digestibility of nutrients in ruminants (29). Lin

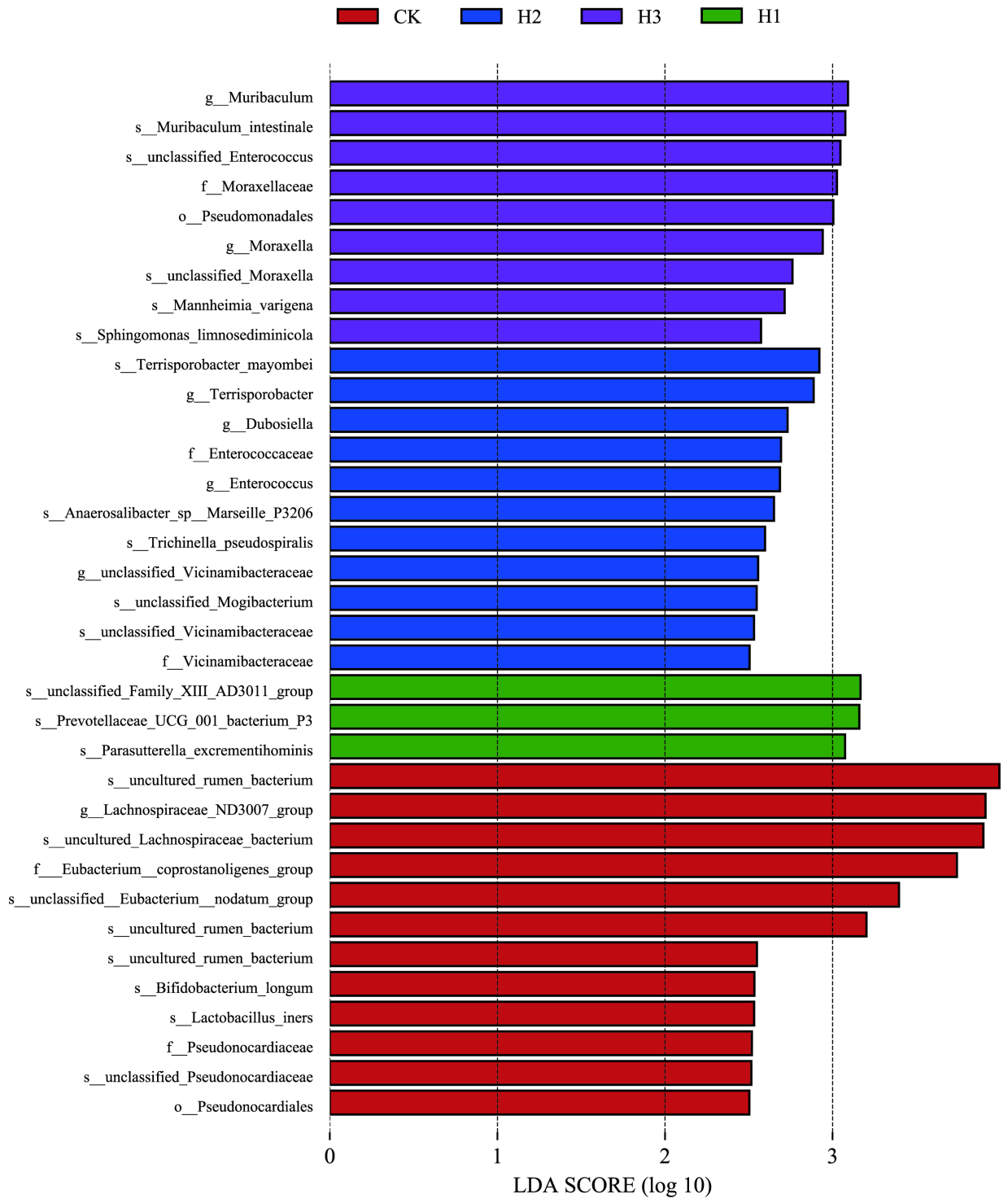

FIGURE 5
Histogram of LDA score calculated for each taxon ranging from phylum to genus.

et al. (30) demonstrated that LBP enhanced the digestive and absorptive processes and immune function of immunosuppressed mice by inhibiting the activation of the MLCK signaling pathway, and regulated the immune system of the intestinal mucosal barrier. The addition of 5% RBWF resulted in a significantly higher apparent digestibility of OM and NDF compared to the control group. Additionally, there was a notable increase in the apparent digestibility of ADF, from 24.93 to 27.74%. However, this difference was not found to be statistically significant. In contrast to the aforementioned findings, Ju et al. (31) observed that the addition of *Lycium barbarum* polysaccharides to the lamb diet resulted in a notable enhancement in the apparent digestibility of DM, OM, and CP. This discrepancy could be attributed to the distinct processing forms of *Lycium barbarum* products. In this experiment, the fruit was not subjected to extraction,

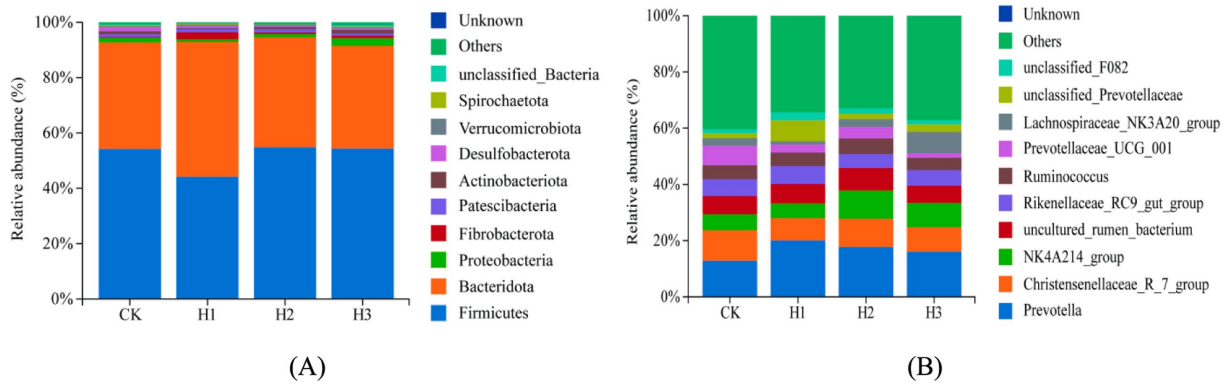

FIGURE 6
Distribution of bacterial taxa averaged under phyla **(A)** and genera **(B)** levels across the different treatment groups (as a percentage of the total sequence).

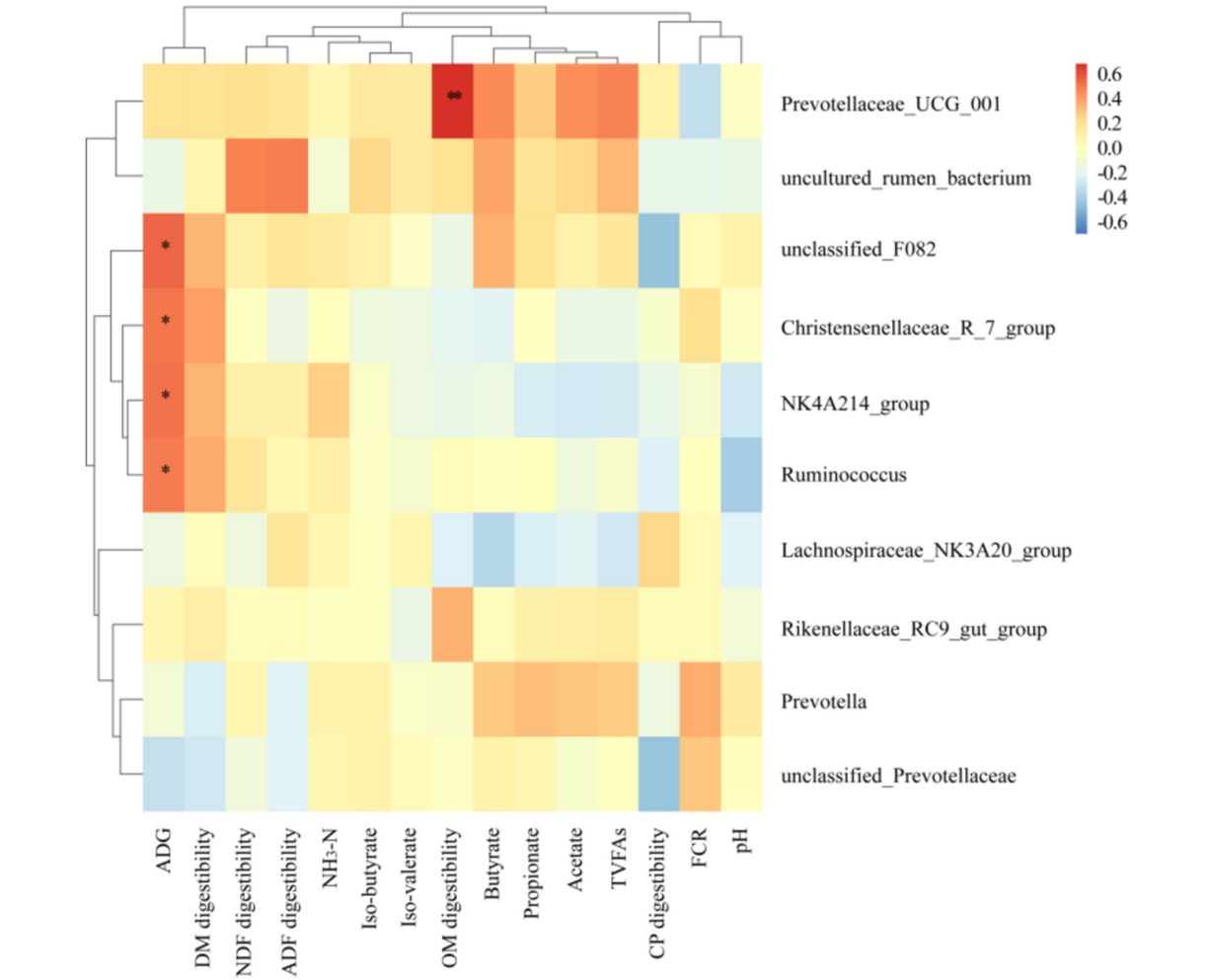

FIGURE 7
Spearman correlation and clustering analysis between growth performance, nutrient apparent digestibility and rumen fermentation parameters with main bacteria in Genus level. Different colors represent positive correlation (red) or negative correlation (blue), and the color shade indicates the magnitude of the correlation, * represents $0.01 < p \leq 0.05$ and ** represents $p \leq 0.01$.

and it was directly incorporated into the lamb diet, consequently influencing the chemical composition of the *Lycium barbarum* additives. In conclusion, the incorporation of RBWF into the diet has been demonstrated to enhance the feed intake of fattening sheep an[illegible] augment the apparent digestibility of OM and NDF, as well as the daily gain of lambs. This is may achieved by elevating the relative abundance of fiber-degrading bacteria within the rumen, which in turn increases [illegible]e feed reward.

TABLE 6 Effects of different levels of RBWF addition on economic benefits of *Duolang* sheep.

Items	CK	H1	H2	H3	SEM	*p*-value[1]		
						Trt	L	Q
Feed Unit price	2.39[a]	2.36[b]	2.30[c]	2.25[d]	0.009	<0.001	<0.001	<0.001
ADFI	1.79[b]	1.73[b]	1.84[a]	1.65[c]	0.043	0.021	<0.001	<0.001
Feed costs	257.12[a]	246.14[c]	242.75[b]	222.32[d]	2.238	<0.001	<0.001	<0.001
Sheep live weight unit price	31.00	31.00	31.00	31.00	0.000	-	-	-
Total gain weight, kg	14.21	13.18	14.89	13.14	0.478	0.563	0.733	0.788
Gain from weight gain	440.51	408.58	461.59	407.34	1.218	0.563	0.733	0.788
Net profit	183.39	162.46	218.86	185.02	1.979	0.789	0.743	0.968

[1]Trt, treatment effect; L, linear; Q, quadratic.
[a, b,c,d] Different letters indicate significant differences between different groups ($p < 0.05$). SEM is the pooled standard error between five groups; the *p*-value indicates significance.
- The unit price for live sheep weight is a fixed value that does not fluctuate.

The compound stomach is the most prominent anatomical feature of ruminants, and the rumen, situated within the four stomachs, serves as the primary site for ruminant digestion of feed. The key indicators for measuring the maintenance of an internal steady state of rumen fermentation in animals are pH, NH_3-N and VFAs. The pH directly affects the growth and interaction of microorganisms in the rumen, and maintaining an appropriate range is closely related to rumen fermentation. At the same time, the concentration of volatile fatty acids (VFAs) and ammonia nitrogen (NH_3-N) within the rumen also exert a direct influence on rumen pH (32). NH_3-N represents the primary nitrogen source for protein synthesis in rumen microorganisms. The microorganisms in the rumen engage in cooperative degradation of nutrients in the feed, converting them into volatile fatty acids (VFAs) that provide energy for body growth (33). The concentration and proportion of VFAs in the rumen are related to dietary composition and energy level, and have an effect on the energy utilization efficiency, growth performance and methane production of the host (34). The addition of 0.6% LBP to the diet of dairy cows has been found to significantly improve the production performance of dairy cows and significantly increase the total volatile fatty acid content of rumen ammonia nitrogen (5). In this study, the concentration of volatile fatty acids (VFAs), including acetate, propionate, iso-butyrate, butyrate and iso-valerate, in rumen fluid demonstrated a linear increase with the elevation of dietary RBWF supplemental level. This outcome aligns with the findings of Duan et al. (35). The pH of rumen fluid in sheep is typically within the range of 5.5–7.0, while the normal range of NH_3-N is 5.0 to 30 mg/dL (36). The rumen fluid pH and NH_3-N of all sheep were maintained within the normal range in this experiment. As a consequence of the considerable rise in VFAs concentration in rumen fluid, the pH in the rumen of *Duolang* sheep in each experimental group declined in a linear fashion with the increase of RBWF supplemental level in the diet, while the concentration of NH_3-N in rumen increased linearly. It may therefore be hypothesized that the addition of RBWF to the diet can increase the protein synthesis of rumen microorganisms, thereby promoting microbial fermentation in the rumen of animals and the absorption and utilization of nutrients in the diet. Further investigation is required to elucidate the precise effects.

The rumen is a digestive organ that contains a diverse range of microorganisms, which work collectively to degrade nutrients in the diet, thereby providing the host with energy (37). Simultaneously, the host provides a stable and conducive environment for the growth of rumen microorganisms, while obtaining energy from the final product (38). The structure and abundance of the rumen microbial flora can exert a direct influence on the health and growth of the host. Conversely, the structure of rumen microbial flora is also influenced by host species, nutritional energy level and environment (39). Prior research has demonstrated that dietary supplementation of *wolfberry* fruit can regulate the composition of gastrointestinal microbiota and cecal fermentation in rabbits (40). The findings of the study conducted indicated that the incorporation of LBP into the weaned piglets diet could potentially enhance the abundance of *Bacteroidetes* in the ileum and cecum, while increasing the levels of *Lactobacillus* and *Bifidobacterium* in the cecum, and the intestinal microflora was shown to be improved (41). Alpha diversity is primarily indicative of the richness and diversity of species distribution within the rumen, whereas beta diversity predominantly reflects the dissimilarities in microbial communities between disparate samples. In this experiment, the rumen microorganisms of *Duolang* sheep were sequenced, and the alpha diversity results demonstrated that the dietary RBWF addition level had no significant impact on the four indexes of Chao1, Ace, Shannon and Simpson in the rumen of *Duolang* sheep. The PCoA and NMDS plots revealed that the bacterial community structures of the different treatment groups were not statistically different from one another, as indicated by the overlap of the respective groups on the plots. The aforementioned results demonstrate that the incorporation of RBWF into the diet does not exert any deleterious effects on the rumen microbial flora structure of *Duolang* sheep.

A substantial body of research has demonstrated that the predominant bacteria in the rumen of ruminants are members of the *Firmicutes* and *Bacteroidota* phyla. These bacteria play a pivotal role in the host's degradation of complex carbohydrates and promotion of the decomposition and absorption of nutrients (42, 43). The results indicate that *Firmicutes* and *Bacteroidota* play an important role in maintaining the stability of the rumen microbial community. Among them, *Bacteroidota* mainly promotes rumen fermentation to degrade cellulose, soluble sugar and carbohydrates to produce small molecules such as VFAs, which are utilized by the host (44). The role of *Firmicutes* is mainly to enhance host lipid metabolism and promote its energy absorption (45). Fujisaka et al. (46) observed that *Firmicutes* and *Bacteroidota* constituted between 70 and 90% of the total bacterial

population. In this experiment, the relative abundance of *Firmicutes* and *Bacteroidota* accounted for approximately 90% of the total bacteria, which was consistent with the aforementioned reports. Furthermore, the relative abundance of *Bacteroidota* and *Fibrobacterota* increased following the addition of RBWF in this experiment, which may facilitate the degradation of cellulose in the rumen and is associated with the enhancement of the apparent digestibility of NDF in this experiment.

In addition to decomposing and metabolizing complex nutrients such as cellulose and protein, *Prevotella* can also engage in cooperative interactions with other microorganisms to facilitate the growth and development of the body (47). The *NK4A214 group* is primarily responsible for the degradation of fibers, which provides energy to the host. Additionally, it has been observed that this process enhances the host's immunity by inhibiting the growth of harmful bacteria (48). The *Lachnospiraceae NK3A20 group* has also been identified as a potentially beneficial bacterium, with involvement in the metabolism of various carbohydrates and the production of acetic acid and butyric acid, which provide energy for the host (49). *Prevotellaceae UCG 001* primarily degrades proteins and amino acids, producing short-chain fatty acids, and facilitates the degradation of fiber in collaboration with cellulose-decomposing bacteria (50). Following the addition of RBWF to the experimental diet, a notable increase was observed in the relative abundance of *Prevotella*, *NK4A214 group*, *unclassified Prevotellaceae* and *Lachnospiraceae NK3A20 group*. These bacteria are capable of decomposing complex dietary fibers, such as cellulose and pectin, into smaller molecular structures that can be utilized by other bacteria within the rumen, resulting in the production of volatile fatty acids (VFAs). Zhu et al. (51) observed that a reduction in the relative abundance of *Prevotellaceae UCG 001* was associated with an improvement in depressive behavior. This finding was hypothesized to be linked to a decrease in short-chain fatty acids and a reduction in intestinal inflammation. The results of this experiment demonstrated a reduction in the relative abundance of *Prevotellaceae UCG 001*, which may be associated with autoimmune regulation. Furthermore, our study elucidated the correlation between growth performance, nutrient apparent digestibility, rumen fermentation parameters and major bacteria at the genus level.

Prior research has demonstrated a positive correlation between *Prevotellaceae UCG 001* and the expression of inflammatory factors (52). In this experiment, *Prevotellaceae UCG 001* was found to be negatively correlated with feed conversion ratio (FCR) and pH, and positively correlated with other indicators. Furthermore, it was significantly positively correlated with organic matter (OM) apparent digestibility. The concentration of acetic acid and butyric acid in the rumen of *Duolang* sheep in the experimental group increased, which enhanced the absorption of nutrients in the rumen and thus promoted growth performance. The abundance of F082, *Christensenellaceae R7 group*, *NK4A214 group* and *Ruminococcus* was significantly and positively correlated with ADG. An increase in the relative abundance of *NK4A214 group* was observed, resulting in an increase in ADG for the experimental group. However, further study is required to elucidate the specific mechanisms through which these strains affect growth performance and rumen fermentation.

In the context of sheep breeding, economic benefits are closely associated with feed costs and growth performance (53). The results of this experiment indicate that an increase in the proportion of RBWF added to the diet is associated with a reduction in feed unit price. This suggests that the inclusion of RBWF in the diet may offer a potential means of reducing feed costs. However, the total weight gain of sheep in the experimental group with RBWF was found to decrease. In contrast, the H2 group exhibited a 4.78% increase in weight gain compared to the control group. Additionally, the gross profit was observed to increase by 17.45% compared to the control group. These findings suggest that the appropriate amount of RBWF can enhance the profitability of mutton sheep breeding. Furthermore, the 5% addition was identified as the optimal dosage. Han et al. (54) demonstrated that the incorporation of *wolfberry* as a functional or nutritional feed ingredient in broiler diets offers a promising economic benefit. Similarly, Wang et al. (55) revealed that the administration of 0.1–0.2% LBP nutrition lick blocks can markedly enhance the economic viability of fattening Tan sheep. Collectively, these studies highlight the potential of *Lycium barbarum* products to enhance livestock productivity.

5 Conclusion

The results of this experiment demonstrate that the administration of RBWF can enhance the growth performance of *Duolang* sheep, optimize rumen fermentation parameters, exert no deleterious effect on the structure and abundance of rumen microbial flora, and augment gross profit. In the context of the experimental conditions, the administration of 5% RBWF yielded more favorable outcomes.

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/, PRJNA1170910, PRJNA1170932.

Ethics statement

The animal studies were approved by the animal study protocol was approved by the Ethics Committee of Institute of Feed Research, Xinjiang Academy of Animal Science (protocol code no. 320230508 and May 10, 2023 of approval). The studies were conducted in accordance with the local legislation and institutional requirements. Written informed consent was obtained from the owners for the participation of their animals in this study.

Author contributions

LH: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Writing – original draft, Funding acquisition, Project administration, Resources, Visualization, Writing – review & editing. PD: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Writing – original draft, Software, Writing – review & editing. YY: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Resources, Validation, Visualization, Writing – review & editing. AS: Supervision, Writing – review & editing. JL: Investigation, Software, Validation, Writing – review & editing. CX: Investigation, Software, Validation,

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Funding

The author(s) declare that financial support was received for the research, authorship, and/or publication of this article. This study was financially supported by the China-Kazakhstan forage basic information sharing platform construction and planting base, diversified processing enterprise technical services (2022E01039) and the Study on the effect of Apocynum venetum on alleviating heat stress in dairy cows (2024D01A44).

Conflict of interest

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

Generative AI statement

The author(s) declare that no Gen AI was used in the creation of this manuscript.

Publisher's note

Supplementary material

The Supplementary material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fvets.2024.1528126/full#supplementary-material

References

1. Dong JZ, Yang JJ, Wang Y. Resources of Lycium species and related research progress. *Zhongguo Zhong Yao Za Zhi*. (2008) 33:2020–7.

2. Zenggen L, Baolong L, Huaixiu W, Yuchuan S. Phytochemical profiles, nutritional constituents and antioxidant activity of black wolfberry (Lycium Ruthenicum Murr.). *Ind Crop Prod*. (2020) 154:112692. doi: 10.1016/j.indcrop.2020.112692

3. Su L, Guzelinur E, Zhang Z, Hou L, Li X, Cui Y, et al. Development and application of nutritional components of Lycium Ruthenicum Murray and its by-products. *Xinjiang Anim Husb*. (2024) 40:37–46. doi: 10.16795/j.cnki.xjxmy.2024.3.008

4. Jianfei L, Yuan G, Yang J-L, Duolong D. Advance on alkaloids of Lycium genus. *Chin Sci Bull*. (2021) 67:332–50. doi: 10.1360/tb-2021-0229

5. Zhao Y, Lei Y. Effects of *Lycium Barbarum* polysaccharides on production performance, rumen fermentation parameters, antioxidant capacity, and immune capacity of dairy cows. *China Feed*. (2024) 29:43–6. doi: 10.15906/j.cnki.cn11-2975/s.20241011

6. Zhang Y, Guo Y, Luo Y, Du M, Yin X, Xu X, et al. Integrated metabolomics and transcriptome revealed the effect of fermented *Lycium Barbarum* residue promoting *Ovis Aries* immunity. *Front Immunol*. (2022) 13:9436. doi: 10.3389/fimmu.2022.889436

7. Liu G, Shenghua Z, Qiong L, Huibi X. A polysaccharide-protein complex from *Lycium Barbarum* upregulates cytokine expression in human peripheral blood mononuclear cells. *Eur J Pharmacol*. (2003) 471:217–22. doi: 10.1016/s0014-2999(03)01827-2

8. Vanessa M, Rita SA, Bruna S, Xiaoying Z, Pires DAC. Comparative studies on the anti-Neuroinflammatory and antioxidant activities of black and red goji berries. *J Funct Foods*. (2022) 92:105038. doi: 10.1016/j.jff.2022.105038

9. Tahidul I, Xiaoming Y, Singh BT, Baojun X. Comparative studies on phenolic profiles, antioxidant capacities and carotenoid contents of red goji berry (*Lycium Barbarum*) and black goji berry (Lycium Ruthenicum). *Chem Cent J*. (2017) 11:59. doi: 10.1186/s13065-017-0287-z

10. Po-Yen C, Tin-Han S, Kai-Chieh C, Jhin-Syuan W, Chi-Ming Y, Yu-Sen C. Potential of galled leaves of goji (*Lycium Chinense*) as functional food. *BMC Nutr*. (2020) 6:26. doi: 10.1186/s40795-020-00351-w

11. William H, George WL. Official methods of analysis of AOAC International. Oxford: Oxford Academic (2006).

12. Van Soest PJ, Robertson JB, Lewis BA. Methods for dietary Fiber, neutral detergent Fiber, and nonstarch polysaccharides in relation to animal nutrition. *J Dairy Sci*. (1991) 74:3583–97. doi: 10.3168/jds.s0022-0302(91)78551-2

13. Mark A, Blaskovich T. Antibiotics special issue: challenges and opportunities in antibiotic discovery and development. *ACS Infect Dis*. (2020) 6:1286–8. doi: 10.1021/acsinfecdis.0c00331

14. Gerard DW. Antibiotic adjuvants: rescuing antibiotics from resistance. *Trends Microbiol*. (2016) 24:862–71. doi: 10.1016/j.tim.2016.06.009

15. Ha DR, Haste NM, Gluckstein DP. The role of antibiotic stewardship in promoting appropriate antibiotic use. *Am J Lifestyle Med*. (2017) 13:376–83. doi: 10.1177/1559827617700824

16. Bojana V, Milinčić Danijel D, Mirjana M, Jelena D, Tijana I, Kostić Aleksandar Ž, et al. Health benefits and applications of goji berries in functional food products development: a review. *Antioxidants*. (2022) 11:248. doi: 10.3390/antiox11020248

17. Yuebang Y, Fang W, Mei Y, Bin T, Yulong Y, Jiashun C, et al. *Lycium Barbarum* polysaccharides as antibiotic substitutes improve growth performance, serum immunity, antioxidant status, and intestinal health for weaned piglets. *Front Microbiol*. (2022) 12:993. doi: 10.3389/fmicb.2021.819993

18. Baoming T, Mengyao L, Wei A, Li Y, Jiawei Z, Yilin L, et al. *Lycium Barbarum* relieves gut microbiota Dysbiosis and improves colonic barrier function in mice following antibiotic perturbation. *J Funct Foods*. (2020) 71:103973. doi: 10.1016/j.jff.2020.103973

19. Duan P, Zhang L, Lihong H, Guishan X. Biological function and application in livestock and poultry production of Lycium Barbarum and its extracts. *Chin J Anim Nutr*. (2023) 35:4207–15. doi: 10.12418/CJAN2023.390

20. Liu YL, Runsheng Y, Saisai L, Yulan D, Jun Y, Xin Y. Effect of dietary *Lycium Barbarum* polysaccharide on growth performance and immune function of broilers. *J Appl Poult Res*. (2017) 26:200–8. doi: 10.3382/japr/pfw063

21. Zhuang H, Zhen L, Jinjin H, Jiandong L, Peng Y. Effects of Chinese wolfberry and Astragalus extract on the antioxidant capacity of Tibetan pig liver. *PLoS One*. (2021) 16:5749. doi: 10.1371/journal.pone.0245749

22. Hao W, Shenfei W, Jing Z, Shao-Ping L. Effects of extraction methods on immunology activity and chemical profiles of *Lycium Barbarum* polysaccharides. *J Pharm Biomed Anal*. (2020) 185:113219. doi: 10.1016/j.jpba.2020.113219

23. Xiaoxue Z, Guoze L, Fadi L, Deyin Z, Lvfeng Y, Yuan Z, et al. Effect of feed efficiency on growth performance, body composition, and fat deposition in growing Hu lambs. *Anim Biotechnol*. (2021) 34:183–98. doi: 10.1080/10495398.2021.1951747

24. Lina L, Baoju K, Qian J, Chen JS. Effects of dietary *Lycium Barbarum* polysaccharides on growth performance, digestive enzyme activities, antioxidant status, and immunity of broiler chickens. *Poult Sci*. (2020) 99:744–51. doi: 10.1016/j.psj.2019.10.043

25. Hao Z, Li Z, Huo J, Chu Y, Li J, Yu X, et al. Effects of Chinese wolfberry and Astragalus extracts on growth performance, pork quality, and unsaturated fatty acid metabolism regulation in Tibetan fragrant pigs. *Anim Sci J*. (2021) 92:e13581. doi: 10.1111/asj.13581

26. Guo Y, Liu J, Tuo Q, Zhang D, Wanapat M, Xin G. The effect of dietary supplementation of *Lycium Barbarum* leaves on the growth performance, organ indexes and intestinal microflora of rats. *Front Vet Sci*. (2024) 11:1416793. doi: 10.3389/fvets.2024.1416793

27. Zhentao H, Shuai L, Xiaolu W, Shuting C, Xianliang Z, Lei H, et al. Effect of mixed meal replacement of soybean meal on growth performance, nutrient apparent digestibility, and gut microbiota of finishing pigs. *Front Vet Sci*. (2024) 11:1486. doi: 10.3389/fvets.2024.1321486

28. Dawei Y, Yamei Y, Jun L. Isolation, structure and biological activity of polysaccharides from the fruits of Lycium Ruthenicum Murr: a review. *Carbohydr Polym*. (2022) 291:119618. doi: 10.1016/j.carbpol.2022.119618

29. Xulei L, Fuyao L, Tianhai Y, Shenghua C, Metha W, Fujiang H. *Cistanche Deserticola* addition improves growth, digestibility, and metabolism of sheep fed on fresh forage from alfalfa/tall fescue pasture. *Animals*. (2020) 10:668. doi: 10.3390/ani10040668

30. Lin R, Lin Y, Wang J, Peng L. Regulation of mouse digestive function, intestinal mucosal barrier function, and inflammatory reaction by *Lycium Barbarum* polysaccharide pathway through myosin light chain kinase. *Heliyon*. (2024) 10:e29795. doi: 10.1016/j.heliyon.2024.e29795

31. Ju H, Hao X, Zhang X, Liu S, Zhang H, Xiang B, et al. Effects of dietary *Lycium Barbarum* polysaccharide on nutrient apparent digestibility, energy metabolism, nitrogen metabolism and serum immune indexes of lambs. *Chin J Anim Nutr*. (2022) 34:7960–9. doi: 10.3969/j.issn.1006-267x.2022.12.044

32. Liu H, Zhou Jianwei A, Degen A, Hongshan L, Xu-Liang C, Lizhuang H, et al. 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*. (2023) 12:77–86. doi: 10.1016/j.aninu.2022.07.015

33. Chuan L, Jiantong Z, Yanjiao L, Xianghui Z, Huan L, Li Kairong Q, et al. Glutamate supplementation improves growth performance, rumen fermentation, and serum metabolites in heat-stressed Hu sheep. *Front Nutr*. (2022) 9:1386. doi: 10.3389/fnut.2022.851386

34. Yao G, Ruochen Y, Chunhui D, Yong W, Qing-Hong H, Shoukun J, et al. Effect of Dioscorea opposite waste on growth performance, blood parameters, rumen fermentation and rumen bacterial Community in Weaned Lambs. *J Integr Agric*. (2023) 22:1833–46. doi: 10.1016/j.jia.2022.10.002

35. Duan P, Rehemujiang H, Zhang L, Mulong L, Li C, Lihong H, et al. *Lycium Barbarum* (wolfberry) branches and leaves enhance the growth performance and improve the rumen microbiota in Hu sheep. *Animals*. (2024) 14:1610. doi: 10.3390/ani14111610

36. Hongjin L, Xu Tianwei X, Shixiao ML, Xueping H, Xungang W, Zhang Xiaoling H, et al. 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*. (2019) 7:e7462. doi: 10.7717/peerj.7462

37. Xin Y, Shoukun J, Chunhui D, Tian Peizhi J, Sisi YH, Yingjie Z, et al. Age-related changes in the ruminal microbiota and their relationship with rumen fermentation in lambs. *Front Microbiol*. (2021) 12:135. doi: 10.3389/fmicb.2021.679135

38. Kansagara YK, Savsani HH, Chavda MR, Chavda JA, Belim SY, Makwana KR, et al. Rumen microbiota and nutrient metabolism: a review. *Bhart Kris Anus Patr*. (2022) 37:320–7. doi: 10.18805/bkap486

39. Zhongyan L, Zhihui X, Zongzhuan S, Yuanchun T, Hong S. Dietary energy level promotes rumen microbial protein synthesis by improving the energy productivity of the ruminal microbiome. *Front Microbiol*. (2019) 10:847. doi: 10.3389/fmicb.2019.00847

40. Paola C, Giulio C, Filippo B, Elisa C, Laura M, Federica R, et al. Dietary supplementation with goji berries (*Lycium Barbarum*) modulates the microbiota of digestive tract and Caecal metabolites in rabbits. *Animals*. (2022) 12:121. doi: 10.3390/ani12010121

41. Jiashun C, Lina L, Qian J, Baoju K, Yinghui L, Jie Y. Effects of dietary supplementation of *Lycium Barbarum* polysaccharides on growth performance, immune status, antioxidant capacity and selected microbial populations of weaned piglets. *J Anim Physiol Anim Nutr*. (2019) 104:1106–15. doi: 10.1111/jpn.13247

42. Xinyu G, Yuzhu S, Lv Weibing P, Xiaoning LX, Luo Yuzhu H, Jiang WJ, et al. Sex differences in rumen fermentation and microbiota of Tibetan goat. *Microb Cell Factories*. (2022) 21:55. doi: 10.1186/s12934-022-01783-8

43. Ruth H, De MMC, Hugo J, Alejandro R, Alejandro C-Q. Functional and phylogenetic characterization of Bacteria in bovine rumen using fractionation of ruminal fluid. *Front Microbiol*. (2022) 13:3002. doi: 10.3389/fmicb.2022.813002

44. Yang X, Fan X, Jiang H, Zhang Q, Basangwangdui ZQ, Dang S, et al. Simulated seasonal diets Alter yak rumen microbiota structure and metabolic function. *Front Microbiol*. (2022) 13:6285. doi: 10.3389/fmicb.2022.1006285

45. Ali A, Ahmad ZJ, Bo ZL, Chao Y, Qudratullah K, Shah Tariq D, et al. Dynamics of rumen bacterial composition of yak (*Bos Grunniens*) in response to dietary supplements during the cold season. *PeerJ*. (2021) 9:e11520. doi: 10.7717/peerj.11520

46. Shiho F, Yoshiyuki W, Kazuyuki T. The gut microbiome: A Core regulator of metabolism. *J Endocrinol*. (2023) 256:111. doi: 10.1530/joe-22-0111

47. Kou X, Ma Q, Liu Y, Khan MZ, Wu B, Chen W, et al. Exploring the effect of gastrointestinal Prevotella on growth performance traits in livestock animals. *Animals*. (2024) 14:965. doi: 10.3390/ani14131965

48. Giuseppe C, Corrado D, Matteo D, Andrea S, Federica M, Breanne MA, et al. Exploring the relationship between bacterial genera and lipid metabolism in bovine rumen. *Animal*. (2022) 16:100520. doi: 10.1016/j.animal.2022.100520

49. Ronald K, Peter R, Eric A, Kenters Nikki J, William K, Joan NS, et al. Rumen Lachnospiraceae isolate Nk3a20 exhibits metabolic flexibility in response to substrate and Coculture with a methanogen. *Appl Environ Microbiol*. (2023) 89:e0063423. doi: 10.1128/aem.00634-23

50. Masami M, Fumiko N, Hiroshi S, Ryuichiro T. *Paraprevotella Clara* gen. Nov., Sp. Nov. and *Paraprevotella Xylaniphila* Sp. Nov., members of the family 'Prevotellaceae' isolated from human Faeces. *Int J Syst Evol Microbiol*. (2009) 59:1895–900. doi: 10.1099/ijs.0.008169-0

51. Hui-Zheng Z, Yubin L, Qingyu M, Wenzhi H, Li Xiaojuan W, Man-Si DL, et al. Xiaoyaosan improves depressive-like behavior in rats with chronic immobilization stress through modulation of the gut microbiota. *Biomed Pharmacother*. (2023) 112:108621. doi: 10.1016/j.biopha.2019.108621

52. Ruizhi H, Ziyu H, Ming L, Jijun T, Hongfu Z, De-Xing H, et al. Dietary Protocatechuic acid ameliorates inflammation and up-regulates intestinal tight junction proteins by modulating gut microbiota in Lps-challenged piglets. *J Anim Sci Biotechno*. (2020) 11:92. doi: 10.1186/s40104-020-00492-9

53. Tekletsadik E, Solomon MB, Mengistu U. The effect of barley bran, linseed meal and their mixes supplementation on the performances, carcass characteristics and economic return of arsi-bale sheep. *Small Rumin Res*. (2013) 114:35–40. doi: 10.1016/j.smallrumres.2013.05.010

54. Han H, Yaozhong W, Chen P. Effects of beer yeast and wolfberry on production performance and economic benefits of broilers. *Shanghai J Anim Husb Vet Med*. (2008) 44:63–5.

55. Wang Z, Ding Y, Yun H, Zongshan W, Mei N, Ding J, et al. Effect of *Lycium Barbarum* polysaccharide nutrition licking brick on fattening effect of tan sheep. *Contemp Anim Husb*. (2019) 44:1–3.

frontiers | Frontiers in Veterinary Science

TYPE Original Research
PUBLISHED 22 January 2025
DOI 10.3389/fvets.2024.1525783

OPEN ACCESS

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RECEIVED 10 November 2024
ACCEPTED 23 December 2024
PUBLISHED 22 January 2025

CITATION
Meng Y, Xian T, Kang G, Wang H and Feng T (2025) Effects of dietary L-carnosine supplementation on the growth, intestinal microbiota, and serum metabolome of fattening lambs.
Front. Vet. Sci. 11:1525783.
doi: 10.3389/fvets.2024.1525783

Effects of dietary L-carnosine supplementation on the growth, intestinal microbiota, and serum metabolome of fattening lambs

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Dietary L-carnosine supplementation has been shown to enhance animal performance and improve meat quality. However, the mechanisms underlying the effects of L-carnosine on the physiological functions of animals have not been fully elucidated. We investigated the effects of dietary L-carnosine supplementation on growth performance, intestinal microbiota diversity, and the serum metabolome in fattening lambs to reveal the molecular mechanism underlying the effect of L-carnosine on the growth performance of sheep. Sixty 3-month-old male crossbred lambs (Dorper ♂ × Small Tail Han ♀) with an average body weight of 30 ± 5 kg were randomly divided into two groups: a control group (group C) fed a basal diet, and an experimental group (group L) fed a basal diet supplemented with 400 mg/kg of L-carnosine. At the end of the 60-day experiment, all sheep were weighed, and fecal and blood samples were collected from 12 random sheep. The fecal microbiota was analyzed using 16S rRNA sequencing, and serum metabolites were analyzed using liquid chromatography–tandem mass spectrometry. Spearman correlation analysis was employed to assess the associations between intestinal microbiota and serum metabolite biomarkers. The results showed that weight gain and daily weight gain were significantly increased in group L compared to group C ($p < 0.01$). The dominant phyla in the intestinal microbiota (Firmicutes and Bacteroidetes) did not significantly differ between the two groups ($p > 0.05$). At the genus level, the abundances of *Syntrophococcus* ($p < 0.01$) and *Butyricimonas* ($p < 0.001$) were higher, whereas those of *Escherichia-Shigella* and *Candidatus Saccharimonas* were significantly lower in group L than in group C ($p < 0.05$). Non-targeted metabolomics identified 68 differentially abundant biomarkers (VIP > 1, $p < 0.05$). The content of pyridine N-oxide glucuronide was significantly downregulated ($p < 0.01$), whereas those of L-histidinol, D-apiose, and isodomedin were significantly upregulated in group L versus group C ($p < 0.001$). *Holdemania* and *Butyricimonas* were positively correlated with L-histidine, D-apiose, and L-erythrulose ($p < 0.001$), whereas *Butyricimonas* was negatively correlated with pyridine N-oxide glucuronide ($p < 0.001$). This study provided new insights into the effects of L-carnosine on the intestinal microbiota and nutrient metabolism in fattening sheep that will be helpful for the future application of L-carnosine in ruminants.

KEYWORDS

fattening lamb, L-carnosine, microbiota, metabolomics, multi-omics analysis

1 Introduction

With the enhancement of human living standards, the demand for livestock products is increasing, which is an excellent opportunity for animal husbandry, including the sheep farming industry (1). Carnosine is a dipeptide found in animals (2), but its content varies among species and animal body parts (3, 4). Carnosine and L-carnosine have the same chemical structure and biological activity, and as the amino acid residues in carnosine have an L-form stereochemical configuration, it can also be referred to as L-carnosine. L-Carnosine is present at high concentrations in the skeletal muscle of most vertebrates and has critical antioxidant functions (5). In porcine myoblasts, it promoted cell proliferation by activating targets of the mammalian the rapamycin signaling pathway and mitigated cellular damage caused by oxidative stress (6). In mice exposed to deoxynivalenol, L-carnosine bound to Keap1, releasing the transcription factor Nrf2 into the nucleus, thereby activating the transcription of downstream genes and increasing antioxidant production to neutralize excess reactive oxygen species and thus reduce the oxidative stress caused by deoxynivalenol (7). Further, L-carnosine helps maintain animal health through anti-glycosylation (8, 9), pH stabilization, and metal chelation (10). Studies have shown that L-carnosine ameliorates the adverse effects of oxidative stress in pregnant ewes, and it may also offer benefits in reducing the combined challenges posed by pregnancy and heat stress during the hot-dry season (11). In finishing pigs, dietary L-carnosine increased body weight, feed intake, and daily gain, increased the secretion of thyroid hormone, and induced the proliferation of satellite cells (12). In chickens, L-carnosine also positively influences weight gain and activates the enzymatic antioxidant system in the blood (13, 14).

The intestinal microbiota plays a vital role in adaptive coevolution with mammals (15). The intestinal tract of sheep is characterized by a diverse microbial ecosystem (16), which is influenced by multiple factors, such as feeding, drinking, the environment, physiology, and disease (17–19). The intestinal microbiota also plays a crucial role in biological processes related to regulating nutrient absorption and maintaining homeostasis (20). It produces various digestive enzymes that convert indigestible plant macromolecules into small molecules that are absorbable by the host (21). For example, certain microorganisms produce pectinases that can degrade pectin in the plant cell wall into small molecules that can be utilized by ruminants (22), whereas many species produce lipases that hydrolyze long-chain fatty acids (23). Once absorbed by the host, these simple metabolites enter the bloodstream, altering serum metabolite levels (24). Microbial homeostasis influences the efficiency with which the host animal utilizes its feed, ultimately affecting productive performance metrics, such as daily weight gain and the feed conversion rate.

Metabolomics plays a crucial role in analyzing metabolic pathways, processes, and gene functions (25, 26), and is widely used in the fields of animal disease diagnosis and food ingredient identification (27). Comprehensive analysis of the correlations between the intestinal microbiota and serum metabolome not only enables identifying the relationship between dietary energy levels and lamb quality (28) but has also been used to investigate how they reflect age and nutritional requirements in Tibetan sheep (29). Furthermore, it has well explained the effects of different diets on intestinal microbes and metabolic pathways in Hu sheep (30).

Our knowledge about the effects of L-carnosine on the intestinal microbiota and serum metabolome in fattening sheep is limited, and published studies are scarce. We investigated the effects of dietary L-carnosine supplementation on the fecal microbiota and metabolite composition in fattening lambs using 16S rRNA sequencing and liquid chromatography–tandem mass spectrometry (LC–MS/MS)-based metabolomics. Additionally, multi-omics analysis was conducted on the fecal microbiota and serum metabolites to provide a reference for the application of L-carnosine in sheep.

2 Materials and methods

2.1 Experimental animals and experimental design

The experiment was conducted at the Sheep Experimental Station of the Beijing Academy of Agriculture and Forestry Sciences in Yangyuan County, Zhangjiakou City, Hebei Province, China. In total, 60 male crossbred lambs (Dorper ♂ × Small Tail Han ♀) of 3 months of age with an average body weight of 30 ± 5 kg were randomly divided into two groups. The lambs were housed in 2 sheltered outdoor paddocks and fed a basal diet consisting of a total mixed ration according to the Chinese sheep feeding standard (NY/T816-2004). The contents of digestible energy, metabolizable energy, crude protein, calcium, and phosphorus in the diet were 11.83 MJ·kg-1, 9.73 MJ·kg-1, 14.61, 0.39, and 0.25%, respectively (31). Animals in the control group (group C) were fed only the basal diet (Table 1). Based on previous studies and the dietary supplementation level of L-carnosine used previously in fattening pigs (12, 32), we determined

TABLE 1 Composition and nutrient levels of the basic diet of fattening sheep.

Item	Content
Ingredients	
Corn silage	30.90
Corn	44.20
Soybean meal	17.00
Wheat bran	5.00
Salt	0.70
Limestone powder	1.00
Calcium hydrogen phosphate	0.20
Premix[1]	1.00
Total	100.00
Nutrientcomponent[2]	
Metabolizable energy	9.73
Digestible energy	11.83
Crude protein	14.61
Calcium	0.39
Phosphorus	0.25

[1]The premix provided the following per kg diets: VA 200 kIU, VD_3 40 kIU, VE 1000 mg, Nicotinamide 1,200 mg, Biotin 70 mg, Fe (FeSO4) 1300 mg, Mn (MnSO4) 1000 mg, Zn (ZnSO4) 1240 mg, Se (Na2SeO3) 8.50 mg, Co (CoSO4) 11 mg. [2]Metabolizable energy was a calculated value, while the others were measured values.

the optimal supplementation level of L-carnosine for fattening lambs to be 400 mg/kg. In the experimental L-carnosine group (group L), L-carnosine (purity ≥98%, Zhengzhou Luyuan Biotechnology Co., Ltd.) was added to the basal diet at a concentration of 400 mg/kg. Both groups were provided with adequate water and salt blocks during the 60-day trial period.

2.2 Sample collection

The morning after the feeding experiment, the 60 sheep were weighed. Fresh feces were individually collected from the rectum of 12 random sheep in each group ($n = 24$ in total), immediately transferred into sterile tubes, and frozen in liquid nitrogen. To prevent contamination, the outer layer of each fecal sample was discarded, and the middle inner portion was sampled for testing. Blood (5 mL) was collected from the jugular veins of the above 24 sheep using vacuum blood collection tubes for serum metabolomics analysis. The blood samples were maintained at room temperature for 4 h, followed by centrifugation at 3000 rpm, 4°C for 10 min to collect the serum. The serum was frozen at −80°C until metabolomics analysis.

2.3 Microbial DNA extraction and sequencing

Microbial DNA was extracted from the fecal using an E.Z.N.A.® Soil DNA Kit (Omega Bio-tek, Norcross, GA,U.S.). The DNA was examined using 2% agarose gel electrophoresis, and the DNA concentration and purity were determined with a Thermo Fisher NanoDrop-2000 spectrophotometer (Thermo Scientific, U.S). The variable regions V3 and V4 of the bacterial 16S rRNA gene were PCR-amplified using primers 338F (5′-ACTCCTACGGG GAGGCAGCAG-3′) and 806R (5′-GGACTACHVGGGGTWTC TAAT-3′). The PCR products were recovered, purified, assayed, and quantified using a microfluorometer (QuantiFluor-ST; Promega, USA). The PCR products were mixed in equimolar amounts, and a sequencing library was constructed using a NEX-TFLEX Rapid DNA-Seq Kit and sequenced on the Illumina MiSeq PE 300 platform (Shanghai Majorbio Bio-Pharm Technology Co., Ltd.).

2.4 Microbiome composition analysis

Operational taxonomic units (OTUs) at a 97% similarity level were clustered and compared to the 16S rRNA database (Silva v138/16S_bacteria) to classify annotations of the OTU sequences, setting the classification confidence to 0.7. All data were analyzed on the Shanghai Majorbio Cloud platform. The alpha diversity index was determined using Mothur. Differences or similarities in the composition of the fecal microbial communities were analyzed using principal coordinates analysis (PCoA). Abundance differences in intestinal microbial composition at various levels between the two groups were analyzed using Student's *t*-test. The linear discriminant analysis effect size (LEfSe) algorithm was used to identify differences among various taxonomic groups. Using the acquired community abundance data, rigorous statistical approaches were used to perform hypothesis testing on species across various microbial communities. This process aims to evaluate the significance of species abundance differences and pinpoint species that exhibit notable disparities between groups. PICRUSt was used to normalize the OTU abundance table to obtain the corresponding GreenGene IDs of the OTUs, and Kyoto Encyclopedia of Genes and Genomes (KEGG) functional annotations were compared against the Clusters of Orthologous Genes database to obtain annotation information of the OTUs and abundance information for each function across the samples.

2.5 Metabolite extraction and metabolomics analysis

For metabolite extraction, methanol was added to 100 μL of serum, after which the samples were pulverized using a cryo-mill and sonicated for 30 min (5°C, 40 kHz). The samples were left to precipitate the proteins and then centrifuged. The supernatants were transferred into sample vials for LC–MS/MS analysis.

The samples were separated by ultra-high performance liquid chromatography system (ACQUITY UPLCHSS T3, Waters Corp., USA) and detected. The raw data included quality control (QC) and detection samples. Data pre-processing for appropriate data analysis encompassed the filtering of the raw data, recoding of missing values, normalization, QC verification, and data conversion and was conducted using the Progenesis QI 2.3 software.

The MS data were matched with the metabolite database HMDB[1] and Metlin,[2] and data analysis was performed on the Shanghai Majorbio Cloud platform. Projected variable importance (VIP) values were calculated using orthogonal least partial squares discriminant analysis (OPLS-DA) modeling, and the validity of the OPLS-DA model was evaluated using R2Y and Q2.

Enrichment analysis was performed using Fisher's exact test. *p*-values were corrected using the Benjamini-HochbergBH method, and the corrected *p*-values were thresholded at 0.05. KEGG pathways meeting this criterion were considered significantly enriched in the metabolite set, and the significantly enriched pathways were accurately examined using the Scipy software v1.0.0 ($p < 0.05$). The raw sequencing reads generated in this study have been deposited in the NCBI Sequence Read Archive (SRA) under BioProject accession number PRJNA1175011.[3]

2.6 Correlation analysis

The Spearman correlation coefficients between fecal microbial taxa and differential serum metabolites were calculated using the Python software. Correlation heatmaps were generated to visualize and analyze the associations. Spearman's correlation analysis is a statistical technique used to assess the relationship and the strength of influence between an independent variable and a dependent variable by analyzing the rank order of two sets of variables. It involves three

1 http://www.hmdb.ca/
2 https://metlin.scripps.edu/
3 https://www.ncbi.nlm.nih.gov/sra/PRJNA1175011

primary steps: first, ranking the data from both sets of variables based on their magnitude; second, replacing the original data with these ranks; and finally, calculating the correlation between these ranks. This method provides insights into the association and impact of the independent variable on the dependent variable.

3 Results

3.1 Growth performance

The effect of L-carnosine on the weight gain of fattening lambs is presented in Table 2. Total weight gain and average daily weight gain (ADG) were significantly increased, by 13.31 and 13.28%, respectively, in group L compared to group C ($p < 0.01$).

3.2 Fecal microbiota richness and diversity

At the 97% similarity threshold, a total of 1,662 OTUs were found in samples from groups C and L (Figure 1). The microbiota of group C and group L samples shared 1,260 OTUs, and 312 and 90 OTUs were uniquely identified in group C and group L samples, respectively. Diversity indices were calculated based on the OTUs of each library. We assessed alpha diversity in the two groups using five indices (Shannon, Simpson, ACE, Chao, and coverage) (Table 3). No significant differences between the two groups in these indicators were found ($p > 0.05$). Beta diversity was evaluated using PCoA. A PCoA plot based on the Bray–Curtis algorithm (Figure 2) showed that groups C and group L differed in terms of beta diversity ($p < 0.05$). Notably, the group C samples exhibited a dispersed distribution in the PCoA plot, indicating substantial variation in the microbial community structures within the group. Conversely, the group L samples clustered more compactly, suggesting a higher degree of similarity in the intestinal microbial community structures among sheep supplemented with L-carnosine.

3.3 Fecal microbiota composition

To investigate the differences in the fecal microbiota composition between groups C and L, we used the Wilcoxon rank-sum test to compare the mean relative abundances of dominant bacteria at the phylum and genus levels. The dominant phyla in the two groups were Firmicutes and Bacteroidota. The abundances of Actinobacteriota and Proteobacteria were decreased, whereas those of Bacteroidota and Spirochaetota were increased in group L compared to group C (Figure 3A). The top three dominant genera in the two groups were *UCG-005*, *norank_f_Muribaculaceae*, and *Rikenellaceae_RC9_gut_group* (Figure 3B). Further analyses revealed that, at the phylum level (Figure 4A), the abundance of Patescibacteria was significantly lower in group L than in group C ($p < 0.01$). At the genus level (Figure 4B), the abundances of *Syntrophococcus* ($p < 0.01$) and *Butyricimonas* ($p < 0.001$) were higher in group L than in group C. In contrast, the abundances of *Escherichia-Shigella* and *Candidatus* Saccharimonas were significantly lower in group L than in group C ($p < 0.05$).

3.4 Metabolomics analysis

Using non-targeted metabolomics technology, serum samples from groups C and L were analyzed to characterize the blood metabolites in fattening lambs with or without L-carnosine supplementation. The differences between the groups were analyzed using OPLS-DA. As shown in the OPLS-DA score plot (Supplementary Figure S1A), the samples of the two groups were clearly distinguished. The values of R2Y (0.995) and Q2 (0.657) were > 0.5 (Supplementary Figure S1B), indicating that the OPLS-DA model had a good fit and good predictive ability, and that VIP values could be calculated based on the data to screen for differential marker metabolites.

Using VIP > 1 and $p < 0.05$ as the optimal thresholds, 68 differential metabolites were identified (Figure 5A). The clustering heatmap of the differential metabolites shows the differential metabolite expression profiles and VIP values for the metabolites with the top 30 VIP values (Figure 5B; Supplementary Table S1). Pyridine N-oxide glucuronide was downregulated ($p < 0.01$), whereas L-histidinol, D-apiose, and isodomedin were upregulated in group L ($p < 0.001$) compared to group C.

Differential metabolites were significantly enriched in eight KEGG pathways ($p < 0.05$): choline metabolism in cancer, ascorbate and alternate metabolism, pyrimidine metabolism, tryptophan

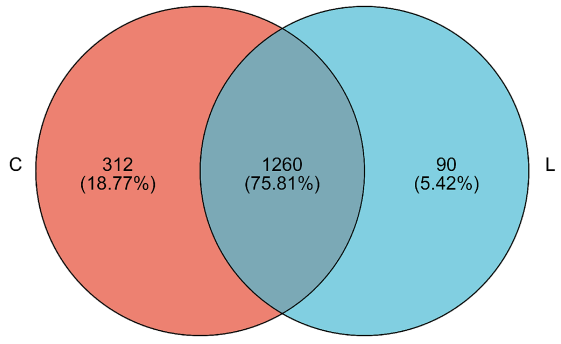

FIGURE 1
Venn diagram showing the OTUs in the fecal microbiota of fattening sheep. "C" indicated the control group, "L" represented the l-carnosine of 400 mg/kg. The same as below.

TABLE 2 Effect of L-carnosine on weight gain in fattening lambs.

Groups	Number	Initial weight/kg	Final weight/kg	Total weight gain/kg	Average daily gain/g
Group C	30	33.02 ± 2.28	47.45 ± 2.95	14.43 ± 1.79	240.56 ± 29.82
Group L	30	31.88 ± 2.65	48.25 ± 1.88	16.35 ± 1.23**	272.50 ± 20.55**

Data are presented as mean ± standard error of mean. **$p < 0.01$ *vs.* group C. "Group C" indicated the control group, "Group L" represented the L-carnosine of 400 mg/kg. The same as below.

TABLE 3 Effect of L-carnosine on five alpha diversity indices related to the fecal microbiota of fattening sheep.

Groups	Shannon	Simpson	Ace	Chao	Coverage
Group C	4.60 ± 0.55	0.035 ± 0.031	765.5 ± 161.3	786.2 ± 165.5	0.9965 ± 0.0006
Group L	4.78 ± 0.45	0.024 ± 0.018	751.6 ± 129.1	766.2 ± 134.0	0.9966 ± 0.0006

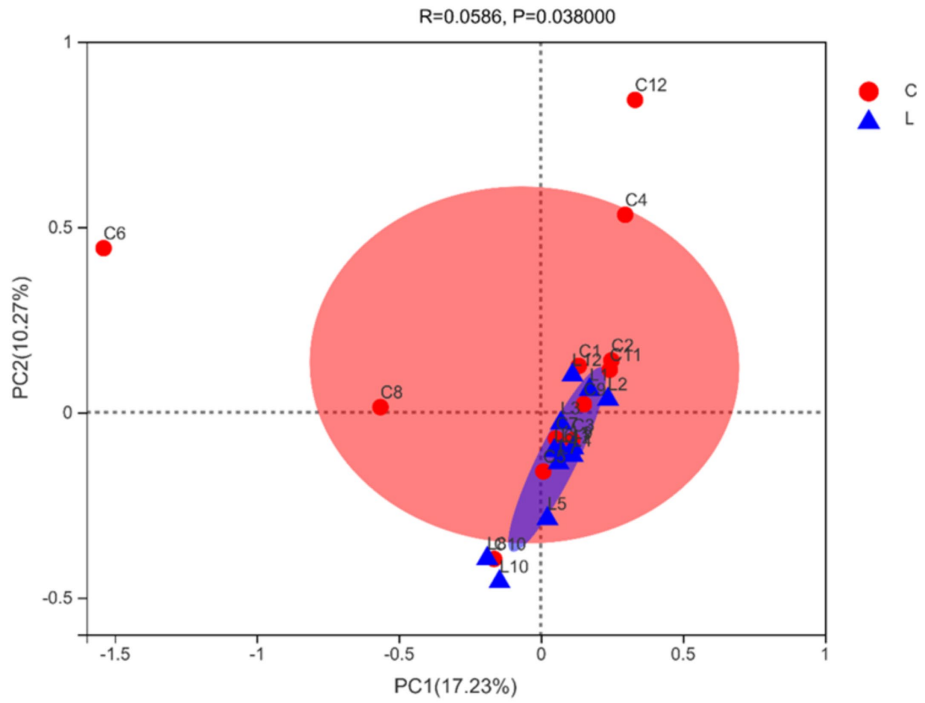

FIGURE 2
PCoA plot of microbial beta-diversity (based on the Bray–Curtis algorithm).

metabolism, histidine metabolism, protein digestion and absorption, lysine degradation, and glycerophospholipid metabolism (Figure 5C).

3.5 Correlations between fecal microbial and serum metabolites

The correlations between 20 genera and 40 metabolites were analyzed using Spearman correlation analysis (Figure 6). *Holdemania* and *Butyricimonas* were significantly positively correlated with L-histidinol, D-apiose, and L-erythrulose ($p < 0.001$). A highly significant negative correlation existed between *Butyricimonas* and pyridine N-oxide glucuronide ($p < 0.001$). *Pygmaiobacter* was positively correlated with phenylacetylglutamine ($p < 0.05$). A significant positive correlation was observed between *Butyricimonas* and stachydrine ($p < 0.05$).

4 Discussion

The dipeptide L-carnosine has been used in pig and chicken production to improve productive performance and antioxidant capacity and to maintain livestock health (33–35). However, reports on its application in sheep are scarce. Because of the unique digestive mechanism and dietary characteristics of ruminants, their intestinal microbiota is crucial for maintaining intestinal health and nutrient absorption. Intestinal microbial communities indirectly but profoundly influence the animal feed conversion efficiency and production performance by regulating the production of endogenous metabolites and various metabolic pathways (36).

The present study investigated the effects of dietary L-carnosine supplementation on the growth performance of fattening lambs. The dietary supplementation induced an increase in the total weight gain and ADG in the fattening lambs. Similar positive effects have been observed in other animal species. L-Carnosine supplementation in pigs increased the final weight, average daily feed intake, and ADG (12), and in chickens, it effectively increased body weight and breast muscle development (14). However, the mechanism by which L-carnosine is able to improve growth performance in fattening sheep remained unclear. Therefore, we analyzed the effects of L-carnosine supplementation on intestinal microbiota composition and serum metabolite profiles to gain insights into the mechanisms underlying its growth-promoting effects in fattening sheep.

The results revealed that the addition of L-carnosine did not affect the diversity and dominant species in the intestinal microbiota of fattening lambs. At the phylum level, the intestinal

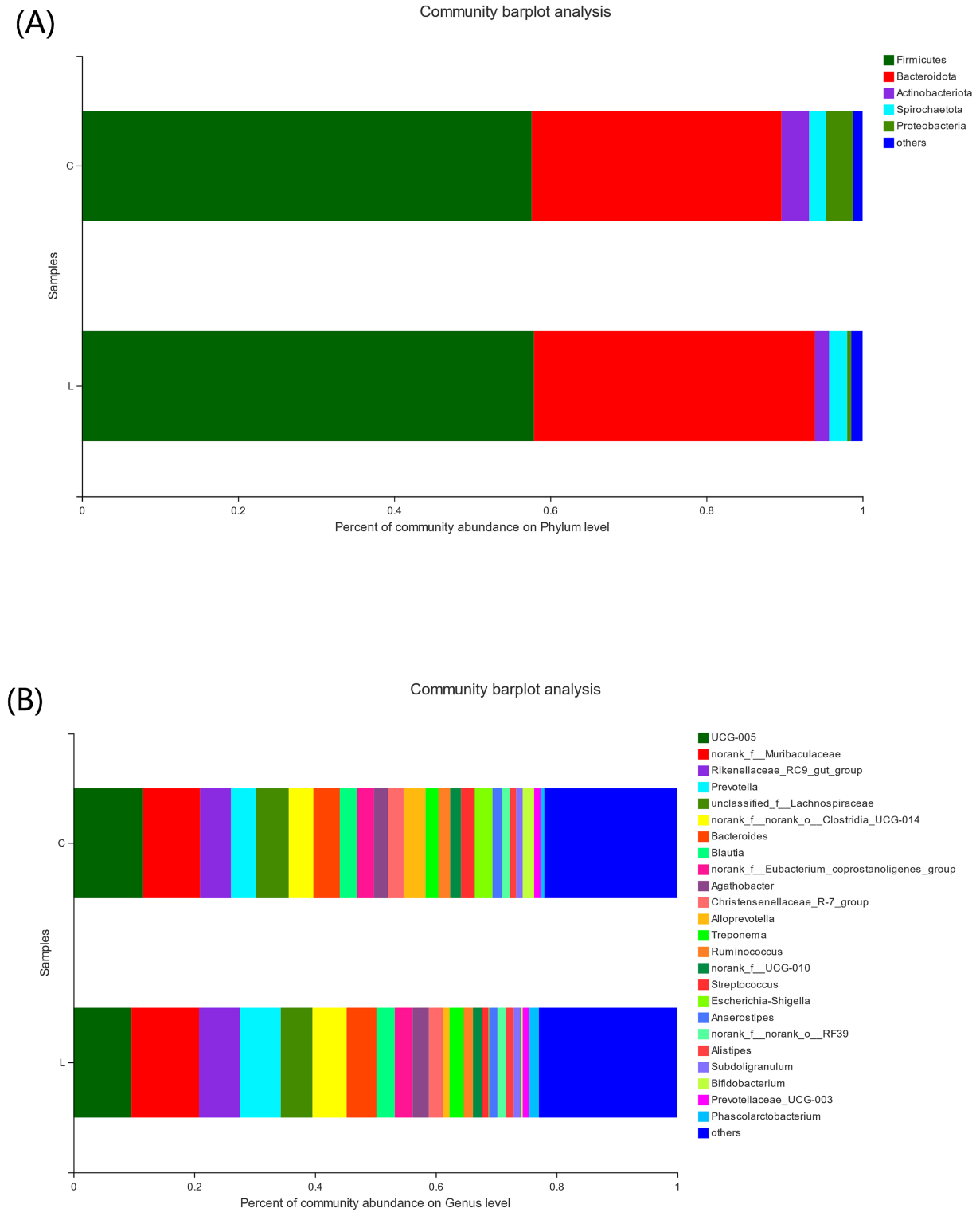

FIGURE 3
Effect of L-carnosine on fecal microbiota composition of fattening lambs. **(A)** Bacterial abundance at the phylum level. **(B)** Bacterial abundance at the genus level.

microbiota of fattening lambs was enriched in Firmicutes and Bacteroidetes both in the C and L groups, which is consistent with the prior findings in herbivores (37, 38). Firmicutes and Bacteroidetes are primarily responsible for carbohydrate, protein, and fiber metabolism (18, 39). At the genus level, L-carnosine supplementation significantly increased the relative abundances

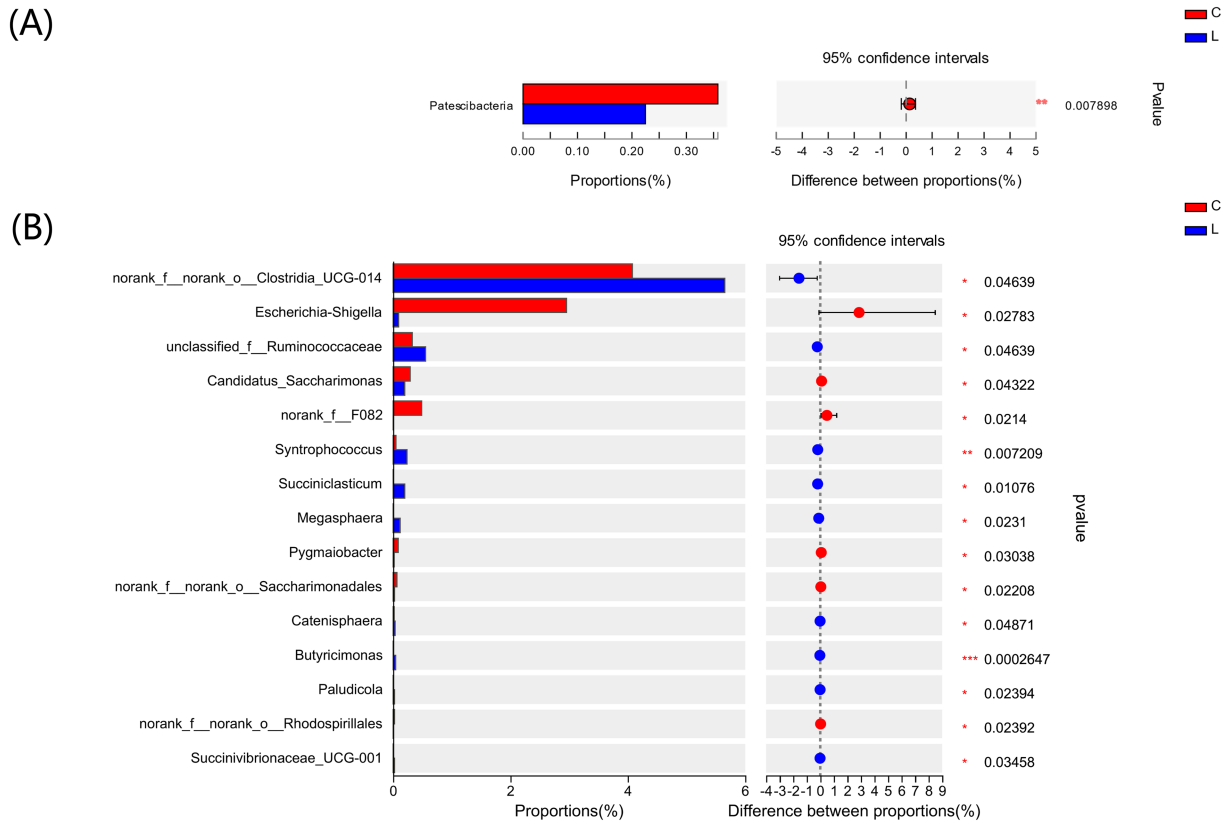

FIGURE 4
Differences in the major bacterial **(A)** phyla and **(B)** genera in lamb of control and L-carnosine group based on Wilcoxon rank-sum tests. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

of *Syntrophococcus* and *Butyricimonas*. *Syntrophococcus*, a member of Firmicutes, can digest various carbohydrates and produce short-chain fatty acids (SCFAs) (40). In a previous study, an increase in solid feed intake was accompanied by an elevation in the relative abundance of *Syntrophococcus*, thereby enhancing the carbohydrate digestive capacity of early-weaned lambs (41). *Syntrophococcus* is involved in the utilization of non-fibrous carbohydrates and the production of acetic acid. *Butyricimonas* is a butyrate producer (42). Acetic acid and butyrate are SCFAs. In the gastrointestinal tract, SCFAs can enhance intestinal barrier integrity, regulate glucose and lipid metabolism and the immune system, modulate inflammatory responses, suppress pathogenic bacterial growth, and manage blood pressure (43, 44). L-carnosine supplementation increased the abundance of *Syntrophococcus* and *Butyricimonas*, which possibly improved the utilization efficiency of non-fibrous carbohydrates and SCFA levels. The abundances of *Escherichia-Shigella* and *Candidatus* Saccharimonas were significantly reduced in the L group versus the C group. *Escherichia-Shigella*, common intestinal pathogens, can cause intestinal diseases (e.g., enteritis, diarrhea, and dysentery) or extraintestinal conditions (e.g., urinary tract infections, meningitis, or sepsis) through attachment to epithelial cells and/or invasion of target host cells (45). *Candidatus* Saccharimonas, an opportunistic pathogen in the intestinal tract, is associated with gastrointestinal disorders and causes inflammatory diseases of the intestinal mucosa (46, 47). A previous study revealed a negative correlation between *Candidatus* Saccharimonas and production performance in geese (48). Our findings support that dietary L-carnosine supplementation in sheep increases the relative abundance of beneficial bacteria in the intestine, while decreasing that of pathogenic bacteria.

The serum concentrations of the metabolites L-histidine, D-apiose, and isodomedin were highly significantly upregulated in group L. D-Apiose is a branched pentose in the cell walls of higher plants (49) that has an antioxidant effect (50). The differential metabolites were significantly enriched in the histidine metabolic pathway, in which L-histidine was upregulated in group L. L-Histidine, as a precursor of histidine synthesis (51), increases the synthesis of ammonia, alanine, and glutamine in muscle and affects the anabolism of proteins (52). Isodomedin belongs to the class of diterpenes (53), which exhibit anti-inflammatory, antioxidant, and antimicrobial properties (54). Pyridine N-oxide glucuronide, a glucuronic acid-derivative, was highly significantly downregulated in group L. It has been shown that glucuronic acid is closely related to inflammation by acting on Toll-like receptor 4 and inducing pain in rats (55). We speculate that the addition of L-carnosine increased the antioxidant capacity in the fattening lambs to a certain extent. In summary, L-carnosine supplementation may have increased the antioxidant capacity and enhanced protein synthesis in the fattening lambs.

Comprehensive analysis of the microbiomes and non-target metabolomes unveiled the relationships between differential microbiota genera and differential metabolites. Notably, our

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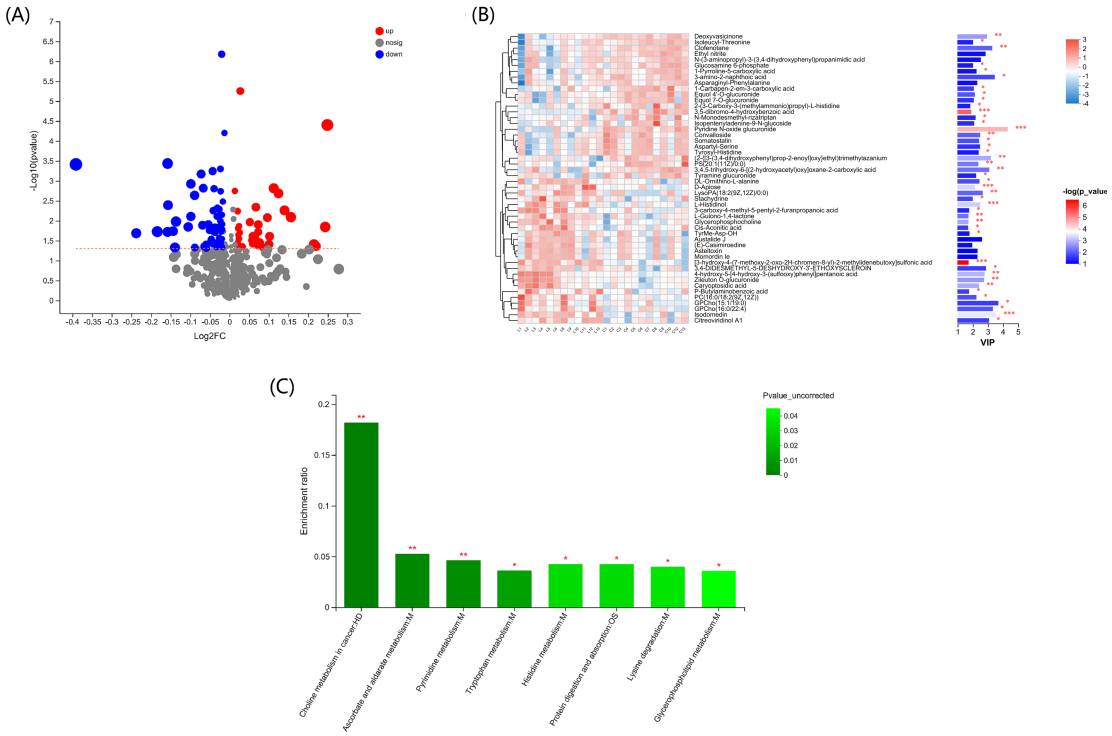

FIGURE 5
Effect of L-carnosine on the serum metabolome in fattening lambs. **(A)** Volcano plots showing the distribution of differential metabolites identified based on $p < 0.05$ and VIP > 1. **(B)** Profiling, VIP score, and p-values for the top 30 differential serum metabolites in groups L and C. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$. **(C)** KEGG metabolic pathway enrichment analysis of the differential serum metabolites in L-carnosine and control groups. *$p < 0.05$, **$p < 0.01$ vs. group C.

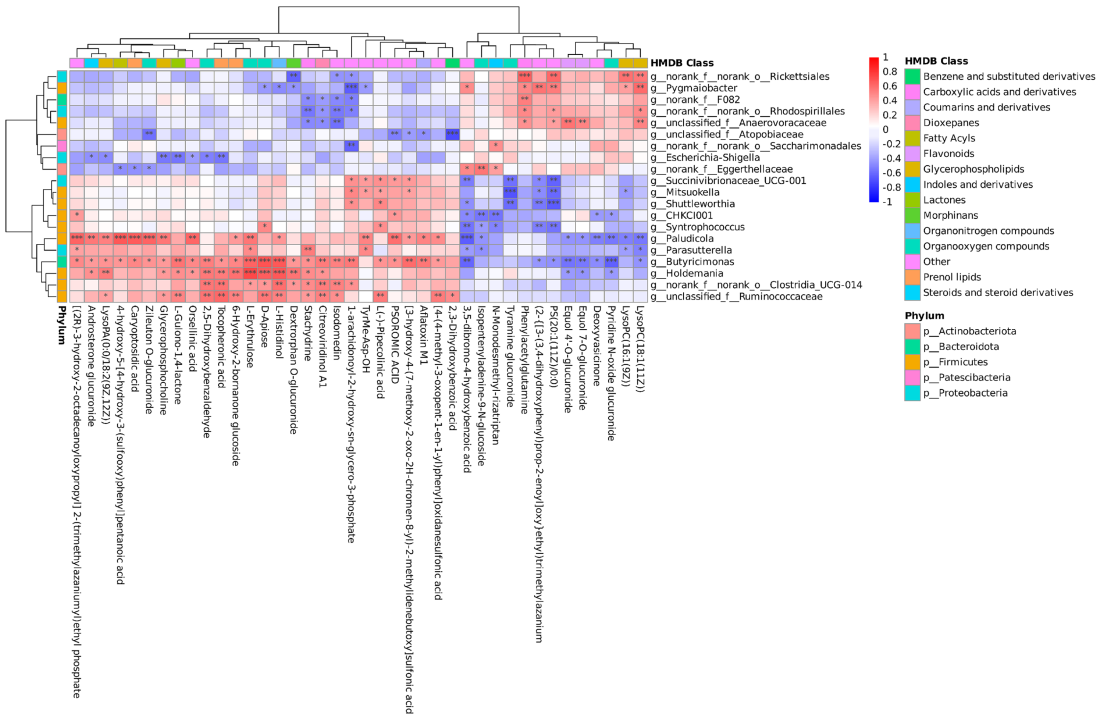

FIGURE 6
Correlation analysis of fecal microbiota and serum metabolites in fattening lambs. Red indicates a positive correlation, blue indicates a negative correlation. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ indicate statistically significant correlation between fecal microbiota and serum metabolites.

results indicated a positive correlation between *Butyricimonas* and L-histidine and D-apiose. SCFAs, produced through microbial fermentation in the intestine, exhibit diverse physiological functions (56). *Butyricimonas* is a putative SCFA producer with potent anti-inflammatory and immunomodulatory effects (57). Amino acids serve as precursors for bacterial SCFA synthesis, demonstrating an interaction between microbial activity and the homeostasis of host amino acids and SCFAs (58). Stachydrine exerts potent anti-inflammatory action via the NF-κB signaling pathway (59) and prevents oxidative stress by increasing the activity of antioxidant enzymes such as superoxide dismutase (60). These findings suggest that *Butyricimonas* may enhance the nutrient conversion capacity and production performance by alleviating inflammatory responses and influencing metabolic processes associated with amino acids and carbohydrates. Phenylacetylglutamine is a metabolic byproduct of intestinal bacteria (41) and serves as a substitute for urea in the urea cycle to facilitate the excretion of nitrogenous waste from the body (61). *Pygmaiobacter* is the main butyric acid producer, and the correlation analysis showed that it was significantly positively correlated with phenylacetylglutamine, which may induce changes in intestinal microbes. We found a negative correlation between *Butyricimonas* and pyridine N-oxide glucuronide. Pyridine N-oxide glucuronide promotes inflammation. These findings corroborate that L-carnosine has anti-inflammatory properties. Comprehensive analysis of the microbiome and non-target metabolomes further showed that L-carnosine promoted weight gain in fattening sheep by exerting anti-inflammatory and increasing antioxidant property.

5 Conclusion

Dietary L-carnosine supplementation in fattening lambs was found to improve growth performance by positively influencing the intestinal microbiota and serum metabolites. Enhanced oxidative capacity, protein synthesis ability, and anti-inflammatory activity may be among the main mechanisms of L-carnosine in increasing weight gain during the fattening period. Therefore, L-carnosine shows promise for application in the sheep industry.

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 studies were approved by Beijing Academy of Agriculture and Forestry Sciences. The studies were conducted in accordance with the local legislation and institutional requirements. Written informed consent was obtained from the owners for the participation of their animals in this study.

Author contributions

YM: Formal analysis, Writing – original draft. TX: Software, Writing – original draft. GK: Data curation, Writing – original draft. HW: Conceptualization, Supervision, Writing – original draft. TF: Conceptualization, Funding acquisition, Supervision, Writing – review & editing.

Funding

The author(s) declare that financial support was received for the research, authorship, and/or publication of this article. This work was supported by the Achievement Transformation Project of Beijing Academy of Agriculture and Forestry Sciences (2020607), and Central Guidance on Local Science and Technology Development Fund (Regional Science and Technology Innovation System Project) (246Z6602G).

Acknowledgments

We thank all staff of the Experimental Station of Beijing Academy of Agriculture and Forestry Sciences (located in the Yangyuan County, Zhangjiakou City, Hebei Province) for their assistance during the sample collection.

Conflict of interest

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

Generative AI statement

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

Publisher's note

Supplementary material

The Supplementary material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fvets.2024.1525783/full#supplementary-material

SUPPLEMENTARY FILE S1
Differences in serum metabolite profiles and changes in serum metabolites.

References

1. Davis TC, White RR. Breeding animals to feed people: The many roles of animal reproduction in ensuring global food security. *Theriogenology*. (2020) 150:27–33. doi: 10.1016/j.theriogenology.2020.01.041

2. Hsieh SL, Li JH, Dong CD, Chen CW, Wu CC. Carnosine suppresses human colorectal cancer cell proliferation by inducing necroptosis and autophagy and reducing angiogenesis. *Oncol Lett*. (2022) 23:44. doi: 10.3892/ol.2021.13162

3. Dunnett M, Harris RC. High-performance liquid chromatographic determination of imidazole dipeptides, histidine, 1-methylhistidine and 3-methylhistidine in equine and camel muscle and individual muscle fibres. *J Chromatogr B Biomed Sci Appl*. (1997) 688:47–55. doi: 10.1016/s0378-4347(97)88054-1

4. Wang CY, Li YR, Pan C, Chen J, Jiang W, Li WN, et al. Quantitative analysis of carnosine, anserine, and homocarnosine in skeletal muscle of aquatic species from east China sea. *Biochem Biophys Rep*. (2020) 25:100880. doi: 10.1016/j.bbrep.2020.100880

5. Turner MD, Sale C, Garner AC, Hipkiss AR. Anti-cancer actions of carnosine and the restoration of normal cellular homeostasis. *Biochim Biophys Acta, Mol Cell Res*. (2021) 1868:119117. doi: 10.1016/j.bbamcr.2021.119117

6. Palin MF, Lapointe J, Gariépy C, Beaudry D, Kalbe C. Characterisation of intracellular molecular mechanisms modulated by carnosine in porcine myoblasts under basal and oxidative stress conditions. *PLoS One*. (2020) 15:e0239496. doi: 10.1371/journal.pone.0239496

7. Zhou JY, Lin HL, Qin YC, Li XG, Gao CQ, Yan HC, et al. L-Carnosine protects against deoxynivalenol-induced oxidative stress in intestinal stem cells by regulating the Keap1/Nrf2 signaling pathway. *Mol Nutr Food Res*. (2021) 65:e2100406. doi: 10.1002/mnfr.202100406

8. Hipkiss AR, Brownson C, Carrier MJ. Carnosine, the anti-ageing, anti-oxidant dipeptide, may react with protein carbonyl groups. *Mech Ageing Dev*. (2001) 122:1431–45. doi: 10.1016/s0047-6374(01)00272-x

9. Tanaka KI, Sugizaki T, Kanda Y, Tamura F, Niino T, Kawahara M. Preventive effects of carnosine on lipopolysaccharide-induced lung injury. *Sci Rep*. (2017) 7:42813. doi: 10.1038/srep42813

10. Kang JH, Kim KS, Choi SY, Kwon HY, Won MH, Kang TC. Protective effects of carnosine, homocarnosine and anserine against peroxyl radical-mediated Cu,Zn-superoxide dismutase modification. *Biochim Biophys Acta Rev Cancer*. (2002) 1570:89–96. doi: 10.1016/s0304-4165(02)00158-7

11. Yaqub LS, Ayo JO, Habibu B, Lawal M, Kawu MU, Rekwot PI. Thermoregulatory, oxidative stress and lipid responses in prepartum ewes administered with L-carnosine during the hot-dry season. *Trop Anim Health Prod*. (2021) 53:388. doi: 10.1007/s11250-021-02832-x

12. Bao Y, Gao C, Hao W, Ji C, Zhao L, Zhang J, et al. Effects of dietary L-carnosine and alpha-lipoic acid on growth performance, blood thyroid hormones and lipid profiles in finishing pigs. *Asian Australas J Anim Sci*. (2015) 28:1465–70. doi: 10.5713/ajas.14.0604

13. Kopec W, Jamroz D, Wiliczkiewicz A, Biazik E, Pudlo A, Korzeniowska M, et al. Antioxidative characteristics of chicken breast meat and blood after diet supplementation with carnosine, L-histidine, and β-alanine. *Antioxidants*. (2020) 9:1093. doi: 10.3390/antiox9111093

14. Zhang F, Jin C, Wang X, Yan H, Tan H, Gao C. Dietary supplementation with pioglitazone hydrochloride and l-carnosine improves the growth performance, muscle fatty acid profiles and shelf life of yellow-feathered broiler chickens. *Anim Nutr*. (2021) 7:168–75. doi: 10.1016/j.aninu.2020.05.011

15. Davenport ER, Sanders JG, Song SJ, Amato KR, Clark AG, Knight R. The human microbiome in evolution. *BMC Biol*. (2017) 15:127. doi: 10.1186/s12915-017-0454-7

16. Zhang H, Shao M, Huang H, Wang S, Ma L, Wang H, et al. The dynamic distribution of small-Tail Han sheep microbiota across different intestinal segments. *Front Microbiol*. (2018) 9:32. doi: 10.3389/fmicb.2018.00032

17. Paz EA, Chua EG, Hassan SU, Greeff JC, Palmer DG, Liu S, et al. Bacterial communities in the gastrointestinal tract segments of helminth-resistant and helminth-susceptible sheep. *Anim Microbiome*. (2022) 4:23. doi: 10.1186/s42523-022-00172-1

18. Wu H, Yuan Y, Pan H. Comparison of fecal microbiota composition of blue sheep fed *Lolium perenne* versus *Sorghum sudanense*. *Can J Microbiol*. (2021) 67:372–80. doi: 10.1139/cjm-2020-0333

19. Zhu Z, Sun Y, Zhu F, Liu Z, Pan R, Teng L, et al. Seasonal variation and sexual dimorphism of the microbiota in wild blue sheep (*pseudois nayaur*). *Front Microbiol*. (2020) 11:1260. doi: 10.3389/fmicb.2020.01260

20. Cui X, Wang Z, Yan T, Chang S, Wang H, Hou F. Rumen bacterial diversity of Tibetan sheep (*Ovis aries*) associated with different forage types on the Qinghai-Tibetan Plateau. *Can J Microbiol*. (2019) 65:859–69. doi: 10.1139/cjm-2019-0154

21. Zhang H, Lang X, Li X, Chen G, Wang C. Effect of Zanthoxylum bungeanum essential oil on rumen enzyme activity, microbiome, and metabolites in lambs. *PLoS One*. (2022) 17:e0272310. doi: 10.1371/journal.pone.0272310

22. Yuan P, Meng K, Wang Y, Luo H, Huang H, Shi P, et al. Abundance and genetic diversity of microbial polygalacturonase and pectate lyase in the sheep rumen ecosystem. *PLoS One*. (2012) 7:e40940. doi: 10.1371/journal.pone.0040940

23. Xu XC, Li BB, Wei X, Yang YX, Wang XL, Chen YL. Differential expression of peroxisome proliferator-activated receptor γ, fatty acid synthase, and hormone-sensitive lipase in fat-tailed and thin-tailed sheep breeds. *Genet Mol Res*. (2015) 14:15624–33. doi: 10.4238/2015.December.1.14

24. Patil RD, Ellison MJ, Wolff SM, Shearer C, Wright AM, Cockrum RR, et al. Poor feed efficiency in sheep is associated with several structural abnormalities in the community metabolic network of their ruminal microbes. *J Anim Sci*. (2018) 96:2113–24. doi: 10.1093/jas/sky096

25. Guijas C, Montenegro-Burke JR, Warth B, Spilker ME, Siuzdak G. Metabolomics activity screening for identifying metabolites that modulate phenotype. *Nat Biotechnol*. (2018) 36:316–20. doi: 10.1038/nbt.4101

26. Schrimpe-Rutledge AC, Codreanu SG, Sherrod SD, McLean JA. Untargeted metabolomics strategies-challenges and emerging directions. *J Am Soc Mass Spectrom*. (2016) 27:1897–905. doi: 10.1007/s13361-016-1469-y

27. Diez R, Diez MJ, Garcia JJ, Rodríguez JM, Lopez C, Fernandez N, et al. Improvement of albendazole bioavailability with menbutone administration in sheep. *Animals*. (2022) 12:463. doi: 10.3390/ani12040463

28. Zhang X, Han L, Hou S, Raza SHA, Gui L, Sun S, et al. Metabolomics approach reveals high energy diet improves the quality and enhances the flavor of black Tibetan sheep meat by altering the composition of rumen microbiota. *Front Nutr*. (2022) 9:915558. doi: 10.3389/fnut.2022.915558

29. Li H, Yu Q, Li T, Shao L, Su M, Zhou H, et al. Rumen microbiome and metabolome of Tibetan sheep (ovis aries) reflect animal age and nutritional requirement. *Front Vet Sci*. (2020) 7:609. doi: 10.3389/fvets.2020.00609

30. Zhang Z, Shahzad K, Shen S, Dai R, Lu Y, Lu Z, et al. 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*. (2022) 8:815358. doi: 10.3389/fnut.2021.815358

31. Feng T, Ding H, Wang J, Xu W, Liu Y, Kenéz Á. Metabolite profile of sheep serum with high or low average daily gain. *Front Vet Sci*. (2021) 8:662536. doi: 10.3389/fvets.2021.662536

32. Kang GL. Effects of carnosine on production performance, fecal flora and serum metabolites in fattening lambs. Handan: Hebei University of Engineering (2023). 55 p.

33. Babizhayev MA, Deyev AI, Yegorov YE. L-carnosine modulates respiratory burst and reactive oxygen species production in neutrophil biochemistry and function: may oral dosage form of non-hydrolized dipeptide L-carnosine complement anti-infective anti-influenza flu treatment, prevention and self-care as an alternative to the conventional vaccination? *Curr Clin Pharmacol*. (2014) 9:93–115. doi: 10.2174/1574884709999140311125601

34. Ma XY, Jiang ZY, Lin YC, Zheng CT, Zhou GL. Dietary supplementation with carnosine improves antioxidant capacity and meat quality of finishing pigs. *J Anim Physiol Anim Nutr*. (2010) 94:e286–95. doi: 10.1111/j.1439-0396.2010.01009.x

35. Cong J, Zhang L, Li J, Wang S, Gao F, Zhou G. Effects of dietary supplementation with carnosine on growth performance, meat quality, antioxidant capacity and muscle fiber characteristics in broiler chickens. *J Sci Food Agric*. (2017) 97:3733–41. doi: 10.1002/jsfa.8236

36. Yang G, Zhang S, Li Z, Huang J, Liu Y, Liu Y, et al. Comparison between the gut microbiota in different gastrointestinal segments of Large-Tailed Han and Small-Tailed Han sheep breeds with High-Throughput sequencing. *Indian J Microbiol*. (2020) 60:436–50. doi: 10.1007/s12088-020-00885-4

37. Chi X, Gao H, Wu G, Qin W, Song P, Wang L, et al. Comparison of gut microbiota diversity between wild and captive bharals (*Pseudois nayaur*). *BMC Vet Res*. (2019) 15:243. doi: 10.1186/s12917-019-1993-7

38. Chang J, Yao X, Zuo C, Qi Y, Chen D, Ma W. The gut bacterial diversity of sheep associated with different breeds in Qinghai province. *BMC Vet Res*. (2020) 16:254. doi: 10.1186/s12917-020-02477-2

39. Cholewińska P, Wołoszyńska M, Michalak M, Czyż K, Rant W, Smoliński J, et al. Influence of selected factors on the Firmicutes, Bacteroidetes phyla and the Lactobacillaceae family in the digestive tract of sheep. *Sci Rep*. (2021) 11:23801. doi: 10.1038/s41598-021-03207-w

40. Rey M, Enjalbert F, Combes S, Cauquil L, Bouchez O, Monteils V. Establishment of ruminal bacterial community in dairy calves from birth to weaning is sequential. *J Appl Microbiol*. (2014) 116:245–57. doi: 10.1111/jam.12405

41. Wang S, Chai J, Zhao G, Zhang N, Cui K, Bi Y, et al. The temporal dynamics of rumen microbiota in early weaned lambs. *Microorganisms*. (2022) 10:144. doi: 10.3390/microorganisms10010144

42. Jang JH, Yeom MJ, Ahn S, Oh JY, Ji S, Kim TH, et al. Acupuncture inhibits neuroinflammation and gut microbial dysbiosis in a mouse model of Parkinson's disease. *Brain Behav Immun*. (2020) 89:641–55. doi: 10.1016/j.bbi.2020.08.015

43. Nogal A, Valdes AM, Menni C. The role of short-chain fatty acids in the interplay between gut microbiota and diet in cardio-metabolic health. *Gut Microbes*. (2021) 13:1–24. doi: 10.1080/19490976.2021.1897212

44. Fabiano GA, Shinn LM, Antunes AEC. Relationship between oat consumption, gut microbiota modulation, and Short-Chain fatty acid synthesis: an integrative review. *Nutrients*. (2023) 15:3534. doi: 10.3390/nu15163534

45. Bi Y, Cox MS, Zhang F, Suen G, Zhang N, Tu Y, et al. Feeding modes shape the acquisition and structure of the initial gut microbiota in newborn lambs. *Environ Microbiol*. (2019) 21:2333–46. doi: 10.1111/1462-2920.14614

46. Wang J, Li P, Liu S, Zhang B, Hu Y, Ma H, et al. Green tea leaf powder prevents dyslipidemia in high-fat diet-fed mice by modulating gut microbiota. *Food Nutr Res*. (2020) 64:10. doi: 10.29219/fnr.v64.3672

47. Butorac K, Novak J, Banić M, Leboš Pavunc A, Čuljak N, Oršolić N, et al. Modulation of the gut microbiota by the Plantaricin-Producing lactiplantibacillus plantarum D13, analysed in the DSS-Induced colitis mouse model. *Int J Mol Sci*. (2023) 24:15322. doi: 10.3390/ijms242015322

48. Ni H, Zhang Y, Yang Y, Li Y, Yin Y, Sun X, et al. Comparative analyses of production performance, meat quality, and gut microbial composition between two chinese goose breeds. *Animals*. (2022) 12:1815. doi: 10.3390/ani12141815

49. Chen Y, Wang Y, Zhu A, Zhang L, Zhang X, Zhang J, et al. UPLC-TOF/MS-based metabolomics reveals the chemical changes and in vitro biological effects in fermentation of white ginseng by four probiotics. *Front Microbiol*. (2022) 13:1022200. doi: 10.3389/fmicb.2022.1022200

50. Carter MS, Zhang X, Huang H, Bouvier JT, Francisco BS, Vetting MW, et al. Functional assignment of multiple catabolic pathways for D-apiose. *Nat Chem Biol*. (2018) 14:696–705. doi: 10.1038/s41589-018-0067-7

51. Zhang J, Yuan K, Zhou WL, Zhou J, Yang P. Studies on the active components and antioxidant activities of the extracts of *Mimosa pudica* Linn. from southern China. *Pharmacogn Mag*. (2011) 7:35–9. doi: 10.4103/0973-1296.75899

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

53. Shah MD, Tani K, Yong YS, Ching FF, Shaleh SRM, Vairappan CS, et al. Antiparasitic potential of chromatographic fractions of nephrolepis biserrata and liquid Chromatography-Quadrupole Time-of-Flight-Mass spectrometry analysis. *Molecules*. (2021) 26:499. doi: 10.3390/molecules26020499

54. Paz MFCJ, Islam MT, Tabrez S, Firoz CK, Jabir NR, Kamal MA, et al. Effect of diterpenes on hepatic system. *Curr Pharm Des*. (2018) 24:4093–100. doi: 10.2174/1381612824666181001152247

55. Lewis SS, Hutchinson MR, Zhang Y, Hund DK, Maier SF, Rice KC, et al. Glucuronic acid and the ethanol metabolite ethyl-glucuronide cause toll-like receptor 4 activation and enhanced pain. *Brain Behav Immun*. (2013) 30:24–32. doi: 10.1016/j.bbi.2013.01.005

56. Fu H, He M, Wu J, Zhou Y, Ke S, Chen Z, et al. Deep investigating the changes of gut microbiome and its correlation with the shifts of host serum metabolome around parturition in sows. *Front Microbiol*. (2021) 12:729039. doi: 10.3389/fmicb.2021.729039

57. Liang X, Fu Y, Cao WT, Wang Z, Zhang K, Jiang Z, et al. Gut microbiome, cognitive function and brain structure: a multi-omics integration analysis. *Transl Neurodegener*. (2022) 11:49. doi: 10.1186/s40035-022-00323-z

58. Wang B, Luo Y, Su R, Yao D, Hou Y, Liu C, et al. Impact of feeding regimens on the composition of gut microbiota and metabolite profiles of plasma and feces from Mongolian sheep. *J Microbiol*. (2020) 58:472–82. doi: 10.1007/s12275-020-9501-0

59. Wu H, Zhang M, Li W, Zhu S, Zhang D. Stachydrine attenuates IL-1β-induced inflammatory response in osteoarthritis chondrocytes through the NF-κB signaling pathway. *Chem Biol Interact*. (2020) 326:109136. doi: 10.1016/j.cbi.2020.109136

60. Bao X, Liu Y, Huang J, Yin S, Sheng H, Han X, et al. Stachydrine hydrochloride inhibits hepatocellular carcinoma progression via LIF/AMPK axis. *Phytomedicine*. (2022) 100:154066. doi: 10.1016/j.phymed.2022.154066

61. Krishnamoorthy NK, Kalyan M, Hediyal TA, Anand N, Kendaganna PH, Pendyala G, et al. Role of the gut Bacteria-Derived metabolite phenylacetylglutamine in health and diseases. *ACS Omega*. (2024) 9:3164–72. doi: 10.1021/acsomega.3c08184

frontiers | Frontiers in Veterinary Science

TYPE Original Research
PUBLISHED 06 February 2025
DOI 10.3389/fvets.2025.1528861

OPEN ACCESS

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RECEIVED 15 November 2024
ACCEPTED 22 January 2025
PUBLISHED 06 February 2025

CITATION
Zhang S, Yimamu M, Ma C, Pan J, Wang C, Cai W and Yang K (2025) Dietary guanidinoacetic acid supplementation improves rumen metabolism, duodenal nutrient flux, and growth performance in lambs.
Front. Vet. Sci. 12:1528861.
doi: 10.3389/fvets.2025.1528861

Dietary guanidinoacetic acid supplementation improves rumen metabolism, duodenal nutrient flux, and growth performance in lambs

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Guanidinoacetic acid (GAA) is the only precursor of creatine, which is an important energy source for growth and metabolism. The degradation of guanidinoacetic acid in rumen plays a decisive role in its application in ruminant diet. Two experiments were conducted to investigate the rumen GAA escape rate and its effects on metabolism, blood metabolites and growth performance of Kazakh male lambs. In the first part of the experiment, 24 Kazakh male lambs equipped with rumen and duodenal fistulas were selected to determine the rumen escape rate of guanidylacetic acid. In the second part, 24 healthy Kazakh male lambs were selected to determine the growth performance. They were divided into 4 groups and fed a basal diet supplemented with 0, 500, 1,000, 1,500 mg/kg GAA, respectively. The results show that ruminal escape rates of 36–56% were achieved in lambs with dietary GAA supplementation at 500–1,500 mg/kg DM. Dietary 1,500 mg/kg DM GAA increased levels of creatine, IGF-I and insulin, and promoted lamb growth.

KEYWORDS

lambs, guanidinoacetic acid, creatine, rumen metabolism, ruminal escape rate

1 Introduction

Guanidinoacetic acid (GAA) is the only precursor of creatine, which is an important energy source for growth and metabolism in vertebrates. The addition of exogenous GAA stimulates creatine biosynthesis (1). Although creatine levels can be increased by direct dietary supplementation, GAA is a more stable and potent inducer of creatine synthesis (2). The addition of GAA to animal diets has been shown to promote muscle energy metabolism and growth performance (3–5). For example, dietary GAA supplementation can improve carcass quality, energy metabolism and meat quality in fattening pigs (6) and promote growth performance in broilers (7–9). Furthermore, the metabolites produced (creatine, creatine phosphate, creatinine) as well as unmetabolized GAA are excreted in the urine, with previous studies confirming that no harmful residues are detected in livestock products (10).

Several studies on GAA supplementation in cattle via abomasum continuous infusion (11) showed increased plasma and urinary creatine concentrations and enhanced creatine synthesis (12). These findings suggest that GAA can act as a precursor and stimulate creatine synthesis in cattle. A previous study showed that the bioavailability of exogenous GAA after infusion into the rumen and abomasum of cattle was approximately 50%, indicating that GAA is degraded in the rumen (13); however, specific degradation rates and changes in nutrient flow

were not reported in detail. GAA degraded in the rumen may be used by microorganisms to support their growth. Furthermore, Li et al. (14) reported that the addition of 0.6 or 0.9 g/kg DM GAA improved growth performance, nutrient digestion and rumen fermentation in Angus beef cattle, although the escape rate of dietary GAA in the rumen and the effect on rumen fermentation remain to be clarified.

As an animal-derived amino acid derivative, GAA is largely absent from whole plant protein-based lamb rations. The effects of GAA on growth performance and rumen metabolism in sheep have not been reported. To bridge a current knowledge gap, we investigated the extent to which GAA added exogenously in the diet is degraded in the rumen and its effects on rumen metabolism and growth performance in lambs.

2 Materials and methods

All experimental procedures involving animals were approved (animal protocol number: 2020024) by the Animal Welfare and Ethics Committee of Xinjiang Agricultural University, Urumqi, Xinjiang, China.

2.1 Experimental materials

GAA (non-rumen protected; purity >98%) was purchased from Genetech Biotechnology Co., Ltd. (Beijing, China).

All lambs used in this study were commercial livestock purchased from a local market and owned by this research team. Before study, the purchased lambs were quarantined and cared by Huikang Animal Husbandry Co., Ltd. (Changji, Xinjiang). After the study, all the lambs were euthanized by carotid bleeding under anesthesia (i.v. Lumianning, main component xylazine hydrochloride, 20 mg/kg BW [injected]) and then treated following harmless disposal procedures.

2.2 Experimental design

Experimental design 1: To investigate the effect of supplemental GAA on ruminal GAA flow rate and rumen metabolism in lambs, 24 Kazakh rams [aged 5 months, weight (34.29 ± 1.95) kg] with ruminal and proximal duodenal fistulas were selected. The test lambs (n = 6 per group) were fed a basal diet (without creatine and GAA) supplemented with GAA at 0 mg (0 mg/kg group), 500 mg (500 mg/kg group), 1,000 mg (1,000 mg/kg group) and 1,500 mg (1,500 mg/kg group) per kg of dry matter (DM) basal diet. A non-isotopic marker (Li, Cr-EDTA) was used to determine the chyme flow rate. The experiment lasted for 23 days, 15 days of adaptation and 8 days of sampling.

Experimental design 2: We then investigated the effect of dietary GAA supplementation on blood metabolites and growth performance in 24 randomly selected healthy Kazakh rams (aged 3 months, (27.35 ± 0.58) kg). The test lambs (n = 6 per group) were fed a basal diet (without creatine and GAA) supplemented with GAA at 0 mg (0 mg/kg group), 500 mg (500 mg/kg group), 1,000 mg (1,000 mg/kg group) and 1,500 mg (1,500 mg/kg group) per kg of DM basal diet. The feeding trial was conducted for 55 d consisting of a 10-day pre-feeding period and a 45-day trial period.

The basal diet was formulated according to the NRC (2007) nutritional requirements. The diet formulations and nutrient levels are shown in Table 1.

2.3 Feeding management

The test lambs were numbered and housed in semi-open sheds (1.2 m × 1.5 m) with good ventilation. The lambs were fed daily in two separate feedings at 08:00 and 20:00. GAA was mixed into the feed concentrate. Lambs were fed and watered ad libitum. Feed intake was recorded daily and each lamb was weighed every 15 days. Dry matter intake (DMI), average daily gain (ADG) and feed weight ratio (F:G) were calculated for each lamb. Disinfection and sterilization of the facility and immunization of the animals were conducted according to routine farm procedures.

2.4 Sample collection and processing

In experiment 1, ruminal fluid was collected on day 16 of the trial period at 0 h (before feeding) and 1, 2, 4, 6 and 8 h after the morning feed, filtered through nylon cloth and then frozen. Duodenal chyme (chyme is a mixture and includes both solids and liquids) were collected in three batches (8 lambs per time-point, 2 lambs per treatment group) on days 17–23 of the trial period according to the

TABLE 1 Feed ingredients and nutrient levels of experimental diets (%).

Ingredient	Ratio
Maize	30.00
Wheat bran	7.20
Soybean meal	12.00
Cottonseed meal	7.80
Alfalfa hay	20.00
Wheat straw	20.00
Premix[A]	3.00
Total	100

Items	Nutrient levels (DM basis)
Organic matter (OM)	89.87
Crude protein (CP)	16.58
Ether extract (EE)	1.53
Neutral detergent fiber (NDF)	46.16
Acid detergent fiber (ADF)	32.62
Calcium (Ca)	0.58
Phosphorus (P)	0.33
Lysine	0.81
Methionine + cysteine	0.58

[A]The premix provided the following per kg of the concentrate supplement: Vitamin A 10,000.00 IU, Vitamin D3 2,550.00 IU, Vitamin E 20.00 IU, niacin 20.00 mg, biotin 0.06 mg, Cu (as copper sulfate) 22.00 mg, Fe (as ferrous sulfate) 94 mg, Mn (as manganese sulfate) 80 mg, Zn (as zinc sulfate) 88 mg, I (as potassium iodide) 0.75 mg, Se (as sodium selenite) 0.5 mg, Co (as cobalt chloride) 0.33 mg, Ca (as calcium carbonate and calcium hydrogen phosphate) 0.35%, P (as calcium hydrogen phosphate) 0.125%, NaCl 0.8%.

disposable perfusion method (15). In brief, samples of duodenal chyme were collected from the test lambs prior to perfusion to determine background values. Each lamb received a perfusion of Chromium-EDTA(Cr-EDTA, 12 g/lamb) delivered using a 100-mL syringe fitted with a fine tube (approx. 20 cm) to facilitate even dispersion to different parts of the rumen. After the perfusion, duodenal chyme samples (20 mL) were collected at 0 h (before the morning feed), and 1, 2, 3, 4, 6, 8, 12, 16, 24, 32, 40 and 48 h, after feeding. At each time-point, 5 g of ruminal or duodenal chyme samples were mixed to produce a mixed sample for the determination of DM, GAA, total nitrogen and total reducing sugar content.

In experiment 2, blood was collected from the jugular vein of lambs at 0 h before the morning feed on day 45 of the test period. The blood was centrifuged at 3,500 × *g* for 15 min to isolate the plasma, which was stored at −20°C.

2.5 Sample analysis and calculations

The diet samples were finely ground, passed through a 1-mm mesh (Thomas-Wiley Laboratory Mill Model 4, Thomas Scientific, Swedesboro, NJ, USA), and analyzed for the contents of dry matter (DM), organic matter (OM), crude protein (CP), fat (EE), acid detergent fibers (ADF), and neutral detergent fibers (NDF). The samples (5.0 g) were dried at 105°C overnight according to the AOAC method (16). The nitrogen content was determined using a nitrogen analyzer combustion method (990.03; AOAC) (Model CNS-2000; LECO Company, St.1990), and CP was calculated as N× 6.25. Ether-like hexane extracts were obtained from the samples using the Ankom Extraction System (Macedonia, New York). The NDF and ADF contents were determined as described by Van Soest et al. (17) and Goering et al. (18). After anerobic hydrolysis, the amino acid content of the diet samples was analyzed using a Sykam S433D Amino Acid Analyzer (Sykam, Germany).

The rumen and duodenal chyme collected were air-dried in an oven at 60°C and then subjected to DM determination at 105°C. The chromium concentration was determined by inductively coupled plasma mass spectrometry (THERMO, USA, model iCAPQ) using the DM of the rumen and duodenal chyme as described above. Method: The sample was weighed into a PTFE digestion tank and 5 mL of nitric acid was added. The sample was left to stand, sealed and placed in a microwave digestion apparatus. After the temperature had cooled to <50°C, the digestion tank was removed and placed in a fume hood; blanks were treated in the same way. Total nitrogen in the contents of the rumen and duodenum was determined using the Kjeldahl method (16). Total reducing sugars were determined by the tetrazolium blue chloride method (19).

The DM of duodenal chyme was weighed and 500 mg was dissolved in 1 mL of distilled water, extracted by ultrasonication for 10 min and centrifuged at 12,000 × *g* for 15 min. The supernatant was then removed for determination of GAA. Ruminal fluid samples were filtered through nylon mesh and centrifuged at 12,000 × *g* for 15 min. The supernatant was then removed for determination of creatine and GAA. The concentrations of GAA and Cr in the supernatants were determined according to methods described by Wada et al. (20). Briefly, to precipitate the protein, 200 μL plasma or supernatant was mixed with 400 μL acetonitrile and incubated for 10 min before centrifugation at 12,000 × *g* for 10 min. Subsequently, 100 μL clear supernatant was mixed with 200 μL phosphoric acid (2 mM). A sample of the mixture (20 μL) was then analyzed using an IC YS-50 weak acid cation exchange column (4.6 mm × 125 mm) under the following conditions: flow rate, 1.0 mL/min; column temperature, 30°C; detection wavelength, 210 nm; elution mode, one-time linear elution.

Ruminal fluid pH was determined using a PE20K Mettler Toledo (METTLER TOLEDO, Shanghai, China) acidity meter.

Volatile fatty acids (VFA) were determined by gas chromatography using 4-methyl-N-valeric acid as an internal standard. Ammoniacal nitrogen (NH_3-N) was determined using an alkaline sodium hypochlorite-phenol spectrophotometric method (21). Protozoa counts were determined using a hemocytometer after adding formaldehyde solution (37% formaldehyde (v/v): 0.9% (w/v) NaCl, 1:9) (22).

Plasma levels of insulin (INS), growth hormone (GH) and insulin growth factor-I (IGF-I) were determined in lambs 0 h before the morning feed on day 45 of the trial by immunoradiometric assay using enzyme-linked immunosorbent assay (ELISA) kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, China).

2.6 Duodenal inflow calculations

The duodenal inflow was calculated after first matching the decreasing curve of chromium concentration in DM per unit of rumen chyme with sampling time according to Equation 1:

$$C = C_0 \times e^{-kt} \quad (1)$$

where C = the concentration of Cr in the duodenal chyme sample, C_0 = the concentration of Cr in the duodenal chyme sample at $t = 0$, k & t = the dilution constants and the sampling time, respectively.

The fitted equations for duodenal inflow obtained from the determination of chyme chromium concentration in experiment 1 are shown in Table 2.

Then, the duodenal inflow volume of the rumen chyme was then calculated using Equation 2:

$$\mathrm{Qr} = \frac{\mathrm{Cr}}{\mathrm{C_0}} \quad (2)$$

Where Qr = duodenal inflow volume (kg/day), Cr = perfusion dose of chromium in the test lambs (mg), C_0 = the concentration of chromium in the duodenal chyme sample at $t = 0$.

TABLE 2 Fitting equations for duodenal chyme chromium concentrations.

Group	C_0	k	Exponential equation	R^2
0 mg/kg	6.8495	0.0885	$y = 6.8495e^{-0.0885x}$	0.9864
500 mg/kg	6.0481	0.0827	$y = 6.0481e^{-0.0827x}$	0.9911
1,000 mg/kg	6.5505	0.0737	$y = 6.5505e^{-0.0737x}$	0.9906
1,500 mg/kg	6.6911	0.0652	$y = 6.6911e^{-0.0652x}$	0.9799

The GAA, total nitrogen and total reducing sugars of duodenal inflow are the multiplications of the GAA, total nitrogen and total reducing sugars concentration values of the mixed duodenal chyme samples and the duodenal inflow values.

2.7 Data analysis

Ruminal fluid pH, VFA, ammonia nitrogen and protozoa counts were calculated using a MIXED model with repeated observations in SAS (Version 9.2; SAS Inst. Inc., Cary, NC, USA). The fixed effects were the treatment (Trt), the sampling time-point (Date) and the interaction between the two (Trt × Date). The interaction between treatment and date was primarily analyzed in terms of its effects on test treatment. Other indicators analyzed using the GLM program of SAS (Version 9.2; SAS Inst. Inc., Cary, NC, USA). Orthogonal polynomial comparison coefficients were used to determine the linear and quadratic effects of the elevated dietary GAA levels on different parameters. The results were presented as least squares means and standard error of mean (SEM), with $p < 0.05$ set as the threshold for statistical significance.

3 Results

3.1 Changes in the level of GAA in the ruminal fluid of lambs

No GAA was detected in the ruminal fluid of lambs in the 0 mg/kg group. Complete ruminal evacuation of GAA was completed after 8 h of dietary GAA supplementation in the 500 mg/kg group, and within 12 h in the 1,000 mg/kg group. In the 1,500 mg/kg group, ruminal evacuation of GAA was incomplete after 12 h of dietary GAA supplementation (Table 3).

3.2 Ruminal fermentation parameters

Compared to the 0 mg/kg group, the pH of the rumen fluid was significantly higher in the 1,500 mg/kg group (Treatment, $p < 0.05$) (Table 4). Dietary GAA supplementation at 500–1,500 mg/kg increased the ruminal ammonia content in lambs (Treatment, $p < 0.05$), and there was a significant interaction between date and treatment (Interaction, $p < 0.05$). Propionic acid, butyric acid and isovaleric acid levels were significantly lower in the 500 mg/kg group compared to those in the 0 mg/kg group (Treatment, $p < 0.05$). Propionic acid levels were significantly lower in the 1,500 mg/kg group and butyric acid levels were significantly higher than those in the 0 mg/kg group (Treatment, $p < 0.05$). The Acetic acid: propionic acid ratio was significantly higher in the 500 mg/kg and 1,500 mg/kg groups than that in the 0 mg/kg group (Treatment, $p < 0.05$). The protozoa counts were significantly higher in the sheep rumen fluid of the 1,500 mg/kg group compared to that in the 0 mg/kg group (Treatment, $p < 0.05$), and there was a significant interaction between date and treatment (Interaction, $p < 0.05$).

TABLE 3 Effect of dietary guanidinoacetic acid supplementation on the guanidinoacetic acid content of rumen fluid in lambs (μmol/L).

Item	Dietary GAA supplement levels, mg per kg DM diet				SEM[A]
	0	500	1,000	1,500	
0 h	–[B]	–	–	118.24	6.68
1 h	–	406.11	827.67	1,315.81	23.43
2 h	–	296.39	612.38	1,076.44	28.91
4 h	–	133.62	328.34	705.79	18.58
6 h	–	72.26	210.10	495.01	20.26
8 h	–	–	136.52	240.69	17.39

[A]SEM = standard error of the least squares means; $n = 6$ lambs/group.
B"–" means that the concentration was not detected under this test condition.

3.3 Nutrition duodenal inflow

There were no significant differences in the DMI and duodenal inflow (DM) between the groups (Linear & Quadratic, $p > 0.05$, Table 5). No GAA was detected in the duodenal chyme of lambs in the 0 mg/kg group. The GAA flux in the duodenal chyme of lambs in the 500 mg/kg, 1,000 mg/kg and 1,500 mg/kg groups reached 393.78 mg/d, 451.55 mg/d and 655.37 mg/d, respectively, and the ruminal escape rates reached 65.63, 37.63 and 36.41%, respectively. As dietary GAA levels increased, duodenal reducing sugar influx increased linearly. The amount of total reducing sugars entering the duodenum of lambs was significantly higher in the 1,000 mg/kg and 1,500 mg/kg groups compared to that in the 0 mg/kg group (Linear, $p < 0.05$). There were no significant differences in the total nitrogen entering the duodenum between the groups (Linear & Quadratic, $p > 0.05$).

3.4 Jugular venous plasma parameters

With increasing levels of dietary GAA supplementation, jugular venous plasma GAA, creatine and IGF-I levels increased linearly (Linear, $p < 0.05$, Table 6) while glucose concentrations decreased linearly (Linear, $p < 0.05$).

3.5 Growth performance

The average day gain of lambs showed a linear increase with dietary GAA supplementation (Linear, $p < 0.05$, Table 7).

4 Discussion

In recent years, the potential benefits of dietary GAA supplementation in ruminants have become a focus of research. In this study, we investigated the ruminal escape of GAA in lambs and its impact on their growth performance. We showed that dietary GAA supplementation was associated with a ruminal escape rate of GAA ranging from 36 to 65%, and the average daily gain of lambs increased linearly with the supplementation of GAA in the diet.

The ruminal GAA escape rate ranged from 36 to 65%, indicating partial degradation of GAA in the rumen, which is consistent with the study reported by Speer et al. (13) showing that GAA bioavailability was approximately 50% after infusion into the rumen and wrinkled

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TABLE 4 Effects of dietary guanidinoacetic acid supplementation on ruminal fermentation parameters in lambs.

Item	Dietary GAA supplement levels, mg·kg^{-1}				SEM[A]	*p*-value		
	0	500	1,000	1,500		Treatment	Date	Interaction
pH	6.32^{b}	6.31^{b}	6.44^{ab}	6.52^{a}	0.03	<0.01	<0.01	0.95
NH_3-N, mg·100 mL^{-1}	21.33^{b}	25.84^{a}	24.96^{a}	24.53^{a}	0.47	<0.01	<0.01	<0.01
Total olatile fatty acids, mmol·L^{-1}	81.51	74.18	77.56	79.34	1.99	0.72	<0.01	0.28
Acetate, %	62.35^{a}	65.87^{b}	62.79^{a}	63.60^{b}	1.23	0.01	<0.01	0.16
Propionate, %	20.62^{b}	17.93^{c}	20.98^{bc}	16.49^{a}	0.23	<0.01	<0.01	0.84
Butyrate, %	14.02	13.14	13.05	16.54	0.32	0.21	<0.01	0.49
Isobutyrate, %	0.98	1.09	1.12	1.21	0.05	0.93	<0.01	0.25
Valerate, %	0.87	1.00	1.01	0.89	0.07	0.03	0.06	0.76
Isovalerate, %	1.15^{a}	0.98^{b}	1.10^{ab}	1.27^{a}	0.06	0.35	<0.01	0.30
A:P[B]	3.31^{b}	3.88^{a}	3.20^{b}	4.20^{a}	0.15	<0.01	<0.01	0.06
Protozoa, ×10^{5}/mL	2.76^{b}	2.88^{ab}	2.92^{ab}	3.01^{a}	0.92	0.11	<0.01	<0.01

[A]SEM = standard error of the least squares means (n = 6 lambs/group).
[B]A:P = acetate: propionate.
[a–c]Least squares mean values within a row with different superscripts differed ($p < 0.05$).

TABLE 5 Effects of dietary guanidinoacetic acid supplementation on the nutrition duodenal inflow in lambs.

Item	GAA supplement levels, mg·kg^{-1} DM diet				SEM [A]	*p*-value	
	0	500	1,000	1,500		Linear	Quadratic
DMI, g/day	1,096.24	1,081.64	1,096.31	1,087.96	11.36	0.84	0.79
Duodenal inflow (DM), g/day	341.67	361.98	352.44	354.25	19.63	0.75	0.64
GAA ruminal escape, mg/day	–[B]	393.78	451.55	655.37	28.75		
GAA percentage ruminal escape, %	–	65.63	37.63	36.41	3.37		
Total reducing sugar flow flux, mg/day	405.99	520.83	568.25	590.48	41.64	< 0.01	0.28
Total nitrogen flow flux, g/day	16.20	16.51	15.58	16.37	0.95	0.92	0.81

[A]SEM = standard error of the least squares means; n = 6 lambs/group.
[B]"–" means that the concentration was not detected under this test condition.

TABLE 6 Effects of dietary guanidinoacetic acid supplementation on jugular vein plasma parameters in lambs.

Item[A]	GAA supplement levels, mg·kg^{-1} DM diet				SEM[B]	*p*-value	
	0	500	1,000	1,500		Linear	Quadratic
GAA, μmol/L	162.96	166.63	180.79	203.75	5.86	<0.01	0.24
Creatine, μmol/L	106.76	113.62	117.48	123.32	2.51	<0.01	0.90
Glucose, mmol/L	5.51	5.28	5.16	5.04	0.12	0.01	0.67
GH, ng/mL	1.88	2.05	1.92	1.69	0.11	0.18	0.09
IGF-I, ng/mL	99.90	114.08	100.14	135.35	7.69	0.02	0.19
INS, mIU/L	20.39	20.81	19.19	24.38	1.11	0.05	0.05

[A]GH = growth hormone; IGF-I = insulin-like growth factor-1; INS = insulin.
[B]SEM = standard error of the least squares means; n = 6 lambs/group.

stomach of cattle. The inclusion of GAA in the diet facilitated its degradation in the rumen, causing an elevation in ammoniacal nitrogen content and an increase in the pH of the rumen fluid. This effect may be correlated with alterations in the protozoa population, particularly in groups receiving 1,000 mg/kg and 1,500 mg/kg of GAA, where their numbers increased. Notably, protozoa consume bacteria for nourishment (23, 24). The heightened protozoa population subsequently increased their consumption of bacteria and starch granules, resulting in a decline in bacterial count and reduced carbohydrate availability. This, in turn, impeded the degradation and utilization of both carbohydrates and proteins (25), further contributing to the increase in pH. Additionally, the expanded protozoa population ingested significant quantities of microbial proteins, thereby enhancing nitrogen excretion via the release of ammonia (26).

The increase in protozoa numbers in the rumen may be due to the use of GAA as a nitrogen source to provide energy and promote division.

TABLE 7 Effects of supplementation with GAA on growth performance in lambs.

Item[A]	GAA supplement levels, mg·kg^{-1} DM diet				SEM[B]	*p*-value	
	0	500	1,000	1,500		Linear	Quadratic
Initial BW, kg	27.83	27.45	27.55	27.38	0.31	0.42	0.75
Day 45 BW, kg	33.95	33.92	34.09	34.72	0.42	0.15	0.52
DMI, g (DM)	901.42	897.45	899.57	913.78	7.14	0.23	0.22
AG, kg	6.12	6.47	6.54	7.34	0.38	0.04	0.57
ADG, g/d	135.93	143.78	145.33	163.04	8.48	0.04	0.57
F:G	6.90	6.37	6.26	5.64	0.44	0.06	0.92

[A]BW = body weight; DMI = dry matter intake; AG = average gain; ADG = average day gain; F:G = feed to gain ratio.
[B]SEM = standard error of the least squares means; $n = 6$ lambs/group.
The least squares means for BW, DMI, ADG, F:G in the table are the results of an analysis of covariance with initial weight as the covariate.

The nitrogen resources in the protozoa are derived partly from bacterial proteins obtained by engulfing bacteria, and partly from protein nitrogen supplied in the diet (27). Proteolytic enzymes are found in ruminal protozoa and are highly active, engulfing insoluble protein particles, free amino acids and bacteria to synthesize protozoal proteins (28, 29).

In this study, we did not observe a significant change in the flow of total duodenal nitrogen material. However, there was a linear increase in the flow of GAA into the small intestinal chyme, indicating an increase in the proportion of available nitrogen entering the small intestine; therefore, we hypothesized that dietary GAA improves nitrogen utilization in sheep. Adding 1,500 mg/kg GAA to lamb diets improved nitrogen retention in lambs and also demonstrated that dietary GAA can improve nitrogen utilization in sheep. In addition, the increase in the number of protozoa, which engulf starch granules and store them as branched chain starch that enters the small intestine with the chyme, increases the flow of reducing sugars from the small intestine chyme (27, 30). This phenomenon may also account for the increase in the flow of reducing sugars from the duodenum after dietary GAA supplementation and the improvement in the utilization of non-structural carbohydrates.

In this study, growth performance was improved lambs receiving 1,500 mg/kg MD GAA, with an increase in average daily weight gain and reduced average feed-to-weight ratio. Significantly elevated jugular vein plasma levels of creatine and GAA were observed with increasing dietary GAA supplementation, indicating incomplete degradation of GAA by ruminal microorganisms. Comparable findings were reported in cattle receiving rumen infusions of GAA (13). Speer et al. (13) stated that approximately 50% of GAA undergoes ruminal degradation, and rumen-infused GAA leads to increased plasma and urinary concentrations of Cr and enhanced Cr synthesis. Increasing creatine levels in animals can conserve the raw materials for protein synthesis and energy sources such as glycine and arginine, to improve energy metabolism, accelerate animal growth and increase feed utilization. Thus, the increase in creatine levels induced by the addition of GAA acid to the diet may be one of the mechanisms by which GAA improves growth performance.

Moreover, the addition of 1,500 mg/kg GAA to the diet increased plasma insulin levels and decreased plasma glucose levels. This may be explained by the presence of a positive charge on the GAA side-chain (31), which affects the depolarization of islet cell membranes, stimulating the secretion of protein kinase A and C-type insulin and improving the sensitivity of the mechanism that regulates insulin secretion (32). Previous studies have shown that exogenous GAA stimulates insulin secretion in rodents (33), and reduces plasma glucose levels (34). This, combined with the increased availability of reducing sugars in the duodenum, suggests that the addition of GAA to the diet increased the rate of glucose metabolism and facilitated glucose utilization, thus exerting a positive effect on the energy supply available to the organism for growth.

The addition of 1,500 mg/kg GAA to the diet increased plasma IGF-I levels, possibly by conserving arginine and stimulating IGF-I secretion. The addition of GAA to the diet of broiler chickens (8) also increased IGF-I secretion in the blood. IGF-I has been reported to improve amino acid utilization and increase net protein gain, thereby promoting body growth (35). Changes in IGF-I and insulin secretion may be one of the mechanisms by which dietary GAA supplementation improves the growth performance of lambs.

Looking ahead, the application of GAA in lamb production holds significant promise. The ability of GAA to enhance growth performance and nutrient utilization, as demonstrated in this study, suggests that it could become a valuable feed additive in the lamb industry. By improving average daily gain and feed efficiency, GAA has the potential to reduce the time required for lambs to reach market weight, thereby increasing the overall productivity and profitability of lamb farming operations. Furthermore, the positive effects of GAA on rumen fermentation and blood metabolites, such as increased microbial protein synthesis and elevated levels of IGF-1, indicate that it can support optimal health and development in lambs. This could lead to improved meat quality and yield, which are critical factors for consumer satisfaction and market competitiveness. Additionally, the synergistic potential of GAA with other feed additives, like betaine, offers an opportunity to further enhance its benefits. For instance, combining GAA with betaine has been shown to improve nutrient digestibility and energy-nitrogen metabolism in lambs, although the combined effect on growth performance may not be significantly greater than the individual effects of each additive. This highlights the importance of continued research to optimize the use of GAA in lamb diets and explore its interactions with other feed components. Moreover, long-term studies are needed to assess the sustained impact of GAA supplementation on physiological parameters and overall health of lambs over extended periods. By addressing these research gaps, the lamb industry can harness the full potential of GAA to drive advancements in production efficiency and animal welfare.

5 Conclusion

Ruminal GAA escape rates of 36–56% were achieved in lambs with dietary GAA supplementation at 500–1,500 mg/kg DM. Dietary supplementation of 1,500 mg/kg GAA increased the ruminal ammoniacal nitrogen concentration and the total reducing sugar flow into the small intestine, promoted the creatine level and glucose utilization in lambs, and increased daily weight gain in lambs.

Data availability statement

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

Ethics statement

The animal study was approved by Animal Welfare and Ethics Committee of Xinjiang Agricultural University, Urumqi, Xinjiang, China. The study was conducted in accordance with the local legislation and institutional requirements.

Author contributions

SZ: Conceptualization, Investigation, Methodology, Software, Writing – original draft, Writing – review & editing. MY: Conceptualization, Data curation, Software, Writing – review & editing. CM: Data curation, Writing – review & editing. JP: Project administration, Supervision, Writing – review & editing. CW:–. WC: Investigation, Writing – review & editing. KY: Conceptualization, Funding acquisition, Methodology, Supervision, Writing – review & editing.

Funding

The author(s) declare that financial support was received for the research, authorship, and/or publication of this article. This research was financed by the R&D and integrated demonstration of key technologies for healthy and efficient breeding of local meat sheep in South Xinjiang Uygur Autonomous Region (grant number 2021YFD1600702-2).

Conflict of interest

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

Generative AI statement

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

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Supplementary material

The Supplementary material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fvets.2025.1528861/full#supplementary-material

SUPPLEMENTARY TABLE 1
Feed ingredients and nutrient levels of experimental diets (%).

SUPPLEMENTARY TABLE 2
Fitting equations for duodenal chyme chromium concentrations.

SUPPLEMENTARY TABLE 3
Effect of dietary guanidinoacetic acid supplementation on the guanidinoacetic acid content of rumen fluid in lambs.

SUPPLEMENTARY TABLE 4
Effects of dietary guanidinoacetic acid supplementation on ruminal fermentation parameters in lambs.

SUPPLEMENTARY TABLE 5
Effects of dietary guanidinoacetic acid supplementation on the nutrition duodenal inflow in lambs.

SUPPLEMENTARY TABLE 6
Effects of dietary guanidinoacetic acid supplementation on jugular vein plasma parameters in lambs.

SUPPLEMENTARY TABLE 7
Effects of supplementation with GAA on growth performance in lambs

References

1. De Oliveira JE, Uni Z, Ferket PR. Important metabolic pathways in poultry embryos prior to hatch. *Worlds Poult Sci J*. (2008) 64:488–99. doi: 10.1017/S0043933908000160

2. Baker DH. Advances in protein-amino acid nutrition of poultry. *Amino Acids*. (2008) 37:29–41. doi: 10.1007/s00726-008-0198-3

3. Farshidfar F, Pinder MA, Myrie SB. Creatine supplementation and skeletal muscle metabolism for building muscle mass-review of the potential mechanisms of action. *Curr Protein Pept Sci*. (2017) 18:1273–87. doi: 10.2174/1389203718666170606105108

4. Wyss M, Kaddurah-Daouk R. Creatine and creatinine metabolism. *Physiol Rev*. (2000) 80:1107–213. doi: 10.1152/physrev.2000.80.3.1107

5. Dinesh OC, Bertolo RF, Brunton JA. Creatine supplementation to total parenteral nutrition improves creatine status and supports greater liver and kidney protein synthesis in neonatal piglets. *Pediatr Res*. (2018) 83:135–41. doi: 10.1038/pr.2017.208

6. Liu Y, Li JL, Li YJ, Gao T, Zhang L, Gao F, et al. Effects of dietary supplementation of guanidinoacetic acid and combination of guanidinoacetic acid and betaine on postmortem glycolysis and meat quality of finishing pigs. *Anim Feed Sci Technol*. (2015) 205:82–9. doi: 10.1016/j.anifeedsci.2015.03.010

7. Bihan-Duval EL, Berri C, Baeza E, Millet N, Beaumont C. Estimation of the genetic parameters of meat characteristics and of their genetic correlations with growth and body composition in an experimental broiler line. *Poul Sci*. (2001) 80:839–43. doi: 10.1093/ps/80.7.839

8. Michiels J, Maertens L, Buyse J, Lemme A, Rademacher M, Dierick NA, et al. Supplementation of guanidinoacetic acid to broiler diets: effects on performance, carcass characteristics, meat quality, and energy metabolism. *Poul Sci*. (2012) 91:402–12. doi: 10.3382/ps.2011-01585

9. Esser AFG, Taniguti TL, da Silva AM, Vanroo E, Kaneko IN, dos Santos TC, et al., Effect of supplementation of guanidinoacetic acid and arginine in vegetable diets for

broiler on performance, carcass yield and meat quality. Semina: Ciências Agrárias (2018); 39: 1307–1318

10. European Food Safety Authority. EFSA panel on additives and products or substances used in animal feed. Safety and efficacy of guanidinoacetic acid for chickens for fattening, breeder hens and roosters, and pigs. *EFSA J*. (2016) 14:4394. doi: 10.2903/j.efsa.2016.4394

11. Ardalan M, Miesner MD, Reinhardt CD, Thomson DU, Armendariz CK, Titgemeyer EC. 1577 Guanidinoacetic acid as a precursor for creatine in steers. *J Anim Sci*. (2016) 94:766. doi: 10.2527/jam2016-1577

12. Ardalan M, Batista ED, Titgemeyer EC. Effect of post-ruminal guanidinoacetic acid supplementation on creatine synthesis and plasma homocysteine concentrations in cattle. *J Anim Sci*. (2020) 98:skaa 072. doi: 10.1093/jas/skaa072

13. Speer HF, Pearl KA, Titgemeyer EC. Relative bioavailability of guanidinoacetic acid delivered ruminally or abomasally to cattle. *J Anim Sci*. (2020) 98:skaa 282. doi: 10.1093/jas/skaa282

14. Li SY, Wang C, Wu ZZ, Liu Q, Guo G, Huo WJ, et al. Effects of guanidinoacetic acid supplementation on growth performance, nutrient digestion, rumen fermentation and blood metabolites in Angus bulls. *Animal*. (2020) 14:2535–42. doi: 10.1017/S1751731120001603

15. Udén P, Colucci PE, Van Soest PJ. Investigation of chromium, cerium and cobalt as markers in digesta. Rate of passage studies. *J Sci Food Agric*. (1980) 31:625–32. doi: 10.1002/jsfa.2740310702

16. Association of Official Analytical Chemists. Official methods of analysis of the Association of Official Analytical Chemists. Washington, DC, USA: AOAC (2007).

17. Van Soest PJ, Robertson JB, Lewis BA. Methods for dietary fiber, neutral detergent fifiber and non-starch polysaccharides in relation to animal nutrition. *J Dairy Sci*. (1991) 74:3583–97. doi: 10.3168/jds.S0022-0302(91)78551-2

18. Goering HK, Van Soest PJ. Forage fiber analysis In: Agriculture Handbook No. 379. Washington, DC: U.S. Government Printing Office (1970).

19. Chong KJ, Lipke PN. Determination of reducing sugars in the nanomole range with tetrazolium blue. *J Biochem Biophys Methods*. (1985) 11:109–15. doi: 10.1016/0165-022X(85)90046-6

20. Wada T, Shimbo H, Osaka H. A simple screening method using ion chromatography for the diagnosis of cerebral creatine deficiency syndromes. *Amino Acids*. (2012) 43:993–7. doi: 10.1007/s00726-011-1146-1

21. Emmet RT. Spectrophotometric determination of urea and ammonia in natural waters with hypochlorite and phenol. *Anal Chem*. (1969) 41:1648–52. doi: 10.1021/ac60281a007

22. Samal L, Chaudhary LC, Agarwal N, Kamra DN. Effects of plants containing secondary metabolites as feed additives on rumen metabolites and methanogen diversity of buffaloes. *Anim Prod Sci*. (2016) 56:472–81. doi: 10.1071/AN15596

23. Burggraaf W, Leng RA. Antiprotozoal effects of surfactant detergents in the rumen of sheep. *New Zealand J Agric Res*. (1980) 23:287–91. doi: 10.1080/00288233.1980.10425358

24. Tansol P, Yu Z. Do ruminal ciliates select their preys and prokaryotic symbionts? *Front Microbiol*. (2018) 9:1710–23. doi: 10.3389/fmicb.2018.01710

25. Itabashi H, Matsui H. Recent advances in microbial ecology of protozoa and fungi in the rumen of herbivores. In Proceedings of the 7th international symposium on the nutrition of herbivores. Nanjing, China: China Agricultural University Press; (2007). pp. 17–22.

26. Wang L, Abu-Doleh A, Plank J, Catalyurek UV, Firkins JL, Yu Z. The transcriptome of the rumen ciliate Entodinium caudatum reveals some of its metabolic features. *BMC Genomics*. (2019) 20:1008. doi: 10.1186/s12864-019-6382-x

27. Coleman GS. A comparison of the cellulolytic activities of rumen bacteria and protozoa. *Appl Biochem Biotechnol*. (1984) 9:347–8. doi: 10.1007/BF02798969

28. Abou Akkada AR, El-Shazly K. Effect of absence of ciliate protozoa from the rumen on microbial activity and growth of lambs. *Appl Microbiol*. (1964) 12:384–90. doi: 10.1128/am.12.4.384-390.1964

29. James Newbold C, Mcewan NR, Calza RE, Chareyron EN, Duval SM, Eschenlauer SCP, et al. An NAD(+)-dependent glutamate dehydrogenase cloned from the ruminal ciliate protozoan, Entodinium caudatum. *FEMS Microbiol Lett*. (2005) 247:113–21. doi: 10.1016/j.femsle.2005.04.034

30. Orpin CG, Letcher AJ. Some factors controlling the attachment of the rumen Holotrich Protozoa Isotricha intestinalis and I. prostoma to plant particles in vitro. *J Gener Microbiol*. (1978) 106:33–40. doi: 10.1099/00221287-106-1-33

31. Charles S, Tamagawa T, Henquin JC. A single mechanism for the stimulation of insulin release and 86Rb+ efflux from rat islets by cationic amino acids. *Biochem J*. (1982) 208:301–8. doi: 10.1042/bj2080301

32. Marco J, Calle C, Hedo JA, Villanueva ML. Glucagon-releasing activity of guanidine compounds in mouse pancreatic islets. *FEBS Lett*. (1976) 64:52–4. doi: 10.1016/0014-5793(76)80246-3

33. Aynsley-Green A, Alberti KGMM. In vivo stimulation of insulin secretion by guanidine derivatives in the rat. *Horm Metab Res*. (1974) 6:115–20. doi: 10.1055/S-0028-1093873

34. Meglasson MD, Wilson JM, Yu JH, Robinson DD, Wyse BM, de Souza CJ. Antihyperglycemic action of guanidinoalkanoic acids: 3-guanidinopropionic acid ameliorates hyperglycemia in diabetic KKAy and C57BL6Job/Ob mice and increases glucose disappearance in rhesus monkeys. *J Pharmacol Exp Ther*. (1993) 266:1454–62.

35. Deldicque L, Louis M, Theisen D, Nielens H, Dehoux M, Thissen JP, et al. Increased IGF mRNA in human skeletal muscle after creatine supplementation. *Med Sci Sports Exerc*. (2005) 37:731–6. doi: 10.1249/01.mss.0000162690.39830.27

frontiers | Frontiers in Veterinary Science

TYPE Original Research
PUBLISHED 17 February 2025
DOI 10.3389/fvets.2024.1463209

OPEN ACCESS

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RECEIVED 11 July 2024
ACCEPTED 22 October 2024
PUBLISHED 17 February 2025

CITATION
Liu Z, Jiang A, Ma D, Liu D, Han X, Zhao M, Zhou C and Tan Z (2025) The impact of rumen microbial composition on apparent digestibility, rumen fermentation and metabolism in Sanhe cows and Holstein cows of different parities under identical dietary conditions.
Front. Vet. Sci. 11:1463209.
doi: 10.3389/fvets.2024.1463209

The impact of rumen microbial composition on apparent digestibility, rumen fermentation and metabolism in Sanhe cows and Holstein cows of different parities under identical dietary conditions

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Previous studies have discussed the association between serum metabolism and lactation performance among Sanhe and Holstein cows of different parities and found that the metabolic profiles of these two breeds vary differently with parity. Since the rumen is the central organ for nutrient absorption and production transformation in dairy cows, it remains unknown whether the differences observed under the same dietary conditions are related to the structure of the rumen microbiome. This study measured the apparent digestibility and rumen fermentation parameters of Sanhe cows (S1/S2/S3/S4) and Holstein cows (H1/H2/H3/H4) across four parities and generated a comprehensive rumen microbiome dataset using high-throughput sequencing technology. Significant differences in dry matter digestibility ($p = 0.001$) and ammonia nitrogen ($p = 0.024$) were observed among the S groups, with higher trends of various VFA contents in S1 ($0.05 < p < 0.1$). The H group showed significant differences in crude protein digestibility ($p = 0.001$), higher isovaleric acid content in H1 ($p = 0.002$), and the lowest acetate to propionate ratio ($p = 0.002$) in H3. Metagenomic sequencing results indicated consistency between rumen microbiome patterns and metabolic changes, with S1 distinctly different from S2/S3/S4, and H1 and H2 different from H3 and H4. The species composition of the rumen microbiome was similar between Sanhe and Holstein cows, but differences in abundance were noted. *Rhizophagus <glomeromycetes>*, *Neocallimastix*, and *Piromyces* were more abundant in S1, H1, and H2, and pathways such as autophagy-animal, plant-pathogen interaction, and endocytosis were significantly enriched in these parities. Multiparous Sanhe cows had higher abundances of ATP-binding cassette transporters pathways. Additionally, CAZymes such as GH84 and GH37 were significantly associated with differential physiological indicators and milk traits. In conclusion, this study reveals the complex relationship between rumen microbiota and metabolic characteristics in Sanhe and Holstein cows of different parities, indicating that changes in the structure of the rumen microbiome may be key factors affecting lactation performance and metabolic differences in dairy cows.

KEYWORDS
rumen microbiome, cow breeds, prity, dairy cattle, lactation association

1 Introduction

In recent years, with the increasing demand for high-quality dairy and beef products, cattle breeding and management have garnered growing attention worldwide. Among the numerous cattle breeds, Holstein cows are one of the most widely distributed and extensively studied dairy breeds globally (1). However, there is also a rich diversity of genetic resources in cattle breeding and production, including indigenous breeds such as the Sanhe cattle in China (2, 3). Through selective breeding, Sanhe cattle have been developed for both dairy and meat production. Nevertheless, compared to widely studied breeds like Holstein, there remains a significant gap in understanding the production performance and physiological characteristics of Sanhe cattle.

Lactation is the most critical production activity in dairy cows. Due to differences in basal metabolic levels and energy requirements among cows of different parities (4), the impact of parity on lactation performance cannot be overlooked under normal feeding and management conditions. In our previous research, we systematically explored the physiological and metabolic characteristics of Sanhe and Holstein cows across parities 1 to 4 (5). We found significant differences in the metabolic patterns between primiparous and multiparous Sanhe cows, indicating substantial changes in nutrient utilization and lactation-related metabolic pathways during continuous lactation periods. In Holstein cows, the metabolic profiles exhibited different trends, with the first and second parities being more similar to each other and distinct from the third and fourth parities (6).

For dairy cows, the composition and activity of the rumen microbiome are crucial factors determining feed efficiency, nutrient utilization, and overall productivity (7). However, most existing studies focus on the development of the rumen microbial community in weaned calves (8) and the differences in rumen microbiome composition between high-and low-producing dairy cows (9, 10). There is limited understanding of the successional patterns of the rumen microbiome in lactating cows of different parities. Xue et al. (11) found significant differences in the relative abundance of *Fibrobacteres* and *SR1* in the rumens of mid-lactation cows from parities 2 to 7, while the main functional metabolic bacteria showed no significant changes. The lactation traits of dairy cows have a complex relationship with the core rumen microbiota (12–14). Therefore, the rumen microbiome is a critical entry point for studying the mechanisms regulating milk quality in dairy cows.

We hypothesize that under identical dietary conditions, the differences in serum metabolism and lactation performance between Sanhe and Holstein cows of different parities are partially attributable to specific changes in their rumen microbial community structure. This study focuses on investigating the impact of rumen microbial composition on rumen fermentation and metabolic activities in Sanhe and Holstein cows across parities 1 to 4 under the same dietary conditions. The aim is to further elucidate the effects of rumen microecology on metabolic adaptability and production performance in specific breeds of dairy cows. Combined with previous research findings, this study can provide a comprehensive framework to clarify the multifaceted interactions among host physiology, rumen microbiota, and metabolic responses. This will enhance the understanding of the Sanhe breed and inform breeding and management strategies in the context of sustainable livestock production.

2 Materials and methods

2.1 Animal management and experimental design

All procedures involving animals in this experiment were approved by the Animal Experiment Ethics Committee of the Institute of Subtropical Agriculture, Chinese Academy of Sciences. Sanhe and Holstein cows in lactation periods from parity 1 to 4 were selected from the same farm, with similar feeding management conditions and identical dietary formulations. The four groups of Sanhe cows were designated as S1 ($N=10$), S2 ($N=9$), S3 ($N=10$), and S4 ($N=10$), while the four groups of Holstein cows were designated as H1 ($N=10$), H2 ($N=7$), H3 ($N=9$), and H4 ($N=9$). Detailed information on feeding management procedures and animal selection can be found in our previously published works, including studies on Sanhe cows (5) and Holstein cows (6).

2.2 Collection and analysis of fecal samples

During the sampling period, rectal fecal samples were collected for five consecutive days, twice daily, 2 h before feeding and 2 h after feeding. The samples were mixed with 10% sulfuric acid and stored at −20°C until analysis for apparent digestibility. Fecal samples were dried at 65°C and ground to pass through a 1-mm sieve. The measurements of dry matter (DM; method 930.15), crude protein (CP; method 2001.11), neutral detergent fiber (NDF; method 2002.04), acid detergent fiber (ADF; method 973.18), and ether extract (EE; method 920.39) were conducted following the methods of the Association of Official Analytical Chemists (55, 56). Gross energy (GE) was determined using a calorimeter (5E-C5508, Kaiyuan Instruments, China). Apparent total-tract digestibility was calculated using the acid-insoluble ash method (15).

2.3 Collection and analysis of rumen samples

Using an oral intubation method, a tube was inserted approximately 120–150 cm deep into the esophagus, and contents from the rumen were aspirated using a syringe at the oral end. To avoid saliva contamination, the initial 100 mL of aspirated content was discarded, and the subsequent 100 to 150 mL of rumen fluid was collected. Immediately on-site, the pH value was measured using a portable pH meter (BPHPOCKET-E, BELL Analytical Instruments

(Dalian) Co., Ltd., China). The fluid was then filtered through two layers of sterile gauze into sterile centrifuge tubes and flash-frozen using liquid nitrogen. As described previously (16), after processing the rumen fluid, volatile fatty acid (VFA) concentrations were analyzed using Gas Chromatography (7890A, Agilent, United States). Ammonia nitrogen concentration was determined using a UV-2300 Spectrophotometer (Shimadzu, Kyoto, Japan) by recording absorbance at 700 nm.

2.4 Extraction of rumen fluid DNA and metagenomic sequencing

The extraction of rumen fluid DNA was performed by Guangdong Magigene Biotechnology Co., Ltd. (Guangzhou, China) using a commercial kit following the manufacturer's instructions. The integrity of the DNA was checked using 1% agarose gel electrophoresis. DNA concentration and purity were assessed simultaneously using the Qubit 2.0 (Thermo Fisher Scientific, Waltham, United States) and Nanodrop One (Thermo Fisher Scientific, Waltham, United States). Sequencing libraries were prepared using the NEB Next® Ultra™ DNA Library Prep Kit for Illumina® (New England Biolabs, Ipswich, MA, United States) following the manufacturer's recommendations, with index codes added. Library quality was evaluated using the Qubit 3.0 Fluorometer (Life Technologies, Grand Island, NY) and the Agilent 4200 (Agilent, Santa Clara, CA) system. Finally, the libraries were sequenced on the Illumina Hiseq X-ten platform, generating 150 bp paired-end reads.

2.5 Bioinformatic processing of rumen metagenomic data

The raw data obtained from sequencing were processed using Trimmomatic (v.0.36, http://www.usadellab.org/cms/index.php?page=trimmomatic) to obtain clean data for subsequent analysis. Clean data were assembled using MEGAHIT (Version v1.0.6, https://github.com/voutcn/megahit). Mixed assembled scaffolds were broken from N connection to obtain scaftigs. Scaftigs ≥500 bp were screened and used for ORF prediction with MetaGeneMark (Version 3.38, http://exon.gatech.edu/GeneMark/metagenome/Prediction), with default parameters filtering out predicted results shorter than 90 nt. Redundancies were removed using CD-HIT (Version 4.7, http://www.bioinformatics.org/cd-hit/), creating a unique initial gene catalogue. Clustering was performed with 95% identity and 90% coverage, selecting the longest representative sequence. Clean data from each sample were mapped to the initial gene catalogue using BBMAP,[1] obtaining gene mapping reads for each sample. Gene abundance information for each sample was calculated based on the number of mapped reads and the gene length. DIAMOND software (Version 0.8.35, https://github.com/bbuchfink/diamond/) was used to align unigenes against bacterial, fungal, archaeal, and viral sequences extracted from the NCBI NR (non-redundant protein sequence database). Results with an *e*-value of 1×10^{-10} were selected for LCA algorithm annotation. Based on the LCA annotation results and gene average depth or gene abundance tables, gene average depth and abundance information tables for each taxonomic level (kingdom, phylum, class, order, family, genus, species) were obtained for each sample. Abundance clustering heatmaps and PCA were based on abundance tables at each taxonomic level. Group differences were tested using ANOSIM analysis. LEfSe analysis (default LDA score of 2) was used to identify different species between groups. Visualization was performed using the R package ggplot2 (version 4.3.1). Clustering heatmaps were generated using the R package Pheatmap. Differential KEGG pathways were visualized using OmicStudio tools.[2] CAZy[3] annotation results were generated by dbCAN[4] to obtain annotation information of carbohydrate-active enzymes. CAZy classification circular heatmaps and bar charts were created using the online analysis tool ChiPlot.[5] Spearman correlation analysis was used to analyze the differential GH enzymes and apparent differential indices, with visualization performed using the linkET and ggplot2 packages.

2.6 Statistical analysis

Data for apparent digestibility and rumen fermentation parameters were initially organized using Excel 2019 (Microsoft Corporation, United States) and then subjected to statistical analysis using Statistical Package for the Social Sciences 22.0 software (SPSS, Inc., United States). The normality of each variable was assessed with the Shapiro–Wilk test. If the data met the normal distribution criteria, comparisons were made using analysis of variance (ANOVA), and multiple comparisons between categorical variables were adjusted using Bonferroni correction. A p-value <0.05 was defined as statistically significant, $0.05 \leq p < 0.10$ was defined as a trend, and $p \geq 0.10$ was defined as no difference.

3 Results

3.1 Nutrients digestibility

The apparent digestibility results for Sanhe cows from parity 1 to 4 are shown in Table 1. The digestibility of DM differed significantly among the four parities, with S4 being lower ($p = 0.038$). However, the digestibility of other nutritional indices, including CP, NDF, ADF, EE, and GE, showed no significant differences between S1 and S4 ($p > 0.05$). The digestibility results for Holstein cows from parity 1 to 4 are presented in Table 2. Except for the CP digestibility, which was significantly higher in H4 compared to H1, H2, and H3, the digestibility of DM, NDF, ADF, EE, and GE showed no significant differences among H1 to H4 ($p > 0.05$).

1 http://jgi.doe.gov/data-and-tools/bbtools/

2 https://www.omicstudio.cn/tool

3 http://www.cazy.org/

4 http://csbl.bmb.uga.edu/dbCAN/

5 https://www.chiplot.online/index.html

TABLE 1 Apparent total-tract apparent digestibility of nutrients in Sanhe cows with 1–4 parities.

Item[1]	Group[2]				SEM[3]	*p*-value
	S1	S2	S3	S4		
DM	86.42[a]	85.59[ab]	85.96[a]	84.03[b]	0.325	0.038
CP	63.80	59.72	62.63	59.88	0.832	0.220
NDF	56.98	52.86	58.96	50.91	1.201	0.059
ADF	47.47	44.49	48.52	40.14	1.642	0.260
EE	85.37	82.49	83.34	80.79	0.707	0.134
GE	58.72	52.41	56.74	52.10	1.061	0.061

[1]DM, dry matter; CP, crude protein; NDF, neutral detergent fiber; ADF, acid detergent fiber; EE, ethanol extract; GE, gross energy.
[2]S1, S2, S3, and S4 represented first-, second-, third-, and fourth-parity Sanhe dairy cattle, respectively.
[3]SEM was standard error of means.
[a,b]Means within a row with different superscripts differ significantly ($p < 0.05$).

TABLE 2 Apparent total-tract apparent digestibility of nutrients in Holstein cows with 1–4 parities.

Item[1]	Group[2]				SEM[3]	*p*-value
	H1	H2	H3	H4		
DM	84.01	83.31	83.07	84.96	0.385	0.279
CP	62.72[b]	62.36[b]	65.40[b]	69.58[a]	0.782	0.001
NDF	57.12	55.76	48.19	55.55	1.498	0.134
ADF	59.55	57.04	50.81	56.55	1.448	0.169
EE	85.65	84.37	83.14	83.79	0.613	0.507
GE	58.22	56.45	53.98	59.93	1.006	0.172

[1]DM, dry matter; CP, crude protein; NDF, neutral detergent fiber; ADF, acid detergent fiber; EE, ethanol extract; GE, gross energy.
[2]S1, S2, S3, and S4 represented first-, second-, third-, and fourth-parity Holstein dairy cattle, respectively.
[3]SEM was standard error of means.
[a,b]Means within a row with different superscripts differ significantly ($p < 0.05$).

TABLE 3 Rumen fermentation variables in Sanhe cows with 1–4 parities.

Item[1]	Group[2]				SEM[3]	*p*-value
	S1	S2	S3	S4		
Rumen pH	6.52	6.77	6.73	6.56	0.040	0.068
Ammonia N (mg/dL)	8.59[a]	5.20[b]	6.67[ab]	7.75[a]	0.423	0.024
MCP (mg/mL)	1.42	1.45	1.37	1.21	0.064	0.547
Total VFA (mmol/L)	92.19	63.74	81.69	88.61	3.971	0.053
Acetate (mmol/L)	57.03	40.41	49.82	53.91	2.300	0.058
Propionate (mmol/L)	19.52	12.89	18.16	20.11	1.086	0.079
Butyrate (mmol/L)	11.91	7.79	10.26	11.08	0.565	0.056
Isobutyrate (mmol/L)	0.87	0.64	0.81	0.80	0.031	0.059
Valerate (mmol/L)	1.48	1.03	1.39	1.43	0.068	0.077
Isovalerate (mmol/L)	1.38	0.98	1.26	1.29	0.055	0.070
Acetate/Propionate	2.99	3.18	2.84	2.89	0.073	0.386

[1]MCP, microbial crude protein; VFA, volatile fatty acid.
[2]S1, S2, S3, and S4 represented first-, second-, third-, and fourth-parity Sanhe dairy cattle, respectively.
[3]SEM was standard error of means.
[a,b]Means within a row with different superscripts differ significantly ($p < 0.05$).

3.2 Rumen fermentation parameters

The rumen fermentation parameters for Sanhe cows from parity 1 to 4 are shown in Table 3. There was a trend for lower rumen pH in S1 ($p = 0.068$). Conversely, ammonia N was significantly higher in S1 and lowest in S2 ($p = 0.024$). Additionally, the mean values for total VFA, acetate, butyrate, isobutyrate, valerate, and isovalerate were higher in S1 ($0.05 < p < 0.1$). There were no significant differences in MCP and acetate/propionate ratios among S1 to S4 ($p > 0.05$). The rumen fermentation parameters for Holstein cows from parity 1 to 4

TABLE 4 Rumen fermentation variables in Holstein cows with 1–4 parities.

Item[1]	Group[2]				SEM[3]	*p*-value
	H1	H2	H3	H4		
Rumen pH	6.71	6.61	6.69	6.83	0.050	0.521
Ammonia N (mg/dL)	9.23	6.99	7.86	6.33	0.489	0.176
MCP (mg/mL)	1.44	1.38	1.28	1.43	0.073	0.864
Total VFA (mmol/L)	96.53	78.20	83.17	74.23	4.590	0.336
Acetate (mmol/L)	60.74	49.22	49.71	46.53	2.744	0.253
Propionate (mmol/L)	20.37	16.61	21.19	16.45	1.221	0.396
Butyrate (mmol/L)	11.76	9.50	9.09	8.69	0.627	0.289
Isobutyrate (mmol/L)	0.88	0.67	0.80	0.61	0.056	0.315
Valerate (mmol/L)	1.40	1.11	1.41	1.08	0.070	0.175
Isovalerate (mmol/L)	1.38^{a}	1.08^{b}	0.96^{bc}	0.88b^{c}	0.054	0.002
Acetate/Propionate	3.05^{a}	3.06^{a}	2.43^{b}	2.84^{a}	0.074	0.002

[1]MCP, microbial crude protein; VFA, volatile fatty acid.
[2]S1, S2, S3, and S4 represented first-, second-, third-, and fourth-parity Holstein dairy cattle, respectively.
[3]SEM was standard error of means.
[a,b]Means within a row with different superscripts differ significantly ($p < 0.05$).

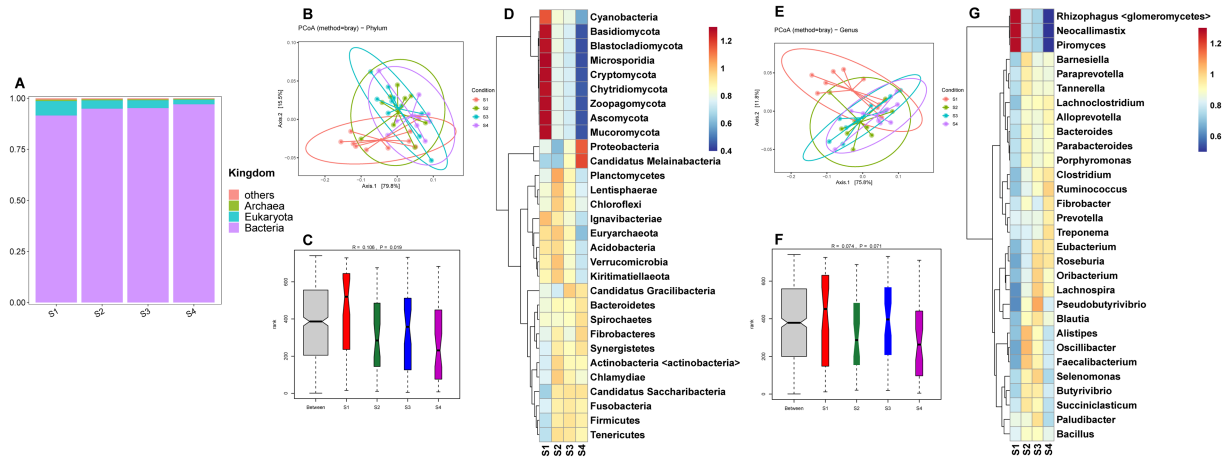

FIGURE 1
(A) Relative abundance distribution of microorganisms at the kingdom level in the rumen of S1–S4. **(B)** PCoA (principal coordinate analysis) clustering analysis of rumen microorganisms at the phylum level in S1–S4 based on the Bray method. **(C)** ANOSIM analysis of rumen microorganisms at the phylum level in S1–S4. **(D)** Heatmap of hierarchical clustering analysis of rumen microorganisms at the phylum level in S1–S4. **(E)** PCoA analysis of rumen microorganisms at the genus level in S1–S4 based on the Bray method. **(F)** ANOSIM analysis of rumen microorganisms at the genus level in S1–S4. **(G)** Heatmap of hierarchical clustering analysis of rumen microorganisms at the genus level in S1–S4.

are presented in Table 4. The acetate/propionate ratio was significantly lower in H3 ($p = 0.002$), whereas isovalerate was significantly higher in H1 ($p = 0.002$). Other rumen fermentation parameters showed no significant differences among H1 to H4 ($p > 0.05$).

3.3 Rumen microbial composition

A total of 2,712,982,832 clean reads were obtained from the 39 rumen samples of Sanhe cows (Supplementary Table S1). Microbial identification at the kingdom level revealed four types: bacteria, eukaryota, archaea, and others (Figure 1A). Among the S1–S4 parities, bacteria had the highest abundance, followed by eukaryota. The abundance of bacteria in S1 was slightly lower than in S3–S4, while eukaryota showed the opposite trend. The PCoA results for the 185 identified phylum-level species are shown in Figure 1B, indicating that the four groups were relatively clustered. However, ANOSIM analysis results demonstrated that inter-group differences were significantly greater than intra-group differences (Figure 1C), with S1 having the highest richness. Hierarchical clustering analysis (HCA) of the top 30 phylum-level microbes revealed that *Cyanobacteria*, *Basidiomycota*, *Blastocladiomycota*, *Microsporidia*, *Cryptomycota*, *Chytridiomycota*, *Zoopagomycota*, *Ascomycota*, and *Mucoromycota* had higher abundances in S1, while *Proteobacteria* and *Candidatus Melainabacteria* were more abundant in S4 (Figure 1D). Further analysis of the 3,528 identified genus-level microbes showed similar PCoA results to the phylum level,

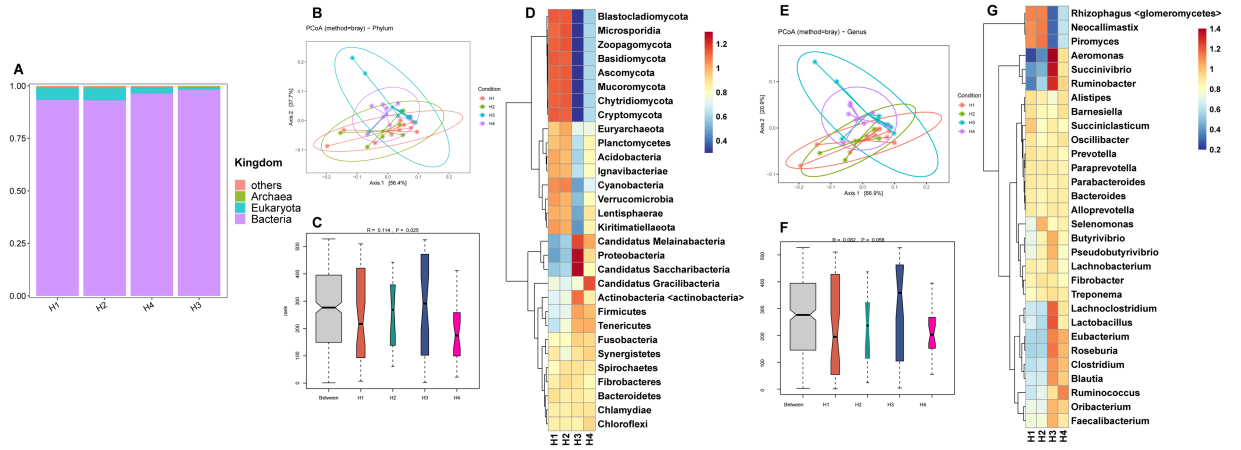

FIGURE 2
(A) Relative abundance distribution of microorganisms at the kingdom level in the rumen of H1–H4. **(B)** PCoA (principal coordinate analysis) clustering analysis of rumen microorganisms at the phylum level in H1–H4 based on the Bray method. **(C)** ANOSIM analysis of rumen microorganisms at the phylum level in H1–H4. **(D)** Heatmap of hierarchical clustering analysis of rumen microorganisms at the phylum level in H1–H4. **(E)** PCoA analysis of rumen microorganisms at the genus level in H1–H4 based on the Bray method. **(F)** ANOSIM analysis of rumen microorganisms at the genus level in H1–H4. **(G)** Heatmap of hierarchical clustering analysis of rumen microorganisms at the genus level in H1–H4.

with less distinct separation (Figure 1E). ANOSIM analysis indicated a trend towards inter-group differences (Figure 1F). The HCA results for the top 30 genus-level species indicated that *Rhizophagus <glomeromycetes>*, *Neocallimastix*, and *Piromyces* were more abundant in S1 (Figure 1G).

The metagenomic sequencing data for rumen fluid from Holstein cows (H1–H4) are provided in Supplementary Table S2. The kingdom-level microbial composition structure was similar to that of Sanhe cows, with bacteria abundance being slightly lower in H1 and H2 compared to H3 and H4, and eukaryota showing the opposite trend (Figure 2A). Analysis of selected phylum-and genus-level microbes in H1–H4 revealed that PCoA showed no distinct separation between groups (Figures 2B,E). ANOSIM analysis indicated significant differences at the phylum level between groups (Figure 2C) and a trend towards differences at the genus level (Figure 2F). The HCA heatmaps of phylum-and genus-level microbes showed that the microbial abundance patterns of H1 and H2 were more similar. Specifically, phylum-level microbes such as *Blastocladiomycota*, *Microsporidia*, *Zoopagomycota*, *Basidiomycota*, *Ascomycota*, *Mucoromycota*, *Chytridiomycota*, and *Cryptomycota* had higher abundances in H1 and H2 (Figure 2D). Similarly, genus-level microbes such as *Rhizophagus <glomeromycetes>*, *Neocallimastix*, and *Piromyces* also had higher abundances in H1 and H2 (Figure 2G).

3.4 Differential rumen microbes and KEGG functional pathways

To further identify microbial composition differences and select representative marker microbes between different parities, we conducted LEfSe analysis on 1–4 parity Sanhe and Holstein cows. According to the LDA and evolutionary analysis results (Figure 3A), 19 marker microbes were identified in S1–S4 ($p < 0.05$). Notably, three of the four microbes enriched in S1 were related to *Prevotella* (*s_Prevotella_sp_tc2_28*, *g_Prevotellaceae_unclassified*, *s_Prevotellaceae_bacterium_MN60*), and the other was *s_Lactobacillus_fructivorans*. S2 had the highest number of marker microbes, including *p_Proteobacteria*, *c_Gammaproteobacteria*, *o_Enterobacterales*, *f_Erwiniaceae*, *p_Actinobacteria*, *g_Pantoea*, *c_Actinobacteria*, *s_Pantoea_agglomerans*, *s_Frigoribacterium_sp_Leaf8*, *f_Microbacteriaceae*, *s_Frigoribacterium_sp_Leaf164*, *g_Frigoribacterium*, and *s_Lactobacillus_amylovorus*, totaling 13. S3 and S4 had one significantly enriched microbe each, *s_Eubacterium_uniforme* and *f_Clostridiaceae*, respectively.

Similarly, in the LEfSe results for H1–H4 (Figure 3B), *s_Fibrobacter_succinogenes* was significantly enriched in H1. In H2, *s_Prevotella_sp_tf2_5*, *g_Selenomonas*, *s_Selenomonas_ruminantium*, *s_Oribacterium_sp_KHPX15*, *acteroidales_bacterium_WCE2008*, and *g_Oribacterium* were significantly enriched. H3 had significant enrichments in *s_Eubacterium_eligens*, *o_Veillonellales*, *f_Veillonellaceae*, and *g_Allisonella*. H4 had significant enrichments in *s_Eubacterium_uniforme* and *g_Roseburia*.

Continuing with the analysis of functional pathways enriched by these rumen microbiota. For the rumen fluid of multiparous Sanhe cows, a heatmap was generated for the top 20 identified KEGG orthologies (KO) number, revealing that, apart from K13412 showing higher expression in S1, the rest exhibited higher abundance in S2–S3 (Figure 4A).

It was also found that K01990, K01992, and K02003 are all related to ABC Transport, while K13412, which has a higher abundance in S1, is associated with calcium-dependent protein kinase (Supplementary Table S3). Stamp analysis was conducted on 425 level 3 pathways mapped to the KEGG database, selecting the top 20 pathways with significant differences in abundance (Figure 4B). Except for autophagy–animal, plant–pathogen interaction, cancer network viewer, and endocytosis showing higher expression in S1, other pathways with differential expression were more abundant in multiparous Sanhe cows.

Concurrently, analysis of microbial functional pathways in Holstein H1–H4 revealed that, apart from K21572 and K05349 showing higher abundance in H1 and H3, the remaining KO pathways exhibited more similarity in abundance between H1 and H2, whereas H3 and H4 showed similar patterns (Figure 4C). The KO pathways with higher abundance in H1 and H2 are mainly related to cell signaling and metabolic regulation, and their activity is influenced by phosphorylation

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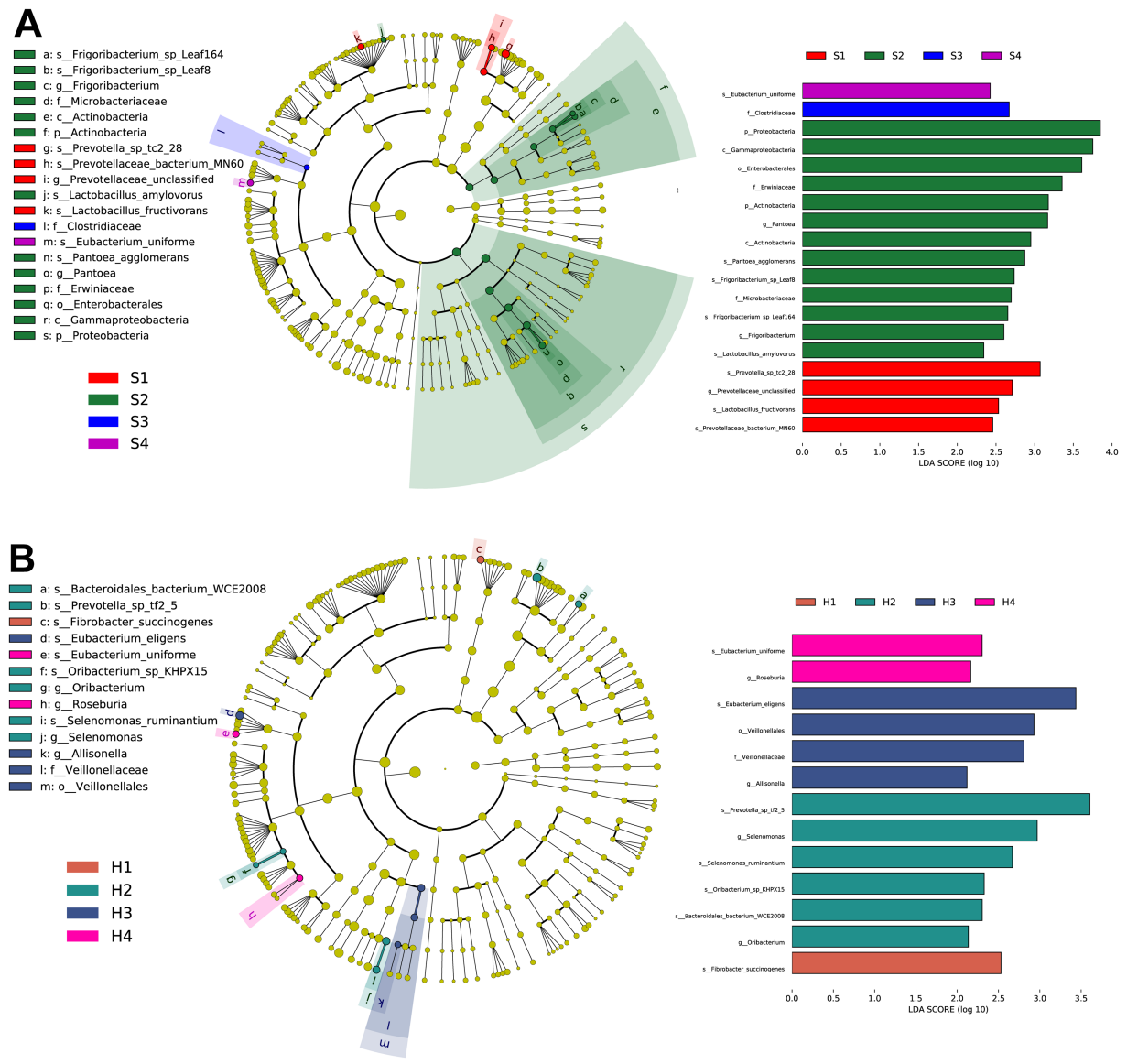

FIGURE 3
(A) Taxonomic tree and biomarker microorganisms of rumen microbiota in S1–S4 based on linear discriminant analysis effect size (LEFSe). **(B)** Taxonomic tree and biomarker microorganisms of rumen microbiota in H1–H4 based on LEFSe. Nodes of different colors represent representative microorganisms in the rumen of cows at different parities, with the specific names of the microorganisms labeled beside the nodes. The selection criteria for biomarkers are linear discriminant analysis (LDA) score >2.0, $p < 0.05$.

status (Supplementary Table S4). Comparative analysis with the KEGG database identified 4 pathways significantly more abundant in S2 and also higher in abundance in H1 and H2, with salmonella infection additionally showing higher abundance in H1 and H2 (Figure 4D).

3.5 The composition of CAZymes in the rumen microbiota

The breakdown of complex carbohydrates by rumen microbiota affects rumen fermentation processes, relying on CAZymes. By comparing with the CAZymes database, we identified six enzyme types in both 1–4 parity Sanhe cows and Holstein cows, including auxiliary activities (AA), carbohydrate-binding modules (CBM), carbohydrate esterases (CE), glycoside hydrolases (GH), glycosyl transferases (GT), and polysaccharide lyases (PL). Among these, GH enzymes were the most abundant in both S and H groups (Supplementary Figure S1). Heatmap analysis of CAZymes abundance distribution in S revealed overall higher abundance in most CAZymes in S4 (Figure 5A). Comparing the inter-group differences in enzymes with higher abundance, we found significant differences in 12 GH enzymes. GH1, GH109, GH112, GH120, GH4, GH42, GH48, and GH50 showed higher abundance in multiparous cows (S2–S4), while GH108, GH37, GH64, and GH84 were more abundant in primiparous cows (S1). Correlation analysis using Spearman's method between previously measured rumen and milk differential indicators and these differential GH enzymes revealed (Figure 5B): ammonia nitrogen showed significant negative correlations with GH109, GH112, and GH120. Milk protein exhibited

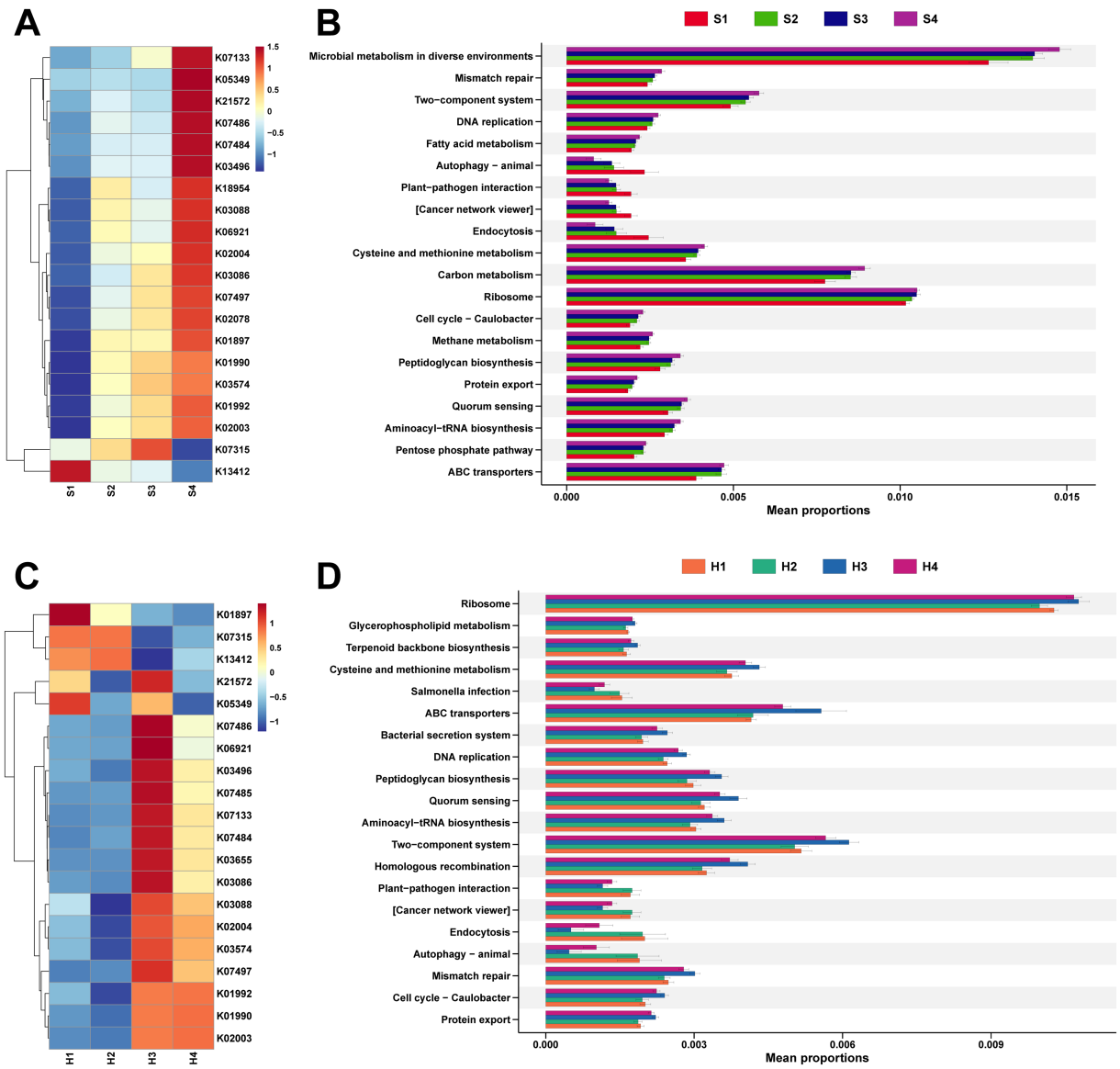

FIGURE 4
(A) Analysis of the top 20 differential KEGG Orthology (KO) identified in the rumen microbiota of S1–S4. **(B)** Analysis of the top 20 differential KEGG functional pathways identified in the rumen microbiota of S1–S4. **(C)** Analysis of the top 20 differential KO identified in the rumen microbiota of H1–H4. **(D)** Analysis of the top 20 differential KEGG functional pathways identified in the rumen microbiota of H1–H4. The stamp plots were created using the rank-sum test (Kruskal test), selecting the top 20 pathways by abundance with $p < 0.05$.

significant positive correlations with GH112 and GH4. Lactose correlated with up to 6 GH enzymes, including significant positive correlations with GH64 and GH84, and significant negative correlations with GH109, GH42, GH48, and GH50. Fat-free dry matter exhibited significant negative correlations with GH48 and significant positive correlations with GH64 and GH84.

For the H group, it can be observed that most CAZymes have higher abundance in H3 (Figure 6A). Additionally, we identified 13 GH enzymes (GH119, GH124, GH18, GH19, GH27, GH37, GH6, GH64, GH65, GH73, GH84, GH85, GH87) with significant inter-group differences (Figure 6B). Among these differential GH enzymes, H3 showed the highest abundance in GH119, GH18, GH6, GH173, and GH85, highlighting differences in enzyme abundance between H1/H2 and H3/H4. Correlation analysis with previously detected differential indicators revealed significant associations with these 13 GH enzymes: acetic/propionic acid showed significant positive correlations with GH37, GH64, GH84, and GH87, and significant negative correlations with the other 7 enzymes. Additionally, isovaleric acid exhibited significant negative correlations with GH6 and GH65, while lactose showed significant positive correlation with GH64.

4 Discussion

To fill the knowledge gap regarding the impact of rumen microecology on the production performance of Sanhe cattle, we focused on comparing the rumen microbiota of Sanhe cattle and Holstein cows across 1–4 parities. The aim was to identify potential differences in

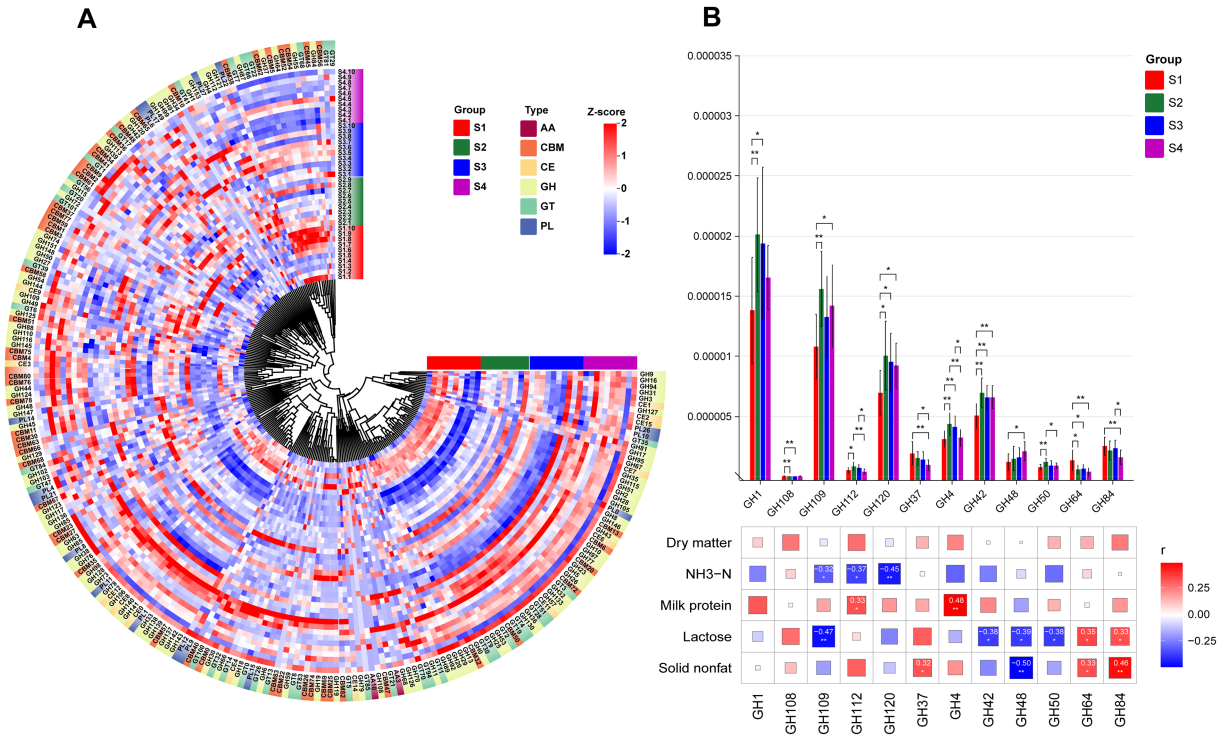

FIGURE 5
(A) HCA of CAZy enzyme classification identified in the rumen microbiota of S1–S4. The inner ring of color blocks represents rumen samples from cows of different groups, while the outer ring of color blocks corresponds to the six types of CAZy enzymes: AA, CBM, CE, GH, GT, and PL.
(B) Spearman correlation analysis between differential GH enzymes identified in the rumen microbiota of S1–S4 and differential physiological indicators. * indicates $p < 0.05$, ** indicates $p < 0.01$, *** indicates $p < 0.001$.

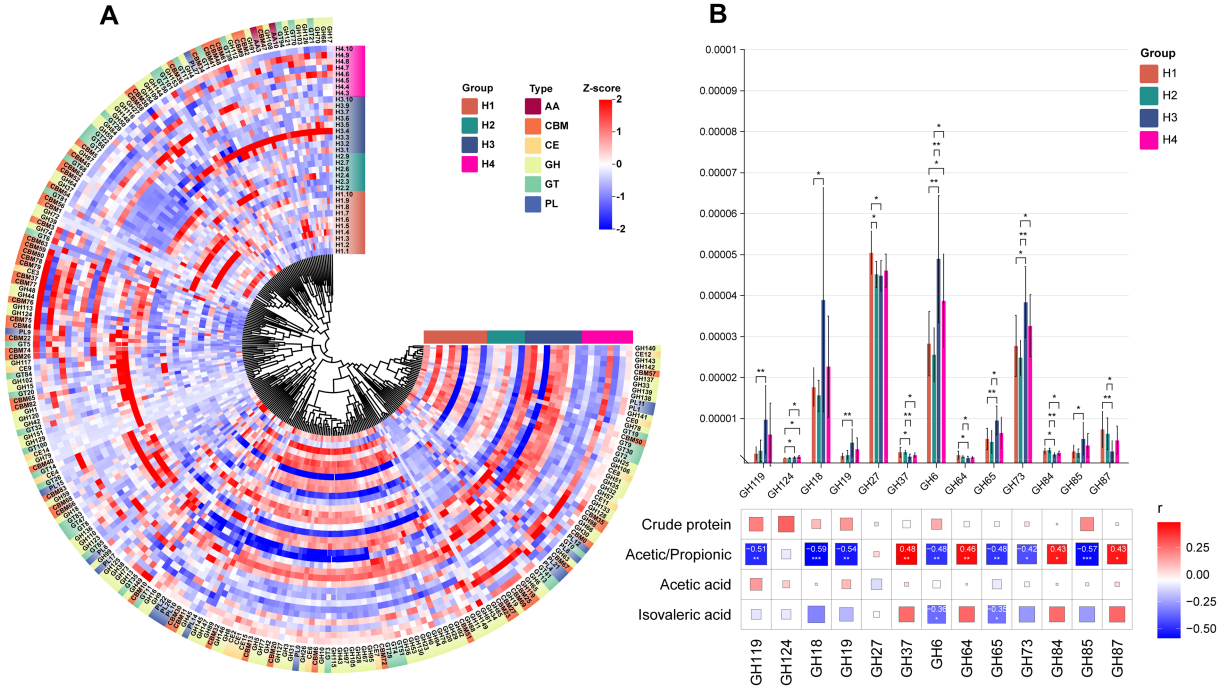

FIGURE 6
(A) HCA of CAZy enzyme classification identified in the rumen microbiota of H1–H4. The inner ring of color blocks represents rumen samples from cows of different groups, while the outer ring of color blocks corresponds to the six types of CAZy enzymes: AA, CBM, CE, GH, GT, and PL.
(B) Spearman correlation analysis between differential GH enzymes identified in the rumen microbiota of S1–S4 and differential physiological indicators. * indicates $p < 0.05$, ** indicates $p < 0.01$, *** indicates $p < 0.001$.

microbial composition and functional characteristics associated with parity and breed under the same dietary conditions, as well as their effects on lactation metabolism.

For Sanhe cattle, our previous studies found that the milk metabolome of S1–S4 displayed a pattern where S1 was significantly different from S2/S3/S4. This aligns with the observed changes in the rumen microecological environment in this study, where the microbial abundance and the types of enriched functional pathways in S1 also significantly differed from those in multiparous cows. We found that among the genera-level microbes significantly enriched in S1, both *Neocallimastix* and *Piromyces* are anaerobic fungi producing cellulase (17), mainly found in the rumen and intestines of ruminants (18, 19). *Neocallimastix* has a very high cellulase activity due to its 89% degradation activity on the cell wall (20). *Piromyces* can ferment cellulose to produce more acetate (21). Previous studies have found that using strains from these two genera for *in vitro* fermentation can increase DM digestibility and VFA content (22), which is consistent with the trends observed in S1 in this *in vivo* experiment. It is currently known that the genus *Prevotella* is the most abundant microbial group detected in ruminants globally (23). On one hand, *Prevotella* can contribute to rumen nitrogen metabolism by hydrolyzing proteins to produce ammonia (24, 25). On the other hand, as an ammonia-producing bacterium, it can generate ammonia through the deamination of amino acids (26), both of which promote the production of ammonia nitrogen. The concentration of NH_3-N in the rumen reflects the relationship between the rate of ammonia nitrogen production and utilization in the rumen. Compared to *Prevotella ruminantium* and *Prevotella bryantii*, which have been verified to have a positive correlation with ammonia nitrogen production (27), the specific functions of the three *Prevotella*-related biomarkers identified in S1 are currently less studied. However, this study suggests that they may still be one of the reasons for the higher ammonia nitrogen levels in S1.

Although there were no significant differences in VFA levels between S1–S4, the total VFA and other short-chain fatty acids showed a higher trend in S1. Among the four biomarkers in S1, *Lactobacillus fructivorans* can decompose fructose to produce more acetic acid (28). The other three biomarkers are related to *Prevotella*, and previous studies have shown that the abundance of *Prevotella* is positively correlated with VFA production, improving fermentation efficiency and promoting the production of short-chain fatty acids (29–31). We also observed that the rumen microbiota in S1 was significantly enriched in KEGG pathways such as autophagy—animal, plant-pathogen interaction, and endocytosis, which are related to immune functions (32), cell metabolism (33), and intracellular material transport (34). These results suggest that the rumen microbiome in S1 undergoes dynamic adjustments to cope with the stress of the first lactation experience, showing more dramatic changes. In contrast, in S2/S3/S4, we observed a higher enrichment of ATP-binding cassette (ABC) transporters (K01990 and K02003) and ABC-2 type transport system permease protein (K01992) in the rumen microbiota, which aligns with the higher mapping of ABC transporters pathways in the KEGG database for S2/S3/S4. ABC transporters in microorganisms mainly participate in the transport of nutrients, especially monosaccharides and amino acids (35). These metabolic activities may indirectly affect the digestion, absorption, and nutrient metabolism of dairy cows, consistent with our previous findings of higher glucose and total protein levels in the serum of S2/S3/S4 cows.

Cows themselves cannot synthesize any enzymes necessary for the deconstruction of plant biomass. They rely primarily on the rumen microbiome to release energy from plant polysaccharides in the form of carbohydrates and sugars (36). Within the CAZyme family, glycoside hydrolases (GHs) are the most abundant and diverse group responsible for breaking glycosidic bonds in plant polysaccharides. They account for 50% of the classified enzymes in the CAZyme database (37) and can decompose cellulose, hemicellulose, and starch into simple carbohydrates that cows can absorb and utilize. Glucose and other substances produced by the breakdown of substrates such as cellulose by GH enzymes are important precursors for lactose synthesis (38, 39). Among the GH enzymes that showed a significant positive correlation with lactose content in Japanese Black cows, GH64 can hydrolyze β-1,3-D-glucan (40), releasing glucose monomers, a process providing the carbon source needed for lactose synthesis. GH84 can participate in the modification of glycoproteins (41), potentially regulating enzymes or substrates related to the lactose synthesis pathway and indirectly influencing lactose production. However, the actual abundance of these GH enzymes in the rumen, the downstream products they produce, and their impact on lactose synthesis through blood circulation still need to be verified through a series of experiments. This is necessary to further explore the mechanisms by which rumen microbial functions affect the biosynthetic pathways of milk components.

Compared to other breeds, Holstein cows are the most widely distributed high-yield dairy cattle globally, and research on their metagenome is both extensive and in-depth (42, 43). However, longitudinal comparisons of rumen microbiota changes with parity and cross-sectional comparisons of rumen microbiota under the same dietary conditions with other cattle breeds are still relatively scarce. Studies on Holsteins have shown that in multiparous cows, parity may be one of the driving factors for host-microbe interactions (44). Previous research mentioned that differences in microbial community composition are mainly attributed to diet, with the host having a smaller impact (23). This study indicates that under the same dietary conditions, the species composition structure of high-abundance phyla and genera in the rumen microbiota of Japanese Black cows and Holstein cows is similar, though the abundance varies slightly between different parities. We found that under the farm conditions we investigated, the trend of changes in the rumen microbiota of Holstein cows is somewhat different from that of Japanese Black cows, where S1 differs from S2/S3/S4. Specifically, H1 and H2 are similar, H3 and H4 are similar, but H1/H2 differ from H3/H4. Interestingly, these changes in the rumen microecology of Holstein cows align with the trends in metabolic profile changes we previously identified, showing similar patterns where H1 and H2 have closer abundance and differ from H3 and H4 on the heatmap (6). These findings suggest that rumen microbiota can influence body metabolism and thereby affect milk traits in different cattle breeds.

In terms of rumen fermentation, unlike S1, which had slightly higher levels of various VFAs than the other parities, the VFA changes in the H group did not follow a regular pattern. We found that although H3 had less acetate and more propionate in the rumen, the differences between groups were not significant. However, the acetate-to-propionate ratio in H3 was significantly lower than in the other parities. This could be related to the significantly higher abundance of *Succinivibrio* and *Ruminobacter* genera found in H3. Previous studies have shown that using a glucogenic

diet as a substrate for *in vitro* fermentation can lead to a reduced acetate/propionate ratio, accompanied by higher abundances of *Succinivibrio* and *Ruminobacter* genera (45). Propionate can be produced directly via the decarboxylation of succinate, a pathway in which the *Succinivibrio* genus plays an important role (46). Moreover, we found that many GH enzymes in H3 had higher abundances and significant negative correlations with the acetate/propionate ratio. These GH enzymes do not directly participate in propionate production but can promote propionate production by providing monosaccharide substrates required for microbial fermentation. Among the GH enzymes highly expressed in H3, GH119 (47), GH6 (48), and GH65 (49) can hydrolyze α-glucan, cellulose, and oligosaccharides to produce glucose, which can promote propionate production (50). *s_Fibrobacter succinogenes* is a major degrader of lignocellulose substances in the intestines of herbivores (51). Previous studies have also found that lower levels of isovaleric acid accompanied by lower abundances of *s_Fibrobacter succinogenes* in the rumen of Altay sheep at different energy feeding levels (52). However, in Nellore calves, it was found that with an increase in concentrate content, rumen isovaleric acid concentration increased, but *s_Fibrobacter succinogenes* decreased (53). This is contrary to the higher isovaleric acid content accompanied by higher abundances of *s_Fibrobacter succinogenes* found in H1 in this study, which might be related to differences in feed composition and the stage of the cattle. *s_Eubacterium uniforme*, identified as a common biomarker in both H4 and S4, is a fiber-digesting bacterium that mainly decomposes cellobiose and xylan and has previously only been isolated from sheep rumen (54).

5 Conclusion

This study explored the differences in rumen microbiota composition between multiparous Sanhe cattle and Holstein cows and found two main points: First, under the same dietary conditions, the species composition of the rumen microbiota was similar between Sanhe cattle and Holstein cows, but their abundances differed. Second, the rumen microecological patterns were highly correlated with milk metabolic patterns, but breed remained a decisive factor influencing dairy cow performance. These results highlight the breed-specific metabolic adaptability of dairy cows, which changes with parity, emphasizing the dynamic interactions between genetic background, physiological state, and metabolic regulation. Understanding these complex metabolic dynamics is crucial for optimizing feeding strategies, improving production efficiency, and ensuring the overall health and welfare of dairy herds.

Data availability statement

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

Ethics statement

The animal study was approved by Animal Experiment Ethics Committee of the Institute of Subtropical Agriculture, Chinese Academy of Sciences. The study was conducted in accordance with the local legislation and institutional requirements.

Author contributions

ZL: Investigation, Methodology, Project administration, Software, Validation, Visualization, Writing – original draft, Writing – review & editing. AJ: Data curation, Investigation, Methodology, Software, Validation, Visualization, Writing – review & editing. DM: Investigation, Writing – review & editing. DL: Investigation, Writing – review & editing. XH: Investigation, Writing – review & editing. MZ: Data curation, Investigation, Writing – review & editing. CZ: Conceptualization, Funding acquisition, Project administration, Resources, Supervision, Writing – review & editing. ZT: Conceptualization, Funding acquisition, Project administration, Resources, Supervision, Writing – review & editing.

Funding

The author(s) declare that financial support was received for the research, authorship, and/or publication of this article. This work was jointly supported by the Projects of International Cooperation and Exchanges NSFC (32261143467), Strategic Priority Research Program of the Chinese Academy of Sciences (XDA26040306), and the National Key Research and Development Program (2022YFD1300805).

Acknowledgments

The authors would like to thank Can Peng, Meimei Geng, Liping Zhang, Xu Liwei, Wen Chen, and Hongzhao Yuan from the Institutional Center for Shared Technologies and Facilities of the Institute of Subtropical Agriculture, Chinese Academy of Sciences, for their technical support. We would also like to extend our gratitude to all the staff at Xiertala Agriculture and Animal Husbandry Farm Co., Ltd., Hulunbuir, Inner Mongolia Autonomous Region, for their assistance during the experiment.

Conflict of interest

DM, DL, XH, and MZ were employed by Hulun Buir State Farm Xieertala Farm and Ranch Co., Ltd.

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

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Supplementary material

The Supplementary material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fvets.2024.1463209/full#supplementary-material

References

1. Ilie DE, Gao YH, Nicolae I, Sun DX, Li JY, Han B, et al. Evaluation of single nucleotide polymorphisms identified through the use of SNP assay in Romanian and Chinese Holstein and Simmental cattle breeds. *Acta Biochim Pol*. (2020) 67:341–6. doi: 10.18388/abp.2020_5080

2. Hu L, Ma Y, Liu L, Kang L, Brito LF, Wang D, et al. Detection of functional polymorphisms in the hsp70 gene and association with cold stress response in inner-Mongolia Sanhe cattle. *Cell Stress Chaperones*. (2019) 24:409–18. doi: 10.1007/s12192-019-00973-5

3. Xu Q, Wang YC, Liu R, Brito LF, Kang L, Yu Y, et al. Differential gene expression in the peripheral blood of Chinese Sanhe cattle exposed to severe cold stress. *Genet Mol Res*. (2017) 16:gmr16029593. doi: 10.4238/gmr16029593

4. Walter LL, Gärtner T, Gernand E, Wehrend A, Donat K. Effects of parity and stage of lactation on trend and variability of metabolic markers in dairy cows. *Animals*. (2022) 12:1008. doi: 10.3390/ani12081008

5. Liu Z, Jiang A, Lv X, Fan D, Chen Q, Wu Y, et al. Combined metabolomics and biochemical analyses of serum and milk revealed parity-related metabolic differences in Sanhe dairy cattle. *Metabolites*. (2024a) 14:227. doi: 10.3390/metabo14040227

6. Liu Z, Jiang A, Lv X, Zhou C, Tan Z. Metabolic changes in serum and milk of Holstein cows in their first to fourth parity revealed by biochemical analysis and untargeted metabolomics. *Animals*. (2024b) 14:407. doi: 10.3390/ani14030407

7. Mizrahi I, Jami E. Review: the compositional variation of the rumen microbiome and its effect on host performance and methane emission. *Animal*. (2018) 12:s220–32. doi: 10.1017/S1751731118001957

8. Amin N, Schwarzkopf S, Kinoshita A, Troscher-Mussotter J, Danicke S, Camarinha-Silva A, et al. Evolution of rumen and oral microbiota in calves is influenced by age and time of weaning. *Anim Microbiome*. (2021) 3:31. doi: 10.1186/s42523-021-00095-3

9. Indugu N, Vecchiarelli B, Baker LD, Ferguson JD, Vanamala JKP, Pitta DW. Comparison of rumen bacterial communities in dairy herds of different production. *BMC Microbiol*. (2017) 17:190. doi: 10.1186/s12866-017-1098-z

10. Sofyan A, Uyeno Y, Shinkai T, Hirako M, Kushibiki S, Kanamori H, et al. Metagenomic profiles of the rumen microbiota during the transition period in low-yield and high-yield dairy cows. *Anim Sci J*. (2019) 90:1362–76. doi: 10.1111/asj.13277

11. Xue M, Sun H, Wu X, Guan Le L, Liu J. Assessment of rumen microbiota from a large dairy cattle cohort reveals the pan and core bacteriomes contributing to varied phenotypes. *Appl Environ Microbiol*. (2018) 84:e00970. doi: 10.1128/AEM.00970-18

12. Jiang BX, Qin CB, Xu YX, Song XH, Fu YH, Li RJ, et al. Multi-omics reveals the mechanism of rumen microbiome and its metabolome together with host metabolome participating in the regulation of milk production traits in dairy buffaloes. *Front Microbiol*. (2024) 15:1301292. doi: 10.3389/fmicb.2024.1301292

13. Lima FS, Oikonomou G, Lima SF, Bicalho MLS, Ganda EK, de Oliveira JC, et al. Prepartum and postpartum rumen fluid microbiomes: characterization and correlation with production traits in dairy cows. *Appl Environ Microbiol*. (2015) 81:1327–37. doi: 10.1128/AEM.03138-14

14. Schären M, Frahm J, Kersten S, Meyer U, Hummel J, Breves G, et al. Interrelations between the rumen microbiota and production, behavioral, rumen fermentation, metabolic, and immunological attributes of dairy cows. *J Dairy Sci*. (2018) 101:4615–37. doi: 10.3168/jds.2017-13736

15. Liu Z, Yan F, Mi H, Lv X, Wang K, Li B, et al. N-Carbamoylglutamate supplementation on the digestibility, rumen fermentation, Milk quality, antioxidant parameters, and metabolites of Jersey cattle in high-altitude areas. *Front Vet Sci*. (2022) 9:848912. doi: 10.3389/fvets.2022.848912

16. Wang Z, He Z, Beauchemin KA, Tang S, Zhou C, Han X, et al. Comparison of two live *Bacillus* species as feed additives for improving *in vitro* fermentation of cereal straws. *Anim Sci J*. (2015) 87:27–36. doi: 10.1111/asj.12346

17. Wei YQ, Yang HJ, Luan Y, Long RJ, Wu YJ, Wang ZY. Isolation, identification and fibrolytic characteristics of rumen fungi grown with indigenous methanogen from yaks (*Bos grunniens*) grazing on the Qinghai-Tibetan Plateau. *J Appl Microbiol*. (2016) 120:571–87. doi: 10.1111/jam.13035

18. Barr DJS, Yanke LJ, Bae HD, McAllister TK, Cheng KJ. Contributions on the morphology and taxonomy of some rumen FUNGI from Canada. *Mycotaxon*. (1995) 54:203–14.

19. Paul SS, Kamra DN, Sastry VRB. Fermentative characteristics and fibrolytic activities of anaerobic gut fungi isolated from wild and domestic ruminants. *Arch Anim Nutr*. (2010) 64:279–92. doi: 10.1080/17450391003625037

20. Sijtsma L, Tan B. Degradation and utilization of grass cell-walls by anaerobic FUNGI isolated from yak, llama and sheep. *Anim Feed Sci Technol*. (1993) 44:221–36. doi: 10.1016/0377-8401(93)90049-P

21. Dijkerman R, Op den Camp HJM, van der Drift C. Cultivation of anaerobic fungi in a 10-I fermenter system for the production of (hemi)-cellulolytic enzymes. *Appl Microbiol Biotechnol*. (1996) 46:85–91. doi: 10.1007/s002530050787

22. Shelke SK, Chhabra A, Puniya AK, Sehgal JP. *In vitro* degradation of sugarcane bagasse based ruminant rations using anaerobic fungi. *Ann Microbiol*. (2009) 59:415–8. doi: 10.1007/BF03175124

23. Mizrahi I, Wallace RJ, Moraïs S. The rumen microbiome: balancing food security and environmental impacts. *Nat Rev Microbiol*. (2021) 19:553–66. doi: 10.1038/s41579-021-00543-6

24. Griswold KE, White BA, Mackie RI. Diversity of extracellular proteolytic activities among *Prevotella* species from the rumen. *Curr Microbiol*. (1999) 39:187–94. doi: 10.1007/s002849900443

25. Wen ZT, Peng LS, Morrison M. The glutamine synthetase of *Prevotella bryantii* B_{14} is a family III enzyme (GinN) and glutamine supports growth of mutants lacking glutamate dehydrogenase activity. *FEMS Microbiol Lett*. (2003) 229:15–21. doi: 10.1016/S0378-1097(03)00764-X

26. Wallace RJ. Ruminal microbial metabolism of peptides and amino acids. *J Nutr*. (1996) 126:1326S–34S. doi: 10.1093/jn/126.suppl_4.1326S

27. Ferme D, Banjac M, Calsamiglia S, Busquet M, Kamel C, Avgustin G. The effects of plant extracts on microbial community structure in a rumen-simulating continuous-culture system as revealed by molecular profiling. *Folia Microbiol*. (2004) 49:151–5. doi: 10.1007/BF02931391

28. Endo A, Futagawa-Endo Y, Sakamoto M, Kitahara M, Dicks LMT. *Lactobacillus florum* sp. nov., a fructophilic species isolated from flowers. *Int J Syst Evol Microbiol*. (2010) 60:2478–82. doi: 10.1099/ijs.0.019067-0

29. Liang JS, Fang W, Chang JN, Zhang GM, Ma WF, Nabi M, et al. Long-term rumen microorganism fermentation of corn Stover in vitro for volatile fatty acid production. *Bioresour Technol*. (2022) 358:127447. doi: 10.1016/j.biortech.2022.127447

30. She YC, Hong JM, Zhang Q, Chen BY, Wei WX, Xin XD. Revealing microbial mechanism associated with volatile fatty acids production in anaerobic acidogenesis of waste activated sludge enhanced by freezing/thawing pretreatment. *Bioresour Technol*. (2020) 302:122869. doi: 10.1016/j.biortech.2020.122869

31. Wu QC, Chen HW, Zhang F, Wang WK, Xiong FL, Liu YY, et al. Cysteamine supplementation in vitro remarkably promoted rumen fermentation efficiency towards propionate production via Prevotella enrichment and enhancing antioxidant capacity. *Antioxidants*. (2022) 11:2233. doi: 10.3390/antiox11112233

32. Münz C. Non-canonical roles of autophagy proteins in endocytosis and exocytosis. *Biochem Soc Trans*. (2021) 49:2841–51. doi: 10.1042/BST20210811

33. Zhang HL, Zhu YM, Zhou XY. Coordination of autophagy and other cellular activities. *Adv Exp Med Biol*. (2019) 1206:697–727. doi: 10.1007/978-981-15-0602-4_30

34. Birgisdottir AB, Johansen T. Autophagy and endocytosis—interconnections and interdependencies. *J Cell Sci*. (2020) 133:jcs228114. doi: 10.1242/jcs.228114

35. Deusch S, Camarinha-Silva A, Conrad J, Beifuss U, Rodehutscord M, Seifert J. A structural and functional elucidation of the rumen microbiome influenced by various diets and microenvironments. *Front Microbiol*. (2017) 8:1605. doi: 10.3389/fmicb.2017.01605

36. Bohra V, Dafale NA, Purohit HJ. Understanding the alteration in rumen microbiome and CAZymes profile with diet and host through comparative metagenomic approach. *Arch Microbiol*. (2019) 201:1385–97. doi: 10.1007/s00203-019-01706-z

37. Cantarel BL, Coutinho PM, Rancurel C, Bernard T, Lombard V, Henrissat B. The Carbohydrate-Active EnZymes database (CAZy): an expert resource for Glycogenomics. *Nucleic Acids Res*. (2009) 37:D233–8. doi: 10.1093/nar/gkn663

38. Al Makishah NH, Elfarash AE. Molecular characterization of cellulase genes in *Pseudomonas stutzeri*. *Electron J Biotechnol*. (2022) 59:55–61. doi: 10.1016/j.ejbt.2022.07.004

39. Urashima T, Fukuda K, Messer M. Evolution of milk oligosaccharides and lactose: a hypothesis. *Animal*. (2012) 6:369–74. doi: 10.1017/S1751731111001248

40. Bai L, Kim J, Son KH, Shin DH, Ku BH, Kim DY, et al. Novel anti-fungal d-Laminaripentaose-releasing Endo-β-1,3-glucanase with a RICIN-like domain from *Cellulosimicrobium funkei* HY-13. *Biomolecules*. (2021) 11:1080. doi: 10.3390/biom11081080

41. Jiang X, Yang Q. Recent advances in glycoside hydrolase family 20 and 84 inhibitors: structures, inhibitory mechanisms and biological activities. *Bioorg Chem*. (2024) 142:106870. doi: 10.1016/j.bioorg.2023.106870

42. Lin LM, Lai Z, Zhang JY, Zhu WY, Mao SY. The gastrointestinal microbiome in dairy cattle is constrained by the deterministic driver of the region and the modified effect of diet. *Microbiome*. (2023) 11:10. doi: 10.1186/s40168-022-01453-2

43. Xue M-Y, Xie Y-Y, Zhong Y, Ma X-J, Sun H-Z, Liu J-X. Integrated meta-omics reveals new ruminal microbial features associated with feed efficiency in dairy cattle. *Microbiome*. (2022) 10:32. doi: 10.1186/s40168-022-01228-9

44. Zhu ZG, Difford GF, Noel SJ, Lassen J, Lovendahl P, Hojberg O. Stability assessment of the rumen bacterial and archaeal communities in dairy cows within a single lactation and its association with host phenotype. *Front Microbiol*. (2021) 12:636223. doi: 10.3389/fmicb.2021.636223

45. Hua D, Zhao Y, Nan X, Xue F, Wang Y, Jiang L, et al. Effect of different glucogenic to lipogenic nutrient ratios on rumen fermentation and bacterial community *in vitro*. *J Appl Microbiol*. (2021) 130:1868–82. doi: 10.1111/jam.14873

46. Jeyanathan J, Martin C, Morgavi DP. The use of direct-fed microbials for mitigation of ruminant methane emissions: a review. *Animal*. (2014) 8:250–61. doi: 10.1017/S1751731113002085

47. Janecek S, Svensson B, MacGregor EA. α-Amylase: an enzyme specificity found in various families of glycoside hydrolases. *Cell Mol Life Sci*. (2014) 71:1149–70. doi: 10.1007/s00018-013-1388-z

48. Chen YX, Zhang Y, Shi X, Xu LX, Zhang L, Zhang LW. The succession of GH_6 cellulase-producing microbial communities and temporal profile of GH_6 gene abundance during vermicomposting of maize stover and cow dung. *Bioresour Technol*. (2022) 344:126242. doi: 10.1016/j.biortech.2021.126242

49. Nakamura S, Nihira T, Kurata R, Nakai H, Funane K, Park EY, et al. Structure of a bacterial α-1,2-glucosidase defines mechanisms of hydrolysis and substrate specificity in GH65 family hydrolases. *J Biol Chem*. (2021) 297:101366. doi: 10.1016/j.jbc.2021.101366

50. Zhang JG, Kawamoto H, Cai YM. Relationships between the addition rates of cellulase or glucose and silage fermentation at different temperatures. *Anim Sci J*. (2010) 81:325–30. doi: 10.1111/j.1740-0929.2010.00745.x

51. Ransom-Jones E, Jones DL, McCarthy AJ, McDonald JE. The *Fibrobacteres*: an important phylum of cellulose-degrading bacteria. *Microb Ecol*. (2012) 63:267–81. doi: 10.1007/s00248-011-9998-1

52. Li H, Song S, Gao L, Lang X, Liu L, Gong X, et al. Effects of feeding level on the gastrointestinal development, rumen fermentation and rumen microbiota in Altay sheep. *Acta Pratacul Sin*. (2021) 30:180–90. doi: 10.11686/cyxb2020184

53. Ribeiro CS, Messana JD, Granja-Salcedo YT, Canesin RC, Fiorentini G, San Vito E, et al. Parameters of fermentation and rumen microbiota of Nellore steers fed with different proportions of concentrate in fresh sugarcane containing diets. *Arch Anim Nutr*. (2016) 70:402–15. doi: 10.1080/1745039X.2016.1206737

54. Van Gylswyk NO, van der Toorn JJTK. *Eubacterium uniforme* sp. nov. and *Eubacterium xylanophilum* sp. nov., fiber-digesting bacteria from the Rumina of sheep fed corn Stover. *Int J Syst Evol Microbiol*. (1985) 35:323–6. doi: 10.1099/00207713-35-3-323

55. AOAC C. *Official methods of analysis of the Association of Analytical Chemists International*. Official Methods: Gaithersburg, MD, USA. (2005).

56. Yang LY, Zhang LM, Zhang PH, Zhou YL, Huang XG, Yan QX, et al. (2022). Alterations in nutrient digestibility and performance of heat-stressed dairy cows by dietary L-theanine supplementation. *Anim. Nutr.* 11, 350–358. doi: 10.1016/j.aninu.2022.08.002

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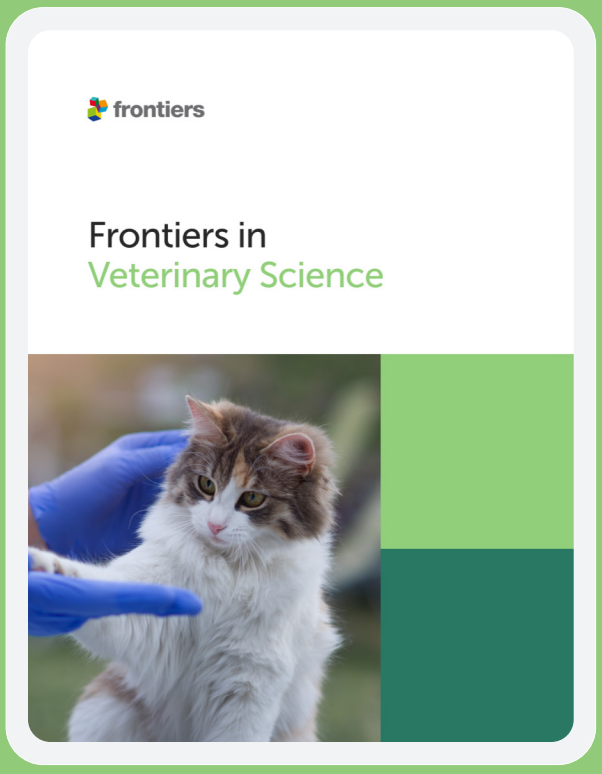

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